MEDICAL UNIVERSITY – PLEVEN

FACULTY OF HEALTH CARE
DEPARTMENT OF CLINICAL LABORATORY,
CLINICAL IMMUNOLOGY AND ALLERGOLOGY

Svetla Ognyanova Blazheva, MD

Study of the endometrial immune profile and endometrial microbiota in women with reproductive failures

ABSTRACT OF A DISSERTATION PAPER
SUBMITTED TO THE MEDICAL UNIVERSITY OF PLEVEN
TO OBTAIN THE EDUCATIONAL AND SCIENTIFIC DEGREE "DOCTOR (PhD)"

Pleven, 2025

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Formal Reviewers:
Prof. Dobroslav Stanimirov Kyurkchiev, MD, D.Sc.
Prof. Iskra Petrova Altankova, MD, D.Sc.

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The dissertation was discussed and approved for defense by the Department of Clinical Laboratory, Clinical Immunology, and Allergology's extended departmental council at the Medical University of Pleven on April 7, 2025. It was then approved for defense before a scientific jury.

The dissertation includes 183 pages, 15 tables, 21 figures. The bibliography comprises 336 publications.

The Scientific Jury is composed of members appointed by Order No. 1222/30.04.2025 of the Rector of MU - Pleven:

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The materials from the dissertation defense are published on the Medical University of Pleven website (http://www.mu-pleven.bg).

Thesis defense presentation will take place on 09.06.2025 in “Louis Pasteur“ hall in “Farmacy Department” – MU Pleven at 13:30 AM.

List of Abbreviations

ASRM	American Society for Reproductive Medicine
BCL6	B-cell lymphoma 6
CBir1	Cytoplasmic Carnitine Acetyltransferase 1
CCR2	C-C chemokine receptor type 2
CD	Cluster of differentiation
CXCR5	Chemokine receptor type 5
EM	Endometrial microbiota
ERK	Extracellular-signal-regulated kinase
ESHRE	European Society of Human Reproduction and Embryology
EPS	Extracellular polymeric substances
EVT	Extravillous trophoblasts
ERA	Endometrial Receptivity Array
FSH	Follicle-stimulating hormone
GATA3	GATA binding protein 3
hCG	Human chorionic gonadotropin
HLA	Human leukocyte antigen
ICOS	Inducible co-stimulator of lymphocytes
ICSI	Intracytoplasmic sperm injection,
IFN	Interferon
IGF	Insulin-like growth factor
IL	Interleukin
ILC	Innate lymphoid cells
IUI	Intrauterine Insemination.
IVF	In vitro fertilization
JNK	c-Jun N-terminal kinase
LH	Luteinizing hormone
MALT	Mucosa associated lymphoid tissue
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
NLRP3	Nucleotide-binding domain, leucine-rich repeat
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Orm2	Ornithine decarboxylase antizyme 2
pbNK	Peripheral blood natural killer cells
PDGF	Platelet-derived growth factor
PRR	Pattern recognition receptor
PD-L1	Programmed death-ligand 1
RIF	Recurrent implantation failure
RPL	Recurrent pregnancy loss
SAA3	Serum Amyloid A3 protein
SCT	Stem cell transplant

SCT	Stem cell transplant
SIGNR1	SIGNR1
STAT3	Signal transducer and activator of transcription 3
Tfh	T follicular helper cells
TLR	Toll-like receptor
TGF	Transforming growth factor
TNF	Tumor necrosis factor
uNK	Uterine natural killer cells
VEGF	Vascular endothelial growth factor
EVT	Extravillous trophoblasts
M1/M2	Macrophage type 1/ Macrophage type 2

CONTENT

I. INTRODUCTION

The development of assisted reproductive technologies (ART) was significantly expanded in recent decades, enhancing the possibilities for infertility treatment. Despite the progress, the rate of failed implantations and early spontaneous abortions remained high, which underscored the need for in-depth research on the factors influencing endometrial receptivity. While embryo quality is essential for successful implantation, a growing body of research suggests that the immunological and microbiome characteristics of the endometrium play a key role in this process.

The concept of an "endometrial immune profile" was proposed by Ledee et al., highlighted the importance of local immune regulation for successful implantation. An imbalance in the cytokine network, as well as changes in the number and function of specific immune cells, could result in an immune response that is either overly active and rejects the embryo, or insufficiently effective to provide a suitable environment for its development. Various mechanisms of immune dysfunction may be factors in recurrent implantation failure and spontaneous pregnancy loss.

In addition to immunological aspects, interest in the role of the microbiome in reproductive medicine has increased in recent years. The endometrial microbiome, its composition and balance have been considered as a potential modulator of endometrial receptivity. Studies showed that the diversity and stability of the microbial environment could influence the processes of adhesion, invasion and early pregnancy development. The heterogeneity of the microbiome community was analyzed using parameters such as alpha and beta diversity, which allowed the assessment of its role in the implantation process.

Despite advances in understanding these mechanisms, a unified approach for standardized assessment of the endometrial immune profile and microbiome in the context of ART has been lacking. The development of integrated diagnostic methods combining immunological and microbiome analyses could contribute to the development of personalized therapeutic strategies to improve implantation success. The current study aimed to add to the existing knowledge in this area by analyzing the interaction between the endometrial immune response and the microbiome in women with recurrent implantation failure and pregnancy loss.

II. AIMS AND OBJECTIVES

The aim of this dissertation is to determine some characteristics of the endometrial immune profile and endometrial microbiota in women with recurrent implantation failure (RIF) and recurrent pregnancy loss (RPL) following ART procedures. By clarifying their role in reproductive success, the goal is to develop diagnostic and therapeutic approaches to improve reproductive outcomes in these patients.

In connection with this goal, the following main tasks have been defined:

1. Selection and grouping of patients (women with RIF and with RPL).
2. Implementation of the method for determination of endometrial leukocyte populations and subpopulations (community lymphocytes, neutrophils, macrophages, T lymphocytes, uNK cells, progenitor NK cells and plasma cells) in women with reproductive failure.
3. Study in women with RIF and RPL who have undergone ART (IVF, ICSI, IUI) to identify endometrial immune cells during the implantation window in the luteal phase.
4. Study women from both clinical groups by DNA analysis of endometrial biopsies to assess endometrial microbiota.
5. Use statistical analysis methods to determine the association between the endometrial immune profile and endometrial microbiota in the clinical group of women with RIF and RPL.To investigate the importance of microbiota composition and immune cell profile in chronic endometritis and antibiotic and probiotic therapy.
6. To study the importance of microbiota composition and immune cell profile for successful delivery in women with RIF and RPL.
7. Based on the study, develop an algorithm for the diagnostic and therapeutic management of women planning ART.

III. MATERIALS AND METHODS

1. CLINICAL GROUP OF STUDIED PATIENTS

The present study encompasses a total of 107 women diagnosed with RIF and 93 women diagnosed with RPL. These subjects were admitted for the purpose of obtaining diagnostic clarity regarding their infertility, and subsequently underwent treatment through ART. The mean age of the patients was found to be 36.26 ± 4.54 years (range: 26–41 years). The selection of patients and the collection of endometrial biopsies were performed at the Medical Center - Clinical Institute for Reproductive Medicine, Pleven. The studies were approved by the Ethics

Committee for Scientific Research at the Medical University - Pleven, with all patients providing written informed consent.

The identification of endometrial immune cells was carried out at the Laboratory of Clinical Immunology at University Hospital "Dr. G. Stranski" - EAD, Pleven. The analysis of endometrial microbiota was conducted at the Medical Center - Clinical Institute for Reproductive Medicine, Pleven. A comprehensive array of clinical data was systematically collected from all participants, encompassing their medical and reproductive histories. Samples that exhibited discrepancies in the phase of the menstrual cycle and serum progesterone levels were excluded from the analysis.

Inclusion Criteria:

- Indication for intracytoplasmic sperm injection (ICSI).
- At least three good-quality embryos transferred in at least two cycles.
- Normal karyotype of partners.
- Preserved ovarian reserve.
- Normal shape of the uterine cavity.
- No antibiotic intake one month prior to sample collection.
- To avoid contamination of endometrial samples, vaginal and cervical secretions were examined. In the presence of pathogens, treatment was administered, and biopsy was performed after control tests demonstrating their absence.
- Presence of a regular menstrual cycle.

Exclusion Criteria:

- Presence of factors contributing to unsuccessful implantation and pregnancy loss.

2. STUDY DESIGN

The study design and sequence of investigations are presented in Figure 1.

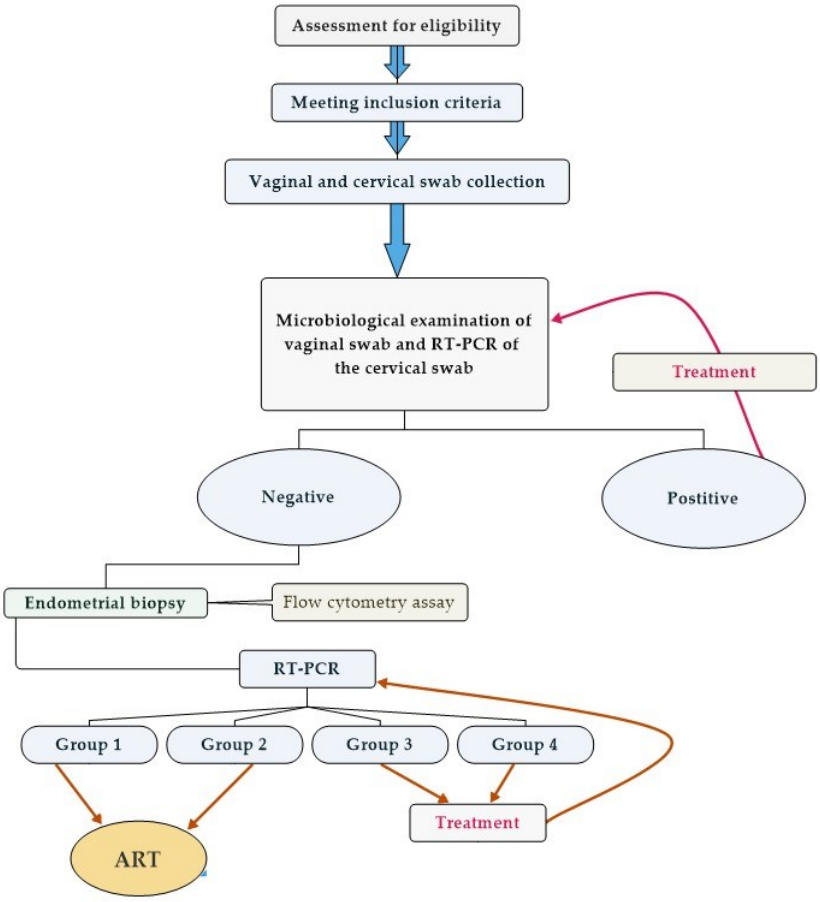

Figure 1. Study design.

3. COLLECTION OF SAMPLES

Endometrial samples were collected during the mid-luteal phase (20–22 days) of the natural menstrual cycle. The vagina and cervix were then meticulously cleansed with 0.9% NaCl. The endometrial tissue was extracted through the use of negative pressure with a sterile catheter. The samples were processed within one hour of their collection. The collected endometrial biopsy was then placed in a sterile tube containing 1 ml of sterile water. Subsequent to the homogenization process, a portion of the resulting homogenate was transferred into a transport medium. The samples were stored at -20 °C for a period of up to one week.

4. HOMOGENIZATION OF ENDOMETRIAL BIOPSY TISSUE

The endometrial biopsy was homogenized using the BD™ Medimachine system, which included a homogenizer, chamber, and filter. The tissue was placed in a sterile chamber with 1 ml of sterile water and then subjected to mechanical disruption by a pulse for 60 seconds. The resulting cell suspension was filtered through a filter with a pore size of 50 µm. After filtration,

the suspension was centrifuged and resuspended in PBS, with the cell concentration adjusted to a range of 5×10^5 to 1×10^6.

5. INVESTIGATION OF ENDOMETRIAL MICROBIOTA

PCR was used as a method for amplifying specific DNA fragments. It is based on the repeated copying of DNA with thermostable DNA polymerase. Specificity is determined by the complementarity between the primers and the DNA matrix.

The Femoflor® 16 REAL-TIME PCR Detection Kit was used for quantitative determination of microorganisms in endometrial biopsies. The kit allows for the evaluation of total bacterial mass and specific microorganisms. The method is based on fluorescent labeling and detection of PCR products. The kit includes controls for validating the process.

The study was conducted with an automatic Real-time thermocycler (model). The software performs quantitative analysis of bacterial DNA. The amount of human DNA is taken into account to exclude false-negative results. Standard samples were used to determine the relative concentration of the target DNA.

Genomic DNA was isolated using the PREP-NA PLUS DNA/RNA Extraction Kit.

6. FLOW CYTOMETRIC ANALYSIS OF ENDOMETRIAL IMMUNE CELLS

Flow cytometry was used for the analysis of cells passing through a laser beam. Optical characteristics such as size, density, and fluorescence were measured. The results were presented using dot plots.

Leukocyte and lymphocyte subpopulations were studied using a FACS Calibur flow cytometer. Monoclonal antibodies labeled with fluorochromes were used to identify cellular markers. Leukocytes, lymphocytes, macrophages, neutrophils, uNK-cells, progenitor NK-cells, T-lymphocytes, and plasma cells were analyzed. The data were presented as a percentage of CD45+ cells and lymphocytes.

The cell suspension was treated with monoclonal antibodies, a lysing solution, and a buffer (PBS). The samples were analyzed using a flow cytometer calibrated with automated software (Cell Quest Pro).

7. STATISTICAL ANALYSIS

Statistical analysis was performed using IBM SPSS software, version 26 (IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY: IBM Corp.) and GraphPad Prism, version 8.4.3 (GraphPad Software, GraphPad Prism, Version 8.4.3. San Diego, California). All parameters were tested for normality of distribution using the Shapiro-Wilk W test. When the level of significance was below 0.05 ($p < 0.05$), the null hypothesis for normal distribution was rejected. Parametric data were analyzed using one-way analysis of variance (ANOVA). Non-parametric data were analyzed using the Mann-Whitney U test for pairwise comparisons. The correlations between different parameters were evaluated using Spearman and Pearson tests. The choice of test was determined by the distribution of the data and adherence to normality assumptions. The Wilcoxon Signed Rank test was used to compare endometrial cells before and after therapy was administered.

IV. RESULTS

1. CLASSIFICATION OF THE ENDOMETRIAL MICROBIOTA IN WOMEN WITH RIF AND RPL

The molecular-genetic study of endometrial biopsies from women with RIF and RPL yielded two main groups, as determined by the following criteria:

1.1. The classification of groups according to the degree of disruption of the endometrial microbiota (EM)

The classification proposed by Iwami Nanako et al. (2022) was employed due to its utility and convenience in clinical practice. This system provides a clear delineation of the microbiota composition, the degree of dysbiosis, and the necessity for therapeutic intervention or monitoring:

Group A: Normal microbiota was characterized by the dominance of *Lactobacillus* spp. (over 90%), and dysbiotic bacteria were found to be absent.

Group B: Low bacterial mass was observed, with a reduction in the number of bacteria, including the absence of *Lactobacillus* spp. and dysbiotic bacteria.

Group C: Moderate dysbiosis was identified, with a reduction in the number of *Lactobacillus* spp. (below 90%) and an increase in the number of dysbiotic bacteria (over 10%).

Group D: Severe dysbiosis was noted, with *Lactobacillus* spp. being absent and dysbiotic bacteria being predominant (over 10%).

1.2. The classification of groups is determined by the type of microorganisms present, which is referred to as the microbial profile:

Group 1: Aerobic microorganisms demonstrated a prevalence in this group.

Group 2: Dominance was exhibited by anaerobic microorganisms.

Group 3: The presence of both aerobic and anaerobic microorganisms was observed.

Group 4: *Candida* spp. exhibited dominance.

Group 5: A combination of aerobic microorganisms and *Candida* spp. was observed.

Group 6: A combination of anaerobic microorganisms and *Candida* spp. was observed.

Group 7: A combination of aerobic, anaerobic microorganisms, and *Candida* spp. was observed.

Group 8: An absence of *Lactobacillus* spp. and dysbiotic microorganisms was recorded.

Group 9: *Lactobacillus* spp. exhibited a predominance that exceeded 90%.

2. AGE DISTRIBUTION OF THE STUDIED WOMEN IN THE DIFFERENT GROUPS

The present study included 200 women with reproductive failures. The mean age of the women was 35.27 ± 4.54 years, ranging from 26 to 41 years. The median age, in addition to the minimum and maximum ages, of the groups that have been categorized according to the type of endometrial microbiota disorder and according to the microbiota profile are presented in the following Figure 2.

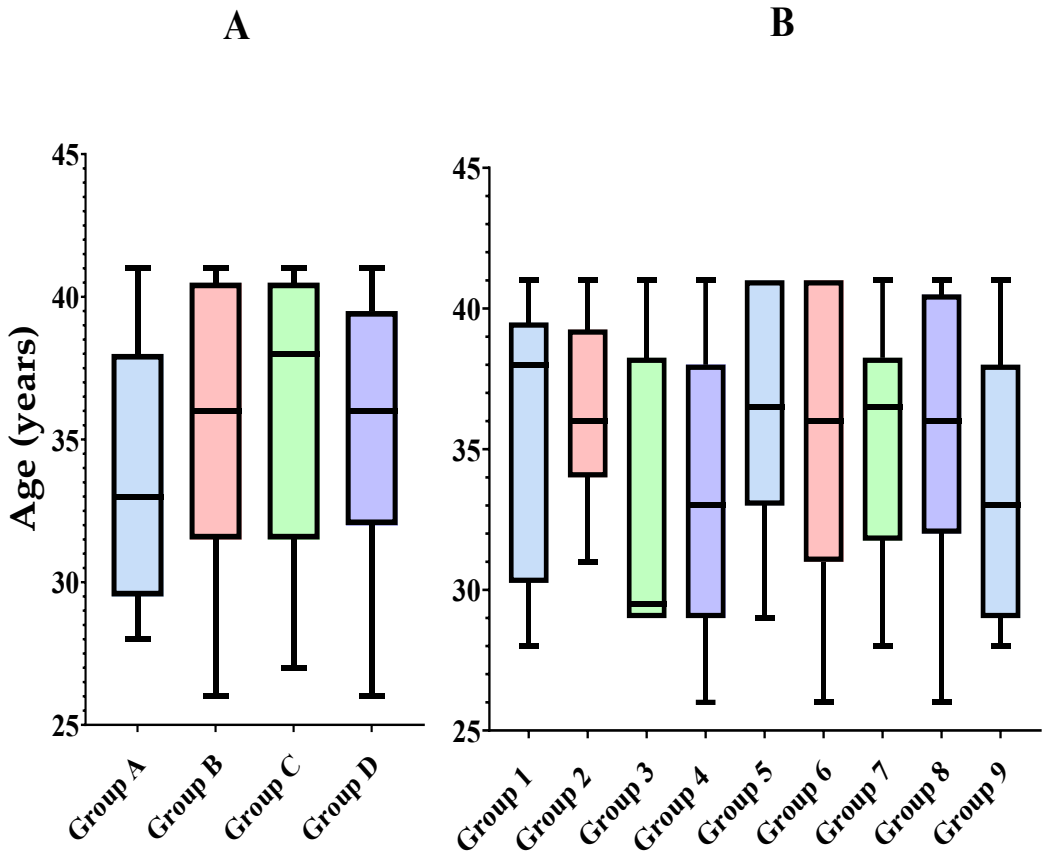

Figure 2. Age distribution in the different groups, represented by median, minimum and maximum: A) in the groups according to EM disorder and B) in the groups according to microbial profile.

