

IMMUNE CELLS INHIBITORY RECEPTORS AND ENDOMETRIOSIS

JOSÉ MANUEL LOURENÇO REIS

**A thesis submitted in fulfilment of the requirements for the Doctoral Degree
in Health Sciences in the specialty of Medicine**

December, 2024

IMMUNE CELLS INHIBITORY RECEPTORS AND ENDOMETRIOSIS

José Manuel Lourenço Reis

**Supervisor: Jorge Natalino Ramos Lima, MD, PhD,
Invited Assistant Professor,
NOVA Medical School, Universidade NOVA de Lisboa**

**Co-Supervisors: Luís Miguel Nabais Borrego, MD, PhD,
Associate Professor with Habilitation,
NOVA Medical School, Universidade NOVA de Lisboa**

**Ana Catarina Silva Gregório da Costa Martins, BSc. PhD,
Assistant Professor
NOVA Medical School, Universidade NOVA de Lisboa**

**A thesis submitted in fulfilment of the requirements for the Doctoral Degree in Health
Sciences in the specialty of Medicine**

December, 2024

Preface

“Endometrial tissue set free by menstruation, therefore, is sometimes not only alive but may actually continue to grow if transferred to situations favorable to its existence.”

Sampson JA. Metastatic or Embolic Endometriosis, due to the Menstrual Dissemination of Endometrial Tissue into the Venous Circulation. Am J Pathol. 1927 Mar;3(2):93-110.43.

Endometriosis is estimated to affect 10% of women of reproductive age and significantly impacts patients' quality of life. Despite extensive research, its etiopathogenesis remains unknown. The theory of retrograde menstruation, proposed in 1927, remains the most widely accepted explanation, though it leaves many questions unanswered.

The origin of endometriosis may involve multiple factors, and our research does not intend to fully unravel this complex puzzle. However, growing evidence suggests that immune dysfunction may play a role in the implantation and growth of endometriotic cells outside the uterine cavity, which could explain why 90% of women experience retrograde menstruation, yet only 10% develop the disease. Insights from studies on cancer cells' immune evasion mechanisms may also prove highly beneficial in unravelling the etiopathogenesis of endometriosis.

Medical therapy for endometriosis primarily relies on hormone-based drugs, which act as contraceptives and are unsuitable for women who wish to conceive. This poses a significant challenge, especially as endometriosis is highly prevalent among women of reproductive age. Additionally, some women cannot tolerate hormone therapy, have contraindications, or may develop resistance, leading to disease progression despite treatment. These issues highlight the need for new non-hormonal therapeutic options, with immunomodulation emerging as a promising area for research.

However, for this to become a reality, the mechanisms behind endometrial cells' ability to evade the immune system must be understood.

According to *Artigo 26º* from *Regulamento do ciclo de estudos conducente ao grau de Doutor em Ciências da Saúde (Regulamento n.º 1137/2022. Publicação: Diário da República, 2.ª série — N.º 227 — 24 de novembro de 2022)*, this includes references to scientific articles published by the doctoral student, whose content was used in full or in part in the preparation of the thesis:

- **Reis JL**, Rosa NN, Ângelo-Dias M, Martins C, Borrego LM, Lima J. Natural Killer Cell Receptors and Endometriosis: A Systematic Review. *Int J Mol Sci.* 2022 Dec 25;24(1):331. doi: 10.3390/ijms24010331. PMID: 36613776; PMCID: PMC9820702.
Impact Factor: 5.6; Q1
- **Lourenço Reis J**, Martins C, Ângelo-Dias M, Rosa NN, Borrego LM, Lima J. TIM-3 Expression in Endometriosis. *Am J Reprod Immunol.* 2024 Jun;91(6):e13887. doi: 10.1111/aji.13887. PMID: 38924299.
Impact Factor: 2.5; Q1
- **Reis JL**, Rosa NN, Martins C, Ângelo-Dias M, Borrego LM, Lima J. The Role of NK and T Cells in Endometriosis. *Int J Mol Sci.* 2024 Sep 21;25(18):10141. doi: 10.3390/ijms251810141. PMID: 39337624; PMCID: PMC11432446.
Impact Factor: 4.9; Q1
- **José Lourenço Reis**, Catarina Martins, Miguel Ângelo-Dias, Natacha Nurdine Rosa, Luís Miguel Borrego and Jorge Lima. PD-1 in Endometriosis: Uncovering Potential Links to Cancer Pathways. Submitted to *Journal of Reproductive Immunology* on October 6, 2024 (Manuscript number: JRI-D-24-00379)

According to the *alínea e)* of *Artigo 26º* from *Regulamento do ciclo de estudos conducente ao grau de Doutor em Ciências da Saúde (Regulamento n.º 1137/2022. Publicação: Diário da República, 2.ª série — N.º 227 — 24 de novembro de 2022)* this PhD research project was approved by *Comissão de Ética da NOVA Medical School/Faculdade de Ciências Médicas - UNL (autorização n.º 177/2024/CEFCM)*, by *Comissão de Ética para a Saúde do HBA (autorização n.º 514_LH n.º 209)* and by *Comissão de Ética para a Saúde do Hospital da Luz (CES/53/2021/ME)*.

Part of the results obtained were presented at the following scientific meetings:

- **José Reis**, Miguel Ângelo-Dias, Catarina Martins, Luís Miguel Borrego, Jorge Lima. Natural killer cells inhibitory receptors and endometriosis. 1.º Congresso de Investigação do Grupo Luz Saúde. May 20, 2022; Lisbon, Portugal. [Oral Communication]
- **José Lourenço Reis**, Catarina Martins, Miguel Ângelo-Dias, Natacha Nurdine Rosa, Luís Miguel Borrego and Jorge Lima. The role of PD-1 and TIM-3 inhibitory receptors in endometriosis. Hospital da Luz Research Congress | 3rd Edition. May 20, 2024; Lisbon, Portugal. [Oral Communication]
- Catarina Martins, **José Lourenço Reis**, Miguel-Ângelo Dias, Luis Miguel Borrego, Jorge Ramos Lima. Using flow cytometry to uncover circulating and peritoneal NK cell profiles in endometriotic women. XVIII Congress of the Iberian Society of Cytometry (SIC 2023). April 19-21, 2023; Madrid, Spain. [Poster]
- **José Reis**, Miguel Ângelo-Dias, Catarina Martins, Luís Miguel Borrego, Jorge Lima. Endometriose & recetores inibidores das células natural killer. XV Congresso Português de Ginecologia. Jun 2-4, 2022; Estoril, Portugal. [Poster]
- **José Reis**, Miguel Ângelo-Dias, Catarina Martins, Luís Miguel Borrego, Jorge Lima. NK cells in endometriosis - could basic research open new therapeutic tools? European Society for Gynaecological Endoscopy (ESGE) 32nd Annual Congress. Oct 1-3, 2023; Brussels, Belgium. [Poster and selected for Oral Communication]

Awarded the Prize for Best ePoster Presentation

- **José Lourenço Reis**, Catarina Martins, Miguel Ângelo-Dias, Luís Miguel Borrego and Jorge Lima. Immune cells, inhibitory receptors and endometriosis. The 1st NMS Research. Oct 16-20; 2023; Lisboa, Portugal. [Oral Communication]

A friend once asked me, “What are you going to do with this PhD?”
Truthfully, I don’t know—perhaps nothing, perhaps everything.
But one thing is certain: our research has advanced knowledge of endometriosis.

Acknowledgements

First and foremost, I would like to express my profound gratitude to all the patients who participated in this study. Their contribution will have an impact on the future of endometriosis care, and without their support, this research would not have been possible.

My sincere thanks go to Dr. Fátima Faustino and Dr. Carlos Veríssimo, with whom my journey in treating endometriosis patients began 12 years ago at Hospital Beatriz Ângelo. I owe them immense gratitude for their unwavering support on a path that has not always been easy.

I am also grateful to my colleagues, Dr. Bruno Nogueira, Dr. Duarte Vilarinho, and Dr. Inês Rodrigues, who work alongside me every day to care for these patients as a dedicated team.

I extend my heartfelt thanks to my colleagues from other specialties and the nurses who worked directly with me: Dr. Luís Féria, Dr. Pedro Amado, Nurse Ana Branquinho, Dr. José Bismarck, Dr. Paulo Roquete, Dr. César Resende, Dr. Bruno Graça, Nurse Ana Teresa, and Nurse Sara Ferreira.

To the Gynaecology Departments of Hospital Beatriz Ângelo and Hospital da Luz, and all the outstanding colleagues within them, I express my gratitude for their dedication to caring for endometriosis patients, often tackling complex cases. Their open communication and cross-departmental collaboration enable seamless patient care.

I would also like to express my appreciation to Professor Luís Borrego and Professor Catarina Martins, from the Immunology Department of NOVA Medical School (Faculdade de Ciências Médicas), for their supervision, tireless commitment, and accessibility. This project belongs to them as much as me, and its success would not have been possible without their involvement.

I am also grateful to Dr. Miguel Ângelo-Dias, whose support with immunological sample analysis and guidance in addressing uncertainties has been invaluable. Additionally, I would like to thank Dr. Natacha Nurdine Rosa for her collaboration in writing and editing the manuscripts.

A special thanks to Dr. Jorge Gonçalves and Dr. Sofia Nunes for their scientific support.

I am grateful to GLSMED Learning Health for their financial support and to the Administration of Hospital da Luz for enabling this research.

I owe a great deal to my family for their constant support and understanding. I would like to thank my parents for instilling core values in me, Patrícia for being my foundation throughout these challenging 5 years, and my daughters Mafalda and Madalena for their daily love and encouragement, which fuel my life.

Finally, I wish to express my heartfelt gratitude to my supervisor, Professor Jorge Lima, for his wisdom, rigor, and invaluable insights. His belief in me and this project from the very beginning, as well as his friendship, motivation, and encouragement, has been a constant source of strength.

Resumo

A endometriose é uma doença inflamatória crónica caracterizada pela implantação e crescimento de células endometriais fora do útero. A investigação tem apontado de forma recorrente para a disfunção imunitária como um fator crucial na fisiopatologia da endometriose, nomeadamente na interação entre as vias imunitárias inata e adaptativa. Tal disfunção permite que as células endometriais escapem à vigilância imunológica e persistam na cavidade peritoneal. Em condições normais, macrófagos, células Natural Killer (NK) e linfócitos T colaboram para eliminar estas células; contudo, as mulheres com endometriose apresentam frequentemente uma vigilância imunológica deficiente, o que permite a implantação e proliferação de células endometriais ectópicas. Este desequilíbrio imunitário suporta a hipótese de que as lesões endometrióticas sobrevivem e proliferam ao escapar aos mecanismos de controlo do sistema imunitário.

Neste trabalho, analisámos os perfis das células imunitárias no líquido peritoneal (LP) e no sangue periférico (SP) para investigar as diferenças nas células NK e linfócitos T entre doentes com endometriose e o grupo controlo. Explorámos ainda, de que forma determinados recetores inibidores das células imunológicas, nomeadamente o *Programmed cell death protein 1* (PD-1) e o *T-cell immunoglobulin and mucin domain 3* (TIM-3), podem contribuir para a disfunção imunitária na endometriose. Tais desequilíbrios podem comprometer a eliminação eficaz das células endometriais ectópicas e promover um estado inflamatório crónico.

A investigação incluiu 62 mulheres recrutadas ao longo de 2 anos, das quais 47 foram diagnosticadas com endometriose e 15 constituíram o grupo controlo, intervencionadas por outra patologia benigna ginecológica que não endometriose. Todas as pacientes foram submetidas a cirurgia laparoscópica neste intervalo de tempo, com confirmação histológica da endometriose no grupo das doentes. Recolheram-se dados demográficos e clínicos e classificou-se a severidade da doença.

Foram obtidas amostras de SP e LP de todas as participantes e realizou-se imunofenotipagem celular por citometria de fluxo nos dois tipos de amostras, para caracterizar subpopulações de células imunitárias. O painel de anticorpos monoclonais permitiu uma análise detalhada das células NK e T presentes nas amostras de SP e LP.

A análise no SP revelou um aumento de células NK CD56^{Hi}CD16^{Dim} nas doentes com endometriose, sugerindo uma redução da citotoxicidade das células NK sistémicas, já que as células NK CD56^{Hi}CD16^{Dim} são conhecidas pelo seu menor potencial citotóxico. Quanto às células T, o tabagismo revelou-se um fator de estilo de vida com impacto nos perfis imunitários circulantes em doentes com endometriose. A percentagem de células T CD4⁺CD16⁺ foi significativamente menor

em fumadoras do que em não fumadoras, sugerindo que o tabagismo pode prejudicar a ativação das células T, promovendo imunossupressão e evasão imunológica nessas pacientes.

Ao analisar os recetores inibidores das células imunológicas, investigámos o TIM-3, um importante recetor inibidor, amplamente estudado em oncologia e em doenças autoimunes. A interação entre o TIM-3 e os seus ligandos é fundamental para a tolerância imunitária. Numa resposta imunológica saudável, a ligação do TIM-3 aos seus ligandos bloqueia as células T e promove a tolerância periférica. Contudo, em condições patológicas como o cancro, uma elevada expressão de TIM-3 pode induzir a anergia das células T, suprimindo a atividade imunitária e permitindo a progressão das células tumorais. Por outro lado, uma baixa expressão de TIM-3 pode permitir que células T escapem à regulação negativa e se tornem autoreativas, contribuindo para um estado de inflamação crónica, como observado no lúpus eritematoso sistémico ou na esclerose múltipla.

A avaliação efetuada mostrou uma redução significativa na proporção de células T CD56⁺ circulantes que expressam TIM-3 nas doentes com endometriose, comparativamente ao grupo controlo. Especificamente, mulheres com endometriose intestinal, uma forma mais grave da doença, apresentaram níveis significativamente mais baixos de células T CD56⁺ TIM-3⁺. Em estádios mais avançados, a percentagem de células T CD56⁺ que expressam TIM-3 também diminuiu, nomeadamente nos estádios III e IV. Estas alterações imunitárias podem contribuir para a progressão da doença, caracterizada por inflamação persistente e fibrose, uma vez que células T com expressão reduzida de TIM-3 se tornam autoreativas. A alteração na expressão de TIM-3 nas células T CD56⁺ é particularmente importante, já que estas células possuem funções imunoestimuladoras, incluindo a produção de citocinas Th1 e um elevado potencial citotóxico.

Relativamente à análise dos recetores inibidores, observámos ainda um aumento significativo de células NK CD56⁺CD16⁺ que expressam PD-1 no LP das doentes com endometriose, em comparação com o grupo controlo. Este aumento é relevante, já que as células NK desempenham um papel essencial na defesa imunitária inicial neste microambiente, onde as células endometrióticas se tentam implantar e proliferar, principalmente na cavidade pélvica. Este aumento específico afeta as células NK CD56⁺CD16⁺, potentes mediadoras da citotoxicidade celular dependente de anticorpos, além das suas funções citotóxicas intrínsecas.

Entre as fumadoras com endometriose, verificou-se também uma elevada percentagem de células NK CD56⁺CD16⁺ PD-1⁺ no LP, o que pode estar na base de uma dificuldade no controlo da progressão da doença nestas pacientes.

A disquesia, frequentemente associada à invasão intestinal, correlacionou-se com níveis mais elevados de células T PD-1⁺ no LP. Em doentes com envolvimento do trato urinário, observou-se um aumento acentuado do total de células NK PD-1⁺ no LP, bem como níveis elevados de células T CD8⁺

PD-1⁺, T CD56⁺ PD-1⁺ e T PD-1⁺ totais em circulação. Estes resultados indicam que a expressão aumentada de PD-1 nas células imunológicas está associada a casos de endometriose mais graves, sugerindo que o papel do PD-1 como recetor inibidor pode contribuir para a disfunção das células NK e T, aumentando a possibilidade de escape imunológico.

Atualmente, a terapêutica da endometriose é predominantemente hormonal e, como tal, tem efeito contraceptivo, o que a torna inadequada para mulheres que desejam engravidar. Além disso, muitas pacientes não toleram ou desenvolvem resistência a esta terapêutica, levando à progressão da doença. A disfunção observada nos recetores imunológicos, especificamente o PD-1 e o TIM-3, sugere novos potenciais alvos para tratamentos não hormonais. A elevada expressão de PD-1 em pacientes sintomáticas e em estádios avançados sugere vias partilhadas com o cancro, onde inibidores de PD-1 já são usados para potenciar a imunidade antitumoral. Futuras investigações devem incluir ensaios funcionais para verificar os níveis de atividade celular, além de análises de citotoxicidade, respostas de citocinas e influência de fatores genéticos. O futuro da terapia para endometriose pode estar na imunomodulação; a investigação pré-clínica já começou, mas será essencial explorar quais as vias específicas que podem ser alvo de intervenção.

Abstract

Endometriosis is a chronic inflammatory condition where endometrial tissue grows outside the uterus. Recent research increasingly points to immune dysregulation as a substantial factor in endometriosis pathophysiology, particularly regarding the interaction between innate and adaptive immune pathways. This dysfunction allows endometrial cells to avoid immune detection and persist within the peritoneal cavity. Normally, immune cells such as macrophages, NK cells, and cytotoxic T lymphocytes work to eliminate these cells. However, women with endometriosis often exhibit altered immune surveillance, which allows ectopic cells to implant and grow. This immune irregularity supports the theory that endometriotic lesions survive and proliferate by escaping immune detection.

Our study examined immune cell profiles in peritoneal fluid (PF) and peripheral blood (PB), investigating NK and T-cell variations between endometriosis patients and controls. We also probed into how immune checkpoint markers like PD-1 and TIM-3 might contribute to immune dysfunction in endometriosis, suggesting that similar mechanisms to those observed in cancer might be engaged. Such imbalances could inhibit the effective clearance of ectopic cells, thereby preserving a chronic inflammatory condition.

The study consisted of 62 women, with 47 diagnosed with endometriosis and 15 serving as a control group not suffering from the disease, all recruited over 2 years. All participants underwent laparoscopic surgery within this time frame. The patient group had histological confirmation of endometriosis, while the control group underwent interventions for other benign conditions. We collected demographic and clinical data and classified the severity of the disease.

We collected PB and PF samples from all participants and conducted immunophenotyping on both types of samples using flow cytometry to characterize immune cell subsets. A comprehensive immunophenotyping panel of monoclonal antibodies facilitated a detailed NK and T-cell analysis in the PB and PF samples.

Our analysis showed an uptick in circulating CD56^{Hi}CD16^{Dim} NK, indicating a possible decrease in systemic NK cell cytotoxicity compared to the general population. CD56^{Hi}CD16^{Dim} NK cells are recognized for their diminished cytotoxic potential. With regards to T cells, it surfaced that smoking status is a lifestyle factor influencing circulating immune profiles among endometriosis patients.

Our results showed that CD16⁺ CD4 T cells were significantly lower in smokers than in non-smokers, suggesting that smoking may impair T-cell activation, thereby contributing to immunosuppression and immune evasion in these patients.

In our examination of the inhibitory receptors of immune cells, we scrutinized TIM-3, a significant immune checkpoint inhibitor heavily researched in oncology and autoimmune disease research. The interaction between TIM-3 and its ligands is vital for inducing peripheral tolerance. In a healthy immune response, the TIM-3 ligand likely aids in blocking effector T cells and promoting peripheral tolerance. On the other hand, in pathological conditions like cancer, high TIM-3 expression can lead to T-cell anergy, suppressing immune activity and permitting cancer cells to proliferate. Conversely, low TIM-3 expression may enable autoreactive and pathogenic T cells to evade negative regulation, contributing to chronic inflammation as observed in autoimmune diseases such as systemic lupus erythematosus and multiple sclerosis.

Our research demonstrated a substantial decrease in the proportion of circulating CD56⁺ T cells expressing TIM-3 in patients with endometriosis compared to controls. Notably, women suffering from bowel endometriosis, a more severe form of the disease, exhibited significantly lower levels of CD56⁺ T cells expressing TIM-3.

Furthermore, as endometriosis advanced in severity, the percentage of circulating CD56⁺ T cells expressing TIM-3 decreased significantly, particularly in stages III and IV.

These immune changes may contribute to disease progression, characterized by persistent inflammation and fibrosis, as T cells with reduced TIM-3 expression can become autoreactive. The change in TIM-3 expression on CD56⁺ T cells is particularly important. This is because these CD56-expressing cells have robust immunostimulatory functions, such as producing cytokines and possessing significant cytotoxic potential.

