To the Chairman of the Scientific Jury
Appointed by the order RD-38-260/27.05.25
of the Rector of Sofia University
“St. Kliment Ohridski”

REVIEW

by assoc. prof. Dr. Tanya Dimova, Institute of Biology and Immunology of Reproduction, Bulgarian Academy of Sciences

On a dissertation for the award of the educational and scientific degree “Doctor”
Professional field: 4.3. Biological Sciences
Scientific specialty: Cell Biology
Author: Rumiana Rumenova Ganeva
Form of doctoral study: Part-time
Scientific organization: Sofia University, Faculty of Biology
Topic: **Importance of the endometrial immune profile for the success of assisted reproduction**
Scientific supervisor: Assoc. Prof. Dr. Vesselina Moskova-Dumanova

1. General presentation of the procedure and the PhD student

The presented set of documents in connection with the defense of the PhD thesis is in accordance with the requirements of the Law on the Republic of Bulgaria on the State Academic Qualifications for the Doctorate, as well as the specific requirements of the Regulations of Sofia University “St. Kliment Ohridski”. The set contains a dissertation and an abstract, a CV, bachelor's and master's degree diplomas, an order for enrollment of the doctoral student, a certificate of exams taken, applications from the doctoral student and the scientific supervisor for the opening of a procedure for the defense of the dissertation, a declaration of originality of the dissertation work with two annexes, an author's reference for scientific activity, a reference for compliance with the minimum national requirements for the doctorate for the field 4. Natural Sciences, Mathematics and Informatics, Professional Direction 4.3. Biological Sciences.

2. Brief biographical data

Rumiana Ganeva is a biologist at the research laboratory, Nadezhda Hospital from 2016 to the present. In 2016, she completed her higher education, a bachelor's degree in Molecular Biology at the Faculty of Biology, Sofia University "St. Kliment Ohridski", and in 2018 she became a

Master in "Developmental Biology" at the same faculty. In 2022 by order RD-20-84/18.01.22 of the Sofia University's Rector, Rumiana Ganeva was enrolled as a full-time PhD student in the specialty "Cell Biology", in the Department of Cytology, Histology and Embryology", Faculty of Biology of Sofia University with a study period of 3 years, starting from 01.02.2022 to 01.02.2025 and a supervisor Assoc. Prof. Dr. Vesselina Svetoslavova Moskova-Dumanova. In February 2022, by decision of the Faculty Council the doctoral program was transformed into a part-time program with a completion date of February 2026. Before the deadline for enrollment, Rumiana Ganeva successfully pass all the exams, completed all the tasks in her individual plan and prepared her dissertation, as a result of which in March 2025 a procedure for defending the dissertation was launched. In addition to her mandatory program as a PhD student, in 2023 and 2025 Rumiana Ganeva conducted online training in "Statistics in Public Health" at John Hopkins University and several Nature Masterclasses online courses such as "Scientific Presentations", "Communication of Scientific Results", "Experiments: from Idea to Design" and "Scientific Writing and Publishing".