3. DISTRIBUTION OF BIOPSIES BY GROUPS ACCORDING TO THE DEGREE OF EM DISORDER IN THE TOTAL GROUP OF WOMEN WITH RIF AND RPL

The results of the molecular genetic analysis of the studied biopsies are presented in Table 1. A significantly disturbed microbiota (group D) is found with the highest frequency in women with RIF and RPL. This particular type of dysbiosis was observed in 77 cases (38.5%) of the studied cohort. The second most prevalent group is group B (28.5%), followed by group A (20.5%). Group C biopsies (12.5%) were the least common.

Table 1. Distribution of biopsies (n = 200) categorized according to endometrial microbiota disruption and according to the profile of microorganisms detected in women with RIF and RPL.

	Endometrial microbiota Number of biopsies (%)			
Microbial Profile	**Group A** 41 (20.5)	**Group B** 57 (28.5)	**Group C** 25 (12.5)	**Group D** 77 (38.5)
Group 1	-	-	3 (1.5)	3 (1.5)
Group 2	-	-	3 (1.5)	15 (7.5)
Group 3	-	-	-	4 (2.0)
Group 4	4 (2.0)	8 (4.0)	1 (0.5)	2 (1.0)
Group 5	-	-	1 (0.5)	11 (5.5)
Group 6	-	-	7 (3.5)	26 (13.0)
Group 7	-	-	10 (5.0)	16 (8.0)
Group 8	-	49 (24.5)	-	-
Group 9	37 (18.5)	-	-	-

4. DISTRIBUTION OF BIOPSIES ACCORDING TO EM MICROBIAL PROFILE IN THE TOTAL GROUP OF WOMEN WITH RIF AND RPL

In Group C (Table 4), the combination of aerobic, anaerobic microorganisms and *Candida* spp. was found with the highest frequency (at 5.0%, Group 7), followed by the combination of anaerobic microorganisms and *Candida* spp. (at 3.5%, Group 6).

In Group D, the combination of anaerobic microorganisms and *Candida* spp. Was identified most frequently (at 13.0%, Group 6), followed by the combination of aerobic, anaerobic microorganisms and *Candida* spp. (at 8.0%, Group 7) and anaerobic microorganisms (at 7.5%, Group 2).
There was no statistically significant difference in the profile of microorganisms between Group C and Group D ($\chi 2(6, n = 102) = 8.757, p = 0.188$).

The data presented in Table 2 illustrate the association between endometrial microbiota disorders and the prevalence of reproductive disorders, including RIF and RPL, in women. The proportion of women exhibiting impaired or severely impaired endometrial microbiota (Group C and Group D) was comparable in women with RIF (52.34%) and RPL (49.46%). A higher frequency of *Candida* spp. as a self-presenting dysbiotic microorganism (Group 4) was identified in biopsies from women with RIF (9.35%) compared to those from women with RPL (5.38%).

Table 2. Distribution of biopsies (n = 200) according to the composition of microorganisms in the uterine microbiota of women with RIF and RPL.

	n	**Aerobic microorganisms (*n*)**	**Anaerobic microorganisms (*n*)**	**Aerobic and Anaerobic microorganisms (*n*)**	***Candida* spp. (*n*)**	**Aerobic microorganisms and *Candida* spp. (*n*)**	**Anaerobic microorganisms and *Candida* spp. (*n*)**	**Aerobic, Anaerobic microorganisms and *Candida* spp. (*n*)**	**Delivery (*n*)**	**Ongoing pregnancy (*n*)**
Group A	22	0	0	0	4	0	0	0	9	2
Group B	29	0	0	0	4	0	0	0	4	3
Group C	15	1	3	0	1	1	5	4	5	1
Group D	41	2	7	2	1	5	17	7	9	7
Total RIF	**107**	**3**	**10**	**2**	**10**	**6**	**22**	**11**	**27**	**13**
Group A	19	0	0	0	0	0	0	0	10	1
Group B	28	0	0	0	4	0	0	0	8	5
Group C	10	2	0	0	0	0	2	6	2	1
Group D	36	1	8	2	1	6	9	9	9	4
Total RPL	**93**	**3**	**8**	**2**	**5**	**6**	**11**	**15**	**29**	**11**

The distribution of biopsies according to the composition of microorganisms demonstrated that in women with RIF and RPL, the endometrial microbiota was most often significantly impaired. The presence of *Candida* spp. was identified in 86 biopsies.

5. FLOW CYTOMETRIC STUDY OF IMMUNE CELLS IN THE ENDOMETRIUM

5.1. Results for the total group of women, with RIF and RPL according to the degree of EM injury

Significant differences in the percentage values of lymphocytes (H (3) = 8.949, p = 0.030), uNK cells (H (3) = 13.846, p = 0.003), and T-lymphocytes (H (3) = 12.860, p = 0.005) were established by the Kruskal-Wallis H test when endometrial immune cell values were compared among the four studied groups.

The mean percentage values and standard deviations of endometrial immune cells in the four groups are presented in Table 3.

Table 3. Endometrial immune cells in different types of endometrial microbiota in women with RIF and RPL. Data are presented with mean and standard deviation.

	RIF ($\bar{x} \pm SD$)					RPL ($\bar{x} \pm SD$)				
	Group A ***n* = 22**	Group B ***n* = 29**	Group C ***n* = 15**	Group D ***n* = 41**	**Total RIF *n* = 107**	Group A ***n* = 19**	Group B ***n* = 28**	Group C ***n* = 10**	Group D ***n* = 36**	**Total RPL *n* = 93**
Leukocytes (%)	17.71 ± 10.98	25.09 ± 15.73	23.12 ± 16.33	25.56 ± 15.29	**18.26 ± 12.88**	19.34 ± 14.99	22.92 ± 13.81	12.13 ± 9.72	16.62 ± 11.04	**23.55 ± 14.85**
Lymphocytes (%)	43.35 ± 19.64	50.77 ± 18.47	33.68 ± 11.42	44.61 ± 24.75	**44.49 ± 21.04**	38.94 ± 20.14	45.96 ± 18.77	38.46 ± 24.79	46.96 ± 21.76	**44.11 ± 20.89**
Macrophages (%)	31.60 ± 19.67	31.22 ± 18.86	36.32 ± 22.01	32.74 ± 21.96	**32.6 ± 20.48**	42.61 ± 27.17	26.72 ± 14.5	29.59 ± 24.63	27.89 ± 17.72	**30.72 ± 20.55**
Neutrophils (%)	21.14 ± 9.74	17.78 ± 2.45	29.75 ± 15.98	22.02 ± 19.84	**21.77 ± 17.72**	17.30 ± 16.73	25.14 ± 18.69	31.12 ± 27.22	24.58 ± 19.72	**23.97 ± 19.82**
T-lymphocytes (%)	34.28 ± 14.85	44.63 ± 15.2	26.86 ± 15.95	35.03 ± 18.09	**36.33 ± 17.17**	34.69 ± 18.54	40.27 ± 16.98	33.41 ± 20.39	34.18 ± 17.46	**36.03 ± 17.79**
uNK-cells (%)	27.93 ± 12.44	23.93 ± 10.69	16.87 ± 9.81	26.78 ± 13.99	**24.85 ± 12.65**	23.08 ± 12.04	25.44 ± 13.74	17 ± 6.19	28.29 ± 15.98	**25.15 ± 14.02**
CD34+ uNK (%)	3.63 ± 4.14	2.41 ± 3.23	1.22 ± 2.18	2.1 ± 2.49	**2.37 ± 3.11**	2.44 ± 3.52	2.46 ± 3.51	2.07 ± 1.59	1.98 ± 2.35	**2.23 ± 2.91**
Plasma cells * (%)	2.70 ± 4.03	3.46 ± 6.48	6.31 ± 10.57	2.26 ± 5.38	**3.24 ± 6.46**	2.2 ± 2.44	1.18 ± 1.52	2.18 ± 5.39	2.12 ± 2.9	**1.86 ± 2.84**
Plasma cells ** (%)	0.64 ± 1.2	0.59 ± 1.19	0.92 ± 2.07	0.56 ± 1.93	**0.64 ± 1.62**	0.39 ± 0.51	0.29 ± 0.54	0.8 ± 2.04	0.53 ± 0.77	**0.46 ± 0.89**

* Plasma cells (CD45+, CD138+) presented as a percentage of all endometrial cells (leukocytes, stromal cells, epithelial cells).

The Mann-Whitney U test results for endometrial immune cells that had statistical significance were presented in Table 4 and Figure 3.

Table 4. Comparison of mean endometrial immune cell percentages among the four EM groups (n = 200) by Mann-Whitney U test. Comparison data are presented by median and interquartile range (IQR).

Endometrial immune cells	Groups	Median	IQR	*U*	*N*	*Z*	*p*
Leukocytes	**B**	23.79	19.58	484.0	57	-2.302	0.021
	C	9.16	24.79		25		
Lymphocytes	**B**	50.14	23.44	401.0	57	-3.138	0.002
	C	36.72	17.95		25		
Lymphocytes	**C**	36.72	17.95	702.0	25	-2.027	0.043
	D	47.08	39.30		77		
Neutrophils	**A**	12.59	24.35	323.0	41	-2.505	0.012
	C	27.89	35.21		25		
T- lymphocytes	**A**	36.12	24.23	852.5	41	-2.276	0.023
	B	42.25	24.43		57		
T- lymphocytes	**B**	42.25	24.43	403.0	57	-3.118	0.002
	C	27.48	21.64		25		
T- lymphocytes	**B**	42.25	24.43	1595.5	57	-2.696	0.007
	D	32.70	22.20		77		
uNK-cells	**A**	21.80	18.07	269.0	41	-3.219	0.001
	C	15.18	10.89		25		
uNK-cells	**B**	23.15	13.64	405.0	57	-3.097	0.002
	C	15.18	10.89		25		
uNK-cells	**C**	15.18	10.89	527.0	25	-3.388	0.001
	D	24.19	22.91		77		
CD34+ uNK	**A**	1.47	3.55	359.5	41	-2.023	0.043
	C	0.71	2.39		25		
Plasma cells*	**A**	0.13	0.56	895.5	41	-1.972	0.049
	B	0.08	0.48		57		

* Plasma cells (CD45+, CD138+) presented as a percentage of all endometrial cells (leukocytes, stromal cells, epithelial cells).

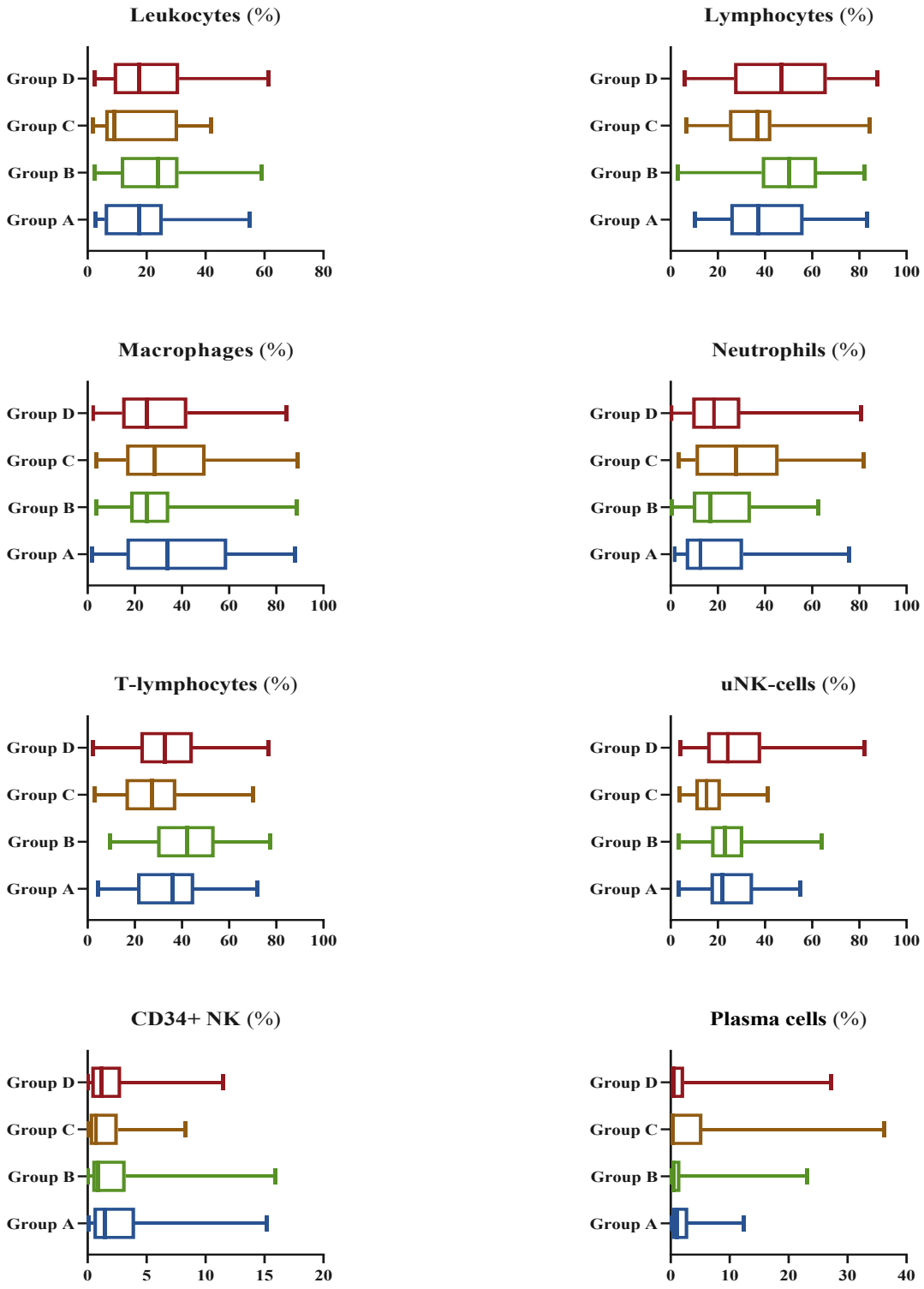

Figure 3. Comparison of mean percentages of endometrial immune cells between the four EM groups (n = 200) by Mann-Whitney U test. Boxes represent median and interquartile range.

A subsequent analysis of the mean endometrial leukocyte percentages revealed no statistically significant differences between the four groups ($p > 0.05$). In Group C, it was found that 11 out of 25 biopsies had a higher percentage of neutrophils than the group average, but an increase in plasma cells was observed in only one biopsy (7.29% of all cells). In Group D, 30 out of 77

biopsies exhibited a higher percentage of neutrophils than the group average; however, increased plasma cells were identified in only two of them (4.76% and 3.23% of all cells, respectively).

5.2. Results of flow cytometric analysis of biopsies in women with RIF in the four groups according to the type of endometrial microbiota disruption

A statistically significant difference in T-lymphocytes between the groups based on the type of endometrial disorder was identified ($F(3, 103) = 4.336$, $p = 0.006$). The percentage value of T-lymphocytes exhibited a normal distribution in the RIF group. A one-way analysis of variance (ANOVA) was employed to compare the mean values between the groups. An additional comparison between the groups was conducted using Tukey's HSD test, which revealed that the mean percentage value of T-lymphocytes in Group B ($M = 44.63$, $SD = 15.20$) was significantly higher than that of Group C ($M = 26.86$, $SD = 15.95$), $p = 0.005$ (Figure 4).

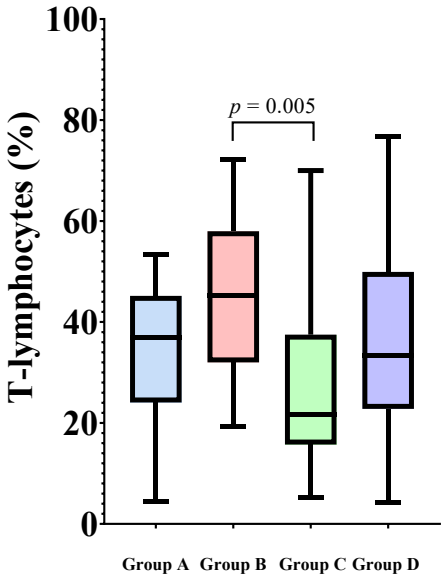

Figure 4. A comparative analysis of the percentage values of endometrial T-lymphocytes among groups based on the type of endometriosis in women with recurrent implantation failure ($n = 107$). The bars represent the median and interquartile range.

The analysis of variation (Kruskal-Wallis test) revealed discrepancies among the four groups and the endometrial cells that did not meet the normality test of distribution ($p < 0.05$). In the context of the study, the following findings were observed with respect to the leukocytes ($H(3) = 8.26$, $p = 0.041$), neutrophils ($H(3) = 10.03$, $p = 0.018$), uNK cells ($H(3) = 9.75$, $p = 0.021$), and CD34+ uNK cells ($H(3) = 8.01$, $p = 0.046$). The Mann-Whitney U test was used to analyze the differences between the groups. This analysis revealed that the percentage values for leukocytes in Group B were significantly higher than those in Group C. Furthermore, the percentage values for neutrophils in Group C were significantly higher than those in Group A. Finally, the percentage values for uNK cells and CD34+ uNK cells in Group A were significantly lower than those in Group C (Figure 5).