In our analysis of inhibitory receptors, we observed a significant increase in CD56⁺CD16⁺ NK cells expressing PD-1 in the PF of endometriosis patients compared to the control group. This increase in PD-1-expressing NK cells in the PF is significant because these cells play a critical role in the initial immune response within this microenvironment where endometriotic cells attempt to implant and spread, especially within the pelvic cavity. This elevation impacts CD56⁺CD16⁺ NK cells known for their potent antibody-dependent cellular cytotoxicity mediation, complementing their intrinsic cytotoxic functions.

Additionally, there was also an elevated presence of PF PD-1⁺ CD16⁺CD56⁺ NK cells among smokers with endometriosis, suggesting potential difficulty in controlling disease progression.

Dyschezia, often linked with bowel involvement, has been correlated with higher levels of PD-1⁺ total T cells in the PF. In endometriosis patients dealing with urinary tract involvement, there was a significant increase in PD-1⁺ total Natural Killer (NK) cells in the PF. Additionally, increased circulating levels of PD-1⁺ CD8⁺ T cells, PD-1⁺ CD56⁺ T cells, and PD-1⁺ total T cells were observed.

These findings suggest that heightened PD-1 expression in immune cells may correspond to more severe or symptomatic endometriosis, implying that PD-1's function as an inhibitory receptor could contribute to NK and T-cell dysfunction, thus promoting immune evasion.

Endometriosis' current treatments lean heavily on hormone-based therapies, which have contraceptive effects. Consequently, these treatments are unsuitable for women aspiring to conceive. Moreover, numerous patients cannot tolerate hormone therapy, or they develop resistance, which leads to disease progression. The apparent dysregulation of immune checkpoints, specifically PD-1 and TIM-3, implies new targets for non-hormonal treatments. The elevated expression of PD-1 in symptomatic patients, especially in advanced stages, may indicate pathways that overlap with cancer, where targeting immune checkpoints is an established approach to boost anti-tumor immunity.

Future research should incorporate functional assays to confirm cell activity levels, along with analyses of cytotoxicity, cytokine responses, and the part that genetic factors play. The future of treatment may reside in immunomodulation. In fact, this future is already underway, as animal studies in this field are currently in progress. For progress, it is crucial to investigate the pathways affected by endometriosis and identify potential targets for intervention.

Table of Contents

Preface	3
Acknowledgements	7
Resumo	9
Abstract	12
Thesis Outline.....	20
CHAPTER I General Introduction	22
1. Overview of endometriosis	23
2. Overview of the immune system	26
2.1 Innate immunity	26
2.2 Adaptive immunity	27
2.3 Immune system coordination	29
3. Endometriosis and the immune system	30
3.1 The immune cells in endometriosis	31
3.1.1 Innate immune system and endometriosis	31
3.1.2 Adaptive immune system and endometriosis	32
3.1.3 The immune system in endometriosis – Future perspectives	34
CHAPTER II Objectives	35
1. General objective	36
2. Specific objectives	36
CHAPTER III Natural Killer Cell Receptors and Endometriosis: A Systematic Review	37
CHAPTER IV The Role of NK and T Cells in Endometriosis	54
CHAPTER V TIM-3 Expression in Endometriosis	79
CHAPTER VI PD-1 in Endometriosis: Uncovering Potential Links to Cancer Pathways	90
CHAPTER VII General Discussion.....	106
1. Strengths and limitations	111
2. Final remarks and future perspectives	112
REFERENCES	114

List of Figures

Figure 1. Potential pathways for the pathogenesis and pathophysiological features of pelvic endometriosis	25
Figure 2. Cells of the innate and adaptive immune system.....	26
Figure 3. Immune system coordination.....	30
Figure 4. Diagrammatic representation of endometriosis and proposed etiology	39
Figure 5. PRISMA flowchart	39
Figure 6. Differences in lymphocytes and lymphocyte subsets of PB and PF samples between healthy controls and endometriosis (EM) patients divided according to race.	59
Figure 7. Differences in lymphocyte subsets of PB between healthy controls and endometriosis (EM) patients divided according to smoking habits, namely, peripheral blood (A) CD16 ⁺ CD4 T cells and (B) CD16 ^{Hi} NK cells/CD56 ^{Hi} NK cells ratios	60
Figure 8. Differences in lymphocyte subsets of PB and PF between healthy controls and endometriosis (EM) patients divided according to the presence of bowel EM.	60
Figure 9. Differences in peripheral blood lymphocyte subsets between healthy controls and endometriosis (EM) patients divided according to the use of hormonal therapy.	61
Figure 10. Differences in lymphocyte subsets of PB and PF between healthy controls and endometriosis (EM) patients divided according to disease severity.	62
Figure 11. Differences in lymphocyte subsets of PB and PF between healthy controls and endometriosis (EM) patients divided according to the presence and severity of dysmenorrhea (A–C), dyspareunia (D, E), and chronic pelvic pain (CPP) symptoms (F–H).	63
Figure 12. Differences in lymphocyte subsets of PB and PF between healthy controls and endometriosis (EM) patients divided according to the presence of rectorrhagia.....	64
Figure 13. Differences in lymphocyte subsets of PB and PF between healthy controls and endometriosis (EM) patients according to the presence and severity of dyschezia symptoms.	65
Figure 14. Gating strategy for lymphocyte subset characterization and marker expression in PB and PF samples. The maturation profile of NK cells was assessed according to the expression of NKG2A, KIR2DL1/CD158a, and CD57.....	73
Figure 15. Gating strategy used to characterize lymphocyte subsets	83
Figure 16. TIM-3 expression in T-cell subsets, both in PB and PF samples, in the control group and women with endometriosis.	85
Figure 17. Variation of TIM-3 expression in circulating CD56 ⁺ T-cell subsets, according to different clinical conditions.	85

Figure 18. Gating strategy used to characterize PD-1 in lymphocyte subsets of peripheral blood and peritoneal fluid samples	94
Figure 19. PD-1 expressing T cells in PF and PB samples between EM and controls	97
Figure 20. PD-1 expression in peritoneal cells according to weight and smoking habits	98
Figure 21. PD-1 expression in peritoneal cells according to fertility status.....	99

List of Tables

Table 1. Summary of methods of selected studies regarding NK cell activating/ inhibiting receptors in endometriosis	41
Table 2. Demographic characteristics	57
Table 3. Disease characteristics.....	57
Table 4. Demographic and clinical characteristics of control group and endometriosis	83
Table 5. Endometriosis characteristics of the included patients	84
Table 6. Demographic and clinical characteristics of control and women with endometriosis	95
Table 7. Endometriosis characteristics of the included patients	96

List of Abbreviations, Acronyms and Symbols

- Negative antigen expression
- + Positive antigen expression
- APC** Antigen-presenting cells
- BMI** Body mass index
- CAR** Chimeric antigen receptor
- CTLA-4** Cytotoxic T-lymphocyte-associated protein 4
- DC** Dendritic cells
- Dim** Low or dim antigen expression intensity
- DNA** Deoxyribonucleic acid
- GnRH** Gonadotropin-releasing hormone
- Hi** High or bright antigen expression intensity
- IFN** Interferon
- IL** Interleukin
- LAG-3** Lymphocyte activation gene 3
- LP** Líquido peritoneal
- MDSCs** Myeloid-derived suppressor cells
- MHC** Major histocompatibility complex
- NK** Natural killer cell
- PB** Peripheral blood
- PD-1** Programmed cell death protein 1
- PF** Peritoneal fluid
- SP** Sangue periférico
- TGF** Transforming growth factor
- Th** T-helper cell
- TIGIT** T-cell immunoreceptor with Ig and ITIM domains
- TIM-3** T-cell immunoglobulin and mucin domain 3
- TNF** Tumor necrosis factor
- Treg** Regulatory T cell
- VEGF** Vascular endothelial growth factor

Thesis Outline

Chapter I provides a general introduction to the current understanding of endometriosis and its relationship with the immune system, exploring their connection and offering future perspectives.

Chapter II presents the main objectives of this thesis.

The subsequent chapters present four studies on which this PhD thesis is based. Each chapter consists of the content of a manuscript that has been published or submitted for publication, subdivided into main sections of abstract, introduction, materials and methods, results, discussion, and conclusion.

Chapter III presents a systematic review of natural killer cell receptors and their association with endometriosis.

- **Reis JL**, Rosa NN, Ângelo-Dias M, Martins C, Borrego LM, Lima J. Natural Killer Cell Receptors and Endometriosis: A Systematic Review. *Int J Mol Sci.* 2022 Dec 25;24(1):331. doi: 10.3390/ijms24010331. PMID: 36613776; PMCID: PMC9820702.

Chapter IV characterizes NK and T cells in the peripheral blood and peritoneal fluid of women with endometriosis compared to a control group, exploring the impact of demographic factors and symptoms on immune profiles.

- **Reis JL**, Rosa NN, Martins C, Ângelo-Dias M, Borrego LM, Lima J. The Role of NK and T Cells in Endometriosis. *Int J Mol Sci.* 2024 Sep 21; 25(18):10141. doi: 10.3390/ijms251810141. PMID: 39337624; PMCID: PMC11432446.

Chapter V examines the role of TIM-3 in endometriosis patients, by investigating its expression as an immune checkpoint in NK and T cells, within the peripheral blood and peritoneal fluid of both endometriosis patients and controls. It further analyzes the relationship between TIM-3 expression and demographic factors, symptoms, and disease severity.

- **Lourenço Reis J**, Martins C, Ângelo-Dias M, Rosa NN, Borrego LM, Lima J. TIM-3 Expression in Endometriosis. *Am J Reprod Immunol.* 2024 Jun; 91(6): e13887. doi: 10.1111/aji.13887. PMID: 38924299.

Chapter VI explores the mechanisms by which endometriotic cells may evade the immune system, focusing on PD-1 expression in immune cells from both peripheral blood and peritoneal fluid in women with endometriosis, and identifying potential links between endometriosis and cancer pathways.

- **José Lourenço Reis**, Catarina Martins, Miguel Ângelo-Dias, Natacha Nurdine Rosa, Luís Miguel Borrego and Jorge Lima. PD-1 in Endometriosis: Uncovering Potential Links to

Cancer Pathways. Submitted to Journal of Reproductive Immunology on October 6, 2024
(Manuscript number: JRI-D-24-00379).

Chapter VII presents a comprehensive discussion of this work, including its strengths and limitations, final remarks, and future perspectives.

CHAPTER I

General Introduction

1. Overview of endometriosis

Endometriosis is a chronic, estrogen-dependent inflammatory condition characterized by the presence of endometrial glands and stroma outside the uterus¹. These ectopic endometrial sites undergo cyclic growth and degradation in response to hormonal stimulation, resembling the activity of the uterine lining². Endometriosis lesions are split into three types: superficial peritoneal endometriosis, ovarian endometrioma, and deep infiltrative endometriosis, defined as those that penetrate more than 5 mm into the peritoneum or those that invade the muscle layer of hollow organs³. The lesions undergo cyclical bleeding and trigger an inflammatory response. With each menstrual cycle, hormonal fluctuations – primarily in estrogen and progesterone – reactivate the endometriotic foci, which in turn leads to lesion transformation, tissue repair, inflammation, angiogenesis, and local neurogenesis. This instigates a series of events that encompass epithelial and mesenchymal changes, fibroblast-to-myofibroblast differentiation, collagen production by ectopic endometrial cells, progressive smooth muscle fiber metaplasia, and fibrosis^{4,5}. This cycle culminates in local inflammation and pain, potentially causing structural and functional changes in affected organs due to scar tissue and adhesions¹.

The condition is estimated to affect approximately 10% of women of reproductive age⁶ and accounts for nearly 50% of infertility cases⁷. Typically, it is diagnosed between the ages of 30 and 40, although diagnostic delays are common due to the non-specific nature of many symptoms^{8,9}. Key clinical features include dysmenorrhea, dyspareunia, and infertility¹⁰. However, as it is a chronic and often debilitating disease, endometriosis impacts many facets of a woman's life, including work, school, sexual health, family, social interactions, and emotional well-being¹¹⁻¹³.

Despite its identification over 160 years ago¹⁴, there are still significant gaps in our understanding of its etiopathogenesis. The disease is believed to stem from a complex interplay of endocrine, immune, pro-inflammatory, and pro-angiogenic factors¹⁰. Multiple theories attempting to explain its origins have been proposed (Figure 1):

1) Sampson's Theory: Retrograde Menstruation

The earliest retrograde menstruation theory, introduced in 1927¹⁵, is widely regarded as the primary explanation for endometriosis. It suggests that during menstruation, small fragments of endometrial tissue flow backward through the fallopian tubes into the peritoneal cavity, potentially forming ectopic endometrial implants¹⁵. However, this theory alone does not fully explain the development of endometriosis, as retrograde menstruation is common in 90% of women^{16,17}, yet only 10% of them develop the disease.

2) Vascular and Lymphatic Metastasis

While Sampson's theory explains the spread of endometrial tissue via retrograde menstruation, it does not account for the endometriotic tissue found outside the peritoneal cavity. The vascular and lymphatic dissemination theory suggests that endometrial tissue may traverse the lymphatic system, allowing it to establish in distant sites, like the pleura or pericardium¹⁸.

3) Coelomic Metaplasia

The Coelomic Metaplasia Theory suggests that cells originating from the primitive peritoneum can transform into endometrial tissue¹⁹. This theory offers an explanation for instances of endometriosis in individuals who do not menstruate, such as men undergoing estrogen therapy for prostate cancer, or girls who have not yet reached menarche.

4) Stem Cell Theory

Stem cells, possessing the ability to self-renew and differentiate into various cell types, are present in endometrial tissue²⁰. According to this theory, stem cells in the basalis layer of the endometrium might travel to ectopic sites through retrograde menstruation, lymphatic or vascular routes, or direct migration, thereby leading to the formation of endometriotic lesions outside of the peritoneal cavity²¹.

5) Genetic/Epigenetic Theory

The genetic/epigenetic theory is a more recent approach to understanding endometriosis, proposing that genetic and epigenetic changes are essential for disease development²². Endometriosis has a heritable component, with heritability estimated at up to 50%, including 26% from common genetic variations²³. This theory suggests that genetic and epigenetic modifications affecting cellular processes may promote changes that contribute to the development of endometriosis²².

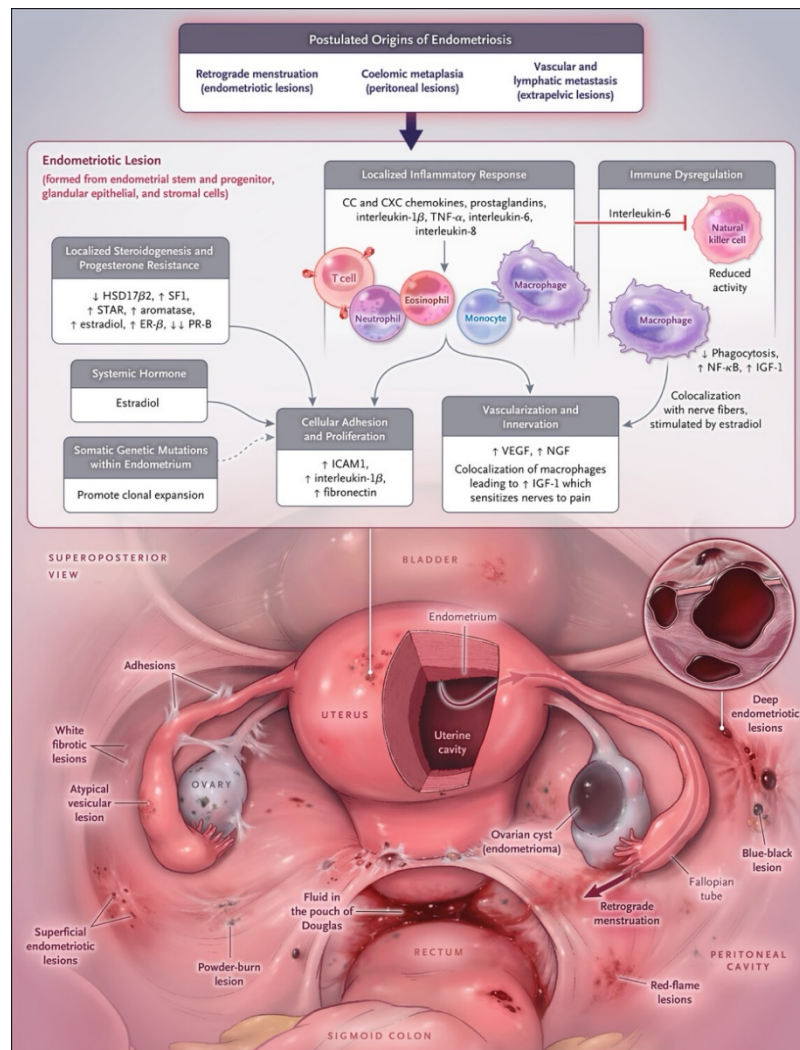


Figure 1. Potential pathways for the pathogenesis and pathophysiological features of pelvic endometriosis

ER, estrogen receptor; HSD17β2, 17β-hydroxysteroid dehydrogenase 2; ICAM, intercellular adhesion molecule; IGF, insulin-like growth factor; NF-κB, nuclear factor κB; NGF, nerve growth factor; PR, progesterone receptor; SF1, steroidogenic factor; STAR, steroidogenic acute regulatory protein; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor. Adapted from Zondervan KT et al, 2020.

The primary goal of treatment is to control the growth of ectopic endometrial tissue, thus reducing bleeding and its subsequent effects. These effects include pain, infertility, and the impairment of non-gynecological organ functions, which are the most severe manifestations. Unless surgery becomes necessary, such as in cases of suspected malignancy, unmanageable symptoms, organ invasion, or specific infertility treatments, medical therapy should be prioritized. The primary treatment options for endometriosis comprise progestins, estroprogestins, and GnRH antagonists.

One significant challenge with medical therapy is that these drugs are often hormone-based and contraceptive, rendering them unsuitable for women seeking to conceive – a critical issue considering the high prevalence of endometriosis among women dealing with infertility, most of whom receive the diagnosis during their reproductive years. Furthermore, some women cannot

tolerate hormone therapy or have contraindications, while others may exhibit resistance, leading to disease progression despite ongoing treatment. These factors underscore the urgent necessity to explore new non-hormonal therapeutic options²⁴⁻³⁰.

2. Overview of the immune system

The immune system is a complex network of cells, tissues, and organs that work in unison to defend the body against foreign pathogens, including bacteria, viruses, fungi, and parasites. This system operates through a multifaceted interaction of components that identify and neutralize harmful agents, thus aiding in protecting the body from infections and diseases. The immune system is composed of two primary branches: innate and adaptive immunity. Each branch has unique roles and strategies for defending against potential threats³¹.

2.1 Innate immunity

The innate immune system serves as the body's first line of defense against a wide range of pathogens. Unlike adaptive immunity, which relies on precise recognition and memory of antigens, innate immunity is non-specific and fast-acting, getting activated within hours of pathogen invasion. Central to this rapid response are specialized cells, such as macrophages, neutrophils, NK cells, dendritic cells, and eosinophils. Each of these cells performs distinct yet complementary functions³¹ (Figure 2).

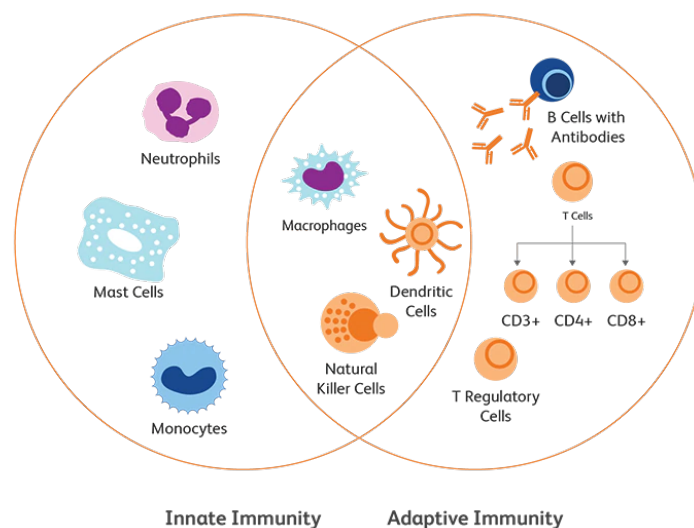


Figure 2. Cells of the innate and adaptive immune system

Adapted from: <https://www.bdbiosciences.com/en-au/learn/research/immunology>.