3. Relevance of the topic and appropriateness of the goal and objectives

Current dissertation is an in-depth research in an extremely important scientific field with a pronounced clinical significance and social impact, namely human reproduction and in particular its inefficiency in patients with recurrent implantation failures. Unfortunately, human reproduction (both natural and assisted) is an inefficient process, since only one in three fertilizations results in a successful pregnancy. In the rest, the embryo is lost in the peri-implantation period or in the period of early pregnancy, with 10% of losses being a result of immunological dysregulation. Successful implantation and pregnancy require a strictly regulated balance between immune tolerance to the different genome of the implanting embryo and immune activation in the case of transplacental infection and uncontrolled trophoblast invasion. Although assisted reproduction has added new opportunities for studying human reproduction such as morphological and genetic evaluation of the embryo, traceability of its transfer to the uterus and the occurrence of implantation, clinicians are observing an increase in the cases of recurrent implantation failure or so-called RIF syndrome after IVF-ET, which by 2024 is close to 20% of the in vitro patients. RIF is still a poorly studied infertility syndrome in otherwise healthy women and it is believed that 50% of these cases have an immunological etiology, and in 82% of these women abnormal endometrial immune regulation is found. The embryo in assisted reproduction can be evaluated morphologically and genetically before transfer to the uterus, but it is only one side in the complex process of implantation. In recent years, there has been considerable interest in the role of the endometrium and the peri-implantation environment on the subsequent development of the embryo. There is increasing evidence that maternal immune cells locally at the implantation site actively contribute to endometrial receptivity and embryo implantation. Intrauterine administration of activated autologous peripheral blood mononuclear cells effectively improves IVF outcomes in patients with RIF. All these facts show that in the context of the current state of knowledge, human implantation is still an unclear process and a real barrier to assisted reproduction techniques introduced many years ago by Edwards and Steptoe and therefore the possibility of clinical intervention is still limited. The present dissertation aims to fill the missing knowledge regarding the characterization of the endometrial immune profile in women with RIF and to identify key immune changes with a role as potential biomarkers for predicting implantation success, as well as for the effectiveness of immunotherapy.

4. Knowledge of the problem

The PhD student knows the state of the problem perfectly not only theoretically and experimentally, preparing her dissertation, but also actively working in the research laboratory of the Nadezhda Hospital where she encounters women with RIF on a daily basis. By critically analyzing the published data by other authors working on the topic, she manages to discover the limits of previous studies and the niche in which to develop her research potential. Therefore, the goal and tasks in the thesis have been adequately set, the planned research has been successfully executed by adequately selected methods and approaches.

5. Evaluation of the dissertation work

The dissertation of R. Ganeva is presented on 193 pages with the following sections: Literature review – 58 pages, Aim and objectives – 1 page, Materials and methods – 19 pages, Results and discussion – 41 pages, Conclusions – 1 page, Contributions – 1 page, Bibliography – 18 pages. Appendices – 6 pcs., Abbreviations used – 5 pages. The thesis has 40 figures and 19 tables. The rich bibliography includes 343 literary sources, of which 37% have been published in the last ten years, which once again emphasizes the relevance of the topic.

THE LITERATURE REVIEW is quite comprehensive and at the same time it purposefully analyzes the role of the endometrium in the embryo implantation from biological and immunological points of view. The PhD student has made a general characterization of the main types of immune cells and the important immune populations locally in the decidua with an emphasis on their role, both in successful implantation and in reproductive pathologies, including RIF. The author draws the reader's attention to the methods for diagnosing immunomediated RIF in *in vitro* patients and summarizes the latest studies in the application of immunotherapies for this disorder. Thus, a critical lack of knowledge in modern reproductive medicine regarding the causes of RIF is naturally addressed, emphasizing the contradictory scientific data, including on the effectiveness of various immunoregulatory treatments. Unfortunately, as the PhD student emphasizes, there are no validated, easily accessible diagnostic tests for confirming immunomediated RIF and immune therapies are often empirical without solid evidence of their efficacy. The lack of purely theoretical knowledge to be used in clinical practice hinders the introduction and validation of evidence-based immune interventions. All this motivates the research in this dissertation, namely the analysis and identification of immune markers for endometrial receptivity, which would allow a personalized approach to RIF treatment and improve the results of the *in vitro* procedures. The literature review is abundantly illustrated with 11 figures. In some of the figures, for example, to illustrate the menstrual cycle, the author has placed her own histological photographs, which is also a good practice. The literature review also includes 4 tables. I admire the presentation of information schematically and in tabular form to facilitate the reader. The goal of the dissertation is clear and specific, namely to establish immune indicators for endometrial receptivity, which would allow an assessment of the effectiveness of local immunotherapy performed in in vitro patients with RIF. Eight tasks have been set to achieve the goal, all of which are expedient and logically related.