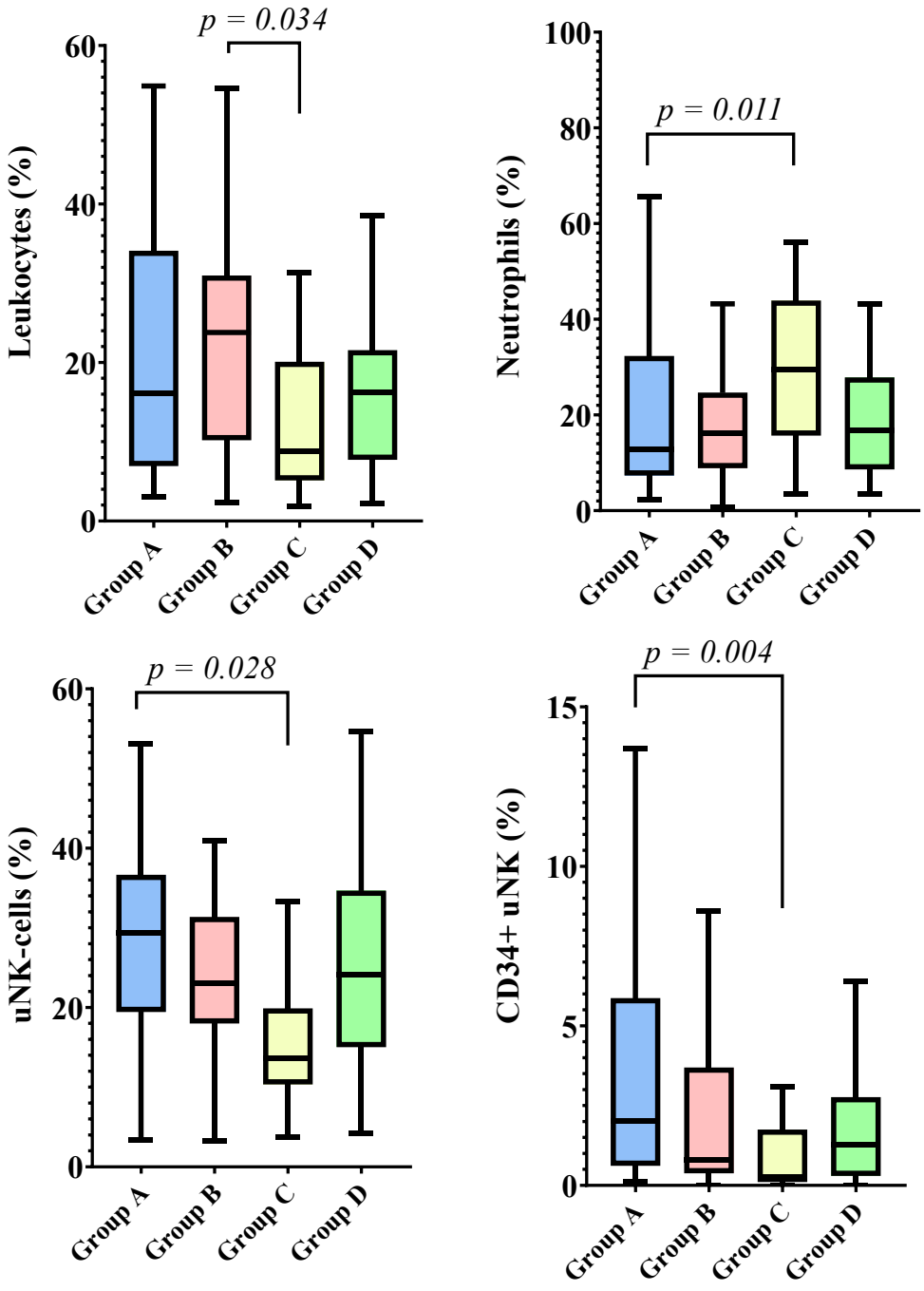

Figure 5. A comparative analysis of the percentage values of leukocytes, neutrophils, uNK cells, and CD34+ uNK cells among the four groups in women with RIF. The boxes represent the median and interquartile range.

5.3. Results of flow cytometric analysis of biopsies in the four groups categorized by the type of endometrial dysfunction in women with RPL

In women diagnosed with RPL, the applied tests did not identify a significant difference in endometrial cells among groups categorized based on the disruption of EM or the microbial profile.

5.4. Results of flow cytometric analysis of biopsies for the overall group of women, categorized by the profile of endometrial microorganisms

The statistical analysis of endometrial immune cells among the nine study groups is presented in Table 5 and Figure 6.

Table 5. A comparative analysis of endometrial immune cells across different groups based on microbial profiles (n = 200) was conducted. Results from the Mann-Whitney U test are presented, with only statistically significant findings being reported.

Endometrial immune cells	Groups	Median	IQR	*U*	*N*	*Z*	*P*
Leukocytes	**2**	10.89	30.19	12	18	-2.043	0.041
	3	38.08	30.61		4		
	3	38.08	30.61	8	4	-2.200	0.028
	4	19.94	18.74		15		
	3	38.08	30.61	1	4	-2.789	0.005
	5	14.08	10.57		12		
	3	38.08	30.61	16	4	-2.446	0.014
	6	16.50	19.43		33		
	3	38.08	30.61	15	4	-2.257	0.024
	7	15.41	20.35		26		
	3	38.08	30.61	21	4	-2.329	0.020
	9	17.48	20.98		37		
	5	14.08	10.57	158	12	-2.467	0.014
	8	24.04	19.42		49		
Lymphocytes	**3**	69.15	41.98	7	4	-2.062	0.039
	5	44.71	30.05		12		
	3	69.15	41.98	24	4	-2.054	0.040
	6	30.31	39.66		33		
	3	69.15	41.98	29	4	-1.977	0.048
	9	35.90	45.95		37		
	4	52.39	22.19	48	15	-2.049	0.040
	5	44.71	30.05		12		
	4	52.39	22.19	132	15	-2.569	0.010
	6	30.31	39.66		33		
	4	52.39	22.19	139	15	-2.797	0.041
	9	35.90	45.95		37		
	8	50.14	26.37	674	49	-2.028	0.028
	9	35.90	45.95		37		
T-lymphocytes	**1**	61.70	35.70	12	6	-2.248	0.005
	5	33.66	19.16		12		
	1	61.70	35.70	42	6	-2.219	0.014
	6	32.54	33.01		33		
	1	61.70	35.70	20	6	-2.800	0.024
	7	27.04	14.58		26		
	1	61.70	35.70	48	6	-2.208	0.020

	9	36.12	24.23		37		
	4	38.68	27.48	109	15	-2.328	0.014
	7	27.04	14.58		26		
	5	33.66	19.16	176	12	-2.141	0.039
	8	42.25	23.72		49		
	7	27.04	14.58	332.5	26	-3.390	0.040
	8	42.25	23.72		49		
	8	42.25	23.72	676.5	49	-2.006	0.048
	9	36.12	24.23		37		
uNK-cells	**2**	18.90	17.82	78	18	-2.061	0.040
	4	30.67	14.56		15		
	4	30.67	14.56	141	15	-2.369	0.010
	6	20.10	17.01		33		
	4	30.67	14.56	218	15	-2.369	0.041
	8	23.08	13.52		49		
Plasma cells*	**2**	0.25	0.42	12	18	-2.045	0.028
	3	3.99	9.04		4		
	3	3.99	9.04	25	4	-2.155	0.048
	9	0.13	0.74		37		
	3	3.99	9.04	20	4	-2.261	0.024
	6	0.11	0.30		33		
	3	3.99	9.04	8	4	-2.204	0.005
	4	0.06	0.18		15		
	3	3.99	9.04	13	4	-2.482	0.014
	7	0.00	0.31		26		
	3	3.99	9.04	28	4	-2.375	0.040
	8	0.13	0.51		49		
	7	0.00	0.31	329.5	26	-2.140	0.010
	9	0.13	0.74		37		
Plasma cells**	**3**	12.76	24.04	26	4	-1.966	0.014
	6	0.62	1.49		33		
	3	12.76	24.04	17	4	-2.227	0.020
	7	0.03	1.48		26		
	3	12.76	24.04	37	4	-2.069	0.039
	8	0.53	2.31		49		
	7	0.03	1.48	310.5	26	-2.409	0.040
	9	0.97	3.80		37		

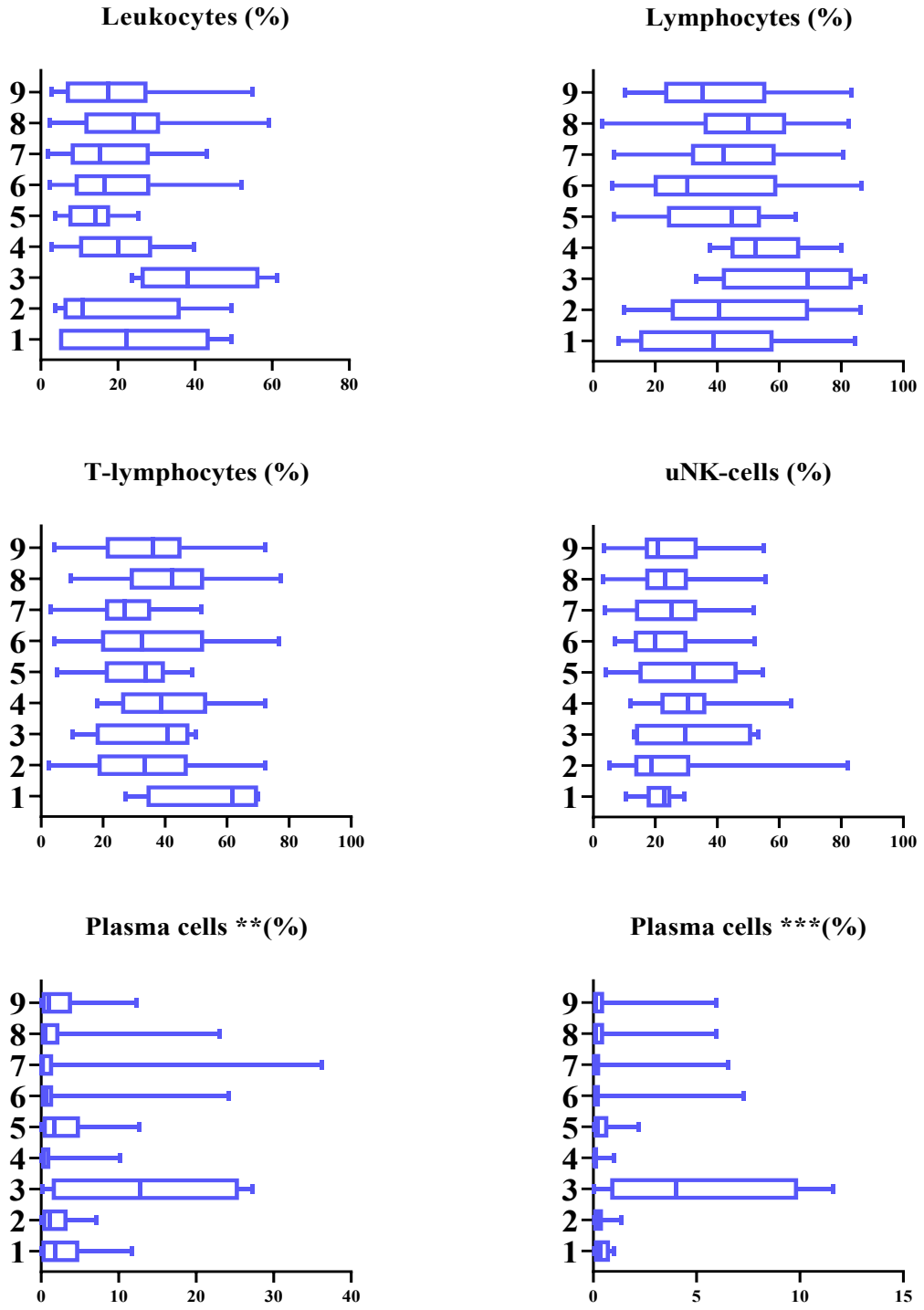

Figure 6. Comparative analysis of endometrial leukocytes, lymphocytes, T lymphocytes, uNK cells, and plasma cells (** shown as percentage relative to total leukocytes and *** shown as percentage relative to total cells), in all women (n = 200), grouped by microbial profile.

6. CORRELATIONS

The objective of the present study was to investigate the correlations between endometrial immune cells in women with RIF and RPL, with the aim of better understanding the role of the

local immune response in the endometrium in the development of reproductive disorders. Given that the sum of the percentages of lymphocytes, neutrophils, and macrophages is constrained to 100%, an increase in one leukocyte type invariably results in a decrease in another. Analogous relationships can be observed between lymphocytes, including T lymphocytes and uNK cells. Consequently, correlation analysis of the percentage values in this cell group was excluded from the present study.

6.1. Results of correlation analysis between endometrial cells in biopsies from women with RIF

The following section presents the findings of the correlation analysis conducted between the various endometrial immune cell types in women diagnosed with RIF (n = 107). The objective of this analysis was to ascertain the prevailing relationships between these cells and to enhance our comprehension of their function within the endometrial milieu. Pearson's correlation analysis was employed to assess the relationship between the variables of interest. The results are displayed in Table 6 and illustrated in Figure 7 – 9.

Table 6. Correlation analysis between endometrial immune cells in women with RIF (n = 107). Only statistically significant results are shown.

Endometrial immune cells, % ($\bar{x}$ ± SD)		***r***	***p***
Leucocytes (18.26 ± 12.883)	**Lymphocytes** (44.49 ± 21.04)	0.379	<0.001
	T-lymphocytes (36.33 ± 17.17)	0.274	0.004
	CD34+ uNK (2.37 ± 3.11)	-0.245	0.011
	Plasma cells* (0.64 ± 1.62)	0.305	0.001
Lymphocytes (44.49 ± 21.04)	**CD34+ uNK** (2.37 ± 3.11)	-0.222	0.022
	Plasma cells** (3.24 ± 6.46)	0.210	0.030
	Plasma cells* (0.64 ± 1.62)	0.317	0.001
Macrophages (32.6 ± 20.48)	**CD34+ uNK** (2.37 ± 3.11)	0.335	<0.001
	Plasma cells* (0.64 ± 1.62)	-0.236	0.014

Plasma cells* relative to all cells (leukocytes, stromal cells and epithelial cells)** and Plasma cells (CD45+, CD138+) represented as percentage relative to all leukocytes.

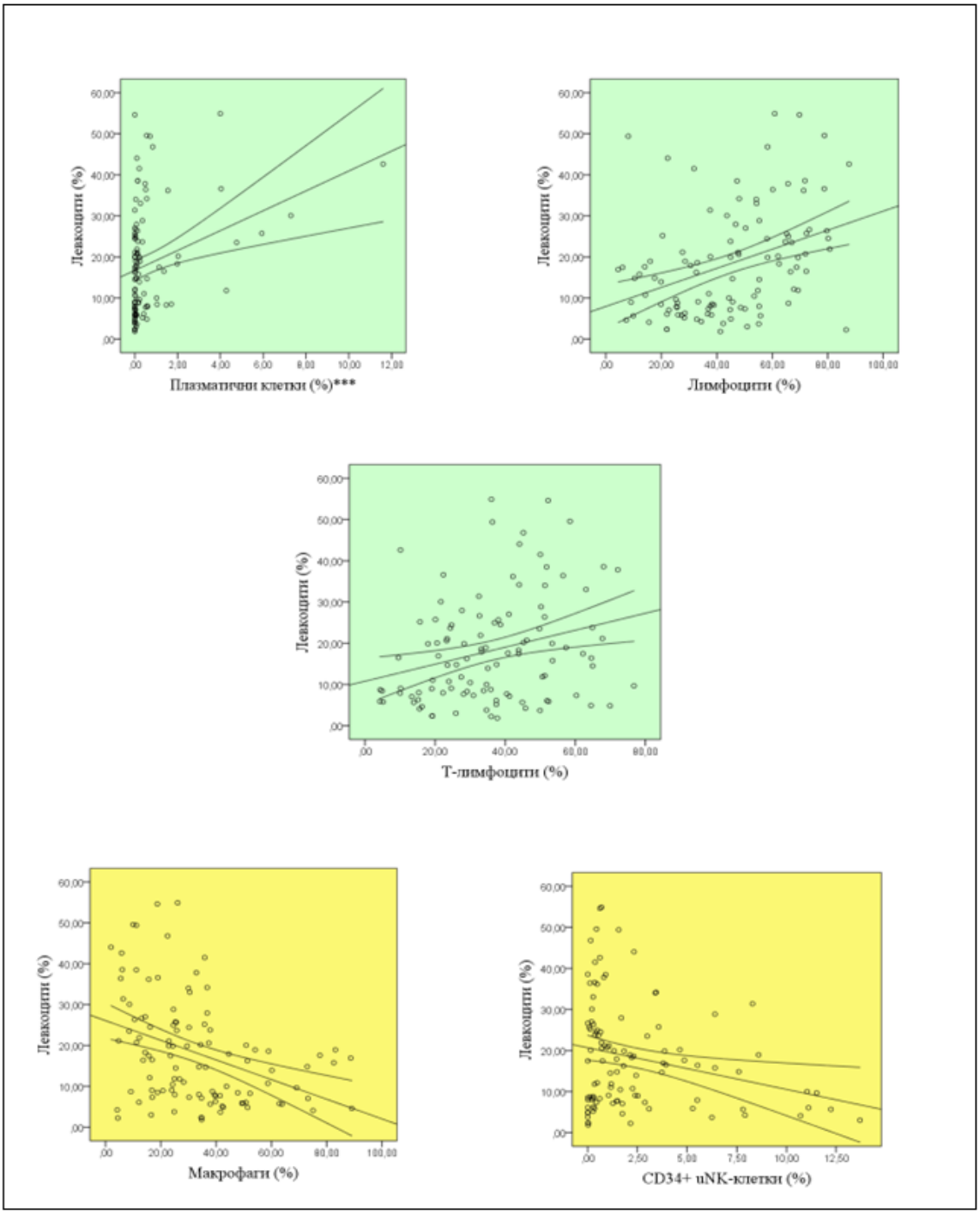

Figure 7. Correlations of endometrial leukocytes in women with RIF. Positive correlations are shown in green, negative correlations are shown in yellow.