Macrophages serve as first responders, recognizing pathogens through pattern recognition receptors, engaging in phagocytosis to engulf them, and presenting antigens to stimulate adaptive immunity. Additionally, they release cytokines to recruit other immune cells^{32,33}. Quickly mobilized by cytokines and chemokines, neutrophils destroy pathogens using antimicrobial peptides, enzymes, reactive oxygen species, and neutrophil extracellular traps. As they are short-lived and undergo apoptosis after responding, this limits the extent of tissue damage³⁴⁻³⁶.

NK cells are specialized lymphocytes of the innate immune system, renowned for their ability to target and eradicate virus-infected cells and certain types of tumor cells without prior sensitization. Unlike other lymphocytes in adaptive immunity, NK cells do not require specific antigen recognition. Instead, they identify stressed cells lacking MHC class I molecules, a common feature of infected or malignant cells³⁷. The ability of NK cells to recognize abnormal cells without prior exposure makes them paramount for controlling infections and malignancies at early stages³⁸. NK cells discharge their cytotoxic function by expelling perforins and granzymes, which create pores in the target cell membrane and initiate apoptosis. Additionally, they discharge cytokines such as interferon-gamma (IFN- γ), which augment the immune response by stimulating macrophages and other immune cells³⁹.

Dendritic cells bridge innate and adaptive immunity by sampling pathogens in peripheral tissues. They then migrate to lymphoid organs and present antigens to T cells, initiating adaptive responses⁴⁰. Eosinophils fight parasitic infections by releasing cytotoxic granules that are activated by cytokines such as IL-5. Basophils release histamine to increase vascular permeability and recruit immune cells. These cells play significant roles in allergic responses⁴¹⁻⁴³. Mast cells are located at barrier sites, and they release histamine and inflammatory mediators to enhance blood flow. These cells also recruit more immune cells, thereby serving critical roles in early pathogen detection and allergic reactions⁴⁴. Combined, these cells coordinate a swift and effective immune response to pathogens.

2.2 Adaptive immunity

The adaptive immune system is responsible for the body's targeted response to pathogens, distinguishing it from the immediate, non-specific actions of innate immunity. This system provides long-term protection through a process known as immunological memory, which enables it to respond more rapidly upon re-exposure to a previously encountered pathogen. The cells of adaptive immunity, primarily B lymphocytes (B cells) and T lymphocytes (T cells) are specialized to recognize specific antigens and generate a tailored response³¹ (Figure 2).

B cells have receptors that recognize specific antigens present on the surface of pathogens. When a B cell encounters an antigen that matches its receptor, it becomes activated with the help of T-helper cells, leading to its proliferation and differentiation into two main types of cells: plasma cells and memory B cells⁴⁵. Plasma cells secrete large amounts of antibodies, specifically designed to bind to the pathogen's antigens. This binding either neutralizes the pathogen directly or marks it for destruction by other immune cells. Each antibody is specific to a single antigen, enabling precise targeting⁴⁶. Memory B cells, on the other hand, do not immediately participate in fighting the current infection but remain in the body for years, ready to respond swiftly if the same pathogen invades again. This function of memory B cells underpins the concept of immunological memory and provides lasting immunity after infection or vaccination⁴⁷.

T cells, which mature in the thymus, are another crucial cell type in adaptive immunity. They are classified into various subsets based on their surface receptors and functions. The primary subsets are helper T cells ($CD4^+$ T cells) and cytotoxic T cells ($CD8^+$ T cells). These cells recognize antigens presented on the surfaces of antigen-presenting cells (APCs) or infected cells through molecules known as the major histocompatibility complex ⁴⁸.

Helper T cells, or $CD4^+$ T cells, play a central role in orchestrating the immune response by releasing cytokines that activate and direct other immune cells, including B cells, cytotoxic T cells, and macrophages ⁴⁸. Upon recognizing an antigen presented by MHC class II molecules on an APC, helper T cells proliferate and differentiate into specialized subtypes, such as Th1, Th2, Th17, or regulatory T cells (Tregs), each of which has a specific function in immunity ⁴⁹. For instance, Th1 cells are essential in combating intracellular pathogens like viruses, whereas Th2 cells are more effective against extracellular parasites like helminths ⁵⁰.

Cytotoxic T cells, or $CD8^+$ T cells, are responsible for directly attacking and killing infected cells. They recognize antigens presented by MHC class I molecules found on all nucleated cells and platelets, allowing them to detect cells infected with intracellular pathogens, such as viruses, or those that have become cancerous ⁵¹. Once activated, cytotoxic T cells release enzymes like perforin and granzymes which induce apoptosis (programmed cell death) in the target cell, effectively eliminating the pathogen along with its host cell ⁵².

A hallmark feature of adaptive immunity is the formation of memory cells following initial exposure to a pathogen. This functionality involves both memory B cells and memory T cells, contributing to long-term immunity that enables a more efficient response from the immune system during any subsequent encounters with the same pathogen⁵³. Memory B cells persist within the body, quickly differentiating into plasma cells that produce specific antibodies upon re-infection⁵³. Memory T cells, either as $CD4^+$ or $CD8^+$ cells, are also retained within the body after an infection has been

cleared. They are divided into central memory T cells, which reside in lymphoid tissues and aid in sustaining the population of memory cells, and effector memory T cells. These cells circulate within blood and tissues, ready for quick response to re-infection⁵⁴.

Regulatory T cells (Tregs), primarily a specialized subset of CD4⁺ T cells, play a crucial role in maintaining immune tolerance by suppressing immune responses to self-antigens and thus, preventing autoimmune reactions. Tregs inhibit the activity of other immune cells that might otherwise target the body's own tissues, thereby safeguarding against autoimmunity. This function is indispensable for immune homeostasis and ensures that the actions of the immune system are balanced and meticulously targeted, minimizing damage to healthy tissues⁵⁵. Tregs accomplish immune suppression through various mechanisms, including the secretion of anti-inflammatory cytokines such as IL-10 and transforming growth factor-beta (TGF- β). These cytokines inhibit the activity of effector T cells and other immune cells. The role of Tregs in modulating immune responses underscores the complexity of the adaptive immune system and the significance of maintaining balance within immune reactions⁵⁶.

2.3 Immune system coordination

Both innate and adaptive immunity depend on an intricate system of signaling molecules, which includes cytokines and chemokines, for communication and coordination of responses across distinct cells and tissues. Cytokines function as messengers that regulate immune responses, enhancing interactions among immune cells and directing them toward infection sites. Chemokines, a subgroup of cytokines, assist in guiding cells to specific locations within the body, ensuring an efficient immune response⁵⁷.

The major histocompatibility complex is a critical element for communication within the immune system, particularly for adaptive immunity. MHC molecules present antigen fragments on the surface of infected cells, allowing T cells to recognize and respond to the pathogen. This process of antigen presentation is crucial for activating T cells and for the ensuing immune response⁵⁸ (Figure 3).

The immune system is designed to protect the body, but when it becomes dysregulated, it can lead to diseases. Autoimmune diseases, such as rheumatoid arthritis and lupus, occur when the immune system mistakenly attacks the body's own tissues. In these instances, the immune system fails to differentiate between self and non-self, resulting in chronic inflammation and tissue damage⁵⁹.

Cancer poses a unique challenge to the immune system as cancerous cells can elude detection and actively inhibit immune responses to secure their survival and proliferation. However, recent

advancements in immunotherapy, such as immune checkpoint inhibitors and chimeric antigen receptor (CAR) T-cell therapy, have showcased significant potential in re-stimulating the immune system to efficiently target and eradicate cancer cells⁶⁰.

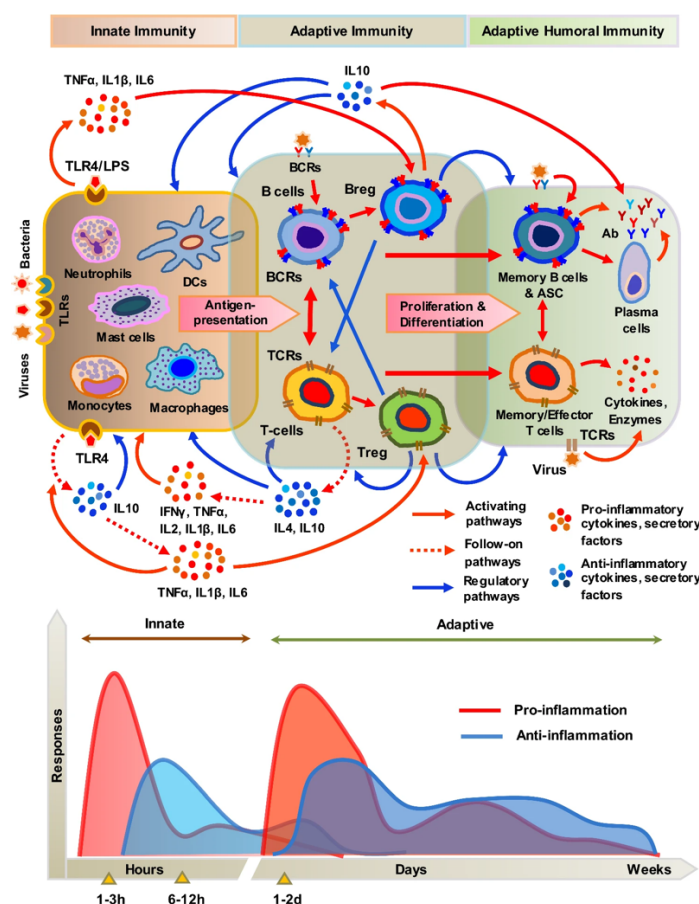


Figure 3. Immune system coordination

TNF, Tumor necrosis factor; IL, Interleukin; TLR4, Toll-like receptor 4, LPS: lipopolysaccharide, DCs, Dendritic cells; IFN γ , Interferon-gamma; BCRs, B cell receptors; TCRs, T cell receptors; Breg, Regulatory B cells; Treg, Regulatory T cells. Adapted from Nguyen T.G., 2020

3. Endometriosis and the immune system

Despite substantial research in this area, the exact cause of endometriosis remains unclear. The retrograde menstruation theory, often considered the primary explanation, does not wholly account for the condition's development. Retrograde menstruation occurs in most women, yet only one in ten develops endometriosis^{16,61}. Building on this, Dmowski and colleagues proposed that patients with endometriosis might have an impaired immune response that fails to clear menstrual debris^{62,63}.

Immunological dysfunction has garnered significant attention in recent years. This is because researchers are investigating “innate-adaptive immune pathways” that contribute to the creation and durability of endometriotic lesions within the peritoneal cavity. In a fully functioning immune system,

immune surveillance mechanisms can identify and get rid of aberrant cells, such as endometrial-like cells at ectopic sites. However, in people with endometriosis, this process seems to be compromised⁶⁴.

Usually, when endometrial cells reach the pelvic cavity through retrograde menstruation, immune cells such as macrophages, NK cells, and cytotoxic T lymphocytes are tasked with removing these cells and preventing their implantation. Studies indicate that women with endometriosis display modified immune surveillance abilities. This allows ectopic cells to avoid destruction and commence implantation on peritoneal surfaces^{64,65}.

3.1 The immune cells in endometriosis

Dmowski and Braun⁶³ propose that polymorphic, genetically abnormal endometrial cells may prefer proliferation over apoptosis in response to local triggers. While normally functioning cells within the same host are eradicated, these abnormal cells could potentially bypass peritoneal defenses and leverage the environment to propagate clonally when relocated into the peritoneal cavity. In the case of endometriosis, peritoneal macrophages, and NK cells exhibit a decreased capacity to eliminate endometrial cells from the peritoneal cavity. An imbalance between Th1 and Th2 cells further interferes with cytokine secretion, culminating in inflammation that enables lesion progression⁶⁶. The discharges from these cells incite a local inflammatory response, fostering cellular invasion and advancement of the disease. This inflammation draws leukocytes into the peritoneal cavity where they get activated and unleash cytokines with diverse impacts. Additionally, these stimulated immune cells may be less efficient at eliminating displaced cells, and their secretions could encourage ectopic cell growth and disease progression, rather than inhibiting it.

3.1.1 Innate immune system and endometriosis

The innate immune cell populations examined in the pathophysiology of endometriosis include neutrophils, macrophages, and NK cells.

Patients with endometriosis exhibit higher peritoneal neutrophil levels compared to individuals without the condition, likely driven by elevated chemotactic factors such as IL-8 present in plasma and PF⁶⁷. Despite this increase, the phagocytic function of peripheral monocytes and neutrophils is reduced⁶⁸.

Macrophages, the most abundant cells in the PF of healthy individuals, are present in increased quantities in patients with endometriosis. However, these macrophages demonstrate diminished functionality and reduced phagocytic capacity. Additionally, their production of inflammatory

mediators aids in the implantation and proliferation of endometrial cells, thus encouraging lesion development⁶⁹.

NK cell populations are found in both the PB and the uterus, predominantly characterized as CD56^{dim}CD16⁺ in the circulation and CD56^{bright}CD16⁻ in the uterus⁷⁰. Oosterlynck et al. were the first to identify the role of NK cells in the pathogenesis of endometriosis, observing reduced cytotoxic activity of NK cells against ectopic endometrial cells, a trend linked to advanced stages of the disease⁷¹. This finding has been consistently observed, with decreased NK cell activity being more evident in the PF, though it is also present in PB⁷². The specific mechanism behind NK cell inhibition in endometriosis remains unclear. Studies have reported abnormal expression of both activating and inhibitory receptors on NK cells in endometriosis compared to healthy individuals^{73,74}.

3.1.2 Adaptive immune system and endometriosis

Several studies have confirmed the presence of anti-endometrial antibodies in the serum and PF of patients with endometriosis⁷⁵. It is thought that these autoantibodies may contribute to endometriosis by activating the immune system and perpetuating inflammation, though this is largely speculative, and there is a lack of evidential support. Notably, there is a robust association between endometriosis and other immune-related or autoimmune diseases such as rheumatoid arthritis, psoriasis, and allergies⁷⁶. With the presence of autoantibodies, B cell populations have been investigated in relation to endometriosis. A meta-analysis of 22 studies focusing on B cells in endometriosis showed that most studies reported an increase in the presence or activation of B cells in affected patients. However, seven studies found no difference in B cell numbers between patients with endometriosis and the control groups⁷⁷. Further research is necessary to better understand the role of B cells and their interactions with other immune cells within the microenvironment of endometriotic lesions.

Research on T cells in patients with endometriosis has revealed a higher CD4/CD8 T-cell ratio and increased concentrations of both cell types in the PF of these patients⁷⁸. Endometrial lesions had more T cells than the eutopic endometrium, though the CD4/CD8 T-cell ratio was similar. No notable changes were observed in the PB⁷⁹. Moreover, the proportion of Th17 cells in the PF was higher in individuals with endometriosis and seemed to be linked with the stage of the disease⁸⁰. Regulatory T cells, generally involved in immune regulation and tolerance, also showed increased numbers in the PF of patients with endometriosis. This increase is suspected to be related to local immunosuppression, potentially inhibiting the clearance of ectopic endometrial cells. However, the exact function of Tregs in the development of endometriosis remains uncertain^{81,82}.

The link between endometriosis, a chronic inflammatory condition, and excessive cytokine secretion has been extensively examined. Cytokines, acting as chemotactic factors, play a role in recruiting macrophages and T cells, thereby driving the inflammatory response associated with the disease. Studies have noted altered cytokine levels in the serum or PF in instances of endometriosis occurring in various locations⁸³. IFN- γ is elevated in the PF of endometriosis patients, which regulates the Th1/Th2 cell balance but does not affect the growth or apoptosis of cells from ovarian-derived ectopic endometrial implants. This suggests potential resistance of endometrial cells to apoptosis upon entering the peritoneal cavity⁸⁴. IL-1, a pro-inflammatory cytokine produced by activated macrophages in the PF, regulates immune responses, and promotes cell growth, differentiation, and angiogenesis, with elevated levels observed in endometriosis patients⁸⁵. IL-6, also produced in response to infection and tissue damage, plays a vital role in host defense by stimulating acute-phase responses, hematopoiesis, and immune activation. Although its expression is usually regulated, continuous IL-6 synthesis can occur in pathological conditions, contributing to chronic inflammation and autoimmunity⁸⁶. In endometriosis, IL-6 promotes ectopic endometrial growth by interfering with normal cellular responses. Macrophages, the primary source of IL-6 in the PF, are more numerous and active in endometriosis patients, releasing IL-6 and other cytokines at elevated levels⁸⁷⁻⁸⁹. This IL-6 secretion enhances haptoglobin production, reducing phagocytosis and thereby shielding endometrial implants from immune detection. This positive feedback loop promotes ectopic endometrium survival and lesion growth. Furthermore, high IL-6 levels inhibit NK cell activity, further facilitating endometriosis progression⁸⁸. IL-8 and TNF- α are among the cytokines with elevated expression levels in the PF of women with endometriosis. These cytokines support endometrial cell adhesion to the peritoneum and disease progression, attracting immune cells to inflammation sites^{90,91}.

IL-10 and IL-4 are anti-inflammatory cytokines that play key roles in immune modulation. Initially thought to be produced mainly by Th2 lymphocytes, IL-10 is now known to be secreted by various cells, particularly Tregs, along with other subsets of B cells, T cells, macrophages, and even non-immune cells like keratinocytes and tumor cells⁹². IL-10 helps control inflammation by inhibiting pro-inflammatory mediators, reducing phagocytosis, and enhancing regulatory T-cell functions, thereby fostering immune tolerance⁹². However, its primary role involves the differentiation of naive T cells following their antigen-stimulation. This process results in the production of activated T cells that can generate helper T cells polarized toward a Th2 cellular response⁹³. In the context of endometriosis, it has been repeatedly demonstrated that disease development is associated with Th2-type immune responses^{66,94,95}. Studies have revealed higher IL-10 levels in the PF and PB of women with endometriosis, related to reduced NK cell cytotoxicity in the endometriosis environment. This

cytokine imbalance may facilitate the implantation and persistence of ectopic endometrial tissue in the peritoneal cavity^{96,97}.

Research on women with endometriosis has revealed elevated TGF- β activity in their PF⁹⁸. This cytokine plays a role in various processes, which include angiogenesis and immune regulation. Its isoform, TGF- β 1, has anti-inflammatory effects. However, TGF- β also promotes the differentiation of regulatory T cells and Th17 inflammatory cells. TGF- β in endometriosis may contribute to inhibiting NK cell activity in the PF⁹⁸. Angiogenesis, the process of creating new capillaries, is thought to be involved in the pathogenesis of endometriosis⁹⁹. High levels of the vascular endothelial growth factor (VEGF) are found in the PF of patients with endometriosis. These levels are driven by estradiol and progesterone, which facilitate increased vascularization in endometriotic tissues and enhance endothelial cell proliferation^{100,101}.

3.1.3 The immune system in endometriosis – Future perspectives

While research on the interplay between the immune system and endometriosis remains limited, an in-depth study could reveal the exact mechanisms of disease pathogenesis. Such understanding will assist us in recognizing potential autoimmune components, which could transform treatment strategies towards those used for autoimmune diseases and potentially enhance patient outcomes. Current research concentrates on decoding these immune pathways to craft targeted therapies that tackle both inflammation and immune dysfunction. The aim is to reduce pain and decelerate disease progression without being overly dependent on hormonal treatments¹⁰².

With a deeper understanding of immunosurveillance, immune evasion, and innate-adaptive immune pathways in endometriosis, we find ourselves in an ideal position to examine the immune microenvironment of endometriotic lesions closely, searching for new therapeutic targets. Furthermore, there is an urgent necessity for groundbreaking diagnostic and treatment approaches that not only treat endometriosis but also allow for the preservation of reproductive potential when desired.

Recognizing endometriosis as an immune-mediated disease underscores the potential for immunomodulatory therapies. For instance, we could control disease progression and alleviate symptoms by targeting pro-inflammatory cytokines, such as TNF- α , or by modulating immune cells like macrophages and Tregs⁶⁴.

CHAPTER II

Objectives

1. General objective

The primary objective of this research was to analyze the populations of NK and T cells, along with their inhibitory immune receptors, in the PB and PF of women with endometriosis, in comparison to a control group without the disease. The study aimed to identify the mechanisms through which endometriosis cells evade the immune system, enabling them to plant and proliferate outside the uterine cavity.