In the MATERIALS AND METHODS section, the PhD student presents the design of the study, performed in two stages. The materials and methods are described in detail and in an understandable way for each stage separately and together with materials used are systematized in infographics. In stage 1, 116 female patients with RIF participate, who are precisely selected to meet 14 criteria. This shows that the PhD student has realized the need for the correct definition and completion of the experimental groups as an element of utmost importance for the success of any study. In stage 1, immune indicators in the endometrium of patients with RIF undergoing embryo transfer were compared in the context of the outcome of implantation - successful or unsuccessful. After a thorough statistical analysis, immune indicators were selected that distinguish cases with successful and unsuccessful implantation. In stage 2, the selected endometrial immune indicators were monitored in women with RIF, subjected to immunotherapy - intrauterine application of autologous PBMC. In phase 2, 18 female patients with RIF were included, who were selected to meet 11 criteria. The autologous PBMCs were immunophenotyped against a control group of female volunteers, with previous deliveries by natural conception (their number was not specified). In these two groups, some markers of systemic inflammation and cytokines were compared with a view to the safety of the application. The hormonal stimulation of the women, the dating and processing of the endometrial biopsies to obtain permanent histological sections, the histological analysis and software-based immunohistochemical cytometry using HALO Image Analysis Software, ELISA method for measuring cytokines, preparation, cultivation and phenotyping of PBMCs with FACS, as well as their application are described in detail. All antibodies used are given in a table with catalog number, manufacturer and dilution. Similar information is provided for all reagents and kits used. The main methods of analysis in these studies are statistical, based on which several immune parameters are selected and their predictive power as markers of endometrial receptivity is assessed. The PhD student shows skills in statistical data processing, applying diverse, but adequately selected statistical methods to test her hypotheses. The statistical packages SPSS and GraphPad Prism are used as the way of presenting the data (mean with standard deviation or median with range) and the comparison tests used (parametric or non-parametric) are consistent with the data distribution model - normal Gaussian or skewed distribution. The correlation between the individual immune indicators is monitored, and their prognostic value for assessing endometrial receptivity and the effectiveness of cell treatment is assessed using ROC analysis. This type of analysis offers a prognostic value based on the compromise between sensitivity and specificity at different threshold settings and is used to evaluate medical diagnostic tests. Considering that no medical test is perfect, the PhD student explaines in detail the interpretation of the data from this analysis. The specialized manipulations as part of the in vitro procedures accompanying the studies in the dissertation and performed by the medical teams, in accordance with the good clinical practices applied at the MC, are also described in detail. All documents required for scientific research with human samples, such as permits from the ethics committee of the Nadezhda Medical Center and the ethics committee of the Sofia University as well as signed informed consent for sample donation, are fuly described and attached. A document for a registered clinical trial for the cell treatment of RIF patients is also attached. An impressive number of endometrial samples, a total of 131, collected in the period 2020-2024, were processed for the research in this dissertation.

RESULTS AND DISCUSSION Section:

The results are described concisely and in accordance with the tasks set. They are divided into two groups according to the two stages of the study and followed by discussion for the given set of results. The style of presentation is strictly scientific and at the same time the information is easy to understand, thanks to the 14 figures and 12 tables. In stage 1 of the study, the quantities and ratios of immune cells in the endometrium of women with RIF who have undergone an *in vitro* procedure and have a result after embryo transfer (successful or unsuccessful implantation) are analyzed. In this way, the opportunities provided by assisted reproduction were used, namely fragmentation of the reproductive process, dating and traceability of its individual stages, in this case, through embryo transfer, the women with RIF having successful or unsuccessful implantation can be tracked. The basic characteristics of the studied female patients in this stage are presented in a table by groups - with successful and unsuccessful implantation after ET in a comparative aspect. These include age, body mass index, type of infertility, previous unsuccessful IVF attempts, number of good quality embryos, number of transferred embryos, serum levels of progesterone and estrogen on the day of transfer. Software-based immunohistochemical cytometry is used for *in situ* phenotyping of the endometrial immune populations as a new and interesting approach. Usually the immunophenotyping is done with flow cytometric analysis. It is a fairly precise method, but difficult to apply to biopsy materials, but more importantly, it could not provide the information about localization and clustering of the immune cells, which can only be shown with immunohistochemical visualization. The numbers of seven immune populations – T lymphocytes, B lymphocytes, helper T cells, cytotoxic T cells, monocytes, macrophages and NK cells – were visualized and compared. Informative microscopic photographs with excellent quality are placed, showing conventionally stained and immunohistochemically labeled histological sections with all needed negative and positive control stainings. The positive control for specificity of immunostaining is made on spleen preparations. The number of the immune cells, positive for a given marker, is presented as a number of the positive cells out of the total number of stromal cells. After statistical processing, these are presented as median and range. Such an approach implies low values due to the relatively small proportion of the positive immune cells to the total proportion of the stromal cells. In this line, the operating with such low values requires precision in data processing, which the PhD student has tried to apply. The results obtained confirm that the immune component in the endometrium is an important player for embryonic implantation. Interesting data are obtained on the significant predominance of T cells *in situ* in the luteal endometrium of RIF women with failed implantation after embryo transfer. Since the remaining endometrial immune cells are comparable in both groups, the PhD student successfully set out to examine how the ratios between the immune cells correlate with the implantation success. She found that failed implantation is associated with significantly lower Tk/T, Th/T and MΦ/T ratios, and higher NK/Mon and T/Mon ratios. These immune ratios plus a few more that were at the border of significance (NK/T, Th/Tk, Th/MΦ) together with the amounts of total T cells and monocytes that are selected to be assessed as prognostic markers for embryo implantation by subjecting them to ROC analysis. High discriminative values of NK/T, Tk/T, Th/T, MF/T, Th/Tk and Th/MF ratios for assessing the success rate after embryo transfer are found. For instance, the MF/T ratio turned out to be an important indicator for improving endometrial receptivity, with a cut-off value with extremely high precision of 66.36%. A possible mechanism of interaction between immune cells in the endometrium during the implantation window in cases of successful or unsuccessful implantation is also systematized. The possibility of using these immune indicators to assess the endometrial receptivity and successful implantation after immunotherapy applied to women with RIF, is verified as well. When the autologous PBMC derived from RIF women and prepared for intrauterine application are immunophenotyped vs those from healthy women, no significant difference is found. However, a slightly unusual CD4/CD8 ratio of 1.44 is obtained in the blood, while the generally accepted one is 2. This study shows that after PBMC application and embryo transfer, the average success rate in RIF patients is 36.4% (4/11, Figure 6.5) and it is similar to that of RIF patients, regardless immunotherapy according to the CDC (The Center for Disease Control and Prevention, CDC, 2021), i.e. no promoting effect of cell treatment is found. On the other hand, however, this success rate is higher than the average success rate after a first IVF attempt in women between 35-40 years of age (age of the RIF women used in the dissertation), which is 26% (CDC, 2021). Although autologous PBMC treatment leads to an improvement in the endometrial immune environment before embryo transfer, it proves that the selected immune indicators of endometrial receptivity could not predict which RIF patients will have a positive effect from cell therapy and that the presence of RIF is not a sufficient reason to apply cell therapy.

The discussion is written in a fascinating way and shows the serious and in-depth knowledge of the PhD student. She skillfully discusses her own data on the immune profile of receptive endometrium associated with successful implantation against the background of data from other authors, visualizing her point of view on graphic abstracts. What made a great impression on me is that the doctoral student skillfully synthesizes her results and by adding an element of discussion, prepares informative graphic abstracts. These present the scientific findings on immune profiles in combination with hypothetical models for mechanisms of immune cell interactions. A hypothetical mechanism of immune interactions after treatment with autologous PBMCs is also described. It is commendable that the majority of the schemes in the figures in the dissertation were generally made by the doctoral student with the illustrator Biorender, and are not borrowed. Some of the limits of the present study are also defined. For me, it makes a very good impression when a doctoral student is aware of the limitations of his own research and is not afraid to point them out. This is evidence of his scientific maturity and a request to overcome these limits in the future studies. In every study, no matter how comprehensive it is, there are unexplored spots, the important thing is for the researcher to be aware of them.