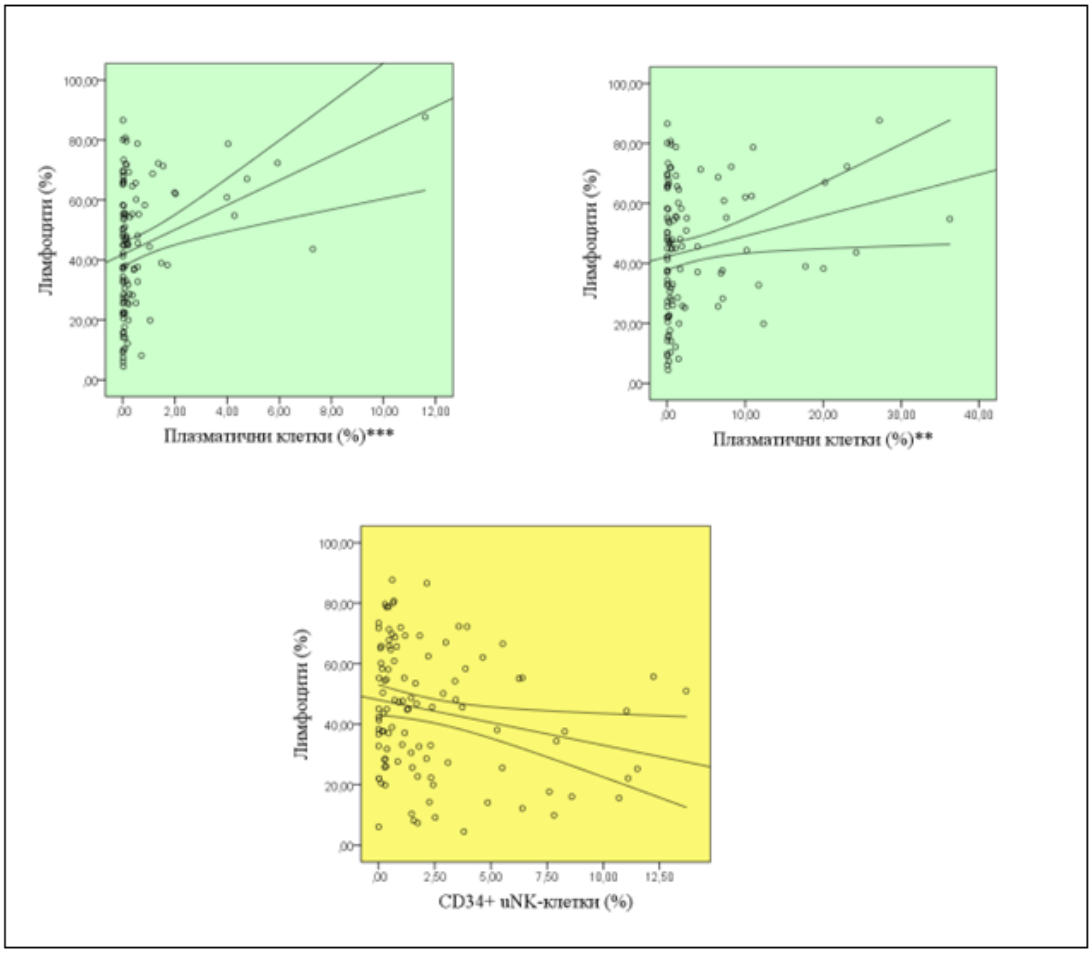

Figure 8. Correlations of endometrial lymphocytes in women with RIF. Positive correlations are shown in green, negative correlations are shown in yellow.

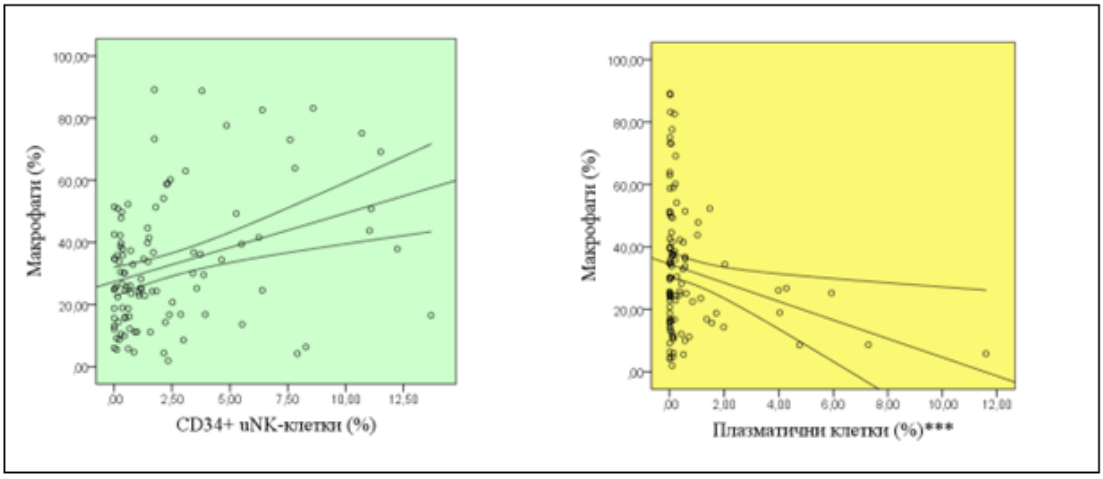

Figure 9. Endometrial macrophage correlations in women with RIF. Positive correlations are shown in green, negative correlations are shown in yellow.

The remaining correlation pairs did not show statistically significant relationships ($p > 0.05$).

6.2. Results of correlation analysis between endometrial cells in biopsies from women with RPL

The results of the correlation analysis between endometrial cells in biopsies from women with RPL are presented in Table 7 and illustrated in Figure 10 – 12.

Table 7. Correlation analysis between endometrial immune cells in women with RPL (n = 93). Only statistically significant results are shown.

Endometrial immune cells, % ($\bar{x}$ ± SD)		***r***	***p***
Leucocytes (23.56 ± 14.85)	Lymphocytes (44.11 ± 20.89)	0.405	<0.001
	Macrophages (30.73 ± 20.55)	-0.418	<0.001
	T- lymphocytes (36.03 ± 17.79)	0.235	0.023
	Plasma cells* (0.46 ± 0.89)	0.348	0.001
Lymphocytes (44.11 ± 20.89)	Macrophages (30.73 ± 20.55)	-0.513	<0.001
	Plasma cells*** (5.15 ± 7.06)	-0.282	0.006
	Plasma cells* (0.46 ± 0.89)	0.269	0.009
	uNK- cells (25.15 ± 14.02)	0.284	0.006
Neutrophils (23.97 ± 19.82)	uNK- cells (25.15 ± 14.02)	-0.207	0.047
CD34+ uNK (2.23 ± 2.91)	uNK- cells (25.15 ± 14.02)	-0.212	0.041

Plasma cells* represented as a percentage of all endometrial cells (leukocytes, stromal cells, epithelial cells); Plasma cells*** represented as a percentage of lymphocytes.

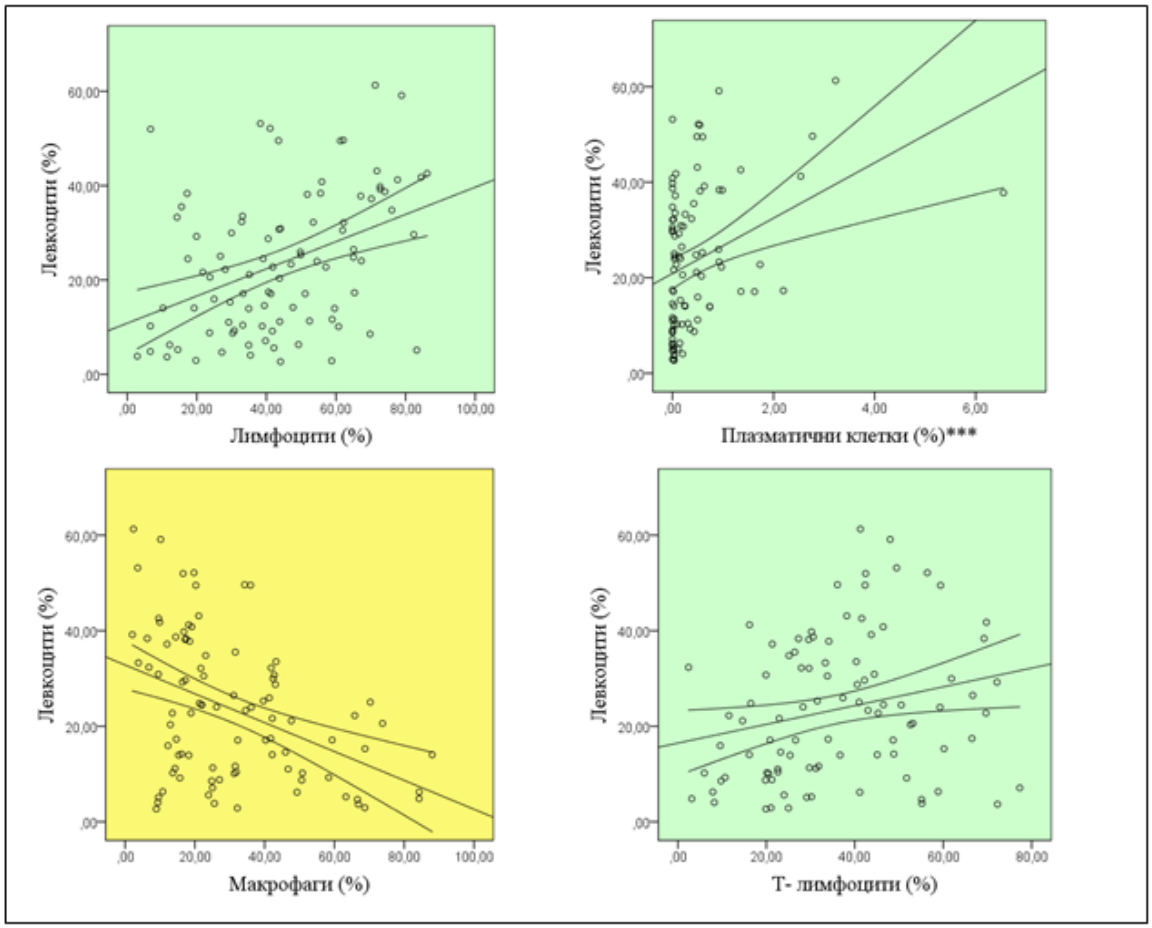

Figure 10. Correlations of endometrial leukocytes in women with RPL (n = 93). Positive correlations are shown in green, negative correlations are shown in yellow.

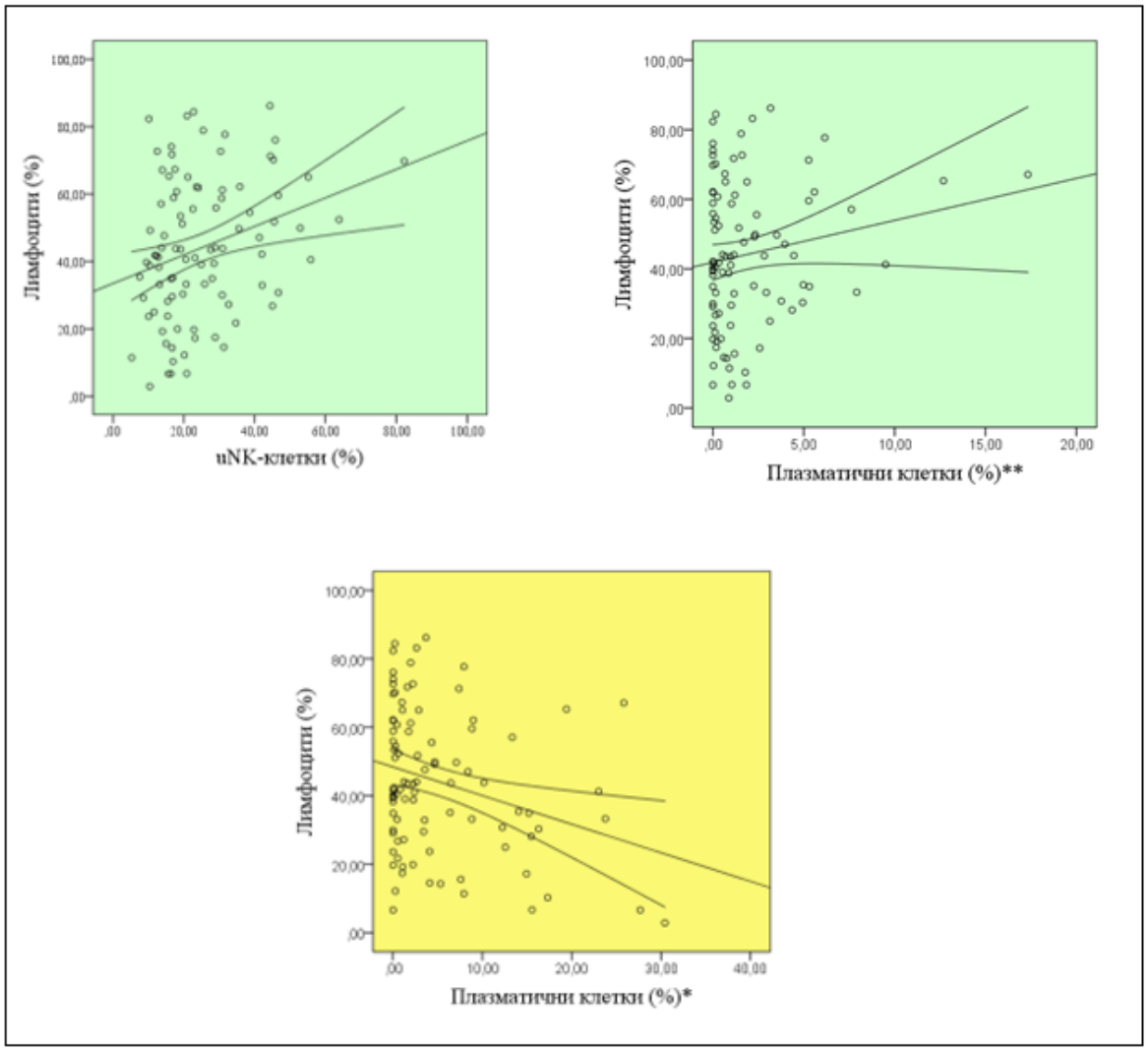

Figure 11. Correlations of endometrial lymphocytes in women with RPL (n = 93). Positive correlations are shown in green, negative correlations are shown in yellow.

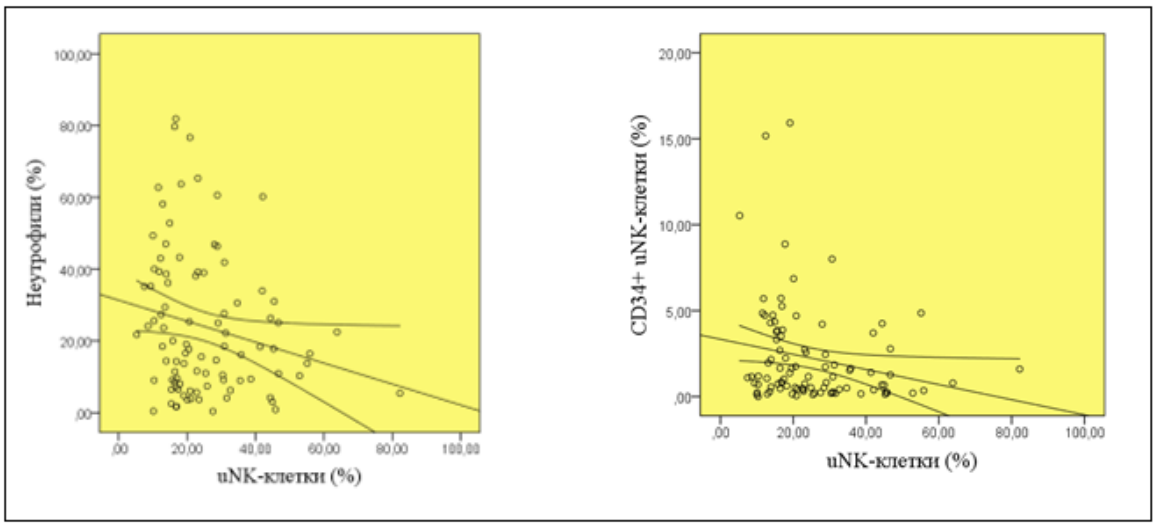

Figure 12. Correlations of endometrial immune cells in women with RPL (n = 93).

The remaining pairs do not show statistically significant correlations ($p > 0.05$).

6.3. Correlations between endometrial cells in the four groups by EM in the studied women

Pearson correlation analysis was utilized to ascertain statistically significant relationships between endometrial cell levels in disparate groups categorized by EM, as illustrated in Table 8.

Table 8. Correlation analysis between endometrial immune cells in the four groups by EM ($n = 200$). Only statistically significant results are shown.