2. Specific objectives

In detail, the specific objectives were as follows:

- Conduct a systematic review to improve understanding of NK cell function in endometriosis, considering the growing research on the role of NK receptors in the disease's pathogenesis and the absence of comprehensive reviews on this topic (**Chapter III**).
- Investigate abnormal NK and T-cell patterns associated with symptoms such as dysmenorrhea, dyspareunia, pelvic pain, rectorrhagia, and dyschezia, while analyzing the influence of demographic factors on immune profiles in endometriosis patients, both in peritoneal fluid and peripheral blood (**Chapter IV**).
- Examine the behavior of TIM-3 in endometriosis patients by investigating its expression as an immune checkpoint in NK and T cells within the peripheral blood and peritoneal fluid of both endometriosis patients and controls. Additionally, analyzes the relationship between TIM-3 expression and demographic factors, symptoms, and disease severity (**Chapter V**).
- Investigate the mechanisms by which endometriotic cells may evade the immune system, with a focus on PD-1 expression in immune cells from both peripheral blood and peritoneal fluid, in women with endometriosis and establish potential links between endometriosis and cancer pathways (**Chapter VI**).

CHAPTER III

Natural Killer Cell Receptors and Endometriosis: A Systematic Review



Review

Natural Killer Cell Receptors and Endometriosis: A Systematic Review

José Lourenço Reis ¹, Natacha Nurdine Rosa ², Miguel Ângelo-Dias ^{3,4}, Catarina Martins ^{3,4},
Luís Miguel Borrego ^{3,4,5} and Jorge Lima ^{1,3,4,*}

- ¹ Department of Obstetrics and Gynecology, LUZ SAÚDE, Hospital da Luz, 1500-650 Lisboa, Portugal
² UCD School of Medicine, University College Dublin, D04 V1W8 Dublin, Ireland
³ Comprehensive Health Research Centre (CHRC), NOVA Medical School | Faculdade de Ciências Médicas (NMS|FCM), Universidade Nova de Lisboa, 1169-056 Lisboa, Portugal
⁴ Immunology Department, NOVA Medical School | Faculdade de Ciências Médicas (NMS|FCM), Universidade Nova de Lisboa, 1169-056 Lisboa, Portugal
⁵ Department of Imunoallergy, LUZ SAÚDE, Hospital da Luz, 1500-650 Lisboa, Portugal
* Correspondence: jorgeramoslima@sapo.pt

Abstract: Endometriosis is a chronic inflammatory disorder, characterized by the presence of endometrial cells outside the uterine cavity. An increasing number of studies correlate the immune system with endometriosis, particularly NK receptors (NKR), which have been suggested to play an essential role in the pathogenesis of the disease. This systematic review aims to enlighten the role of NKR in endometriosis. A literature search was performed independently by two reviewers, to identify studies assessing the role of NKR in endometriosis. In total, 17 studies were included. Endometriosis pathogenesis seems to be marked by the overexpression of NK inhibitor receptors (KIRS), namely, CD158a+, KIR2DL1, CD94/NKG2A, PD-1, NKB1, and EB6, and inhibiting ligands such as PD-L1, HLA-E, HLA-G, and HLA-I. Concurrently, there is a decrease in NK-activating receptors and natural cytotoxicity receptors (NCRs), such as NKp46, NKp30, and NKG2D. The immune shift from NK surveillance to NK suppression is also apparent in the greater relative number of ITIM domains compared with ITAM domains in NKRs. In conclusion, NK receptor activity seems to dictate the immunocompetency of women to clear endometriotic cells from the peritoneal cavity. Future research could explore NKRs as therapeutic targets, such as that which is now well established in cancer therapy through immunotherapy.

Keywords: endometriosis; natural killer cells (NK cells); NK receptors; endometrioma; ectopic endometrium; immune dysfunction; NK inhibition; NK suppression



Citation: Reis, J.L.; Rosa, N.N.; Ângelo-Dias, M.; Martins, C.; Borrego, L.M.; Lima, J. Natural Killer Cell Receptors and Endometriosis: A Systematic Review. *Int. J. Mol. Sci.* **2023**, *24*, 331. <https://doi.org/10.3390/ijms24010331>

Academic Editor:
Dariusz Szukiewicz

Received: 9 November 2022
Revised: 12 December 2022
Accepted: 21 December 2022
Published: 25 December 2022



Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Endometriosis is a chronic gynecological disorder characterized by the presence of endometriotic tissue outside the uterine cavity. It is a very incapacitating disease affecting 1 in 10 women during reproductive age [1], having a great impact on their quality of life. Although endometriosis was first noted by Carl von Rokitansky more than one hundred years ago, the pathogenesis of this condition is still not clear. Regarding pathogenesis, the theory of retrograde menstruation and implantation of ectopic endometrium is the most accepted [2]. Retrograde menstruation occurs in almost all women, but only 10% of them develop endometriosis [1]. Thus, the question arises as to what facilitates ectopic endometriotic cell implantation and growth. The hypothesis of immunological dysfunction has gained greater evidence in recent years. In the peritoneal cavity microenvironment, immune dysfunction enhances the capacity of ectopic endometrial cells to adhere and invade, leading to poor clearance of the ectopic endometrium and increased proliferation and angiogenesis [3]. NK cells have been suggested to play an important role in the pathogenesis of the disease by either tolerating or inhibiting the survival, implantation, and

proliferation of endometrial cells [4]. An increasing number of studies [5–20] address the role of NK receptors (activating and inhibiting) in endometriosis pathogenesis (Figure 1). To the best of our knowledge, there are no systematic reviews concerning the involvement of NK cell receptors in endometriosis; therefore, this assessment is important for a better understanding of the topic.

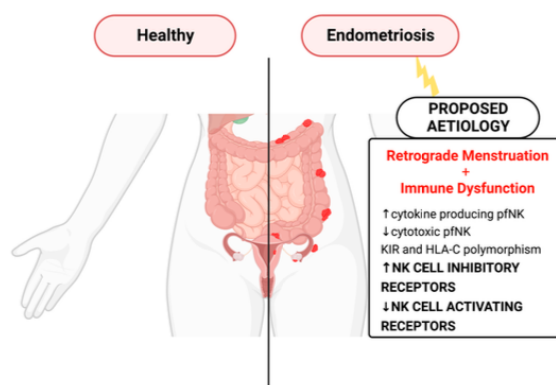


Figure 1. Diagrammatic representation of endometriosis and proposed etiology.

2. Methods

This systematic review identified and analyzed human studies which assess the role of NK cell receptors in women suffering from endometriosis compared with healthy women, and was conducted according to the PRISMA guidelines (Preferred Reporting Items for Systematic Reviews and Meta-analyses) [21,22] (Figure 2) and registered in PROSPERO (CRD42020205783).

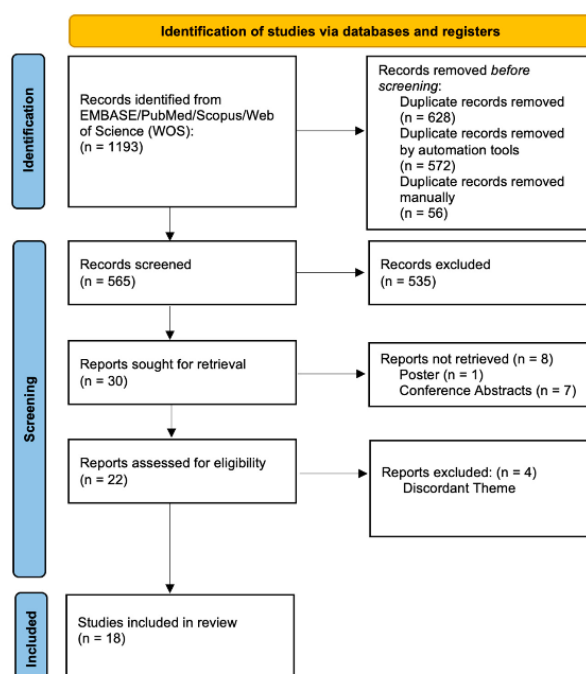


Figure 2. PRISMA flowchart [22].

2.1. Search Strategy

An extensive computerized literature search strategy was performed to retrieve the studies included in this review. The searches were performed in PubMed/Medline, EMBASE, Scopus, and Web of Science databases, using database-specific subject heading terms and all its variants in free-text words, according to the specificities of each database (Table S1). To ensure literature saturation, we performed a supplementary search of grey literature and of the reference sections of the selected studies and reviews, to identify any additional relevant missing publications as well as conference papers not retrieved in the electronic search. No date or language limits were imposed on the search.

2.2. Eligibility Criteria and Study Selection

The study selection was performed independently by two individuals (J.L.R. and N.N.R.), who screened the titles and abstracts of all suitable articles from the queries according to the eligibility criteria (Table S2). No restrictions to geographical location, language of publication, or year of publication were applied, and all non-primary studies were excluded.

Studies that did not meet eligibility criteria were excluded and the remaining articles were thoroughly analyzed and read in full and included or discarded according to the eligibility criteria. Any disagreement was resolved after discussion with a third reviewer (J.L.). All decisions, including reasons for exclusion and the number of selected articles in each step, were recorded and depicted in a flow chart, following the PRISMA 2020 guidelines.

EndNote version 20 was used to store, organize, and manage all the references arising from the literature search, including the management and removal of duplicates, and scanning of the titles/abstracts of all the records.

2.3. Data Extraction and Quality Assessment

All relevant data were extracted from each selected study by two independent reviewers and any disagreement was referred to a third reviewer (J.L.) to reach consensus. The following data were extracted:

- Identification of the study—title, authors, year of publication, journal title, country of origin, study design, number of participants, and recruitment procedure and duration.
- Participant characteristics—sample size, age, BMI, parity, rAFS classification of endometriosis [23], previous treatments, and preoperative painful symptoms scores—of subjects in endometriosis and control groups.
- Methodological features—sample characteristics (endometriotic tissue, peritoneal fluid, peripheral venous blood, and eutopic endometrium), methodology used for NK cell marker characterization (^{51}Cr cytotoxicity assay, ELISA, flow cytometry, immunohistochemistry, in situ hybridization, RNA extraction, and quantitative real-time Western blotting) and NK cell markers.
- Outcomes—comparison of NK cell markers between the different samples studied.

The methodological quality of each individual study was assessed independently by two reviewers on 20 October 2022 (J.L.R. and N.N.R.), using the NHLBI quality assessment tool of case-control studies (<https://www.nhlbi.nih.gov/health-topics/study-quality-assessment-tools>). This scale evaluates 12 components of a study to determine its methodological quality. Each study evaluation criterion was assigned “Yes”, “No”, “NR” (not reported), “NA” (not applicable), or “Unc” (Unclear). Subsequently, studies were graded as “Poor”, “Fair”, or “Good”. The level of bias influences the evidence and results obtained in this systematic review. Conflicts regarding study quality were resolved with a third reviewer (J.L.).

2.4. Data Synthesis

Due to the high heterogeneity of the selected studies evaluating several NK cell receptors in different types of samples (peripheral blood, peritoneal fluid, and eutopic and

ectopic endometrium), a quantitative meta-analysis was not feasible to perform. Data from the selected studies were narratively and qualitatively summarized.

3. Results

3.1. Study Selection

In total, 1193 studies were obtained. Upon the exclusion of 628 duplicates, titles and abstracts were screened, and 535 studies that did not meet eligibility criteria were excluded. There were 30 reports sought for retrieval, where an additional 8 studies were excluded because they were classified as conference abstracts/posters. The remaining 22 articles were thoroughly analyzed and read in full, leading to the exclusion of 4 more studies due to research theme incongruency. In total, 18 studies [5–20,24,25] were included in the final qualitative analysis, as per Figure 2.

3.2. Study Quality Assessment and Risk of Bias

Quality assessment was conducted on the 18 selected studies as presented in Table S3 (following NHLBI Assessment Tool guidelines). Among these studies, 15 were deemed “good quality” [5,6,8–10,14–20,24,25]. This assessment was based on the sample size, consideration/adjustment to key confounding variables (demographic factors, menstrual cycle stage, hormonal therapy, and disease staging as per revised American Society for Reproductive Medicine (rASRM) classification) [23], and appropriateness of the methodology according to the research hypothesis. None of the studies included sample size justification or blinded evaluators of outcomes.

3.3. Heterogeneity of the Studies Assessed

NK cell receptors (and their ligands) were evaluated in 18 case–control (observational) studies in the context of endometriosis, all using a human model. One of these was also classified as quasi-experimental [24], analyzing the impact of 17 β -estradiol treatment on results. Heterogeneity was noted in the samples evaluated in each study, as presented in Table 1. Studies used a wide array of samples, including peritoneal fluid [6–11,13,14,16–19,24,25], endometriotic lesions/eutopic endometrium samples [12,15,18,19,24], and peripheral blood [7–10,18,20,24].

Additionally, each study employed different techniques to evaluate the NK cell receptor activity and expression in endometriosis. Flow cytometry [7–11,14,16,19,24,25] was most commonly used, followed by Western blotting [6–10,19,24], immunohistochemistry [12,15,18,24], polymerase chain reaction (PCR) [14,19,20], enzyme-linked immunosorbent assay (ELISA) [13,17,18], RNA in situ hybridization [12], and ^{51}Cr cytotoxicity assays [5].

The study results are summarized in Table 1.

Table 1. Summary of findings and methods of selected studies regarding NK cell-activating/inhibiting receptors in endometriosis.

Samples	Design	n	Comparison	Methods	Markers	Results	Reference
PF	Case–control	11 I/II EDT; 22 III/IV EDT; 11 controls	NK cytotoxicity and NK inhibition receptors in early and late EDT	FC cytotoxicity assay ^{51}Cr	FITC anti-CD45/PE-anti-CD14 γ_1 FITC/ γ_{2a} PE, FITC-anti-CD3/PE-anti-CD19, FITC-anti-CD3/PE-anti-CD56, FITC-anti-CD3/PE-anti-NKB1/PerCP-anti-CD56, FITC-anti-CD3/PE-anti-GL183/PerCP-anti-CD56, FITC-anti-CD3/PE-anti-EB6/PerCP-anti-CD56.	↓ peritoneal cytotoxicity against K562 EDT (I/II/III/IV) vs. controls; ↑ KIR expression (NKB1, EB6) in III/IV EDT vs. controls; ↑ KIR expression (NKB1, EB6) in III/IV EDT vs. I/II EDT	Wu et al., 2000 [5]

Table 1. *Cont.*

Samples	Design	n	Comparison	Methods	Markers	Results	Reference
PF	Case-control	10 EDT; 10 controls	HLA-G inhibitory ligand expression in women with and without EDT	Western blotting	mAb aa 61-83 α 1 domain of HLA-G	No statistical significance between groups	Hornung et al., 2001 [6]
PB, PF	Case-control	11 I/II EDT; 17 III/IV EDT; 6 controls	ICAM-1 and KIR expression in women with and without EDT	FC Western blotting	FITC-labeled anti-CD3 mAb, anti-CD4 mAb, PE-labeled anti-CD8 mAb, PE-labeled anti-CD19 mAb, FITC-labeled anti-CD16 mAb, FITC-labeled anti-CD14 mAb, PE-labeled anti-CD54 (ICAM-1) mAb, PE-labeled anti-CD158a, anti-CD158b, and CD94	↓ ICAM in PF macrophages EDT vs. controls; ↑ KIR2DL1+ NK among CD16+ NK in PB and PF of EDT vs. controls (more pronounced in III/IV EDT)	Maeda, Izumiya, Oguri et al., 2002 [7]
PB, PF	Case-control	12 I/II EDT; 30 III/IV; 40 controls	KIR2DL1+ NK cell expression in women with and without EDT	FC Western blotting	FITC-anti-CD16 mAb PE-labeled anti-CD158a mAb, anti-CD158b mAb, PE anti-CD94 mAb	↑ KIR2DL1+ NK cells in PB and PF of EDT vs. controls (more pronounced in III/IV EDT)	Maeda, Izumiya, Yamamoto et al., 2002 [8]
PB, PF	Case-control	18 I/II EDT; 70 III/IV EDT; 104 controls	CD158a+ cells (KIR subtype) expression in women with and without EDT	FC Western blotting	FITC-labeled anti-CD16 mAb, PE-labeled anti-CD158a and anti-CD158b mAbs, PE-labeled anti-CD94 mAbs	↑ CD158a+ cells in PF of EDT (I/II/III/IV and III/IV) vs. controls; ↑ CD158a+ cells in PB of EDT (I/II and III/IV)	Maeda et al., 2004 [9]
PB, PF	Case-control	6 I/II EDT; 18 III/IV; 25 controls	ITIM and ITAM KIR expression in women with and without EDT	FC Western blotting	FITC-labeled anti-CD56 mAb PE-labeled anti-CD158a mAb, anti-CD158b mAb, Anti-CD158a mAb, anti-CD158b mAb	ITIM-KIR > ITAM-KIR in PB EDT; ↑ CD158a+CD56+ NK cells in PB and PF EDT vs. controls	Matsuoka et al., 2005 [10]
Endometrium Peritoneal EDT	Case-control	15 EDT and 12 controls (IHC); 24 endometrium EDT and 14 peritoneal fluid EDT and 17 controls (RNA ISH)	HLA-G expression in eutopic and ectopic endometrium in women with and without EDT	IHC RNA ISH	mAb4H84 cDNA probe	HLA-G protein and gene transcripts found in >90% glandular peritoneal fluid EDT but not in stromal endometrial epithelium (controls and EDT)	Barrier et al., 2006 [12]
PF	Case-control	26 I/II EDT; 20 III/IV EDT; 24 controls	CD56+ cell expression, Fas antigen CD95 and early activation molecule CD69 in PF of women with and without EDT	FC	Anti-CD45FITC/CD14PE, IgG1FITC/IgG2aPE, anti-CD69FITC, anti-95FITC and anti-CD56PE	↓ CD56+ dim expression in I/II EDT vs. controls; ↑ CD95 in I/II EDT vs. controls; ↑ CD69+CD56+ in I/II EDT vs. controls; ↑↑ CD69+CD56+ in III/IV EDT vs. controls	Eidukaite et al., 2006 [11]

Table 1. *Cont.*

Samples	Design	n	Comparison	Methods	Markers	Results	Reference
PF	Case-control	17 I/II EDT; 14 III/IV EDT; 27 controls	HLA-G expression in PF of women with and without EDT	ELISA	Anti-sHLA-G	No statistical significance between groups	Eidukaite and Tamosiunas, 2008 [13]
PB, PF, Endometrium Peritoneal EDT	Case-control	20 III/IV EDT; 13 controls	(HLA)-E receptor CD94/NKG2A expression in women with and without EDT	FC, RT-PCR	Anti-CD56 (clone C218), anti-NKG2A (clone Z199), PE-conjugated anti-NKG2A, PE-conjugated anti-NKG2C, fluorescein-conjugated anti-CD56, peridinin chlorophyll protein-conjugated anti-CD3, anti-CD45, cytokeratin 20	↑ CD94/NKG2A cells in PF in EDT vs. controls; ↑ HLA-E mRNA in peritoneal EDT	Galandrini et al., 2008 [14]
Endometrium	Case-control	15 EDT; 15 controls	HLA-I and HLA-II expression in women with and without EDT	IHC	IgG2a (HLA-I), IgG1 (HLA-II)	↑ HLA-I and HLA-II expression in EDT endometrial stroma and glands vs. controls	Baka et al., 2011 [15]
PF	Case-control	3 I/II EDT; 18 III/IV EDT; 28 controls	Expression of NK Cell surface antigens (CD16 and CD56+ cells), NCRs (Nkp46/40/30) and cytokine production (TNF- α , IFN- γ etc.) of PF NK cells in women with and without EDT	FC	Anti-CD45 PerCP-Cy5.5/anti-CD56 PE/anti-CD16 fluorescein isothiocyanate, anti-CD45 PerCP-Cy5.5/anti-CD56 FITC/anti-CD335 (Nkp46) PE, anti-CD45 PerCP-Cy5.5/anti-CD56 FITC/anti-CD336 (Nkp44) PE, and anti-CD45 PerCP-Cy5.5/anti-CD56 FITC/anti-CD337 (Nkp30) PE	↓ Nkp46+ NK cells in III/IV EDT vs. controls; ↓ CD56dim/Nkp46+ cells in III/IV EDT vs. controls; ↑ TNF- α producing NK cells in III/IV EDT vs. controls; ↑ IFN- γ producing NK cells in III/IV EDT vs. controls	Funamizu et al., 2014 [16]
PF	Case-control	121 EDT; 81 controls	Levels of soluble NKG2D ligands (MICA, MICB and ULBP-2) in women with and without EDT	ELISA	ELISA (R&D Systems, Inc., Minneapolis, MN, USA)	↑ MICA in EDT vs. controls; ↑ MICB in EDT vs. controls; ↑ MICA, MICB, ULBP-2 in deep infiltrating EDT	González-Foruria et al., 2015 [17]
Serum, PF, Endometrium EDT	Cross-sectional observational /Case-control	60 I/II EDT; 83 III/IV EDT; 77 controls (ELISA) 26 EDT; 22 controls (IHC)	Soluble HLA-G expression in endometrium, EDT, PF, and serum in women with and without EDT	ELISA IHC	mAb 4H84 MEM-G/9 mouse mAb	↑ sHLA-G in serum but not PF of III/IV EDT vs. controls; ↑ HLA-G protein expression in EDT but not eutopic endometrium	Rached et al., 2019 [18]