Based on the results obtained, 6 conclusions are drawn. I accept the CONCLUSIONS formulated by the doctoral student with some very small remarks. In my opinion, the conclusions are artificially divided into conclusions from stage 1 and conclusions from stage 2. Conclusion 1 is confirmatory, but is slightly speculative without data from healthy controls in the present study.

6. Contribution and significance of the dissertation for the science and practice

The data on immune ratios as indicators of endometrial receptivity, established in the dissertation, could be used in clinical practice to assess the endometrial receptivity of in vitro patients with RIF. In this way, as the author suggests, once low receptivity and immune imbalance are established, efforts can be taken to treat this condition instead of performing new unsuccessful in vitro procedures, which adversely affects the patients both emotionally and financially. For the first time in Bulgaria, immune indicators have been derived for studying the effects of the application of systemic or local immune therapies on the endometrial immune profile and endometrial receptivity in in vitro patients with RIF. The effect of one of the most commonly used immunological treatments in in vitro patients – intrauterine application of autologous PBMCs – on both the endometrial immune profile and the implantation success of patients with RIF is studied.

7. Assessment of publications on the dissertation

There are 3 articles published on the dissertation, one in Q1 and one in Q2, with a total impact factor of 5.6, the third article is published in the Sofia University Yearbook. In two of the publications, the PhD student is the first author, in the third - second. These publications are an excellent attestation of the importance of the topic, the quality of the dissertation and the excellent scientific supervision. Rumiana Ganeva has been participated in 18 scientific forums reporting results from her dissertation - 4 reports as oral presentations and the rest as posters.

8. Personal participation of the PhD student, originality and authenticity of the dissertation

The dissertation is the personal work of the PhD student, the data are original and authentic, as proved by the plagiarism check and the report of her supervisor.

9. Abstract

The abstract, presented in Bulgarian and English fully meets the requirements regarding its content and structure. It comprehensively and accurately reflects the content of the dissertation. It is written on 58 pages.

10. Critical remarks and recommendations

I have a few critical remarks, some of which are purely technical or stylistic, others are incorrectly translated terms. In principle, CD8 T cells are defined as cytotoxic rather than killer cells as they are described in the dissertation. On p. 17 "a watery mucosa is produced", it should be mucus because mucosa (in latin) means mucous membrane; "the dominant follicle releases an egg", it is correct that it releases an oocyte. On p. 18 "progesterone prepares the corpus luteum and endometrium", it should be progesterone is released from the corpus luteum and prepares the endometrium; "if pregnancy occurs, the fertilized egg is implanted in the endometrium", it is correct to say that if fertilization occurs, the embryo (at the blastocyst stage) is implanted in the endometrium. On p. 20 “the embryo slides along the lumen of the endometrium” - the lumen is of the uterus, not of the endometrium, and the embryo is rather moved along the apical surface of the epithelial endometrial cells by the movements of their cilia. On p. 48 “adult monocytes”, it is more correct to say mature or differentiated monocytes. On p. 56 “anti-inflammatory factors such as IFNg TNFa”, they are pro-inflammatory factors. The same applies to IL-17, it is a pro-inflammatory factor. On p. 88 – preparation for immunohistochemical staining includes deparaffinization and hydration in addition to deparaffinization, p. 90 – usually histological sections are observed with an upright microscope, the inverted one is used for cultures, is it a technical error or are you really observing histological sections with an inverted microscope. In the Results and Discussion section: Table 4.2 and Figure 4.4, as well as Table 6.2. and Figure 6.1. contain the same data presented in two different ways – tabular and graphical. I think the correlation coefficient is misinterpreted as high at around 0.5, this is a moderate correlation coefficient. For me personally, the software-based immunohistochemical in situ cytometry is a very interesting approach for analysis. However, I think that it misses a very important population of immune cells, uterine NK cells with a CD56++ phenotype, which cannot be defined. The other thing I miss in the study is the comparison of the immune cells and ratios as predictors of endometrial receptivity between women with RIF and healthy women, although I realize how difficult it is to obtain biopsies from luteal endometrium from healthy women.