Groups	Endometrial immune cells, % ($\bar{x} \pm SD$)		*r*	*p*
Group A ($n = 41$)	Leucocytes (18.58 ± 13.15)	Plasma cells* (0.53 ± 0.94)	0.499	0.001
	Lymphocytes (41.31 ± 19.75)	Plasma cells* (0.53 ± 0.94)	0.420	0.006
	T-lymphocytes (34.47 ± 16.44)	Plasma cells*** (6.47 ± 10.71)	-0.337	0.031
	Plasma cells* (6.47 ± 10.71)	uNK-cells (25.68 ± 12.35)	-0.352	0.024
Group B ($n = 57$)	Leucocytes (23.99 ± 14.70)	Lymphocytes (48.41 ± 18.61)	0.478	<0.001
	Leucocytes (23.99 ± 14.70)	Macrophages (29.01 ± 16.86)	-0.355	0.007
	Leucocytes (23.99 ± 14.70)	Plasma cells*** (5.74 ± 11.19)	-0.311	0.019
	T-lymphocytes (42.49 ± 16.11)	Plasma cells** (2.34 ± 4.84)	-0.273	0.040
	Macrophages (29.01 ± 16.86)	CD34+ uNK (2.43 ± 3.34)	0.308	0.020
Group C ($n = 25$)	Leucocytes (16.53 ± 13.61)	Lymphocytes (35.60 ± 17.67)	0.424	0.035
	Leucocytes (16.53 ± 13.61)	Macrophages (33.63 ± 22.83)	-0.598	0.002
	Lymphocytes (35.60 ± 17.67)	T-lymphocytes (29.48 ± 17.75)	0.493	0.012
Group D ($n = 77$)	Leucocytes (20.80 ± 13.85)	Lymphocytes (45.71 ± 23.28)	0.326	0.004
	Leucocytes (20.80 ± 13.85)	Macrophages (30.47 ± 20.11)	-0.472	<0.001
	Leucocytes (20.80 ± 13.85)	Plasma cells* (0.55 ± 1.49)	0.336	0.003
	Lymphocytes (45.71 ± 23.28)	CD34+ uNK (2.43 ± 3.34)	-0.369	0.001
	Lymphocytes (45.71 ± 23.28)	Plasma cells** (2.19 ± 4.37)	0.285	0.012

	Lymphocytes (45.71 ± 23.28)	Plasma cells* (0.55 ± 1.49)	0.317	0.005
	Lymphocytes (45.71 ± 23.28)	uNK-клетки (27.49 ± 14.87)	0.287	0.011
	Macrophages (30.47 ± 20.11)	CD34+ uNK (2.43 ± 3.34)	0.464	<0.001
	Macrophages (30.47 ± 20.11)	Plasma cells** (2.19 ± 4.37)	-0.243	0.033
	Macrophages (30.47 ± 20.11)	Plasma cells* (0.55 ± 1.49)	-0.279	0.014

Plasma cells* as a percentage of total cells (leukocytes, stromal cells and epithelial cells); Plasma cells** as a percentage of total leukocytes; Plasma cells*** as a percentage of total lymphocytes.

The remaining pairs do not show statistically significant relationships ($p > 0.05$).

6.4. Correlations between endometrial cells in the groups according to the microbial profile of the studied women

Pearson analysis applied to the nine groups according to microbial profile showed the following relationships between endometrial cells (Table 9).

Table 9. Correlations between metrics in the 9 EM groups ($n = 200$). Only statistically significant results are shown.

Groups	**Endometrial immune cells, % ($\bar{x}$ ± SD)**		***r***	***p***
Group 1 (n = 6)	Neutrophils (36.61 ± 30.86)	T-lymphocytes (54.47 ± 18.23)	-0.926	0.008
	Neutrophils (36.61 ± 30.86)	CD34+ uNK (0.99 ± 1.07)	0.838	0.037
	T-lymphocytes (54.47 ± 18.23)	CD34+ uNK (0.99 ± 1.07)	-0.934	0.006
Group 2 (n = 18)	Leucocytes (19.00 ± 15.70)	Plasma cells* (0.47 ± 0.63)	0.549	0.018
	Lymphocytes (44.67 ± 23.67)	uNK- cells (24.76 ± 18.50)	0.486	0.041
	Lymphocytes (44.67 ± 23.67)	CD34+ uNK (2.24 ± 3.03)	-0.499	0.035
	Macrophages (30.40 ± 18.92)	CD34+ uNK (2.24 ± 3.03)	0.747	0.000
Group 3 (n = 4)	Neutrophils (20.20 ± 9.21)	T-lymphocytes (35.40 ± 17.36)	0.957	0.043
Group 4 (n = 15)	Leucocytes (20.08 ± 12.34)	CD34+ uNK (4.21 ± 4.84)	-0.755	0.001
	Plasma cells * (2.67 ± 5.75)	CD34+ uNK (4.21 ± 4.84)	0.518	0.048
	Plasma cells** (1.30 ± 2.55)	CD34+ uNK (4.21 ± 4.84)	0.537	0.039

Group 5 (n = 12)	Leucocytes (13.70 ± 6.90)	T-lymphocytes (30.32 ± 12.74)	0.582	0.047
	Lymphocytes (39.04 ± 18.56)	Plasma cells** (3.04 ± 3.85)	0.709	0.010
	Lymphocytes (39.04 ± 18.56)	Plasma cells* (0.47 ± 0.63)	0.694	0.012
	Macrophages (36.89 ± 23.25)	Plasma cells*** (7.67 ± 8.65)	-0.594	0.042
	Neutrophils (24.06 ± 20.08)	Plasma cells*** (7.67 ± 8.65)	0.661	0.019
Group 6 (n = 33)	Leucocytes (19.11 ± 11.84)	Macrophages (33.23 ± 24.36)	-0.579	0.000
	Leucocytes (19.11 ± 11.84)	Neutrophils (28.20 ± 23.89)	0.421	0.015
	Leucocytes (19.11 ± 11.84)	T-lymphocytes (34.64 ± 20.30)	0.415	0.016
Group 7 (n = 26)	Leucocytes (18.73 ± 13.38)	Lymphocytes (45.54 ± 19.48)	0.72	0.000
	Leucocytes (18.73 ± 13.38)	Macrophages (31.28 ± 16.67)	-0.472	0.015
Group 8 (n = 49)	Leucocytes (24.47 ± 15.00)	Lymphocytes (47.79 ± 19.63)	0.53	0.000
	Leucocytes (24.47 ± 15.00)	Macrophages (29.24 ± 17.56)	-0.372	0.009
	Leucocytes (24.47 ± 15.00)	Plasma cells* (6.09 ± 11.68)	-0.318	0.026
	Macrophages (29.24 ± 17.56)	CD34+ uNK (2.16 ± 3.18)	0.341	0.017
Group 9 (n = 37)	Leucocytes (19.28 ± 13.43)	Plasma cells*** (0.57 ± 0.98)	0.493	0.002
	Lymphocytes (39.80 ± 20.02)	Plasma cells** (2.58 ± 3.52)	0.348	0.035
	Lymphocytes (39.80 ± 20.02)	Plasma cells*** (0.57 ± 0.98)	0.474	0.003
	T-lymphocytes (34.35 ± 16.67)	Plasma cells* (6.89 ± 11.20)	-0.348	0.035
	uNK-cells (24.96 ± 12.63)	Plasma cells* (6.89 ± 11.20)	-0.346	0.036

Plasma cells* (CD45+, CD138+) as a percentage of total lymphocytes; Plasma cells** as a percentage of total leukocytes; Plasma cells*** as a percentage of total cells (leukocytes, stromal cells and epithelial cells).

The remaining pairs do not show statistically significant relationships ($p > 0.05$).

6.5. Correlations between endometrial cells in biopsies from group 1 to group 9 in all studied women

Correlation analysis was performed between endometrial immune cells in biopsies with more than 90% lactobacilli and without bacterial and mycotic agents (group 9) and all other biopsies. The data are presented in Table 10.

Table 10. Correlations between endometrial cells in biopsies with lactobacilli above and below 90% (*n* = 200). Only statistically significant results are shown.

Groups	Endometrial immune cells, % ($\bar{x} \pm SD$)		*r*	*p*
Group 9 (*n* = 37)	Leucocytes (19.28 ± 13.43)	Plasma cells* (0.57 ± 0.98)	0.493	0.002
	Lymphocytes (39.80 ± 20.02)	Plasma cells** (2.58 ± 3.52)	0.348	0.035
	Lymphocytes (39.80 ± 20.02)	Plasma cells* (0.57 ± 0.98)	0.474	0.003
	T-lymphocytes (34.35 ± 16.67)	Plasma cells* (6.89 ± 11.20)	-0.348	0.035
Groups 1-8 (*n* = 163)	Leucocytes (21.04 ± 14.21)	Lymphocytes (45.33 ± 21.04)	0.394	0.000
	Leucocytes (21.04 ± 14.21)	Macrophages (30.32 ± 19.23)	-0.448	0.000
	Leucocytes (21.04 ± 14.21)	T-lymphocytes (36.61 ± 17.61)	0.234	0.003
	Leucocytes (21.04 ± 14.21)	CD34+ uNK (2.23 ± 2.95)	-0.197	0.012
	Leucocytes (21.04 ± 14.21)	Plasma cells* (0.55 ± 1.40)	0.254	0.001
	Lymphocytes (45.33 ± 21.04)	T-lymphocytes (36.61 ± 17.61)	0.166	0.034
	Lymphocytes (45.33 ± 21.04)	uNK-cells (25.00 ± 13.45)	0.265	0.001
	Lymphocytes (45.33 ± 21.04)	CD34+ uNK (2.23 ± 2.95)	-0.204	0.009
	Lymphocytes (45.33 ± 21.04)	Plasma cells** (2.60 ± 5.45)	0.165	0.035
	Lymphocytes (45.33 ± 21.04)	Plasma cells* (0.55 ± 1.40)	0.268	0.001
	Macrophages (30.32 ± 19.23)	T-lymphocytes (36.61 ± 17.61)	-0.185	0.018
	Macrophages (30.32 ± 19.23)	CD34+ uNK (2.23 ± 2.95)	0.261	0.001
	Macrophages (30.32 ± 19.23)	Plasma cells* (0.55 ± 1.40)	-0.219	0.005
	Neutrophils (23.57 ± 18.66)	uNK-cells (25.00 ± 13.45)	-0.192	0.014

7. COMPARATIVE ANALYSIS OF ENDOMETRIAL IMMUNE CELLS BEFORE AND AFTER ANTIBIOTIC AND PROBIOTIC THERAPY

A subsequent flow cytometric study of endometrial immune cells before and at least one month after antibiotic and probiotic therapy of 46 followed women showed that there was a statistically significant difference in uNK cells before and after treatment. The Wilcoxon signed-rank test was utilized, yielding a value of $Z = -2.059$ and $p = 0.039$. To facilitate a comparative analysis of uNK cells before and after treatment, the differences in their percentage value for each woman systematically ranked and scored. The mean rank for each group was subsequently calculated to estimate the average degree of change. The mean rank of uNK cells following treatment (Mdn = 26.04) exceeded the mean rank prior to treatment (Mdn = 19.56). This finding suggests that the treatment resulted in a significant increase in uNK cells in the patients.

Furthermore, mean levels of leukocytes, lymphocytes, macrophages, T-lymphocytes, and progenitor natural killer (NK) cells were elevated after therapy, though this did not reach statistical significance. Subsequent to the therapeutic intervention, there was a reduction in the mean neutrophil and plasma cell counts, though this observation did not attain statistical significance (Figure 13).

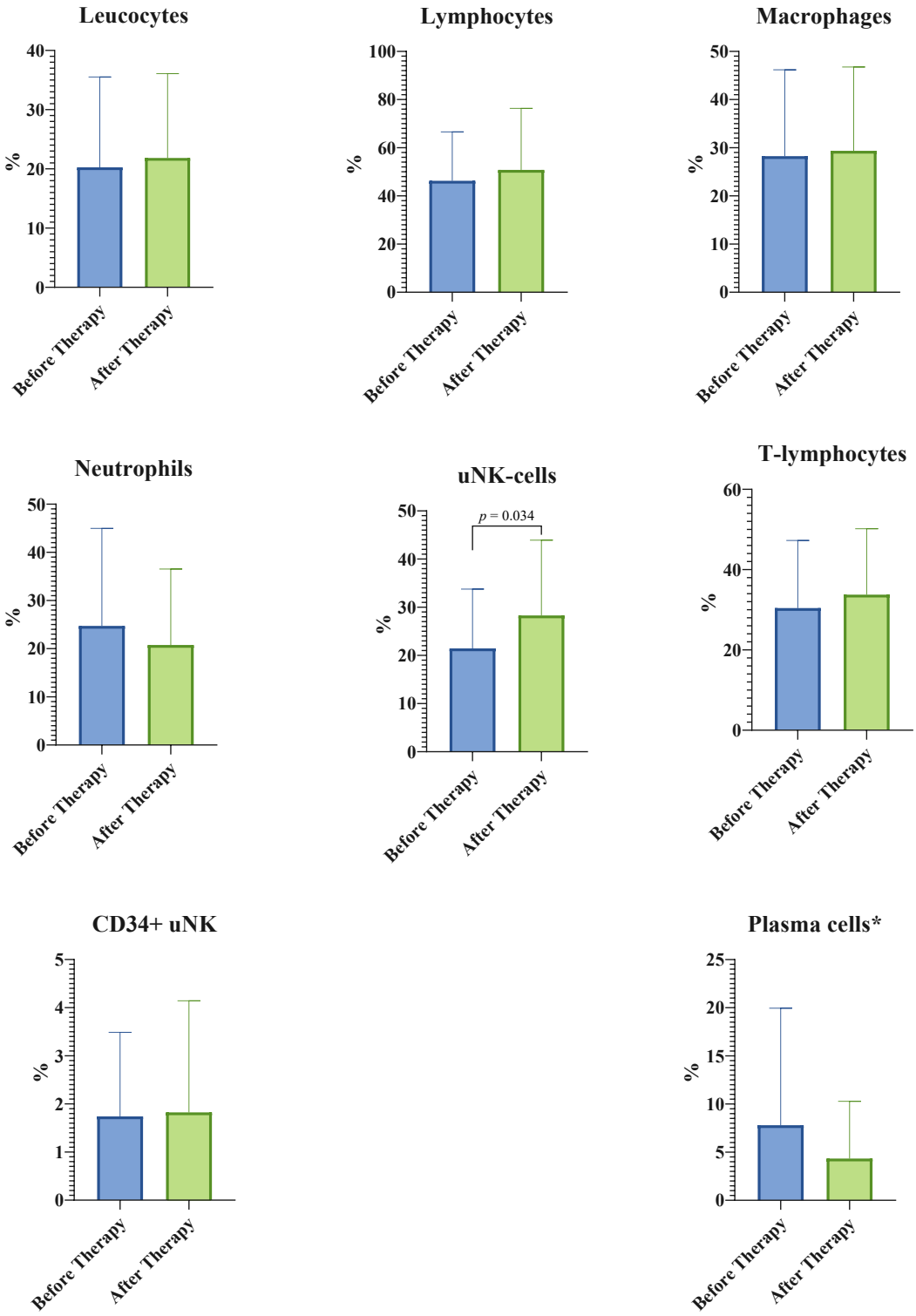

Figure 13. Results of flow cytometric analysis of endometrial immune cells before and after therapy in women with reproductive failure ($n = 46$). Plasma cells* (CD45+, CD138+) presented as percentage of total lymphocytes.

8. FERTILITY AFTER THE FIRST ART PROCEDURE OF THE ANTIBIOTIC AND PROBIOTIC THERAPY

Following the completion of antibiotic and probiotic therapy for endometrial dysbiosis, patients were observed for a period of time to ascertain the effect of endometrial microbiota disruption on reproductive outcome. The data concerning fertility subsequent to the initial ART procedure (ICSI) are exhibited in Table 11 andTable ***12***, which present a comparison of diverse patient cohorts according to the presence of endometrial microbiota disorder. The following tables present the significance of normal endometrial microbiota (EM) on pregnancy outcome.

Table 11. Comparison of the results of the first ART (ICSI) procedure after antibiotic and probiotic therapy between the group with normal EM (Group A) and the other groups with endometrial microbiota disorder. The fertility data are valid until 2024.

Patients	**Negative hCG test** ***n*** (%)	**Spontaneous abortion** ***n*** (%)	**Ongoing pregnancy** ***n*** (%)	**Births** ***n*** (%)
Group A ***n* = 41**	**18** (43.9)	**1** (2.44)	**3** (7.32)	**19** (46.34)
Groups B - D ***n* = 159**	**83** (52.2)	**18** (11.3)	**21** (13.2)	**37** (23.3)
Total ***n* = 200**	**101** (50.5)	**19** (9.5)	**24** (12.0)	**56** (28.0)

Women with an endometrial biopsy showing normal EM had statistically significantly higher fertility rates compared to other women ($\chi 2(2, n = 200) = 10.053, p = 0.007$).

Table 12. Comparison of the results of the first ART (ICSI) procedure after antibiotic and probiotic therapy between different groups according to the type of endometrial microbiota disorder. Birth rate data is updated to the end of 2024.

Patients	**Negative hCG test** ***n*** (%)	**Spontaneous abortion** ***n*** (%)	**Ongoing pregnancy** ***n*** (%)	**Births** ***n*** (%)
Group A ***n* = 41**	**18** (43.9)	**1** (2.44)	**3** (7.32)	**19** (46.34)
Group B ***n* = 57**	**29** (50.9)	**8** (14.0)	**8** (14.0)	**12** (21.05)
Group C ***n* = 25**	**13** (52.0)	**3** (12.0)	**2** (8.0)	**7** (28.0)
Group D ***n* = 77**	**41** (53.2)	**7** (9.1)	**11** (14.4)	**18** (23.37)
Total ***n* = 200**	**101** (50.5)	**19** (9.5)	**24** (12.0)	**56** (28.0)

9. ALGORITHM FOR DIAGNOSTIC AND THERAPEUTIC MANAGEMENT OF WOMEN PLANNING ART

9.1. After passing an eligibility assessment, a vaginal and cervical examination follows:

9.1.1. Microbiological examination of vaginal discharge;

9.1.2. Performing RT-PCR (Femoflor screen) of cervical swab.

9.2. If positive for dysbiotic microorganisms:

9.2.1. Therapy of dysbiosis with antibiotics and probiotics;

9.2.2. Follow-up examination of vaginal and cervical swab after completion of therapy.

9.3. After a negative test for dysbiosis, a mid-luteal biopsy of the uterine lining is performed.

9.4. Study of the endometrial microbiota to identify the species diversity and quantity of microorganisms.

9.4.1. Assessment of the local immune profile;

9.4.2. Therapy of dysbiosis with antibiotics and probiotics;

9.4.3. Antibiotic and probiotic therapy for chronic endometritis (when plasma cells are more than 1% of all cells).

9.5. Discussion of additional tests for the diagnosis of chronic endometritis (DNA analysis for EBV, CMV, HSV-1,2).

9.6. Discuss appropriate immunomodulatory therapy if an imbalance between endometrial immune cell populations is identified (corticosteroids, IVIg, Intralipid).