Table 1. *Cont.*

Samples	Design	n	Comparison	Methods	Markers	Results	Reference
Endometrium EDT PB	Case-control Quasi-experimental	15 EDT; 15 controls	PD-1/PDL-1 expression in women with and without EDT, post and prior estrogen and cytokine treatment	IHC FC Western blotting	Rabbit anti-human PD-1, CD4, CD8, PD-L1. anti-GAPDH CD279 (PD-1)-PE, CD274 (B7-H1)-PE, CD8-FITC, and CD4-PerCP-Cyanine5.5	↑ PD-1/PDL-1 and PD-1 in eutopic and ectopic endometrium EDT vs. controls; ↑ PD-1/PDL-1 and PD-1 in PB EDT vs. controls; ↑ PD-1/PDL-1 and PD-1 in eutopic endometrium EDT post 17β-estradiol treatment vs. controls	Wu et al., 2019 [24]
Endometrium EDT PF	Case-control	20 EDT; 13 controls	NKG2D expression and its ligands in women with and without EDT	FC RT-PCR Western blotting	FITC-conjugated mouse, anti-human CD16 mAb, PE-Cy5-conjugated mouse, anti-human CD56 mAb PE-mouse, anti-human NKp30 mAb, PE-mouse, anti-human NKp44 mAb, PE-mouse, anti-human NKp46 mAb, PE-mouse, and anti-human NKG2D mAb	↓ NKp30 CD56+ in PF in EDT; ↑ NKp46 CD16+ in PF in EDT; ↓ NKG2D CD56+ in PF in EDT; ↓ ULBP-2 in eutopic endometrium in EDT vs. controls; ↓ ULBP-3 in ectopic endometrium in EDT vs. controls and eutopic EDT	Xu, 2019 [19]
PB	Case-control	147 EDT; 117 controls	HLA-C and KIR polymorphism combinations in women with and without EDT	PCR	Commercial kits (HLAssure SE kit, Accutype Software, SSO typing kit)	↑ HLA-C*03:01 occurrence in EDT vs. controls; ↑ KIR centromeric A/A haplotypes in EDT vs. controls; ↓ KIR2DS2-positive individuals in EDT vs. controls	Chou et al., 2020 [20]
PF	Case-control	32 EDT; 30 controls	NKp46 co-expression patterns in women with and without EDT	FC	anti-TNF- α -BV421, anti-IFN- γ -PE- Cy7, anti-IL-4-PerCP-Cy5, anti-IL-10- APC, anti-TGF- β -PE	↓ CD56+ /NKp46+ in PF in EDT; ↓ NKG2C+ /NKp46+ in PF in EDT; ↓ CD16+ /NKp46+ in PF in EDT; ↑ NKG2D+ /NKp46+ in PF in EDT No significant co-expression of inhibitory receptors (CD158a and NKG2A) and NKp46 ↑ IFN-γ-producing NK cells in PF in EDT	Saeki et al., 2022 [25]

aa, amino acids; EDT, endometriosis; FC, flow cytometry; IHC, immunohistochemistry; ISH, in situ hybridization; ITAM, tyrosine-based activation motifs; ITIM, tyrosine-based inhibitory motifs; KIR, natural killer inhibitor receptor; mAb, monoclonal antibody; PB, peripheral venous blood; PF, peritoneal fluid. Scoring and staging of EDT (I/II/III/IV) as per American Fertility Society Guidelines [23].

3.4. NK Cell Receptors in Peritoneal Fluid

Fourteen studies evaluated the activity and expression of NK cell receptors in the peritoneal fluid of endometriosis subjects [5–11,13,14,16–19,25]. The role of immune dysfunction in the progression of endometriosis was highlighted by Maeda and colleagues [7–9], who found, by using flow cytometry, that the levels of NK cell inhibitory receptors such as CD158a+ and KIR2DL1+ were significantly higher in the peritoneal fluid of endometriosis subjects than controls. This increase was even more pronounced in stage III/IV endometriosis (KIR2DL1+ 24.2% $\pm$ 16.1% vs. 12.3% $\pm$ 6.9%, $p = 0.007$ [8], 17.5% $\pm$ 8.7% vs. 11.7% $\pm$ 5.5%, $p = 0.008$ [7], CD158a+ 17.0% $\pm$ 12.3% vs. 12.3% $\pm$ 7.1%, $p = 0.017$ [9]) Matsuoka et al. [10] corroborated these findings, also reporting an increased percentage of CD158a+ in CD56+ NK cells in the peritoneal fluid of the endometriosis group (13.9% $\pm$ 7.5% vs. 7.6% $\pm$ 3.0%, $p = 0.028$). Galandrini et al. [14] conducted a similar study using cytofluorometric analysis and reported an increased frequency of human leukocyte antigen-E inhibitory receptor CD94/NKG2A-expressing peritoneal NK cells in subjects with endometriosis (75.9% $\pm$ 8.2% vs. 52.1% $\pm$ 16.3%, $p < 0.0001$). Wu et al. [5] also explored NK inhibitor receptor (KIR) expression in endometriosis with flow cytometry, noting a significant increase in the mean fluorescence intensity of receptors NKB1 (183 $\pm$ 56 vs. 97 $\pm$ 45, $p < 0.001$) and EB6 (82 $\pm$ 45 vs. 53 $\pm$ 14, $p = 0.021$) in the peritoneal fluid of subjects with severe (III/IV) endometriosis compared with the controls. The increased inhibition of NK cell activity at the peritoneal level in endometriosis was also explored through NK cell inhibitory ligands in three studies [6,13,18], which analyzed HLA-G expression in peritoneal fluid using different methods (ELISA, Western blotting, and immunohistochemistry). All the studies reached the same result: there were no significant statistical differences in HLA-G in the peritoneal fluid of endometriosis subjects compared with the controls. These results were different in other samples ((serum and endometrium) [12,18]), as expressed in the following sections of this report.

NK cell-activating receptors were also investigated in the studies selected. Using flow cytometry, Xu [19] reported a decrease in Nkp30 ($p = 0.006$) and NKG2D ($p = 0.01$) levels in CD56+ NK cells in the peritoneal fluid of endometriosis subjects. In this same study, however, Xu noted a significant increase in activating receptor Nkp46 in CD16+ NK cells ($p = 0.04$) [19]. This finding is in contrast to that of Funamizu et al. [16], who reported a decrease in peritoneal activating receptor Nkp46 in advanced (III/IV) endometriosis: 22.84% (11.58–46.34) vs. 30.20% (18.72–51.20), $p < 0.05$. Furthermore, these authors noted a decrease in peritoneal CD56dim/Nkp46 cells, 16.67% (8.95–33.09) vs. 26.12% (14.60–41.14, $p < 0.05$), as well as an increase in TNF- α (33.87% (25.21–60.73) vs. 25.42% (17.56–30.43), $p < 0.05$) and IFN- γ (50.04% (26.98–71.33) vs. 19.41% (6.01–38.37), $p < 0.05$) producing NK cells [16]. This decrease in peritoneal cytotoxic cells (CD56dim/Nkp46) in endometriosis is complemented by the findings of Wu et al. [5], whose ^{51}Cr release assay showed a decrease in cytotoxicity against K562 in the peritoneal fluid of subjects with severe (III/IV) (LU₂₀ 4.6 $\pm$ 3.6 vs. 8.0 $\pm$ 4.0 $p = 0.011$) and early (I/II) (LU₂₀ 4.7 $\pm$ 2.7 vs. 8.0 $\pm$ 4.0 $p = 0.028$) endometriosis. The decreased activation of NK cell activity at the peritoneal level in endometriosis was also explored through ligands. González-Foruria et al. [17] noted an increase in soluble forms or the shedding of NK-activating receptor ligands (namely, MICA ($p = 0.015$), MICB ($p = 0.003$), and ULBP-2 (0.045)) in the peritoneal fluid of subjects with deep infiltrating endometriosis relative to controls. Further elucidating the role of NK cell-activating receptors, Saeki et al. [25] noted a decrease in co-expression of CD56+/Nkp46+ and CD56dim/Nkp46+ cells ($p < 0.05$) with a negatively correlated increase in IFN- γ -producing cells ($p < 0.01$) in subjects with endometriosis. Contrastingly, they also observed an increase in the co-expression of activating receptor NKG2D+/Nkp46+ cells in endometriosis subjects ($p < 0.05$).

NK cell activity may also be suppressed through the loss of NK cells (apoptosis). Eidukaite et al. [11] implicated the Fas–FasL mechanism in the pathophysiology of early endometriosis by reporting a cytometric decrease in CD56dim NK cells (24.0% $\pm$ 4.9% vs. 36.3% $\pm$ 15.9%, $p < 0.02$), and an increase in CD95 (Fas antigen that promotes NK

apoptosis) ($66.6\% \pm 10.6\%$ vs. $42.4\% \pm 10.1\%$, $p < 0.01$) in the peritoneal fluid of subjects with early endometriosis. They also reported an increase in NK cells containing the Fas early activation molecule (i.e., CD69+ CD56+ NK cells) in the peritoneal fluid of subjects with early ($66.8 \pm 16.5\%$ vs. $43.3 \pm 13.7\%$, $p < 0.01$) and advanced ($74.2\% \pm 7.9\%$ vs. $43.3\% \pm 13.7\%$, $p < 0.01$) endometriosis [11].

3.5. NK Cell Receptors in Endometrium and Endometriotic Lesions

Endometrium samples and endometriotic lesions were assessed for the expression of a variety of NK receptors/ligands in the studies selected, to ascertain which features promoted immune evasion and ectopic proliferation/implantation. Two studies [12,18] explored HLA-G (inhibitory NK cell ligand) protein expression in the endometrium and endometriotic lesion samples via immunohistochemistry. As per Barrier et al. [12], HLA-G protein was found in the glandular epithelia of 14 out of 15 peritoneal endometriotic lesion samples (93.3%) (none in stroma), whereas none was found in eutopic endometrium of both control and endometriosis samples. Similarly, Rached et al. [18] found a significant increase in the HLA-G protein expression in endometriotic lesions of advanced-stage endometriosis compared with the eutopic endometrium (both control and endometriosis) ($p = 0.018$). Barrier et al. [12] went further to analyze HLA-G gene transcripts in endometrial samples using RNA in situ hybridization. With this technique, they found that the glandular epithelia of 13 out of 14 peritoneal lesions (92.8%) contained the HLA-G gene transcript, but this was not present in the eutopic endometrium (control and endometriosis samples). The phenomenon of NK cell inhibition was also explored through the expression of programmed-death 1 (PD-1) and its ligand (PD-L1) in endometriosis using immunohistochemistry and Western blotting [24]. Immunohistochemistry results showed an increase in PD-1 and PD-L1 expression in the eutopic and ectopic endometria of endometriosis samples compared with the controls ($p < 0.05$ and $p < 0.01$, respectively) [24]. PD-L1 of the eutopic endometrium of endometriosis samples was upregulated by 17β -estradiol treatment ($p < 0.01$). Regarding Western blot results, ectopic endometriotic lesions showed a significant increase in PD-1 and PD-L1 expression compared with the controls and the eutopic endometrium from endometriosis samples ($p < 0.01$); eutopic endometrium from endometriosis also differed significantly from the controls, exhibiting greater PD-L1 expression ($p < 0.01$) [24]. Additionally, a significant increase in CD4+/CD8+ T cell infiltrate occurrence and size was reported in endometriosis samples (eutopic and ectopic) compared with controls ($p < 0.01$) [24]. In contrast, in the context of activation, NK cell-activating receptor NKG2D ligands were assessed via real-time PCR and Western blotting. Although Xu [19] did not find any statistically significant differences in ligand mRNA expression, changes at a translational level were observed, namely, a significant decrease in ULBP-2 protein expression in the eutopic endometrium of endometriosis samples (relative to the eutopic endometrium of control samples and ectopic lesions ($p < 0.05$)), and a decrease in ULBP-3 protein expression in the ectopic endometrium samples compared with the eutopic endometrium (control and endometriosis) ($p < 0.05$). Factors of immune evasion were also measured [15]. Using immunohistochemistry on endometrial samples obtained via curettage from endometriosis samples and controls, Baka et al. [15] noted a significant increase in HLA-I and HLA-II expression in both the glandular (HLA-I 87.5% vs. 56.3%, $p < 0.02$, HLA-II 46.7% vs. 20.0%, $p < 0.04$) and stromal (HLAI 100% vs. 87.5%, $p < 0.02$, HLAI 66.7% vs. 40.0%, $p < 0.007$) epithelia of endometriosis samples compared with the controls.

3.6. NK Cell Receptors in Peripheral Blood and Serum

Several studies [7–10,18,20,24] have explored NK cell receptor expression in the peripheral venous blood of endometriosis patients. Chou et al. [20] investigated the impact of HLA-C (KIR ligand) and KIR genotype/polymorphisms on the risk of endometriosis in a Han Chinese cohort using a DNA-based method of PCR amplification. They showed that a genotype combination HLA-C*03:03:01 was associated with an increased risk of

endometriosis (OR = 2.811, $p = 0.0473$), while the presence of KIR2DS2 acted as a protective factor against endometriosis with an odds ratio of 0.5577 ($p = 0.0394$). Moreover, the number of KIR centromeric A/A haplotypes was significantly increased in the endometriosis group compared with the control group (OR = 1.793, $p = 0.0394$) [20]. Maeda et al. [9] studied the expression of NK inhibitory receptor CD158a+ in the peripheral blood of endometriosis subjects using flow cytometry and observed a statistically significant increase of 3.8% ($17.5\% \pm 9.9\%$ vs. $13.8\% \pm 7.7\%$, $p = 0.009$) in the endometriosis group compared with the control group. Matsuoka et al. [10] observed similar flow cytometry results, reporting a significant increase in CD158+ CD56+ NK cells in the endometriosis group compared with the control group ($18.8\% \pm 8.2\%$ vs. $13.4\% \pm 5.8\%$, $p = 0.021$). Additionally, the authors also noted a trend whereby KIRs possessing ITIM domains were predominant over KIRs possessing ITAM domains ($65.6\% \pm 32.1\%$ vs. $3.9\% \pm 2.7\%$) in the endometriosis group's peripheral blood samples [10]. Other NK cell inhibitory receptors such as KIR2DL1 + NK cells were also explored in the studies selected [8]. Similarly to CD158a+, endometriosis KIR2DL1 + NK cell levels were significantly higher than those of the control groups ($17.4\% \pm 8.4\%$ vs. $11.7\% \pm 5.5\%$, $p = 0.011$) [8]. Another study by Maeda et al. [7] corroborated this, demonstrating higher levels of KIR2DL1 + NK cells among CD16 + NK cells in the endometriosis group compared with the control group; however, the results were only statistically significant in advanced endometriosis ($24.3\% \pm 15.6\%$ vs. $11.6\% \pm 6.8\%$, $p = 0.008$). Soluble levels of the NK cell inhibitory ligand HLA-G were also investigated by Rached et al. [18] in serum, using ELISA, reporting significantly higher levels of sHLA-G in sera in the endometriosis group compared with the controls (9.3 U/mL ($1.2\text{--}192.6$) vs. 6.1 U/mL ($1.0\text{--}36.9$), $p = 0.013$). Lastly, natural cytotoxicity receptors (NCRs) and NKG2D (NK cell-activating receptors) were studied in the peripheral blood of endometriosis patients by Wu et al. [24]. Unlike the significant differences observed in the peritoneal fluid mentioned above, peripheral blood yielded no statistically significant differences regarding NCRs or NKG2D expression between endometriosis and control groups [24].

4. Discussion

Endometriosis is a multi-factorial disease, characterized by genetic propensity, environmental insult, and, as highlighted in this review, immune dysfunction [26]. Following Sampson's theory of retrograde menstruation, tissue fragments and cells must not only escape the uterine cavity, but also evade apoptosis, adhere to ectopic tissue, generate a local vascular supply, and evade immune surveillance systems to develop into endometriotic lesions [2,26]. NK cell suppression via receptor-mediated dysfunction plays a crucial part in evading immunosurveillance and apoptosis, enabling the proliferation and adhesion of ectopic endometrial cells [27]. Secondary to inflammation associated with endometriosis, the NK cell-mediated release of cytokines and pro-angiogenic factors such as TNF- α and IFN- γ creates an optimal microenvironment for the proliferation of ectopic endometriotic lesions [16]. The studies explored in this review [5–20,24] corroborate this notion, detailing a pathological local environment of endometriosis whereby the aberrant expression/activity of NK cell receptors results in less cytotoxicity (via the increased inhibition, decreased activation, and apoptosis of NK cells themselves) and contribute to a pro-inflammatory state which promotes proliferation and angiogenesis. NK cell suppression has been implicated in the pathogenesis of endometriosis, given its role in immunocompetency and the clearance of atypic debris.

The increased apoptosis of NK cell populations (through aberrant expression of NK cell death receptors) results in increased immune evasion, leaving dysregulated cellular mechanisms and proliferation unconstrained. This is observed in endometriosis, as evidenced by Eidukaite et al. [11] through their flow cytometry results: endometriosis samples do not only contain decreased levels of CD56+ cytotoxic NK cells in peritoneal fluid, but also exhibit an increased presence of Fas antigen (CD95) and increased levels of early-activation molecules (i.e., CD69) in the initial stages of endometriosis (I/II). This suggests that, in the early stages of endometriosis, the Fas–FasL mechanism of induced

cell death is overexpressed, leading to a decrease in NK cell populations, and thus, the increased immune evasion of ectopic endometriotic cells [11].

NK cell inhibitor receptors have also been implicated in immune dysfunction associated with endometriosis. Inhibitor receptor overexpression appears to be a feature of endometriosis, evidenced by several of the studies explored. Matsuoka et al. [10] observed that ITIM (inhibition) receptor domains were more prevalent than ITAM (activation) receptor domains in NK cells found in the peripheral blood of endometriosis subjects. This suggests that the immune balance of activation/inhibition is shifted in endometriosis, favoring NK cell suppression, and thus promoting ectopic endometrial cell survival. This study, however, had the limitation of only having analyzed ITIM/ITAM domains in the peripheral blood of subjects. Most ectopic implantation sites are in the peritoneal cavity; therefore, the peritoneal fluid analysis of ITAM and ITIM domain levels of local NK cells may be more accurate in depicting endometriotic disease profiles than the peripheral blood. Matsuoka et al. [10] also identified a significantly greater expression of NK inhibitor receptor CD158a in CD16+ NK cells in the peripheral blood and peritoneal fluid of endometriosis subjects compared with controls. This finding is supported by Maeda et al. [7,8], who also reported an increase in inhibitor receptor CD158a+ NK cells in the peritoneal fluid and peripheral blood of endometriosis subjects [9]. Unlike Matsuoka et al. [10], however, Maeda et al. [7,8] also found a significant overexpression of NK inhibitor receptor KIR2DL1; the results yielded by Matsuoka et al. [10] did not show significant results for KIR2DL1 levels. This could be due to variability in study populations—it may be that subjects studied by Maeda et al. [7,8] had a greater relative disease severity than those studied by Matsuoka et al. [10], because it is recognized that KIR2DL1 overexpression appears to be progressive. Stage III/IV endometriosis exhibits significantly greater KIR2DL1+ NK levels than stage I/II samples. As for other inhibitor receptors, Galandrini et al. [14] reported a significant increase in CD94/NKG2A receptors in the peritoneal fluid of endometriosis subjects compared with controls. All these results support the hypothesis of NK cell inhibition being one of the underlying factors promoting the immune evasion of ectopic endometrial cells. Maeda et al. [8] also propose that the level of inhibition is potentially correlated with disease severity. A limitation of these studies, however, is that although they report greater KIR expression, the methodologies did not explore whether this indeed reduces NK cell activity. A complementary cytotoxicity assay would be appropriate to confirm the hypothesis. Wu et al. [5] bridged this knowledge gap by conducting a ^{51}Cr cytotoxicity assay against K562 cells. They found that in all stages of endometriosis, there was significantly less peritoneal cytotoxicity against K562 cells than in controls [5]. Additionally, they observed a significant increase in KIR (NKB1, EB6) expression, which appeared to be correlated with disease severity (III/IV was significantly greater than I/II endometriosis) [5]. These findings together support the notion that NK cell activity is suppressed via KIR overexpression in endometriosis. Notably, in these studies, it is unclear whether KIR overexpression and reduced cytotoxicity were the cause or the result of endometriosis. To elucidate this, the influence of the genetic background on KIR expression/activity could be considered. As reported by Chou et al. [20], specific gene polymorphisms which affect inhibitor receptors such as HLA-C*03:03:01 and KIR centromeric A/A haplotypes occurred more often in subjects with endometriosis than controls. This finding suggests that altered KIR expression and immune dysfunction in endometriosis is associated with individual genetic backgrounds; specific genetic profiles seemed to be risk factors, associated with the higher occurrence of endometriosis. This defends the notion that KIR overexpression has a causal relationship with endometriosis, rather than occurring because of the disease. It is pertinent to consider, however, that this study was conducted in a Han Chinese cohort [20]. Whether these genetic findings would also apply to the general population is uncertain. Studies with more diverse samples are necessary to ascertain whether the extrapolation of findings to the general population would be accurate.