11. Personal Impressions

My personal impressions of Rumiana Ganeva are wonderful. An exceptionally well-mannered, warm, responsive and intelligent young woman. I have had the opportunity to see her in a working environment in Nadezhda Medical Center and am delighted with her professionalism, competence and responsibility.

12. Recommendations for future use of the results and contributions of this dissertation

I am convinced that Rumiana will use the data from her dissertation in the clinical practice of the Nadezhda Medical Center, considering that the current work is dictated precisely by the need to help the women with RIF. She points out the future application of the immune ratios as predictors of the endometrial receptivity as part of an algorithm for assessing the endometrial immune profile and the state of endometrial receptivity in the in vitro patients. These immune ratios could be used in studies investigating the effects of the immunological treatments in these patients or for the evaluation of already performed systemic or local therapy incl. immunotherapy. The use of immune indicators of endometrial receptivity, which show the different aspects of immune imbalance in each individual patient, is a step towards a personalized medicine, a term that we hear constantly lately. I would recommend comparing these immune indicators between RIF women and healthy women who conceived naturally, as well as between luteal and proliferative phase endometrium in RIF patients and healthy women. It would be interesting to try to improve the immunohistochemical cytometry by introducing a multiplex approach.

CONCLUSION: The PhD thesis submitted to me for review is an exellent and timely scientific work in an area of high clinical importance and strong social impact, namely human reproduction and in particular its ineffectiveness in women with recurrent implantation failures. The work has reasonably set goals and objectives, relevant methodology, logical structure and excellent execution. The dissertation manages to provide answers to most of the questions posed and open up new fields for fundamental and clinical research. The work meets all the requirements set in the ZRASRB and its Regulations, as well as the specific requirements of the Regulations of Sofia University "Kliment Ohridski". Based on everything said so far, my assessment is POSITIVE and I strongly recommend to the esteemed members of the scientific jury to award the educational and scientific degree "Doctor" to Rumyana Rumenova Ganeva in the scientific specialty "Cell Biology", professional field: 4.3. Biological Sciences, field of higher education: 4. Natural Sciences, Mathematics and Informatics.

08.07.2025 Sincerely yours

Sofia Tanya Dimova

Questions to the PhD student:

1. When assessing the dating of endometrial tissue by steroid receptor expression, have you tried to digitize the positive signal for a more objective assessment?

2. In the software-based quantitative in situ analysis, the results are presented as the number of positively stained immune cells for a given marker out of the total number of stromal cells. The cells for analysis are also defined by the diameter of the nucleus. What do you include in the term "stromal cells" – stromal cells per se (fibroblasts or decidual stromal cells, DSC), or cells in the stromal compartment - stromal cells plus negative immune cells.

3. Would you interpret the relatively higher correlation coefficient between NK (CD56+) cells and cytotoxic T-cells in women with RIF with successful implantation?

4. How would you explain the higher amount of T-lymphocytes in the endometrium of RIF women with failed implantation and lack of difference in the T-helper and T-cytotoxic subsets (Table 4.2)?

5. Intrauterine application of PBMC in RIF women leads to an increase of helper T-cells and, accordingly, the Th/T, Th/Tk and Th/MF ratios, which, as you show, leads to an improvement in the endometrial receptivity. Do you have a hypothesis how this could happen?

6. Did you find immune cell clusters in the endometrium of women with RIF? Many authors have suggested that there is a DALT, decidua-associated lymphoid tissue, similar to other mucosa-associated lymphoid systems? Immune clusters are the sites where the immune cells are activated and differentiated.