9.7. Proceeding to the ART procedure.

V. Discussion

1. Age distribution of the studied women in the different groups

The notion of age as a factor impacting the reproductive potential of women is a salient one in the study of human biology. As women undergo the process of aging, there is an observed decline in the quantity and quality of their oocytes, which can lead to challenges in conceiving (Kawamura, Tomari et al. 2020, Lucas, Vrljicak et al. 2020). A mounting body of research has indicated that after the age of 35, there is a change in the expression of genes related to endometrial lining function, which can potentially affect embryo implantation (Devesa-Peiro, Sebastian-Leon et al. 2022). Consequently, it is imperative to consider the age distribution of different groups in such studies. The present study found no significant difference was found between age and the type of endometrial microbiota disorder or microorganism profile. The absence of a statistically significant correlation between age groups precludes the possibility that age might influence the results obtained. This finding serves to substantiate the validity of the conclusions and analyses, thereby enabling the focus to be shifted to other potential factors or mechanisms that may be associated with reproductive failures in the groups under study.

2. Discussion of the results of the distribution of biopsies by groups of endometrial microbiota. Relationship between microbiota and reproductive health

The objective of this study was to investigate the endometrial microbiota in Bulgarian patients with RIF and RPL and to assess its influence on endometrial immune cells.

In contrast to the vagina, the endometrium has received comparatively less research attention as an environment conducive to the growth of commensal bacteria. Historically, the uterus was considered sterile, and the presence of bacteria in samples from the endometrium, placenta, or amniotic fluid was regarded as pathological (Harris and Brown 1927). The challenge of obtaining adequate endometrial samples further restricts the scope of research in this domain. The advent of novel microbiological methodologies, which differ from conventional techniques based on cultivation, has resulted in the accumulation of mounting evidence that substantiates the heterogeneity and variability of bacterial communities within the endometrium. The implications of these communities on reproductive health are a subject of active research and ongoing debate (Koedooder, Mackens et al. 2018). The uterine microenvironment is distinguished by intricate and dynamic interactions among the microbiota, the immune system, and the endometrium. A disruption in any of these components can initiate a sequence of events that disrupts this delicate balance, leading to a number of pathological changes (Zhu, Yang et al. 2022). The present study reflects the current status of this microenvironment during the mid-luteal phase, which represents the specific area from which the endometrial biopsy was obtained.

A molecular genetic analysis of 77 biopsies revealed a significantly disturbed microbiota, characterized by a predominance of dysbiotic bacteria and a prevalence of less than 90% Lactobacillus spp. The present findings provide substantiation for the correlation between alterations in the uterine cavity's bacterial composition and reproductive complications, including RPL and RIF (see Kitaya, Nagai et al. 2019, Hernandes, Silveira et al. 2020, Diaz-Martínez, Bernabeu et al. 2021, Lozano, Bernabeu et al. 2021, Ravel, Moreno et al. 2021).

In the present study, the lowest mean uNK cell counts were observed in the third EM group, where the highest mean plasma cell percentages were identified. These results are consistent with those of a previous study involving 23 women with unexplained infertility, in which uterine mucosa was sampled during the follicular and late secretory phases. The analysis of immune cells in the samples was conducted through the utilization of a flow cytometry technique. The women were divided into two groups: a cohort of nine women diagnosed with chronic endometritis and a cohort of fourteen women without chronic endometritis. The results demonstrated that women afflicted with chronic endometritis exhibited a reduced proportion of specific immune cell types, including CD56+ CD16- and CD56bright CD16- cells, concurrent with an elevated percentage of CD3+ cells within the endometrium when compared to women not afflicted with chronic endometritis. This finding stands in contrast to the results reported by Matteo et al. (2009).

The results of the present study demonstrate that an increase in total leukocyte count is observed in cases of endometrial inflammation (Disep, Innes et al. 2004). This outcome is consistent with the known pathophysiology of inflammation, which involves the migration of immune cells to the affected tissue. It is noteworthy that while the total number of leukocytes increases in all patients with endometritis, the composition of these cells can vary considerably. In the preceding study, endometrial tissue was examined from 79 women diagnosed with endometritis and 22 women who served as controls and had normal histological results. Leukocytes were characterized using immunohistochemistry for CD45, CD20, CD68, CD3, and CD56, and their numbers were analyzed semiquantitatively on a scale of 0 to 4. In a multitude of instances pertaining to endometritis, an elevated total count of leukocytes has been observed. While the absolute numbers of macrophages, T lymphocytes, and uNK cells remain unchanged between endometritis samples and control groups, the majority of endometritis cases exhibit a substantial increase in B lymphocytes, which typically constitute 1% or less of the endometrial leukocyte population. The present study identified substantial disparities in the percentages of lymphocytes, uNK cells, and T lymphocytes among the various groups, whereas the previous study found no significant differences in the numbers of macrophages, T lymphocytes, and endometrial NK cells.

This finding indicates that the immune response in cases of dysbiosis and endometritis can exhibit significant heterogeneity, contingent on various factors, including the stage of the disorder, the causative agent of inflammation, and individual patient characteristics. Furthermore, the present study observed an increase in neutrophils in specific groups, while the previous study primarily focused on B lymphocytes. This underscores the necessity of conducting a comprehensive investigation into diverse immune cell types to obtain a

comprehensive understanding of the immune response in endometritis (Disep, Innes et al. 2004).

The present study corroborates the hypothesis that endometrial inflammation induces significant alterations in the immune response. However, a thorough examination of the available evidence reveals a more complex scenario than initially anticipated. In contrast to prior investigations that primarily focused on the augmentation of B-lymphocytes, the current study identifies a broader spectrum of immune cells implicated in the pathogenesis of endometritis. The observed disparities in the immune profile may bear substantial clinical ramifications, such as influencing treatment responsiveness and disease prognosis.

When analogous results are compared, the divergence in the methodology for assessing immune cells must be duly noted. Although immunohistochemical analysis is a valuable instrument, it has the potential to impede the precise quantification of antigen expression levels.

The immune profile analysis of patients with RIF and RPL (as presented in **Error! Reference s ource not found.** of this study) does not fully align with the findings of a more extensive investigation encompassing 455 patients. Although the preceding study indicated heightened levels of natural killer (NK) cells and B-lymphocytes in patients with renal interstitial fibrosis (RIF), the present study does not corroborate this finding (Marron and Harrity 2019). The potential causative factors may be attributed to variations in methodology, sample size, and inclusion criteria.

Conversely, congruities have been identified within a subset of the obtained results. For instance, the examination of endometrial immune cells following antibiotic therapy has demonstrated statistically significant elevations in uNK cells and reductions in plasma cells. This finding aligns with the observed higher uNK cell counts and diminished B-lymphocyte levels in control biopsies (Marron and Harrity 2019). The reduced percentage of plasma cells post-therapy, though not statistically significant, corroborates the trend towards normalization of the endometrial immune profile, which may be indicative of treatment efficacy.

The endometrial microbiota has the potential to influence reproductive outcomes through its interactions with the vaginal microbiota and systemic immune responses. Dysbiosis of the vaginal microbiota has been associated with adverse pregnancy outcomes. Evidence suggests the presence of cross-talk between the vaginal and endometrial microbiota, which can impact the local and systemic immune milieu within the reproductive tract (Skafte-Holm, Humaidan et al., 2021). The findings of this study affirm the pivotal role of a normal endometrial microbiota, characterized by the predominance of >90% *Lactobacillus* spp., in achieving optimal implantation and embryonic development (Espinos, Fabregues et al. 2021). A subset of *Lactobacillus* species, classified as probiotics, have been shown to possess the capacity to impede the proliferation of other bacterial species. This phenomenon is attributed to their capacity to generate H_2O_2. Hydrogen peroxide is toxic to microorganisms that lack the capacity for its degradation (e.g., catalase deficiency). The hypothesis posits that the absence of H_2O_2-

producing lactobacilli may precipitate the proliferation of catalase-negative bacteria, which are refractory to this toxin (Bhattacharya, Dutta et al. 2023).

3. DISCUSSION OF THE RESULTS FROM FLOW CYTOMETRIC ANALYSIS OF IMMUNE CELLS IN THE ENDOMETRIUM OF WOMEN WITH REPRODUCTIVE DISORDERS IN GROUPS ACCORDING TO EM DISORDER

The mean percentage value of leukocytes, as observed in the biopsies, corresponds to that reported in previous studies, thereby validating the sampling and processing procedures. During the mid-luteal phase, the proportion of leukocytes within the stromal cell population can reach up to 30% (Salamonsen and Woolley 1999).

In the present study, a comparative analysis of the immune profile of endometrial biopsies obtained during the mid-luteal phase of the menstrual cycle from women with varying degrees of EM is conducted. The endometrium is distinguished by a diverse population of innate immune cells, predominantly macrophages and neutrophils, in addition to adaptive immune cells, including T- and B-lymphocytes (Kitazawa, Kimura et al. 2020). These cells have been found in significant numbers within the lymphoid aggregates of the basal layer, as well as between the stromal and epithelial cells (Vallvé-Juanico, Houshdaran et al. 2019). During the proliferative and secretory phases of the menstrual cycle, endometrial immune cells undergo a process of gradual maturation. This maturation is critical for maintaining the physiological immune microenvironment of the uterus and for participating in the processes of endometrial remodeling, decidualization, and embryo implantation (Agostinis, Mangogna et al. 2019, Kitazawa, Kimura et al. 2020). The functions of immune cells are diverse. The regulation of their type, number, and activation status is primarily influenced by the hormonal milieu (Vallvé-Juanico, Houshdaran et al., 2019; Kitazawa, Kimura et al., 2020). In the initial phases of pregnancy, these cells can account for up to 40% of the total cell count in the uterus. T-lymphocytes, specifically Tregs, play a pivotal role in the establishment of maternal-fetal immune tolerance. The most prevalent decidual immune cells are uNK cells. These cells comprise 70% of the total number of local immune cells. In essence, natural killer (NK) cells function by regulating trophoblast invasion and enhancing vascular remodeling through the actions of extravillous trophoblasts, macrophages, and dendritic cells. Concurrently, these cells maintain their capacity to protect against infections (Zhu, Yang et al. 2022).

In a previous study of the endometrial immune profile in patients with RPL and RIF who achieved live birth (RPL, $n = 24$; RIF, $n = 14$), significantly higher mean percentage values of uNK cells are found in women with RIF compared to those with RPL ($p = 0.008$) (Braun, Vomstein et al. 2023). The present study did not identify any significant disparities in the expression levels of uNK cells or any of the other cell types that were examined.

A subsequent analysis of endometrial cells between the four groups according to endometrial microbiota disorder reveals some statistically significant differences. To date, analogous comparisons are lacking. As indicated by extant literature, the microbiota has been demonstrated to exert influence on the immune system, modulating the local immune response

(D'Ippolito, Di Nicuolo et al. 2018). The alterations in the immune microenvironment have been demonstrated to influence the processes of endometrial remodeling, decidualization, and embryo implantation, thereby elucidating the reproductive disorders observed in the women from the studied groups.

Group A exhibited the highest mean percentage values of CD34+ uNK cells, with a statistically significant difference being established between Group A and Group C. In the scientific literature, progenitor uNK cells are considered in relation to the conflicting data on the origin of uNK cells in the decidua. It has been demonstrated that the decidual tissue contains CD34+ precursors that have the capacity to differentiate in vitro into mature natural killer (NK) cells. These differentiated cells exhibit a phenotype and function that is analogous to that of dNK cells (Vacca, Vitale et al. 2011). This finding indicates that these CD34+ cells are directly committed to the NK cell lineage. Furthermore, the decidual microenvironment attracts NK cells from the peripheral blood during pregnancy (Carlino, Stabile et al. 2008; Santoni, Carlino et al. 2008), where they acquire the characteristics of decidual NK cells under the influence of local factors (Vacca, Chiossone et al. 2019). The origin of uNK cells is likely heterogeneous, with two possible sources: CD34+ precursors in the decidua and NK cells from peripheral blood that adapt to the local environment. The elevated levels of CD34+ uNK cells observed in group A imply a more pronounced activation of uNK cells from CD34+ precursors within the decidual tissue. It is conceivable that Group A is exposed to a more potent microecological stimulus, one that promotes the differentiation of CD34+ precursors into uNK cells. The paucity of data concerning the role of the endometrial microbiota in the development of CD34+ uNK cells represents a significant area for future research. The question of whether this result is due to lactobacilli and their metabolites is of interest. This finding aligns with the conclusions of seminal studies conducted in the early 20th century, which demonstrated that the gut microbiota plays a pivotal role in the proliferation of epithelial stem cells (Abrams, Bauer et al. 1963; Lesher, Walburg et al. 1964). A similar outcome is demonstrated by a study on dysbiosis in the gut microbiota and its impact on the activity of intestinal stem cells and the pathogenesis of necrotizing enterocolitis. Kim et al. conducted a single-cell analysis in mice treated with antibiotics. The gut microbiota has been shown to stimulate the differentiation of stem cells into Paneth cells. However, the mechanisms through which this occurs, involving the regulation of macrophages and the proliferation of mesenchymal cells, differ from the findings reported in this study. In the event of a disruption in the development of these stem cell components due to dysbiosis, the result can be the onset of necrotizing enterocolitis in newborn mice. Moreover, the analysis of the microbiome reveals a significant decrease in *Lactobacillus* in cases of necrotizing enterocolitis and/or antibiotic treatment. However, treatment with *Lactobacillus* results in a partial restoration of the condition in mice with necrotizing enterocolitis (Kim, Li et al. 2022).

In summary, the findings of this study suggest that the endometrial microbiota may have a significant impact on the regulation of progenitor uNK cells, which are a crucial component of the uterine immune system.

The markedly elevated levels of leukocytes and lymphocytes observed in group B in comparison to group C suggest the occurrence of inflammation in group B. This finding

underscores the significance of lactobacilli in establishing local immune homeostasis. Lactobacilli constitute a pivotal element of the endometrial microbiome in healthy women of reproductive age. These bacteria have been demonstrated to possess anti-inflammatory properties and the capacity to modulate the immune response. Their role in immune regulation is associated with the secretion of lactic acid, which inhibits the production of pro-inflammatory cytokines and chemokines through the activation of TLRs (Rautava, Collado et al. 2012; Esmaeili, Mahmoudi et al. 2018). These bacteria have been observed to reduce the expression of co-stimulatory molecules and increase the expression of immunoregulatory molecules on dendritic cells (DCs). Furthermore, lactobacilli have been observed to impede pro-inflammatory gene expression in endometrial cells (Liu, Feng et al., 2022).

The obtained results support the data from a study on *Lactobacillus delbrueckii* and *Lactobacillus rhamnosus*, proving the generation of tolerogenic effects on the immune system (Esmaeili, Mahmoudi et al. 2018). The absence of lactobacilli can affect the immune system in several ways, including:

- An increased expression of pro-inflammatory markers (HLA-DR, CD86, CD80, CD83, and IL-12) has been observed in immature DCs. This can result in heightened immune system activation and an escalation in inflammatory responses.
- A decrease in the expression of anti-inflammatory cytokines, including IL-10, IL-2, and IDO, has been observed. These molecules play an important role in regulating the immune response and maintaining immune tolerance. The reduction in their levels has been demonstrated to induce an imbalance in the immune system and an increased susceptibility to inflammatory and autoimmune reactions.
- Alterations in the function of DCs that, in the absence of adequate regulation by lactobacilli, may exhibit excessive activity, presenting antigens to T-lymphocytes in a manner that promotes inflammation rather than inducing immunological tolerance.

In a separate study, *Lactobacillus rhamnosus* GR-1 was examined in bovine endometrial epithelial cell cultures that had been pre-infected with E. coli. The results demonstrate that *Lactobacillus rhamnosus* GR-1 has the capacity to suppress the expression of pro-inflammatory genes in these cells. In comparison with the group that received a placebo, the effect was found to be significantly more pronounced (Liu, Feng et al., 2022). In the context of endometrial cells, the absence of *Lactobacillus rhamnosus* GR-1 has been demonstrated to increase the risk of inflammatory diseases of the endometrium due to the lack of regulation of genes with a pro-inflammatory function.

Lactic acid, a byproduct of lactobacilli, has been demonstrated to function as an immune modulator, operating at diverse levels within the immune response (Li, Zang et al. 2020). At low pH, L-lactic acid has been shown to inhibit the production of pro-inflammatory cytokines and chemokines induced by TLRs in cervical and vaginal epithelial cells (Delgado-Diaz, Tyssen et al. 2020). Lactic acid has been demonstrated to induce the secretion of anti-inflammatory IL-10, reduce the production of pro-inflammatory IL-12 in DCs, and decrease the activity of NK cells (Ilhan, Łaniewski et al., 2019). Organic acids, which are part of the microbiota, have been shown to increase the production of IL-1RA, inhibit the IL-1 signal, and

lower the secretion of IL-6 and MIP-3α (Delgado-Diaz, Tyssen et al. 2020). Therefore, the interaction between microorganisms, their metabolites, and immune components in the reproductive tract is of significant importance for maintaining reproductive health.

The comparison of cells reveals a marked increase in neutrophil levels ($p = 0.012$) in biopsies from subjects in Group C, characterized by a disturbance in the microbiota, as compared to those in Group D, exhibiting normal EM. The present findings align with those from prior studies, which revealed a substantially lower percentage of endometrial neutrophils from endometria with normal microbiota compared to those from samples with disturbed EM (Franasiak, Werner et al. 2016, Baker, Chase et al. 2018, Benner, Ferwerda et al. 2018). Neutrophils are a component of the innate immune system and play a role in inflammatory responses. As demonstrated in the research, the peptidoglycan of the bacterial cell wall is not only essential for bacterial survival but also for the initiation of inflammation, thereby affecting the function of neutrophils and the innate immune response (Li, Zang et al. 2020). Furthermore, the disruption of the epithelial barrier and the presence of pathogenic bacteria lead to an increase in neutrophils and aim to enhance the innate immune defense of the mucosa (Reis Machado, da Silva et al. 2014). Consequently, in group C, the mechanisms of innate immunity predominate, as evidenced by an increase in neutrophil presence.

The absence of a statistically significant difference for neutrophils in Group D compared to the other groups, despite having significantly disturbed EM, may be associated with the dynamics of immune activation associated with the reduced amount of lactobacilli and the composition of the dysbiotic microorganisms.