Ligands that bind to NK inhibitor receptors are also upregulated in endometriosis. This is evidenced, for instance, by the finding of significantly higher levels of HLA-E

mRNA (which binds to the C-type lectin protein HLA-I-specific inhibitory NK receptor CD94/NKG2A) in the peritoneal fluid of endometriosis subjects compared with controls [14]. Similar results have been reported on ligands which bind to the other family of HLA-I-specific inhibitory NK receptors or killer immunoglobulin-like receptors. HLA-I is an established inhibitory ligand that binds to KIRs on NK cells to prevent autoimmune responses; cells missing an HLA-I complex are targeted by NK cells for cell death following the “missing-self hypothesis” [28]. As per Baka et al. [15], another mechanism of endometrial immune evasion is the overexpression of HLA-I and HLA-II in both the glandular and stromal epithelia of endometrial tissue. The higher levels of HLA-I and HLA-II favor the suppression of cellular immunity in the peritoneal cavity, providing optimal conditions for the establishment of ectopic lesions. Nevertheless, this experiment is not without fault; Baka et al. only investigated samples of eutopic endometrium (found within the uterine cavity); therefore, it is unclear whether these expression trends are maintained in ectopic sites [15].

HLA-G, another NK receptor inhibitory ligand, has a stress-inducible gene; therefore, it is commonly found in tumors or in states of cellular stress, facilitating immune evasion and atypical cellular survival [29]. The selected studies explored this molecule extensively, mainly noting the absence of significant expression in the peritoneal fluid of endometriosis subjects [6,13]. Rached et al. [18] contradicted these findings by noting an increase in soluble HLA-G levels in the peritoneal fluid of endometriosis subjects in the menstrual phase compared with the secretory phase, although this increase was not statistically significant. These results align with Sampson’s theory [2] of retrograde menstruation, whereby endometrial cells escape to the peritoneal cavity during menstruation via patent fallopian tubes. Rached et al. [18] also observed significantly increased levels of soluble HLA-G in the serum of III/IV endometriosis subjects. Additionally, a significant increase in HLA-G protein and gene expression in ectopic peritoneal endometrial lesions’ glandular epithelium, but not the eutopic endometrium, was observed [12,18]. According to these results, it is suggested that ectopic endometrial lesions, upon encountering a new local immune environment, express HLA-G to promote cell survival and immune evasion via NK cell inhibition: this expression appears to be localized to the tissue fragments only. Furthermore, the absence of HLA-G expression in peritoneal fluid highlights that immune dysfunction associated with endometriosis derives from both NK inhibitor receptor and ligand overexpression.

NK immune checkpoint/inhibitory receptor programmed death-1 (PD-1) and its ligand, PD-L1, have also been investigated [30]. As per Wu et al. [24], increased proportions of PD-1- and PD-L1-positive cells were found in the endometriosis endometrium and blood samples compared with controls. The PD-1/PD-L1 axis has been thoroughly investigated in the oncological setting, and these molecules seem to be upregulated in a variety of cancer cells (ovarian/gastric carcinoma/leukemia) [31–33]. Thus, it has been postulated that PD-1 and PDL-1 serve as immunoinhibitory mechanisms contributing to tumor evasion from host defensive responses. These results suggest that this type of immune dysfunction also occurs in the pathogenesis of endometriosis. Further supporting this notion is the fact that PD-1/PDL-1 and PD-1 levels are upregulated in the eutopic endometrium of endometriosis samples following 17 β -estradiol treatment. These results align with the established knowledge of endometriosis being an estrogen-dominant condition, whereby estrogen exposure promotes established ectopic endometrial lesion growth and exacerbates associated pain and inflammation [34]. Additionally, Wu et al. [24] noted a significant increase in the occurrence and size of CD4⁺/CD8⁺ T cell infiltrates in endometriosis samples. This observation is congruent with the inflammatory landscape of endometriosis, where the increased T cell-mediated release of mediators results in higher levels of circulating cytokines. Funamizu et al. [16] also explored the endometriotic cytokine environment, observing an increase in TNF- α - and IFN- γ -producing NK cells in III/IV endometriosis compared with controls. Saeki et al. [25] corroborates these findings, having also observed an increase in IFN- γ -producing NK cells in endometriosis. This aligns with the pathogenic setting of

endometriosis as cytokines promoting an inflammatory state, optimal for angiogenesis and tissue invasion required for ectopic lesion establishment and survival.

The suppression of NK cell-activating receptors has also been implicated in immune dysfunction associated with endometriosis. Funamizu et al. [16] investigated this phenomenon, observing differences in the expression of several NCRs such as NKp46, NKp40, and NKp30. Of these, only NKp46 receptors exhibited a significant decrease in III/IV endometriosis compared with the controls [16]. Nevertheless, this decrease in receptor expression suggests that NK cell cytotoxicity suppression in endometriosis is also achieved by decreased activation. Xu [19] examined NKG2D receptor levels and respective ligands (MICA, MICB, ULBP-2, and ULBP-3), finding a significant decrease in NKG2D in CD56+ NK cells in the peritoneal fluid of endometriosis subjects, decreased expression of ULBP-2 in the eutopic endometrium of endometriosis subjects, and decreased ULBP-3 expression in ectopic endometrial lesions. Furthermore, González-Foruria et al. [17] explored NKG2D ligands. Unlike Xu [19], these authors found a significant increase in MICA, MICB, and ULBP-2 in the peritoneal fluid of subjects with deep infiltrating endometriosis compared with controls, and proposed that this increase was due to the shedding of ligands from endometrial cells onto the peritoneal space, resulting in the lower expression of these ligands on ectopic endometrial cell surfaces [17]. In turn, this promotes immune evasion and decreased NK cell cytotoxicity (less activation/triggering of NK cells by the ectopic lesions). It is possible that the results may not be fully representative of the ligand environment in endometriosis, whereby some allelic variants of MICA are not shed into the peritoneal space, and thus compensate for the absence of other allelic forms. This, although unlikely, cannot be confirmed due to the lack of literature on NKG2D ligands in endometriosis. Chou et al.'s [20] methodology may also be used to ascertain whether this apparent decrease in NK-activating receptors is the cause or the result of endometriosis. As per the study's observations, there were fewer KIR2DS2-positive individuals in the endometriosis cohort compared with the controls [20]. KIR2DS2 (a gene encoding for an NK-activating receptor) appears to be a protective factor against endometriosis, suggesting that the inherent presence of fewer activating receptors creates an optimal environment for the development of endometriosis. However, as mentioned before, given the specificity of the cohort chosen in this study [20], it is unclear whether these results can be extrapolated to the general population. Saeki et al. [25] observed an increase in co-expression of NKp46+ and activating receptor NKG2D+. This prompts the hypothesis that aberrant or excess expression of NK cell-activating receptors may also be involved in the immune dysfunction of endometriosis. When chronically stimulated, NKG2D may exert inhibitory effects, decreasing missing-self signaling, as observed in murine models [25,35].

5. Conclusions

The overall objective of this systematic review was to assess the role of NK receptors (activating and inhibiting) in endometriosis pathogenesis, as depicted in the current literature. This was achieved by analyzing a total of 18 studies [5–20,24,25]. As per their results, endometriosis pathogenesis is marked by the overexpression of NK inhibitor receptors (KIRs), namely, CD158a+, KIR2DL1, CD94/NKG2A, PD-1, NKB1, and EB6, and inhibiting ligands such as PD-L1, HLA-E, HLA-G, and HLA-I in ectopic endometrial lesions. Concurrently, there is also a decrease in NK-activating receptors and NCRs such as NKp46, NKp30, and NKG2D, and a decreased expression (or increased shedding into the peritoneal space) of NK-activating ligands such as MICA, MICB, ULBP-2, and ULBP-3. Additionally, aberrant/excess expression of NKG2D+ has also been suggested to decrease immune response to ectopic endometrial cells. The notion of an immune shift from NK surveillance to NK suppression is also apparent in the greater relative number of ITIM domains compared with ITAM domains in NK receptors found in endometriosis subjects. Further exacerbating conditions for immune evasion and ectopic proliferation are NK cell death via the Fas–FasL mechanism in early endometriosis, decreased NK cell cytotoxicity, and increased NK cell-mediated cytokine release of TNF- α and IFN- γ , a state which promotes inflammation and

angiogenesis required for ectopic endometrial cell lesion survival. Immune dysfunction associated with endometriosis also appears to have a genetic component—certain genotypes, namely, those which cause NK receptor dysfunction, have been associated with a greater risk of endometriosis. Overall, NK receptor activity dictates the immunocompetency of women to clear ectopic endometrial lesions from the peritoneal cavity. The suppression of NK cell activity (either by over-inhibition or decreased activation) creates optimal conditions for the development of endometriosis because it promotes lesion immune evasion, proliferation, and survival. If it is proven that the immune escape of endometriosis cells, allowing their implantation and growth, is related to the function of NK cell receptors, this may open the door to the application of immunotherapy in the treatment of severe endometriosis, as is already the case today with NK inhibitor receptor blockers in cancer treatment [36]. Further research and understanding on NK cell receptor involvement in endometriosis potentiates the development of novel therapeutic strategies, bringing about improvements in patients' quality of life (clinical significance) and a novel approach towards endometriosis-associated infertility. Future research could explore NK inhibitor receptors as therapeutic targets, counteracting their overexpression in endometriosis with a local antagonist.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms24010331/s1>.

Author Contributions: Conceptualization: J.L.; data curation: J.L.R.; investigation: N.N.R. and J.L.R.; methodology: J.L. and M.Â.-D.; project administration: J.L.R. and J.L.; resources: J.L.; supervision: J.L., C.M. and L.M.B.; validation: M.Â.-D.; visualization: M.Â.-D.; writing—original draft preparation: J.L.R. and N.N.R.; writing—review and editing: M.Â.-D., C.M., L.M.B. and J.L. All authors have read and agreed to the published version of the manuscript.

Funding: This work in the context of the “Women’s Health and Maternal & Fetal Research Group” was partially co-financed by Hospital da Luz Lisboa under the initiative “Luz Investigação”.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

- Shafir, A.; Farland, L.; Shah, D.; Harris, H.; Kvaskoff, M.; Zondervan, K.; Missmer, S. Risk for and consequences of endometriosis: A critical epidemiologic review. *Best Pract. Res. Clin. Obstet. Gynaecol.* **2018**, *51*, 1–15. [CrossRef] [PubMed]
- Sampson, J.A. Metastatic or Embolic Endometriosis, due to the Menstrual Dissemination of Endometrial Tissue into the Venous Circulation. *Am. J. Pathol.* **1927**, *3*, 93–110. [PubMed]
- Riccio, L.D.G.C.; Santulli, P.; Marcellin, L.; Abrão, M.S.; Batteux, F.; Chapron, C. Immunology of endometriosis. *Best Pract. Res. Clin. Obstet. Gynaecol.* **2018**, *50*, 39–49. [CrossRef]
- Thiruchelvam, U.; Wingfield, M.; O’Farrelly, C. Natural Killer Cells: Key Players in Endometriosis. *Am. J. Reprod. Immunol.* **2015**, *74*, 291–301. [CrossRef] [PubMed]
- Wu, M.-Y.; Yang, J.-H.; Chao, K.-H.; Hwang, J.-L.; Yang, Y.-S.; Ho, H.-N. Increase in the expression of killer cell inhibitory receptors on peritoneal natural killer cells in women with endometriosis. *Fertil. Steril.* **2000**, *74*, 1187–1191. [CrossRef] [PubMed]
- Hornung, D.; Fujii, E.; Lim, K.-H.; Vigne, J.-L.; McMaster, M.T.; Taylor, R.N. Histocompatibility leukocyte antigen-G is not expressed by endometriosis or endometrial tissue. *Fertil. Steril.* **2001**, *75*, 814–817. [CrossRef] [PubMed]
- Maeda, N.; Izumiya, C.; Oguri, H.; Kusume, T.; Yamamoto, Y.; Fukaya, T. Aberrant expression of intercellular adhesion molecule-1 and killer inhibitory receptors induces immune tolerance in women with pelvic endometriosis. *Fertil. Steril.* **2002**, *77*, 679–683. [CrossRef] [PubMed]
- Maeda, N.; Izumiya, C.; Yamamoto, Y.; Oguri, H.; Kusume, T.; Fukaya, T. Increased killer inhibitory receptor KIR2DL1 expression among natural killer cells in women with pelvic endometriosis. *Fertil. Steril.* **2002**, *77*, 297–302. [CrossRef]
- Maeda, N.; Izumiya, C.; Kusum, T.; Masumoto, T.; Yamashita, C.; Yamamoto, Y.; Oguri, H.; Fukaya, T. Killer Inhibitory Receptor CD158a Overexpression Among Natural Killer Cells in Women With Endometriosis is Undiminished by Laparoscopic Surgery and Gonadotropin Releasing Hormone Agonist Treatment*. *Am. J. Reprod. Immunol.* **2004**, *51*, 364–372. [CrossRef]

10. Matsuoka, S.; Maeda, N.; Izumiya, C.; Yamashita, C.; Nishimori, Y.; Fukaya, T. Expression of Inhibitory-Motif Killer Immunoglobulin-Like Receptor, KIR2DL1, is Increased in Natural Killer Cells from Women with Pelvic Endometriosis. *Am. J. Reprod. Immunol.* **2005**, *53*, 249–254. [\[CrossRef\]](#)
11. Eidukaite, A.; Siaurys, A.; Tamosiunas, V. Aberrant Expression of CD95 and CD69 Molecules among CD56+ Cells in Women with Endometriosis. *Am. J. Reprod. Immunol.* **2006**, *55*, 276–281. [\[CrossRef\]](#)
12. Barrier, B.; Kendall, B.; Ryan, C.; Sharpe-Timms, K. HLA-G is expressed by the glandular epithelium of peritoneal endometriosis but not in eutopic endometrium. *Hum. Reprod.* **2005**, *21*, 864–869. [\[CrossRef\]](#)
13. Eidukaite, A.; Tamosiunas, V. Soluble HLA-G in the peritoneal fluid of women with endometriosis. *Fertil. Steril.* **2008**, *89*, 465–467. [\[CrossRef\]](#) [\[PubMed\]](#)
14. Galandrini, R.; Porpora, M.G.; Stoppacciaro, A.; Micucci, F.; Capuano, C.; Tassi, I.; Di Felice, A.; Benedetti-Panici, P.; Santoni, A. Increased frequency of human leukocyte antigen-E inhibitory receptor CD94/NKG2A-expressing peritoneal natural killer cells in patients with endometriosis. *Fertil. Steril.* **2008**, *89*, 1490–1496. [\[CrossRef\]](#) [\[PubMed\]](#)
15. Baka, S.; Frangou-Plemenou, M.; Panagiotopoulou, E.; Makrakis, E.; Kaltsakas, G.; Hassiakos, D.; Kondi-Pafiti, A. The expression of human leukocyte antigens class I and II in women with endometriosis or adenomyosis. *Gynecol. Endocrinol.* **2010**, *27*, 419–424. [\[CrossRef\]](#) [\[PubMed\]](#)
16. Funamizu, A.; Fukui, A.; Kamoi, M.; Fuchinoue, K.; Yokota, M.; Fukuhara, R.; Mizunuma, H. Expression of Natural Cytotoxicity Receptors on Peritoneal Fluid Natural Killer Cell and Cytokine Production by Peritoneal Fluid Natural Killer Cell in Women with Endometriosis. *Am. J. Reprod. Immunol.* **2014**, *71*, 359–367. [\[CrossRef\]](#) [\[PubMed\]](#)
17. González-Foruria, I.; Santulli, P.; Chouzenoux, S.; Carmona, F.; Batteux, F.; Chapron, C. Soluble Ligands for the NKG2D Receptor Are Released during Endometriosis and Correlate with Disease Severity. *PLoS ONE* **2015**, *10*, e0119961. [\[CrossRef\]](#)
18. Rached, M.R.; Coelho, V.; Marin, M.L.C.; Pincerato, K.; Fujita, A.; Kalil, J.E.; Abrão, M.S. HLA-G is upregulated in advanced endometriosis. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **2019**, *235*, 36–41. [\[CrossRef\]](#)
19. Xu, H. Expressions of natural cytotoxicity receptor, NKG2D and NKG2D ligands in endometriosis. *J. Reprod. Immunol.* **2019**, *136*, 102615. [\[CrossRef\]](#)
20. Chou, Y.-C.; Chen, C.-H.; Chen, M.-J.; Chang, C.-W.; Chen, P.-H.; Yu, M.-H.; Chen, Y.-J.; Tsai, E.-M.; Yang, P.-S.; Lin, S.-Y.; et al. Killer cell immunoglobulin-like receptors (KIR) and human leukocyte antigen-C (HLA-C) allorecognition patterns in women with endometriosis. *Sci. Rep.* **2020**, *10*, 1–9. [\[CrossRef\]](#) [\[PubMed\]](#)
21. Page, M.J.; McKenzie, J.E.; Bossuyt, P.M.; Boutron, I.; Hoffmann, T.C.; Mulrow, C.D.; Shamseer, L.; Tetzlaff, J.M.; Akl, E.A.; Brennan, S.E.; et al. The PRISMA 2020 statement: An updated guideline for reporting systematic reviews. *Int. J. Surg.* **2021**, *88*, 105906. [\[CrossRef\]](#) [\[PubMed\]](#)
22. Page, M.J.; Moher, D.; Bossuyt, P.M.; Boutron, I.; Hoffmann, T.C.; Mulrow, C.D.; Shamseer, L.; Tetzlaff, J.M.; Akl, E.A.; Brennan, S.E.; et al. PRISMA 2020 explanation and elaboration: Updated guidance and exemplars for reporting systematic reviews. *BMJ* **2021**, *372*, n160. [\[CrossRef\]](#) [\[PubMed\]](#)
23. Andrews, W.C.; Buttram, V.C., Jr.; Weed, J.C.; Hammond, C.B.; Thomas, H.H.; Behrman, S.J. Revised American Fertility Society classification of endometriosis. *Fertil. Steril.* **1985**, *43*, 351–352.
24. Wu, L.; Lv, C.; Su, Y.; Li, C.; Zhang, H.; Zhao, X.; Li, M. Expression of programmed death-1 (PD-1) and its ligand PD-L1 is upregulated in endometriosis and promoted by 17β-estradiol. *Gynecol. Endocrinol.* **2018**, *35*, 251–256. [\[CrossRef\]](#) [\[PubMed\]](#)
25. Saeki, S.; Fukui, A.; Mai, C.; Takeyama, R.; Yamaya, A.; Shibahara, H. Co-expression of activating and inhibitory receptors on peritoneal fluid NK cells in women with endometriosis. *J. Reprod. Immunol.* **2022**, *155*, 103765. [\[CrossRef\]](#) [\[PubMed\]](#)
26. Ahn, S.H.; Monsanto, S.P.; Miller, C.; Singh, S.S.; Thomas, R.; Tayade, C. Pathophysiology and Immune Dysfunction in Endometriosis. *BioMed Res. Int.* **2015**, *2015*, 795976. [\[CrossRef\]](#)
27. Fukui, A.; Mai, C.; Saeki, S.; Yamamoto, M.; Takeyama, R.; Kato, T.; Ukita, Y.; Wakimoto, Y.; Yamaya, A.; Shibahara, H. Pelvic endometriosis and natural killer cell immunity. *Am. J. Reprod. Immunol.* **2021**, *85*, e13342. [\[CrossRef\]](#)
28. Brooks, A.G.; Boyington, J.C.; Sun, P.D. Natural killer cell recognition of HLA class I molecules. *Rev. Immunogenet.* **2000**, *2*, 433–448.
29. Ibrahim, E.C.; Morange, M.; Dausset, J.; Carosella, E.D.; Paul, P. Heat shock and arsenite induce expression of the nonclassical class I histocompatibility HLA-G gene in tumor cell lines. *Cell Stress Chaperon.* **2000**, *5*, 207–218. [\[CrossRef\]](#)
30. Quatrini, L.; Mariotti, F.; Munari, E.; Tumino, N.; Vacca, P.; Moretta, L. The Immune Checkpoint PD-1 in Natural Killer Cells: Expression, Function and Targeting in Tumour Immunotherapy. *Cancers* **2020**, *12*, 3285. [\[CrossRef\]](#)
31. Rusak, M.; Eljaszewicz, A.; Bołkun, Ł.; Łuksza, E.; Łapuć, I.; Piszcz, J.; Singh, P.; Dąbrowska, M.; Bodzenta-Lukaszyk, A.; Kłoczko, J.; et al. Prognostic significance of PD-1 expression on peripheral blood CD4+ T cells in patients with newly diagnosed chronic lymphocytic leukemia. *Pol. Arch. Intern. Med.* **2015**, *125*, 553–559. [\[CrossRef\]](#) [\[PubMed\]](#)
32. Dong, M.; Wang, H.Y.; Zhao, X.X.; Chen, J.N.; Zhang, Y.W.; Huang, Y.; Xue, L.; Li, H.G.; Du, H.; Wu, X.Y.; et al. Expression and prognostic roles of PIK3CA, JAK2, PD-L1, and PD-L2 in Epstein-Barr virus-associated gastric carcinoma. *Hum. Pathol.* **2016**, *53*, 25–34. [\[CrossRef\]](#) [\[PubMed\]](#)
33. Webb, J.R.; Milne, K.; Kroeger, D.R.; Nelson, B.H. PD-L1 expression is associated with tumor-infiltrating T cells and favorable prognosis in high-grade serous ovarian cancer. *Gynecol. Oncol.* **2016**, *141*, 293–302. [\[CrossRef\]](#) [\[PubMed\]](#)
34. Chantalat, E.; Valera, M.-C.; Vaysse, C.; Noirrit, E.; Rusidze, M.; Weyl, A.; Vergriete, K.; Buscail, E.; Lluel, P.; Fontaine, C.; et al. Estrogen Receptors and Endometriosis. *Int. J. Mol. Sci.* **2020**, *21*, 2815. [\[CrossRef\]](#)

35. Coudert, J.D.; Scarpellino, L.; Gros, F.; Vivier, E.; Held, W. Sustained NKG2D engagement induces cross-tolerance of multiple distinct NK cell activation pathways. *Blood* **2008**, *111*, 3571–3578. [[CrossRef](#)]
36. Evert, J.H.-V.; Paap, R.; Nap, A.; van der Molen, R. The Promises of Natural Killer Cell Therapy in Endometriosis. *Int. J. Mol. Sci.* **2022**, *23*, 5539. [[CrossRef](#)]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.

CHAPTER IV

The Role of NK and T Cells in Endometriosis



Article

The Role of NK and T Cells in Endometriosis

José Lourenço Reis ^{1,†}, Natacha Nurdine Rosa ^{2,†}, Catarina Martins ^{3,4}, Miguel Ângelo-Dias ^{3,4},
Luís Miguel Borrego ^{3,4,5} and Jorge Lima ^{1,3,4,*}

¹ Department of Obstetrics and Gynecology, Hospital da Luz Lisboa, Luz Saúde, 1500-650 Lisboa, Portugal; jose.mlreis@gmail.com

² UCD School of Medicine, University College Dublin, D04 V1W8 Dublin, Ireland; natachagarciarosa@gmail.com

³ CHRC, NOVA Medical School, Faculdade de Ciências Médicas, Universidade NOVA de Lisboa, 1169-056 Lisboa, Portugal; catarina.martins@nms.unl.pt (C.M.); miguel.dias@nms.unl.pt (M.Â.-D.); borregolm@gmail.com (L.M.B.)

⁴ Immunology Department, NOVA Medical School, Faculdade de Ciências Médicas, Universidade NOVA de Lisboa, 1169-056 Lisboa, Portugal

⁵ Department of Immunology, Hospital da Luz Lisboa, Luz Saúde, 1500-650 Lisboa, Portugal

* Correspondence: jorgeramoslima@sapo.pt

† These authors contributed equally to this work.

Abstract: Endometriosis, a debilitating condition, affects one in ten women of reproductive age. Its pathophysiology remains unclear, though deficiencies in immune surveillance are thought to create an environment conducive to the evasion of ectopic endometrial cells from the immune system. Our research explores the immunological impact of endometriosis both locally and systemically, emphasizing natural killer (NK) and T cell subpopulations. We incorporated 62 female patients who underwent laparoscopic surgery; of those, 47 had endometriosis, and 15 were controls. We collected peritoneal fluid (PF) and peripheral blood (PB) samples which were tagged with monoclonal antibodies and subsequently scrutinized using flow cytometry. Our findings revealed significant differences in immunological profiles based on demographic factors and symptomatology. In the endometriosis cohort, there was an increase in PB CD56^{Hi}CD16^{dim} and PF CD8⁺ CD56^{dim}CD16^{Hi} NK cells. CD16⁺ CD4 T cell levels were significantly lower in the PB of endometriosis patients who smoke. Individuals with more severe disease displayed significantly higher levels of PB CD16⁺ CD8 T cells, which also increased in those with non-menstrual pelvic pain. Dysmenorrhea severity correlated with a progressive increase in PF CD8⁺ CD56^{dim}CD16^{Hi} NK cells. These variations in specific lymphocyte subsets, namely, within NK and T cells, suggest potential immunological mechanisms in the evolution and clinical presentation of endometriosis.

Keywords: endometriosis; NK cells; T cells; peritoneal fluid; peripheral blood



Citation: Reis, J.L.; Rosa, N.N.; Martins, C.; Ângelo-Dias, M.; Borrego, L.M.; Lima, J. The Role of NK and T Cells in Endometriosis. *Int. J. Mol. Sci.* **2024**, *25*, 10141. <https://doi.org/10.3390/ijms251810141>

Academic Editor: Giovanni Tossetta

Received: 15 August 2024

Revised: 8 September 2024

Accepted: 20 September 2024

Published: 21 September 2024



Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Endometriosis (EM) is a chronic gynecological condition characterized by the presence of endometrial tissue outside the uterine cavity, typically in the pelvic peritoneum, ovaries, and bowel. These ectopic lesions result in symptoms including dysmenorrhea, dyspareunia, pelvic discomfort, and infertility. Despite the fact that EM impacts one in ten women of reproductive age [1], the pathophysiology remains unclear, hence, effective diagnostic and treatment strategies are still yet to be established.

The most accepted theory regarding pathogenesis is that of retrograde menstruation [2]. However, though this process occurs in almost all women, only 10% develop EM [1], suggesting that additional factors must be present for EM to develop. The recent literature implicates immunological dysfunction in EM pathophysiology [3–20]. It is suggested that immune surveillance deficiencies in the peritoneal cavity create a conducive

microenvironment for ectopic endometrial cell immune evasion and adherence. This environment promotes proliferation and angiogenesis, allowing for abnormal tissue survival and settlement [3–20]. Many studies suggest that changes in the immune profile, particularly in natural killer (NK) cells and T cells, underpin the compromised immune response observed in women with EM, resulting in poor clearance of ectopic endometrium [3–20]. To our knowledge, 18 studies [3–20] have investigated NK cell and T cell activity or phenotypic changes in the peripheral blood (PB) and peritoneal fluid (PF) of EM patients. According to the literature, NK cell cytotoxicity is diminished in EM, reflecting a functional defect. This defect is characterized by an overexpression of NK cell inhibitory receptors and a decreased expression of activating receptors [6,8,10,21,22]. For lymphocyte subpopulations, both quantitative and functional defects were observed [4,12,19], but the existing literature provides limited clarity. More specifically, Iwasaki et al. [19] reported an increase in suppressive T cells and a decrease in cytotoxic T cells in PB and PF samples of EM patients. Contrastingly, Hassa et al. [7] observed no significant changes in lymphocyte subsets in EM patients. More recently, however, Meggyes et al. [5] highlighted quantitative differences in specific lymphocyte subpopulations, including decreased T, CD4⁺ T, and regulatory T cells. They also noted functional differences, such as differential TIM3/Galectin-9 T cell expression [5].

Further research is needed to understand the immune aspect of EM, thus enabling the development of more targeted and effective treatments. This study intends to contribute insights to a field in which understanding is far from comprehensive. It distinguishes itself by examining the connection between immunology, demographic factors, and symptoms. To this end, we explore the immunological impact of EM both locally (PF) and systemically (PB), specifically focusing on NK and T cell subpopulations. We establish a baseline in control and case groups. Our approach offers a unique clinical perspective from previous studies in the literature, with noticeable methodological differences. In more detail, we identify abnormal patterns in NK and T cell profiles relating to clinical presentation and symptoms such as dysmenorrhea, dyspareunia, pelvic pain, rectorrhagia, and dyschezia. Moreover, we examine the interplay between immunology and demographic factors, like race and smoking status, on both systemic and local NK and T cell populations of control and EM patients. By contributing to a deeper understanding of the interaction between EM and immune and demographic factors, we hope to lay the groundwork for the creation of personalized treatments and interventions for individual clinical presentations. This approach may enhance treatment efficacy while potentially minimizing adverse or off-target effects.

2. Results

2.1. Demographic and Clinical Characteristics of Patients

The demographic and clinical data for both enrolled groups are summarized in Table 1. In the control group, the average age was 38.5 years, with a standard deviation (SD) of 9.2, and the age ranged from 20 to 55 years. In the EM group, the average age was 36.2 years, with an SD of 6.34, and the ages ranged from 31 to 42 years. Most participants in both groups were White (>85%). There were no significant differences in the demographic and clinical characteristics between the two groups, apart from the history of infertility, which was significantly more common in women with EM ($p = 0.003$). With regards to hormone therapy, the difference in its usage between 33% of the control group and 66% of the EM group was not statistically significant ($p = 0.09$).

2.2. Endometriosis Characteristics

Table 2 provides detailed information on the characteristics of EM in the participating patients. The average time between the EM diagnosis and surgery was 2.8 years. A majority of patients had severe forms of the disease (78.7%), corresponding to stages III/IV. The most frequently experienced symptom was dysmenorrhea, reported by 93.6% of participants, with severe dysmenorrhea (≥ 7) reported by 76.6%. Other symptoms included dyspareunia

(59.6%), chronic pelvic pain (40.4%), dyschezia (34.0%), bowel symptoms (31.9%), urinary tract symptoms (10.6%), and rectal bleeding (8.5%). Furthermore, about 4.3% of cases involved extra-pelvic EM.

Table 1. Demographic characteristics.

	Control ^a (n = 15)	Endometriosis ^a (n = 47)	p-Value ^b
Age, years			
Mean (SD)	38.8 (9.2)	36.2 (6.34)	0.434
Min, Max	20, 55	31, 42	
Race, n (%)			0.113
White	15 (100)	40 (85.1)	
Black	0 (0)	7 (14.9)	
BMI, kg/m²			0.430
Mean (SD)	23.2 (2.97)	25.2 (5.60)	
Min, Max	17.28	20.8, 28.0	
Fertile, n (%)			0.003
Yes	14 (93.3)	24 (51.1)	
No	1 (6.7)	23 (48.9)	
Smoker, n (%)			0.087
Yes	0 (0)	8 (17.0)	
No	15 (100)	39 (83.0)	
Hormonal therapy, n (%)			0.090
Yes	5 (33.3)	13 (27.7)	
No	10 (66.7)	34 (72.3)	

^a The results are presented as mean and standard deviation; mean (SD). ^b p-values are based on the Mann–Whitney non-parametric *U* test. Statistically significant results are indicated in bold.

Table 2. Disease characteristics.

	Endometriosis n = 47 ^a
Endometriosis diagnosis, years ago	
Mean (SD)	2.8 (2.52)
Min, Max	1, 14
Dysmenorrhea complaints, n (%)	44 (93.6)
Dysmenorrhea severity, n (%)	
None/Mild/Moderate (<7)	11 (23.4)
Severe (≥7)	36 (76.6)
Dyspareunia complaints, n (%)	28 (59.6)
Dyschezia complaints, n (%)	16 (34.0)
Rectorrhagia n (%)	4 (8.5)
Chronic pelvic pain, n (%)	19 (40.4)
Bowel symptoms, n (%)	15 (31.9)
Urinary tract symptoms, n (%)	5 (10.6)
Extra-pelvic endometriosis, n (%)	2 (4.3)

^a The results are presented as mean and standard deviation; mean (SD).

2.3. Tissue-Resident and Circulating Lymphoid Cells: Differences between Peripheral Blood and Peritoneal Fluid

As expected, the lymphoid cellular content differed significantly between PB (circulating) and PF (tissue-resident) samples. PF samples displayed a significantly larger

proportion of lymphocytes within total leukocytes ($p < 0.001$), despite having lower absolute cell counts (when considering total lymphocytes, as well as T and NK cells).

Regarding T cells, the ratios of CD4/CD8 T cells were considerably higher in PB than in PF samples ($p < 0.001$). PB displayed increased percentages of CD4 T cells (representing 67% of total T cells, $p < 0.001$) and lower percentages of CD8 T cells (constituting 33% of total T cells, $p < 0.001$). In contrast, PF CD4 T cells accounted for only 37% of total T cells, whereas CD8 T cells were more abundant, with mean values of 63%. CD56⁺ T cells also varied between compartments, amounting to roughly 15% in PF compared to only 10% in PB ($p < 0.001$). Additionally, tissue-resident lymphocytes exhibited higher percentages of CD16 in both CD4 and CD56⁺ T cells ($p < 0.001$ for both) and higher percentages of CD56 in CD8 T cells when compared to circulating samples ($p < 0.001$).

Interestingly, no significant differences were observed in total NK cells between PB and PF samples. However, in PB, two major circulating NK subsets were identified: CD56^{dim}CD16^{Hi}, which was the most abundant, and the less represented CD56^{Hi}CD16^{dim}. In PF samples, four distinct subsets were found: CD56^{+/dim}CD16^{Hi} and CD56^{Hi}CD16⁻ NK cells were the most abundant, while CD56^{Hi}CD16⁺ and CD56^{dim}CD16⁻ NK cells were less common. It is worth noting that a larger percentage of circulating NK cells expressed CD8 in comparison to PF NK cells ($p < 0.001$).

The complete statistical data for the results discussed in this section can be found in Table S2 of the Supplementary Materials.

2.4. Patients with Endometriosis: Impact on Circulating and Local Immune Profiles

The next step in our analysis involved further identifying the differences in both sample types between EM patients and the control group.

Initially, only modest differences between controls and EM patients were observed in major circulating subsets. For example, there was a trend towards higher absolute T cell counts in the PB of EM patients ($p = 0.054$) and lower percentages of circulating NK cells ($p = 0.081$). Within the NK cells, despite being the most abundant subset, cytotoxic CD56^{dim}CD16^{Hi} NK cells were slightly decreased in EM patients ($p = 0.073$). Conversely, there were significantly increased percentages ($p = 0.019$) and absolute counts ($p = 0.047$) of CD56^{Hi}CD16^{dim} NK cells compared to controls. Therefore, EM patients showed lower CD56^{dim}CD16^{Hi} NK cells/CD56^{Hi}CD16^{dim} NK cells ratios than controls ($p = 0.019$).

The analysis of PF samples was, however, less effective in identifying differences between EM patients and controls. In fact, the only notable difference was the higher percentages of CD8⁺ CD16^{Hi}CD56^{dim} NK cells observed in EM patients compared to controls ($p = 0.029$).

The complete statistical data for the results discussed in this section can be found in Table S3 of the Supplementary Materials

2.5. Race and Endometriosis: Impact on Circulating and Local Immune Profiles

Some of the reported differences were more pronounced in Black EM patients compared to White patients. According to Figure 1, Black EM patients demonstrated significantly higher percentages of total lymphocytes ($p = 0.008$), CD56^{Hi}CD16^{dim} NK cells ($p = 0.032$), consequently leading to lower ratios of CD56^{dim}CD16^{Hi} NK cells/CD56^{Hi}CD16^{dim} NK cells ($p = 0.025$) in PB. Moreover, pertaining to peritoneal fluid, Black patients exhibited higher percentages of CD8⁺ CD56^{dim}CD16^{Hi} NK cells ($p = 0.033$) and CD8⁺ CD56^{Hi}CD16^{dim} NK cells ($p = 0.009$) compared to their White counterparts.

2.6. Age and BMI Effect on Endometriosis: Impact on Circulating and Local Immune Profiles

In contrast to race, age and body mass index (BMI) appeared to have no significant impact in the subsets studied. On the periphery, the most notable variation identified was that absolute counts of CD56^{Hi}CD16^{dim} NK cells were higher in younger EM patients (age < 37.5 years) compared to a similar subgroup of control women (age < 37.5 years)

($p = 0.020$). Locally, the group of younger women with EM exhibited significantly higher percentages of CD56⁺ T cells ($p = 0.017$) than young controls.

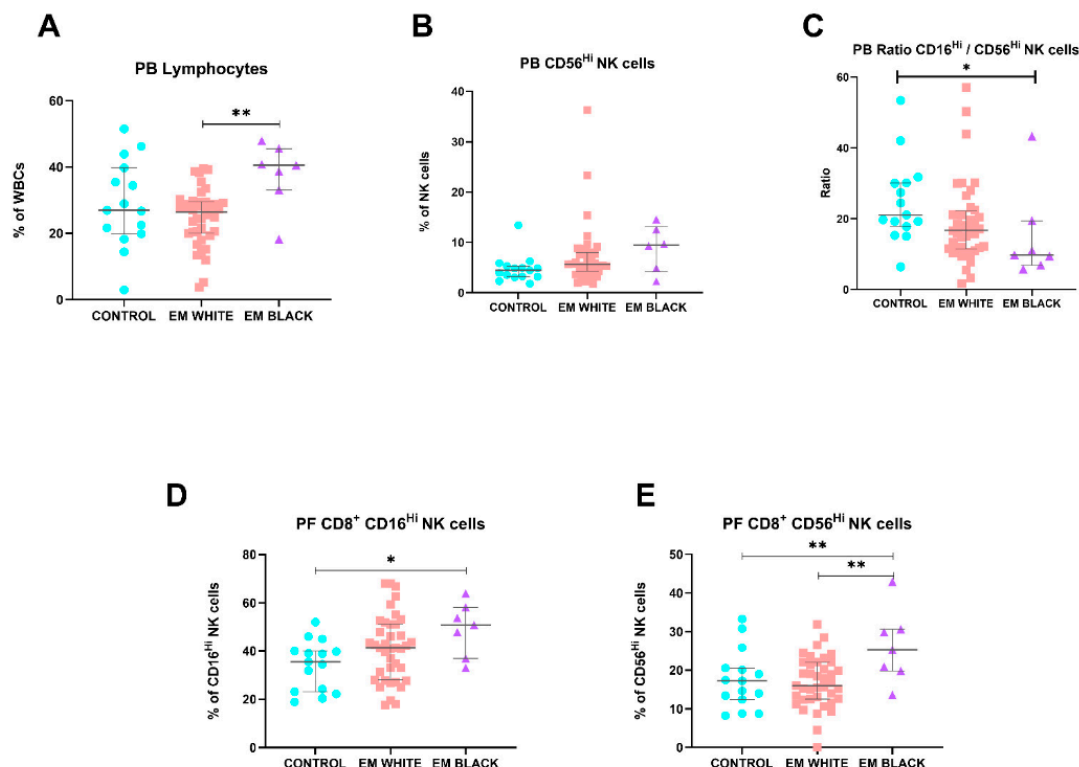


Figure 1. Differences in lymphocytes and lymphocyte subsets of PB and PF samples between healthy controls and endometriosis (EM) patients divided according to race. (A–C) Peripheral blood subsets: total lymphocytes, CD56^{Hi} NK cells, and CD16^{Hi} NK cells/CD56^{Hi} NK cells ratios, respectively. (D,E) Peritoneal fluid CD8⁺ CD16^{Hi} NK cells and CD8⁺ CD56^{Hi} NK cells. Graphs are presented as scatter dot plots with lines referring to medians and interquartile ranges. p -values obtained from KW—Kruskal–Wallis statistical analysis followed by multiple comparison tests. EM, endometriosis; PB, peripheral blood; PF, peritoneal fluid. * $p < 0.05$, ** $p < 0.01$.