The comparative analysis shows that the group with low biomass (group B) exhibits a significantly higher proportion of T-lymphocytes compared to the group with normal microbiota and all other groups. A substantial body of research has demonstrated the pivotal function of Lactobacillus spp. in modulating immune responses and enhancing protection against infections. In the present study, the absence of Lactobacillus spp. has been associated with an elevated proportion of T-lymphocytes, which may offer a potential explanation for the increased immune activity observed in women with a disturbed vaginal microbiome and a heightened risk of HIV infection (Martin, Richardson et al. 1999, van de Wijgert, Morrison et al. 2008, Petrova, van den Broek et al. 2013, Borgdorff, Tsivtsivadze et al. 2014, Stapleton 2016). The absence of these cells can result in an elevated proportion of T-lymphocytes, which, in turn, can increase the risk of infections or inflammatory conditions of a similar nature. These results corroborate the hypothesis that *Lactobacillus* spp. play a regulatory role in T-lymphocytes.

A similar set of results was obtained in a study that utilized flow cytometric analysis of uterine samples from mice. Following treatment with lactobacilli, a significant decrease in CD8+ T-lymphocytes was observed (Silvia Ventimiglia, Jimena Valeff et al. 2021). This phenomenon may be attributed to crosstalk between uNK cells, decidual macrophages, and T-lymphocytes (Vacca, Cantoni et al. 2010). In this particular context, the relationship between natural killer (NK) cells and T-lymphocytes plays an indispensable role. Uterine natural killer cells are recognized for their capacity to modulate the immune response, encompassing the activity of

T-lymphocytes. In the absence of lactobacilli within the endometrial microenvironment, NK cells have been shown to influence the activity of T-lymphocytes. This influence is mediated through the secretion of galectin and glycodelin, which has been observed to increase the percentage of T-lymphocytes (D'Ippolito, Di Nicuolo et al., 2018).

Uterine NK cells play an important role in the regulation of embryo implantation and the modulation of a tolerant immune response in the endometrium. These cells differ from blood NK cells in several ways. Firstly, they exhibit reduced cytotoxic activity. Secondly, they have a smaller number of activating receptors and a larger number of inhibitory receptors. Galectin-1 and glycodelin, molecules with immunomodulatory activity, are selectively expressed. Galectin exerts a regulatory effect on the proliferation and survival of T-lymphocytes, modulating their production of TNF-α, IL-2, IFN-γ, and the secretion of IL-12 by macrophages. Glycodelin has been demonstrated to reduce the activation of T-lymphocytes (D'Ippolito, Di Nicuolo et al., 2018).

In conclusion, the comparative analysis of endometrial cells among the four groups according to endometrial microbiota disorder demonstrates that disturbances in the microbiota are associated with alterations in the immune response and inflammation in the endometrium. These results should be taken into account when developing strategies for the diagnosis and treatment of endometrial microbiota disorders.

Although no statistically significant difference in the profile of microorganisms is observed between Group C and Group D, in Group C, the most numerous are biopsies with a microbial profile that includes a combination of aerobic (*Enterobacteriaceae*, *Streptococcus* spp., and *Staphylococcus* spp.) and anaerobic bacteria (*G. vaginalis*, *P. bivia*, *Porphyromonas* spp., *Eubacterium* spp., *Sneathia* spp., *Leptotrichia* spp., *Fusobacterium* spp., *Megasphera* spp., *Veilonella* spp., *Dialister* spp., *Lachnobacterium* spp., *Clostridium* spp., *Mobiluncus* spp., *Corynebacterium* spp., *Peptostreptococcus* spp., *A. vaginae*) and *Candida* spp. In Group D, biopsies with a microbial profile that includes a combination of anaerobic bacteria and *Candida* spp. are predominant. Further studies are needed to determine whether the difference in the microbial profile between the two groups is associated with significantly lower mean percentage values of uNK cells and T-lymphocytes in Group C compared to Group D.

The cases from Group C have the lowest frequency of confirmation by molecular genetic analysis. This raises the question of the reasons for such a low frequency. A balanced endometrial microbiota typically functions as a protective barrier, producing substances that hinder the attachment and proliferation of pathogenic microorganisms. In the presence of such microorganisms, it can initiate a dynamic protective immune response by producing inflammatory cytokines, chemokines, and antibacterial substances (Zhu, Yang et al. 2022).

In Group C, 11 out of 25 biopsies show a higher percentage of neutrophils than the group average, while only one biopsy shows an increase in plasma cells. Similarly, in Group D, 30 out of 77 biopsies show a higher percentage of neutrophils than the group average, but only two of them have increased plasma cells. Data from previous studies indicate that chronic endometritis is not associated with the presence of pathogens originating from the upper genital

tract. These results coincide with the observation that an increased presence of neutrophils is not always accompanied by an increased number of plasma cells, suggesting different mechanisms of the inflammatory response. Furthermore, the lack of correlation between chronic endometritis and pathogens from the upper genital tract suggests that factors other than the microbial flora may play a role in the development of this type of inflammation (Haggerty, Hillier et al. 2004).

Neutrophils are the primary cells involved in the acute inflammatory response and are among the first to be mobilized during bacterial infections. The high levels of neutrophils in the biopsies from both groups confirm the presence of bacterial infection or an acute inflammatory process. On the other hand, plasma cells increase during chronic inflammatory processes and in the presence of antigens that elicit a humoral immune response.

It is possible that the observed differences between the groups are due to a different phase of the immune response at the time of the biopsy. In the acute phase of infection, neutrophils, which are characteristic of the innate immune response, are dominant at the site of inflammation. If biopsies from early stages of inflammation predominate in the studied groups, this would explain the high percentage of neutrophils. On the other hand, plasma cells are typical of the humoral immune response, and their appearance in the endometrium is associated with the later stages of the inflammatory process. The small number of biopsies with an increased number of plasma cells may reflect a more chronic phase of inflammation or individual differences in the immune response. It is important to note that different phases can coexist in an inflammatory process, which complicates the interpretation of biopsy results taken at different time points.

The diagnosis of chronic endometritis is gaining increasing importance in recent years. Much attention is paid to this presumed aspect in infertility. In Austria, Germany, and Switzerland, the diagnosis and therapy of chronic endometritis are included in the current guidelines for recurrent spontaneous abortions (Hennessy, Dennehy et al. 2021). Chronic endometritis is defined as a chronic inflammatory disease characterized by the infiltration of plasma cells in the area of the endometrial stroma (ESPC). Although endometrial plasma cells are considered an important diagnostic criterion for chronic endometritis, studies show that they are not specific only to this pathology. They are present in hormonally determined endometrial disorders related to changes in gland structure (impaired proliferative and anovulatory pattern) and stromal breakdown processes (Espinos, Fabregues et al. 2021).

Through culture and conventional PCR of endometrial tissue, Kitaya et al. fail to detect microorganisms in more than half of infertile women with chronic endometritis (Kitaya, Matsubayashi et al. 2017), while similar levels of pathogenic microorganisms are reported in women with and without chronic endometritis (Moreno, Codoñer et al. 2016). Similarly, in the current study, a significantly higher mean rank of plasma cells is found in Group A than in Group B. Therefore, the absence of dysbiotic microorganisms is not a sufficient condition to exclude chronic endometritis characterized by the presence of plasma cells. Conversely, the presence of dysbiosis in Group C and Group D does not mean that the number of plasma cells will be increased.

The immunological assessment of etiological factors in reproductive disorders should include the investigation of additional factors and components that may be involved in the etiology of chronic endometritis, such as viruses, autoimmune factors, or disorders in the regulation of the innate immune response. Furthermore, both innate and adaptive immune pathways can be independently engaged under conditions of dysbiosis and acute or chronic endometritis. The causal relationship between chronic endometritis and dysbiosis remains unclear – it is uncertain whether an unregulated immune response in the endometrium leads to a higher frequency of dysbiosis, or whether dysbiosis is the cause of chronic endometritis.

Two studies find that the prevalence of chronic endometritis is 13% in women with RPL (Kitaya 2011) and 30% in women with RIF (Johnston-MacAnanny, Hartnett et al. 2010), respectively. In the current study, biopsies in which more than 5% plasma cells are proven out of the lymphocytes are found in 27.1% of women with RIF and in 31.18% of women with RPL, but the mean percentage value of endometrial plasma cells in women with RIF is higher than that in women with RPL. These results confirm that chronic endometritis is a significant factor in women with RPL and RIF, with its frequency being relatively high in both groups. It is interesting to note that although its frequency is higher in RPL, the severity of the condition may be more pronounced in RIF. The difference in the mean percentage value of plasma cells may reflect different pathophysiological mechanisms in RPL and RIF. For example, in RPL, inflammation may be more common but of lower intensity, while in RIF, inflammation may be less frequent but more pronounced. This suggests the need for a differentiated approach in the diagnosis and treatment of chronic endometritis depending on the specific reproductive problem. On the one hand, higher mean percentage values of plasma cells in RIF may require more intensive treatment of inflammation, while in RPL, the application of broader screening coverage is more important.

Compared to Group C (disturbed microbiota), the group with significantly disturbed microbiota (Group D) has significantly higher levels of lymphocytes and uNK cells. Similar data are documented, with a significant positive correlation being established between uNK cells and *Sphingomonas* (Chen, Chen et al. 2021). This confirms the dynamic nature of the immune response, which is associated with the significant disturbance of the microbiota. This result indicates that it is necessary to differentiate the subpopulations of uNK cells according to their functional characteristics.

4. DISCUSSION OF THE RESULTS FROM FLOW CYTOMETRIC ANALYSIS OF IMMUNE CELLS IN THE ENDOMETRIUM OF WOMEN WITH REPRODUCTIVE DISORDERS IN GROUPS ACCORDING TO THE EM PROFILE

The molecular diagnostic method for the analysis of the endometrial microbiota in this study groups the bacteria according to the different atmospheric requirements for their development. This allows for the grouping of biopsies according to the composition of microorganisms into nine different groups and the analysis of the composition of immune cells in the different groups. The analysis of endometrial cells between the groups shows some statistically

significant differences, which indicates the importance of microbial communities for the immune response.

It is known that some bacterial communities are capable of forming structures called bacterial biofilms. These biofilms represent communities of bacteria densely attached to an inert surface or biological tissue, surrounded by a mucous substance called EPS. It is composed of extracellular polymeric substances that provide structural integrity to the biofilm and protect the bacteria from adverse conditions (Costerton, Stewart et al. 1999). This community is often characterized by a complex internal architecture and contains channels allowing the circulation of nutrients (de Beer, Stoodley et al. 1994). Individual areas within the biofilm can contain genetically identical cells that exhibit different patterns of gene expression (Costerton, Lewandowski et al. 1995). This leads to increased tolerance to adverse conditions and better resistance in a hostile environment, providing protection against chemical disinfection, antimicrobial treatment, and human immune reactions (Costerton, Stewart et al. 1999).

The significantly higher number of leukocytes in Group 3, according to the microbial profile, compared to groups 2, 4, 5, 6, and 7 emphasizes the importance of microbial communities in the context of the immune response. Analogous data are lacking in the scientific literature, but this does not contradict current observations. It is known that aerobic microorganisms can create a microenvironment that supports anaerobic microorganisms (Percival, Malone et al. 2018). Due to the small number of cases in Group 3, according to the microbial profile, generalized conclusions cannot be drawn.

The significant difference in the number of leukocytes between Group 5 and Group 8 shows that some types of microorganisms elicit a stronger inflammatory response than others. This may be due to different virulence factors of the microorganisms or different mechanisms by which they interact with the host's immune system.

Studies using sensors to track oxygen concentrations show that *C. albicans* biofilms create a significant oxygen concentration gradient. Oxygen levels decrease from the environment at the top of the biofilm to very low levels at its bottom. This gradient remains constant whether *C. albicans* is grown alone or in combination with anaerobic bacteria such as *C. perfringens* or *B. fragilis* (Fox, Cowley et al. 2014). *C. albicans* biofilms create locally hypoxic conditions that can favor the growth of anaerobic bacteria. In the analyzed 9 groups, it is observed that the combination of *Candida* spp. and anaerobic microorganisms occurs with the highest frequency.

Epithelial cells are the first line of defense of the female genital tract (Czechowicz, Nowicka et al. 2022). This determines local immunity, rather than systemic immunity, as critical for host defense against infection. Their surface is covered with PRRs responsible for recognizing fungi of the genus *Candida*. Numerous studies emphasize that epithelial cells are able to distinguish and categorize fungi on the vaginal mucosa, defining them as commensal or pathogenic.

Studies related to the pathogenicity of *Candida* spp. and the immune response are mainly focused on vulvovaginal candidiasis. Along with the host's immune response, the virulence factors of *C. albicans*, especially hyphae and Candidalysin, are key to the development of

vulvovaginal candidiasis (Peters, Palmer et al. 2014). The ability of *C. albicans* to transform into hyphae is essential for pathogenesis, as well as for the vaginal migration of polymorphonuclear leukocytes. Candidalysin is a secreted cytolytic peptide toxin whose genes are among the most highly expressed in vaginitis and is essential for the immunopathogenic response.

Upon recognition of pathogenic fungi, epithelial cells activate annexin A1, S100 alarmins, and IL-1 in response to *Candida* via TLR4 and SIGNR1 and the NLRP3 inflammasome (Roselletti, Perito et al. 2019). The inflammatory response in vulvovaginal candidiasis is dependent on the threshold level of *Candida* and the reaction of epithelial cells. Susceptible women react with inflammation at low levels of *Candida*, while resistant women do not. In susceptible women, *Candida* stimulates epithelial cells to produce alarmins and pro-inflammatory cytokines to attract neutrophils. In resistant women, epithelial cells do not produce alarmins/cytokines but instead interact with annexin A1, suppressing the growth of *Candida* (Yano, Peters et al. 2018).

The present study finds that the presence of *Candida* spp. in the endometrium (Group 4) is associated with opposite changes in the number of leukocytes and lymphocytes. The significantly lower mean number of leukocytes compared to Group 3 suggests that the presence of *Candida* spp. may suppress leukocyte activity in the endometrium. On the other hand, Group 4 has a higher mean number of lymphocytes (55.27%) compared to Group 5 (39.04%, $p = 0.040$), Group 6 (38.37%, $p = 0.010$), and Group 9 (39.80%, $p = 0.005$). These findings do not contradict current data on neutrophil anergy induced by various pathogens and inflammatory conditions (Yano and Fidel Jr 2022).

The mean number of T-lymphocytes in Group 4 is significantly lower compared to Group 7, which suggests that the sole presence of *Candida* spp. in the endometrium does not elicit a T-cell response and supports the importance of innate immune defense (Czechowicz, Nowicka et al. 2022).

Corresponding to the results for T-lymphocytes, the analysis of plasma cells shows that Group 4 (with *Candida* spp.) has significantly lower mean percentage values compared to Group 3 (without *Candida* spp.). This confirms the key role of the innate immune response in infections with *Candida* spp. (Peters, Palmer et al. 2014).

In the present study, it is proven that the number of uNK cells is significantly lower in Group 2 compared to Group 4 (The statistical analysis of endometrial immune cells among the nine study groups is presented in Table 1, Table 5 and Figure 6). Furthermore, the number of uNK cells is significantly higher in Group 4 compared to Group 6 and Group 8. No statistically significant differences are observed in the number of uNK cells between the remaining groups.

These results suggest that the number of uNK cells can vary significantly between the different groups, with the highest levels observed in Group 4 and the lowest in Group 2. Uterine NK cells are an important component of the immune system in the endometrium and may play a role in embryo implantation and pregnancy development. Similarly, Matteo et al. observe that infertile women with chronic endometritis have a reduced percentage of uNK cells and T-

lymphocytes compared to women with unexplained infertility (Matteo, Cicinelli et al. 2009). Although numerous studies have been conducted on the effect of *Candida* spp. on the immune system, a thorough analysis focused on the comparison of uNK cells in the endometrium in patients with and without *Candida* spp. infection is lacking. Further research is needed to establish the significance of these observations and to determine whether the difference in the number of uNK cells may be related to reproductive outcome.

The present analysis compares the number of plasma cells in nine groups to investigate the immune response in the different groups. The observed higher mean percentage values of plasma cells in Group 3 and the lower number of plasma cells in Group 9 confirm the results of a similar study, which shows that *Lactobacillus* spp. is more prevalent in the microbiota of women without chronic endometritis (Liu, Ko et al. 2019). The lack of statistical significance between the groups can be explained by the small number of cases in some of them.

The conducted therapy leads to a reduction in plasma cells (below 5% of lymphocytes) in 63% of women with chronic endometritis. This improvement is associated with a 20% increase in the live birth rate. A study involving 95 women shows that 56.8% of them have chronic endometritis. After antibiotic treatment, 82.3% of the patients are cured, while in 17.6%, the disease persists. Women with successfully treated chronic endometritis have a significantly higher rate of pregnancy and childbirth compared to those in whom the disease persists, as well as women without this diagnosis (Cicinelli, Matteo et al. 2018).

Despite the differences in the design of the two studies, the results are similar. Both show that endometritis can negatively affect the success of ART, and that its treatment can improve reproductive outcomes.

The present analysis confirms these findings and the results of a previous study on 80 women undergoing IVF. The results of the present study, similar to the previous one, do not show a significant association between the number of plasma cells and successful pregnancy (Herlihy, Klimczak et al. 2022).Обсъждане на корелациите в групите с RIF и RPL

5. DISCUSSION OF CORRELATIONS IN GROUPS OF WOMEN WITH RIF AND RPL

The results from the correlation analysis of immune cells in women with RIF and RPL show similarities and differences in the interaction between different cell types in the two groups. Regarding the similarities, it is observed that in women from both groups, there is a strong positive correlation between the number of leukocytes and lymphocytes, and a weak but significant positive correlation between the number of leukocytes and plasma cells. A strong negative correlation is also observed between the number of macrophages and neutrophils.

Differences in the interactions between immune cells are observed in women with RIF and RPL. In cases of RIF, the number of leukocytes is inversely proportional to the number of CD34+ uNK cells. Such a relationship is not observed in RPL. The situation is similar with lymphocytes: in RIF, with an increase in lymphocyte values, the values of CD34+ uNK cells decrease. In RPL, this relationship is weaker. These differences suggest that immune regulation

in RIF and RPL proceeds differently. Similar results are obtained in a study that includes women with infertility. Endometrial samples from 58 women are taken during surgical intervention for the diagnosis of endometriosis. After an average of 9.5 months after surgery, 33 of them underwent ART (IVF, ICSI, or IUI). Uterine NK cells and hematopoietic progenitor cells from the endometrium are analyzed by flow cytometry. In women with successful implantation, the populations of endometrial CD34+ cells are higher (3.97% vs 0.69%; $p < 0.0004$), and the co-expression of the NK cell marker CD56 is increased (81.1% vs 60.9%; $p < 0.034$) compared to patients in whom implantation was not successful (Glover, Crosby et al. 2018).

The decrease in the number of CD34+ uNK cells or the imbalance between CD34+ uNK cells and uNK cells in RIF could lead to impaired angiogenesis and remodeling of the utero-placental arteries, which is a key factor for successful placentation.

In the present study, a weak but significant positive correlation is found between the number of lymphocytes and uNK cells in women with RPL, while in women with RIF, such a correlation is absent. The results of another study show a strong positive correlation between CD45+ leukocytes and uNK cells in the control group, but no correlations are found in the RPL groups (Chiokadze, Bär et al. 2020).

In both studies, a correlation is observed between the number of lymphocytes or CD45+ leukocytes and the number of uNK cells or CD56+ cells, but the correlation is stronger compared to that in the present study. Furthermore, the correlation is present only in the control group in the other study, while in the present study, it is present only in women with RPL. It is possible that the women included in the two studies had different characteristics that influenced the correlational relationships between lymphocytes and uNK cells. Another difference is the immunohistochemical method used for the analysis of immune cells.

The results from both studies show that the relationships between lymphocytes and uNK cells are complex and may differ in women with RPL and RIF. Further research is needed to better understand these relationships and their role in reproductive disorders.

The clinical significance of the quantity and function of uNK cells for infertility and adverse pregnancy outcomes remains controversial in the scientific literature. Some studies show a decrease in the number of uNK cells in endometrial biopsies taken in the secretory phase in women with infertility (Klentzeris, Bulmer et al. 1992, Kofod, Lindhard et al. 2017), as well as a decrease in uNK cells in women who experienced miscarriage after ART compared to those who had a successful live birth after ART (Fukui, Fujii et al. 1999). Other studies do not find a difference in the abundance of uNK cells (McGrath, Ryan et al. 2009, Giuliani, Parkin et al. 2014) or even report an increase in progenitor uNK cells in the endometrium of infertile women in biopsies taken during planned surgery (Lynch, Golden-Mason et al. 2007).

6. DISCUSSION OF CORRELATIONS BETWEEN IMMUNE CELLS IN THE FOUR EM GROUPS

The results from the Pearson correlation analysis, presented in Figure 7, show statistically significant relationships between the levels of endometrial immune cells in the different groups categorized by EM. These data do not contradict another study in women with reproductive problems and chronic endometritis, where changes in the transcriptional regulation of cytokines, growth factors, and apoptotic proteins are observed (Di Pietro, Cicinelli et al. 2013). Changes in the inflammatory response can lead to dynamics in the levels of endometrial immune cells. There is no contradiction with the literature data, which show that in the endometrium affected by chronic endometritis, a disturbed pattern of lymphocyte subpopulations is found, associated with an aberrant local microenvironment and altered secretion of paracrine factors.

The results of this study do not contradict a previous one, which shows that the isolation of endometrial microorganisms does not necessarily correlate with endometrial inflammation (Vitagliano, Noventa et al. 2017).

Based on these results, it can be suggested that changes in the levels of endometrial immune cells may be a useful biomarker for assessing the specific immunobiological disorder.

7. DISCUSSION OF THE RESULTS FROM THE ANALYSIS OF ENDOMETRIAL IMMUNE CELLS BEFORE AND AFTER ANTIBIOTIC AND PROBIOTIC THERAPY

The present study, albeit with a limited sample size, suggests that antibiotic and probiotic therapy may positively influence the number of uNK cells. These data are consistent with a previous study that links the imbalance of the endometrial microbiome to changes in the immune system, including impaired differentiation of uNK cells (Al-Nasiry, Ambrosino et al. 2020). These findings are important as they suggest that modulating the endometrial microbiome may be a potential therapeutic strategy for improving uNK cell function and possibly improving reproductive outcomes.

However, the interpretation of the results from these studies is complicated by the fact that the assessment of uNK cells as a diagnostic tool is hindered by the lack of unified reference values. A study involving 215 women shows significant differences in the mean percentages of uNK cells: 2.5% in fertile women, 3.2% in women with recurrent miscarriage (RM), and 3.1% in women with recurrent implantation failure (RIF) (Chen, Mariee et al. 2017). These data highlight the complexity of interpreting uNK cell levels and the need for standardization of methods for their measurement and evaluation.

Interestingly, when a lower limit of 1.2% is established, 16% of women with RM and 18% with RIF have uNK cell levels below this value (Chen, Mariee et al. 2017). This fact raises the question of the possible harmful effects of low uNK cell levels on the endometrium, an area that has been largely neglected by researchers. Most studies focus on the evaluation of higher levels of uNK cells, without paying attention to lower levels, leading to a limited number of studies in this area.

In addition to these challenges, another study finds that in 53% of women with idiopathic RIF, the number of CD56+ NK cells is above 250 per high power field at 400x magnification, while in a control group of women without RIF, this number is only 5% (Santillán, Lozano et al. 2015). These results suggest that an increased number of NK cells in the endometrium may play a role in the development of RIF, but the mechanism by which this occurs is not yet fully elucidated.

In conclusion, the comparative analysis of endometrial immune cells before and after antibiotic and probiotic therapy, as well as studies on uNK cells in women with reproductive problems, provide important information for potential therapeutic strategies. Although further research is needed, these findings may lead to the development of new methods for the diagnosis and treatment of reproductive disorders, as well as to the improvement of women's reproductive health.

8. Discussion of the birth rate after the first ART procedure following antibiotic and probiotic therapy

The results of the present study show a link between endometrial microbiota disorders and reproductive outcome. They are consistent with the data presented by Moreno et al. (Moreno, Garcia-Grau et al. 2022). Their research emphasizes the importance of a balanced composition of the endometrial microbiota for successful reproductive function, or more specifically, that the presence of pathogenic bacteria such as *Atopobium*, *Bifidobacterium*, *Chryseobacterium*, *Gardnerella*, *Haemophilus*, *Klebsiella*, *Neisseria*, *Staphylococcus*, [1] and *Streptococcus* in the endometrium, combined with a reduced amount of *Lactobacillus* spp., is associated with impaired reproductive function. These findings support the hypothesis that endometrial dysbiosis may be an important factor for failed implantation and/or pregnancy loss.

Our results, showing a lower frequency of births in patients with disturbed endometrial microbiota (Groups B, C, and D) compared to those with normal microbiota (Group A), are consistent with the findings of Moreno et al. This reinforces the idea that an imbalance in the composition of the endometrial microbiota can have a negative effect on reproductive outcome.

Punzon-Jimenez et al. focus on the role of *Lactobacillus* spp., finding that *Lactobacillus*-rich vaginal and endometrial microbial profiles are associated with better reproductive outcomes (Punzón-Jiménez and Labarta 2021). This highlights the importance not only of the absence of pathogens but also of the presence of a sufficient amount of *Lactobacillus* for maintaining a healthy reproductive environment. The present study, through the use of antibiotic and probiotic therapy, aims precisely at restoring the balance of the microbiota, including *Lactobacillus* spp., which may explain the improvement (albeit not in all cases) of reproductive outcomes.

It is important to note that the study by Moreno et al. focuses on the identification of specific pathogenic bacteria associated with reproductive problems, while the present study evaluates the overall state of the endometrial microbiota and the effect of its correction through antibiotic

and probiotic therapy on the local immune environment. Despite these differences in approach, both studies emphasize the importance of maintaining a balanced endometrial microbiota for successful pregnancy.

VI. Summarized Conclusions

1. A normal endometrial microbiota, characterized by *Lactobacillus* spp., is important for effective blastocyst implantation.
2. A reduced quantity or the absence of lactobacilli can lead to local inflammation, as well as to quantitative changes in the distribution of immune cells, including uNK cells, T-lymphocytes, and neutrophils.
3. Dysbiosis of the endometrial microbiota may be part of a pathogenetic mechanism underlying RIF and RPL.
4. High levels of leukocytes and lymphocytes in Group B (with low biomass) compared to Group C (with moderate dysbiosis) are indicative of inflammation, thus emphasizing the importance of lactobacilli for establishing local immune homeostasis.
5. The absence of lactobacilli can lead to increased local inflammation, as well as to quantitative changes in endometrial immune cells, including uNK cells, T-lymphocytes, and neutrophils.
6. Probiotics can modulate the endometrial microbiota and may be a novel therapeutic strategy in the treatment of RIF and RPL.

VII. Contributions

Contributions with Original Character

1. A methodology for the flow cytometric determination of endometrial lymphocyte populations is developed through the analysis of endometrial biopsies during the implantation window in the luteal phase in women with reproductive disorders.
2. For the first time in Bulgaria, a complex approach for the investigation of endometrial biopsies is applied, combining molecular genetic and flow cytometric analysis.
3. For the first time in Bulgaria, the influence of the endometrial microbiota on the local immune response is analyzed through the differences in immune cells between groups with different microbiota compositions in women with RIF and RPL.
4. For the first time in the present study, a method for grouping endometrial biopsies based on the composition of the microbiota is used.
5. For the first time in Bulgaria, a study that evaluates the pathogens from the upper genital tract and their role in chronic endometritis and endometrial dysbiosis in women with RIF and RPL is performed.

Contributions with Confirming Character

1. The mean percentage values of leukocytes, observed in the biopsies, correspond to data from previous studies, thus proving the validity of the sampling and processing procedure.
2. The increased frequency of dysbiosis and chronic endometritis in women with RPL and RIF is confirmed.
3. The influence of the endometrial microbiota on reproductive outcomes is confirmed.
4. An endometrial microbiota dominated by *Lactobacillus* spp. is confirmed to be essential for optimal implantation.
5. The role of neutrophils in the endometrial immune response in cases of endometrial dysbiosis is confirmed.
6. The therapeutic effect of antibiotic therapy on chronic endometritis and reproductive outcome is confirmed.

VIII. Appendices

10. List of scientific publications related to the dissertation

1.1. Ivanov P., Lukanov T., Konova E., **Blajeva S.**, Rilcheva V., Tsvyatkovska T., Kovacheva K. *Activation of peripheral natural killer cells in women with repeated early pregnancy loss*. Akusherstvo i ginekologià, 2015, 54(8): 3-7; ISSN: 0324-0959; Web of Science, Scopus

1.2. **Blazheva S.**, Pachkova S., Bodurska T., Ivanov P., Blazhev A., Lukanov T., Konova E. *Unlocking the Uterine Code: Microbiota, Immune Cells, and Therapy for Recurrent Reproductive Failure*. Microorganisms, 2024, 12(3): Article number 547; e-ISSN: 2076-2607; Web of Science, Scopus

1.3. Pachkova S., Gincheva D., **Blazheva S.**, Konova E. *Frequency of urogenital mycoplasmas in women with reproductive problems*. Akusherstvo i ginekologià, 2019, 58(3): 18-22; ISSN: 0324-0959

1.4. **Blazheva S.**, Bodurska T., Ivanov P., Pachkova S., Konova E. *Endometrial immune cells and endometrial microbiome in women with recurrent implantation failure*. Bulgarian Journal of Clinical Immunology, 2022, 15(1): 3-12; ISSN: 2738-7046

2. List of Participations in Scientific Conferences Related to the Dissertation

2.1. Participations in Scientific Forums in Bulgaria

2.1.1. **Blazheva S.**, Bodurska T., Ivanova S., Ivanov P., Konova E. *Research of the endometrial immune cells in women with reproductive failure and impaired endometrial microbiota.* 4th International World of Microbiome Conference. 26 – 28.10.2023, Sofia.

2.1.2. **Блажева С.,** Бодурска Т, Иванов П, Пачкова С, Конова Е. *Проучване на ендометриалните имунни клетки при жени с нарушен ендометриален микробиом и репродуктивни неуспехи*. XXIV Конгрес по стерилитет и репродуктивно здраве, 06 – 09.04.2023 г., к.к. Боровец.

2.1.3. Бодурска Т., **Блажева С.**, Иванова С., Конова Е., Тотев Т. *Пилотни данни от изследване на ендометриален микробиом при пациентки с повтарящи се репродуктивни неуспехи.* XXIV Национален конгрес по стерилитет и репродуктивно здраве с международно участие, 06 – 09.04.2023 г., к.к. Боровец.

2.1.4. **Блажева С.**, Пачкова С., Иванов П., Бодурска Т., Конова Е. *Влияние на ендометриалния микробиом върху ендометриалните имунни клетки при жени с репродуктивни неуспехи.* Годишна научна конференция по имунология, 16.12.2022 г., гр. София.

2.1.5. Пачкова С., **Блажева С.,** Иванов П., Бодурска Т., Конова Е. *Ендометриален микробиом при жени с репродуктивни проблеми*. XX Юбилеен Национален Конгрес по Клинична Микробиология и Инфекции на БАМ, 16 – 18.09.2022 г., гр. Пловдив.

2.1.6. **Блажева С.,** Иванова С., Гинчева Д., Иванов П., Бодурска Т., Луканов Ц., Конова Е. *Проучване на ендометриалния имунен профил и ендометриалния микробиом при жени с имплантационни неуспехи.* Юбилейна Конференция по Клинична Имунология, 06 – 07.11.2020 г., гр. София.

2.1.7. **Blazheva S.,** Pachkova S., Gincheva D., Ivanov P., Gencheva I., Lukanov T., Konova E. *Research on herpes virus carrier and local immune responce in endometrial biopsys*

in women with reproductive failure. Fifth national congress of immunology, 25 – 28.10.2018, Plovdiv.

2.1.8. **Блажева С.**, Пачкова С., Луканов Ц., Конова Е. *Проучване на обща популация NKклетки в периферна кръв при жени с микоплазмена инфекция и репродуктивни неуспехи.* Седма работна среща "Репродуктивна медицина 2017 – противоречия и консенсус", 28 – 30. 04.2017 г., гр. Плевен

2.2. Participations in Scientific Forums Abroad

2.2.1. **Blazheva S.,** Bodurska T., Lukanov T., Atanasova M., Kyurkchiev D., Konova E. *Intravenous immunoglobulin therapy for recurrent pregnancy loss in women with high DFS 70 autoantibodies: a case report*. 17 – 20.05.2024 Ljubljana, Slovenia.

2.2.2. Atanasova M., Konova E., **Blazheva S.,** Bodurska T. *Serum levels of anti-elastin and anti-fibrillin-1 autoantibodies in recurrent pregnancy loss (RPL) patients*. 16th Dresden symposium on autoantibodies, 12 – 15.09.2023 Dresden, Germany.

3. Scientific Projects

3.1. №22/2022 Проучване на ендометриалния имунен профил при жени с повтарящи се имплантационни неуспехи.

3.2. №10/2017 Проучване честотата на носителство на HSV-1,2, CMV и EBV/ херпесвируси в ендометриум при жени с неуспешни асистирани репродуктивни техники.

Patient Information Sheet

Project Title:

Study of the Endometrial Immune Profile in Women with Recurrent Implantation Failure

Explanations and Information for the Patient Regarding the Nature of the Project:

Dear Madam,

We would like to ask you to participate in a research project with the aforementioned title, as you have been diagnosed with unexplained infertility and have not achieved a successful pregnancy with assisted reproductive techniques to date. Participation is entirely voluntary, and you are not required to participate if you do not wish to.

It is important that you read this information carefully before deciding whether to participate in the project. After reviewing the information, you have the right to ask questions, and if you receive satisfactory answers, please complete the form. By doing so, you will confirm your voluntary desire to participate in the project.

If you decide to decline your participation or withdraw from the study, which you have the right to do at any time without giving explanations, your treatment will not be affected by your decision. It will also not affect the attitude of the medical staff towards you, nor the overall care for your health. If you withdraw from the project, please inform the research team.

The project will be carried out by physicians from UMHAT "Dr. G. Stranski" EAD, Medical Center - Clinical Institute for Reproductive Medicine, Pleven, and MU - Pleven. For any questions you may have, please contact the leading researcher, Assoc. Prof. Emiliyana Ilieva Konova, MD, PhD, telephone _____, e-mail _____.

We would like to ask you to participate in the project to answer the question of what the significance of the endometrial cellular composition is for implantation disorders. By completing this project, we will gain new knowledge about lymphocytes, monocyte-macrophages, and neutrophils, as well as some basic lymphocyte populations such as endometrial NK cells, total T-lymphocytes, and progenitor NK cells. This would define the individual endometrial profile more accurately than currently existing methods, in order to determine the most appropriate treatment approach for each patient.

The direct benefit for you from participating in the project is that you will personally be provided with information about the result of the study itself. If necessary, you will be offered a follow-up and treatment algorithm, and the opportunity for consultation with a psychologist and relevant specialists.

The project will last one year.

At the beginning of the project, a physician member of the research team will ask you questions about the illnesses you currently suffer from or have suffered from in the past, as well as some personal data - your date of birth and telephone number - so that contact can be made with you

regarding the result obtained. The data will be entered into a Patient Card – a document to which only members of the research team will have access.

In addition to the usual tests mandatorily required for patients with infertility, you will need to complete a questionnaire with several questions. Taking a sample for this study does not pose a risk. It is performed with a sterile curette by a specialist obstetrician-gynecologist, Assoc. Prof. Dr. Petar Ivanov or Dr. Tatyana Bodurska, under conditions that meet the requirements defined by the standard in obstetric-gynecological practice.

No funds are provided in the project to cover your transportation costs.

If you decide to participate, all information from your participation in this project will remain confidential and will be stored at Medical Center – Clinical Institute of Reproductive Medicine "St. Elisaveta", Pleven. Certain authorized persons, Dr. Svetla Ognyanova Blazheva, will have access to your medical record, but under strict confidentiality.

Informed Consent Form

Project Title:

Study of the Endometrial Immune Profile in Women with Recurrent Implantation Failure

Please highlight Yes or No for all statements below (the correct statement is highlighted).

I was asked to consent myself **Yes** No

I have read the Patient Information Sheet **Yes** No

I was given the opportunity to ask all important questions for me and to discuss this project **Yes** No

I received satisfactory answers to all my questions **Yes** No

I received sufficient information regarding the project **Yes** No

The project was explained to me, I asked my questions and received answers to them from …….. (name of the researcher)

…… (Signature of the Researcher)

I understand that I am free to withdraw from participation in the project at any time without giving explanations for my refusal and without this affecting my future medical care.

Date:…….

Name, middle name, surname of the patient:

……

Signature of the patient:…….