2.7. Smoking and Endometriosis: Impact on the Circulating Immune Profile

Smoking habits seem to significantly affect the circulating immune profiles of patients with EM. As depicted in Figure 2, both circulating T cell and NK cell populations are impacted. For T cells, CD16⁺ CD4 T cells were markedly lower in EM patients who smoked compared to those who did not. The ratios of CD56^{dim}CD16^{Hi} NK cells to CD56^{Hi}CD16^{dim} NK cells, which have been previously reported to decrease in EM patients compared to controls, were even more conspicuously lower in EM patients who did not smoke. All individuals in the control group were non-smokers.

2.8. Extrauterine Locations of EM: Cause or Consequence of Altered Immune Profiles

A detailed characterization of the studied EM group allowed the assessment of local and systemic immune profiles based on the presence of extrauterine endometriotic tissue, specifically in the bowel. Women with bowel EM (identified during surgical intervention) demonstrated lower percentages of circulating CD56⁺ CD8 T cells ($p = 0.033$). Conversely, they exhibited higher percentages of CD56^{Hi}CD16^{dim} NK cells ($p = 0.038$). This led to a noticeable decrease in the circulating CD56^{dim}CD16^{Hi} NK cells/CD56^{Hi}CD16^{dim} NK cells ratios ($p = 0.039$) in these women compared to the control group (Figure 3).

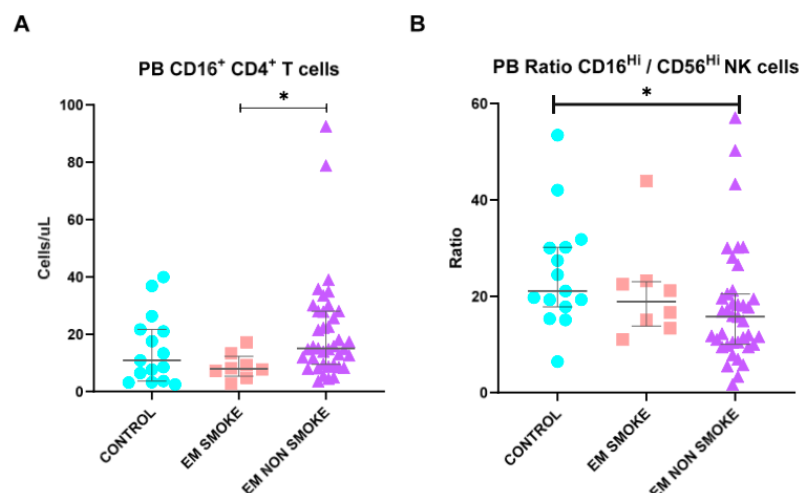


Figure 2. Differences in lymphocyte subsets of PB between healthy controls and endometriosis (EM) patients divided according to smoking habits, namely, peripheral blood (A) CD16⁺ CD4⁺ T cells and (B) CD16^{Hi} NK cells/CD56^{Hi} NK cells ratios. Graphs are presented as scatter dot plots with lines referring to medians and interquartile ranges. *p*-values obtained from KW—Kruskal–Wallis statistical analysis followed by multiple comparison tests. EM, endometriosis; PB, peripheral blood; PF, peritoneal fluid. * *p* < 0.05.

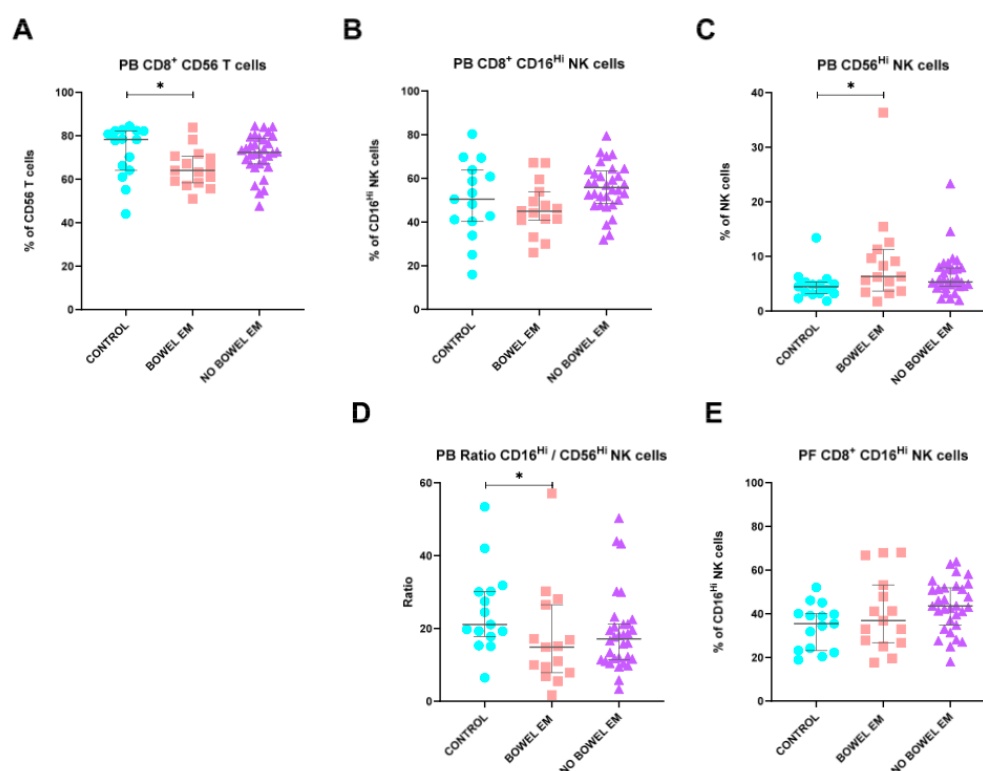


Figure 3. Differences in lymphocyte subsets of PB and PF between healthy controls and endometriosis (EM) patients divided according to the presence of bowel EM. (A–D) Peripheral blood subsets, CD8⁺

CD56⁺ T cells, CD8⁺ CD16^{Hi} NK cells, CD56^{Hi} NK cells, and CD16^{Hi} NK cells/CD56^{Hi} NK cells ratios. (E) Peritoneal fluid CD8⁺ CD16^{Hi} NK cells. Graphs are presented as scatter dot plots with lines referring to medians and interquartile ranges. *p*-values obtained from KW—Kruskal–Wallis statistical analysis followed by multiple comparison tests. EM, endometriosis; PB, peripheral blood; PF, peritoneal fluid. * *p* < 0.05.

2.9. Hormonal Contraception and Endometriosis: Impact on Circulating Immune Profile

As observed in Figure 4, hormonal contraception interferes with CD8 expression in NK cells. Lower values of circulating CD8⁺ NK cells and CD8⁺ CD16^{Hi} NK cells were observed in the control group under therapy compared to all other groups, specifically with EM patients. However, their impact on EM remains unclear, as no differences were observed between EM with and without contraception. Furthermore, despite a slight decrease in both EM subgroups, the percentages of CD8⁺ NK cells were similar to those of controls without hormonal contraception.

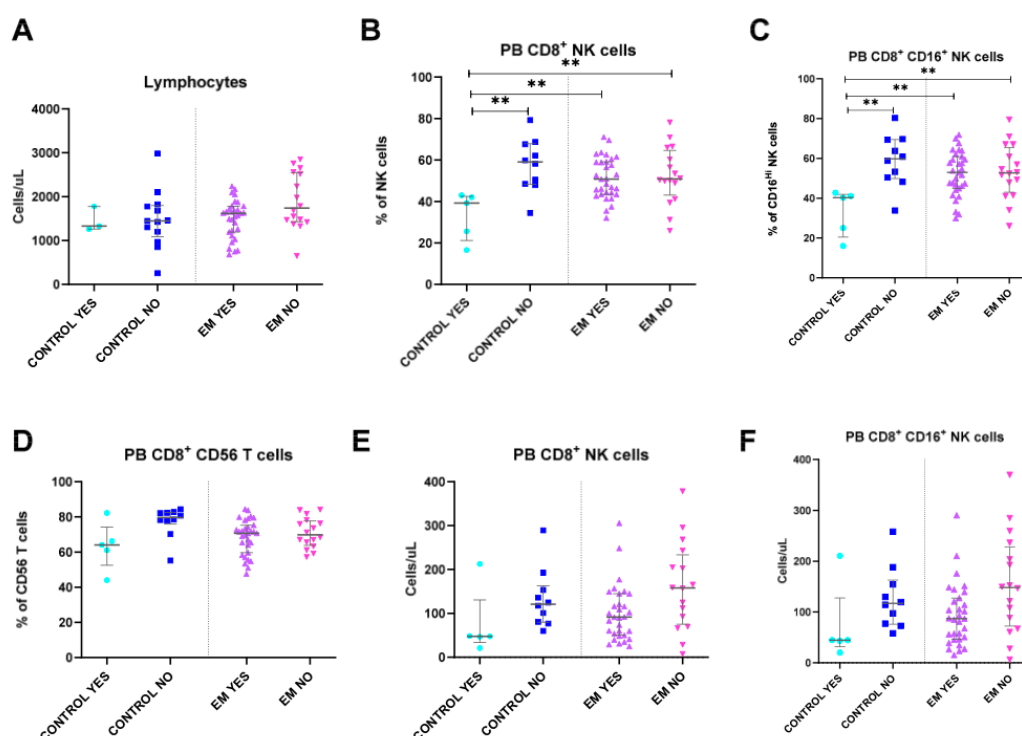


Figure 4. Differences in peripheral blood lymphocyte subsets between healthy controls and endometriosis (EM) patients divided according to the use of hormonal therapy. (A–F) Peripheral blood subsets, total lymphocytes, CD8⁺ CD56⁺ T cells, CD8⁺ NK cells, and CD8⁺ CD16^{Hi} NK cells. Graphs are presented as scatter dot plots with lines referring to medians and interquartile ranges. *p*-values obtained from KW—Kruskal–Wallis statistical analysis followed by multiple comparison tests. EM, endometriosis; PB, peripheral blood; PF, peritoneal fluid. YES, use of hormonal therapy. NO, no use of hormonal therapy. ** *p* < 0.01.

2.10. Endometriosis Severity and Length of Exposure: Impact on Circulating and Local Immune Profiles

The grade of EM and the median years since diagnosis were also considered in the investigation of local and systemic immune profiles. Patients with lower EM grades (i.e., grades 1 and 2) and those diagnosed up to 2 years before enrollment exhibited increased percentages of PF CD8⁺ CD56^{dim}CD16^{Hi} NK cells compared to controls (*p* = 0.033 for grade 1/2 patients; *p* = 0.008 for patients with <2 years of EM diagnosis). In addition,

patients diagnosed less than 2 years prior showed higher percentages of CD16⁺ CD8 T cells ($p = 0.027$), while those diagnosed more than 2 years prior may have seen these values decrease to levels akin to controls.

Disease severity appears to impact peripheral cell populations as well. Figure 5 shows that EM patients with grades 1/2 begin with lower levels of circulating CD16⁺ CD8 T cells, which then progressively increase with disease severity ($p = 0.031$). However, CD8⁺ CD56⁺ T cell percentages remain comparable between controls and EM patients with grades 1/2. Interestingly, these values decrease in EM patients with increasing disease severity, specifically in grades 3 and 4 ($p = 0.050$). A similar pattern is observed in PF samples for CD8⁺ CD16^{Hi} NK cells. Despite decreases in EM grades 3 and 4, patients with grades 1/2 present higher percentages than controls ($p = 0.033$).

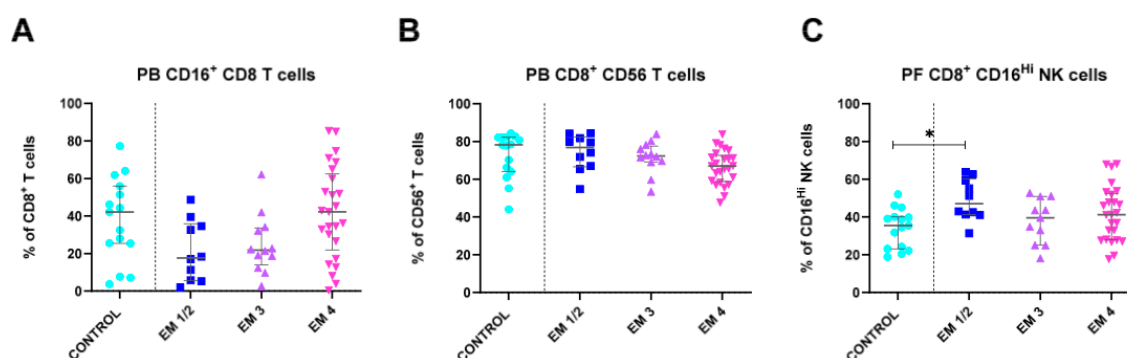


Figure 5. Differences in lymphocyte subsets of PB and PF between healthy controls and endometriosis (EM) patients divided according to disease severity. (**A,B**) Peripheral blood subsets, CD16⁺ CD8 T cells, and CD8⁺ CD56 T cells. (**C**) Peritoneal fluid CD8⁺ CD16^{Hi} NK cells. Graphs are presented as scatter dot plots with lines referring to medians and interquartile ranges. p -values obtained from KW—Kruskal–Wallis statistical analysis followed by multiple comparison tests. EM, endometriosis; PB, peripheral blood; PF, peritoneal fluid. * $p < 0.05$.

2.11. Clinical Features of EM: The Relationship between the Immune System and Symptomatology

Finally, we examined the relationship between discrete clinical features and specific symptomatology concerning the immune parameters evaluated in both peritoneal and circulating lymphoid compartments.

2.11.1. Dysmenorrhea

Concerning dysmenorrhea (Figure 6), EM patients who did not have this complaint showed higher percentages of both CD16⁺ CD8 T cells and CD56⁺ T cells in their peritoneal fluid ($p = 0.033$, and $p = 0.037$, respectively). Furthermore, when dysmenorrhea was classified according to its severity grade, a rise in PF CD8⁺ CD56^{dim}CD16^{Hi} NK cells was observed among patients with increased severity ($p = 0.031$).

2.11.2. Dyspareunia

Furthermore, no significant differences were observed in circulating lymphoid cell populations when comparing patients based on complaints of dyspareunia. Interestingly, however, EM patients without symptoms of dyspareunia had increased levels of circulating CD56^{Hi}CD16^{dim} NK cells ($p = 0.034$), thereby demonstrating significantly lower ratios of CD56^{dim}CD16^{Hi} NK cells to CD56^{Hi}CD16^{dim} NK cells ($p = 0.036$) compared to controls (Figure 6).

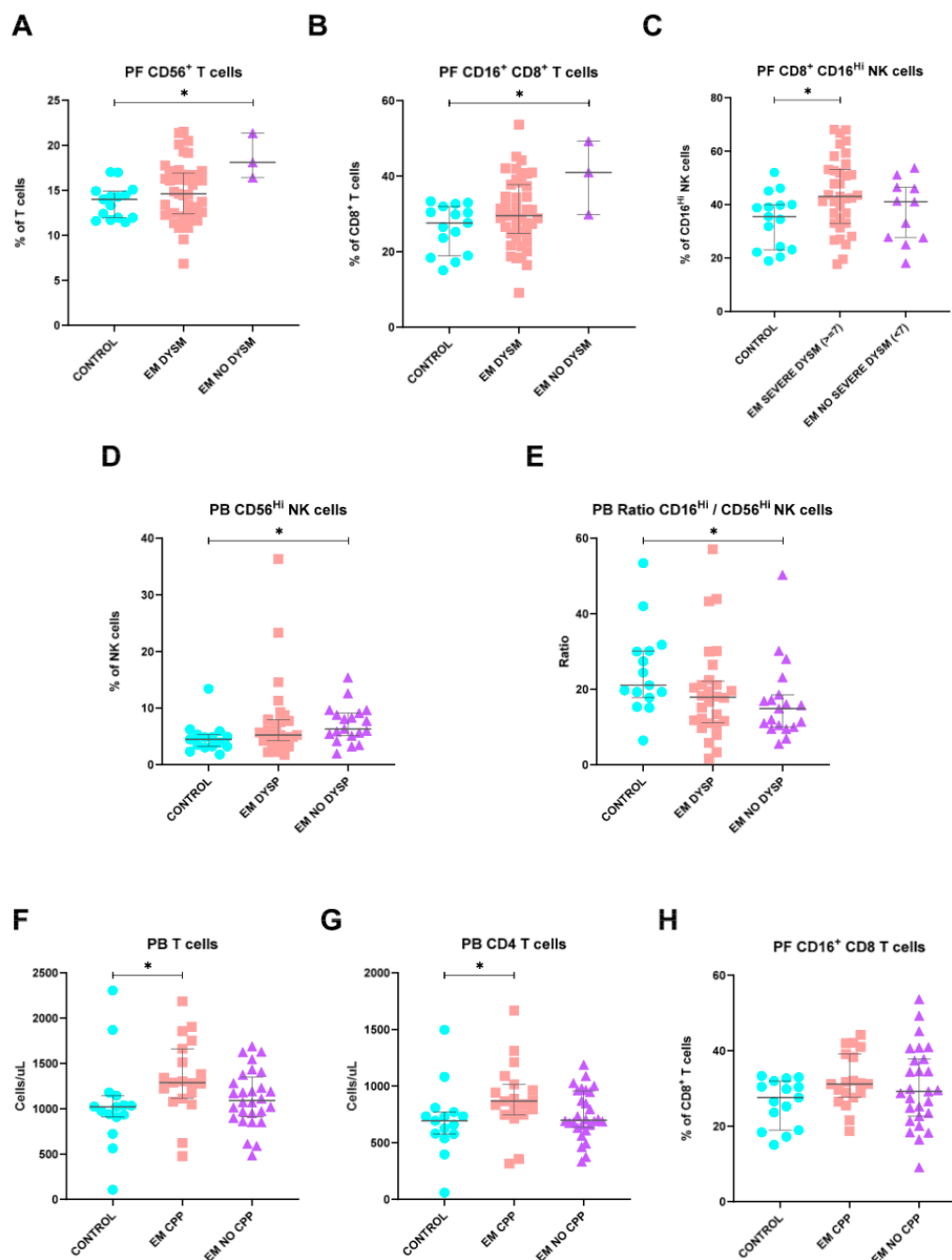


Figure 6. Differences in lymphocyte subsets of PB and PF between healthy controls and endometriosis (EM) patients divided according to the presence and severity of dysmenorrhea (A–C), dyspareunia (D,E), and chronic pelvic pain (CPP) symptoms (F–H). Graphs are presented as scatter dot plots with lines referring to medians and interquartile ranges. *p*-values obtained from KW—Kruskal–Wallis statistical analysis followed by multiple comparison tests. CPP, chronic pelvic pain; DYSM, dysmenorrhea; DYSM, dyspareunia; EM, endometriosis; PB, peripheral blood; PF, peritoneal fluid. * *p* < 0.05.

2.11.3. Non-Menstrual Pelvic Pain

In patients with EM suffering from non-menstrual chronic pelvic pain, significant differences were recognized in circulating lymphocyte levels. More precisely, these patients showed higher CD4 and total T cell counts in comparison to controls ($p = 0.015$ and $p = 0.029$, respectively) while also displaying elevated percentages of CD16⁺ CD8 T cells in PF ($p = 0.046$) (Figure 6).

2.11.4. Rectorrhagia

In the context of rectorrhagia, several differences were observed in immune parameters (Figure 7). Firstly, EM patients who reported rectorrhagia had significantly fewer circulating CD8⁺ NK cells than both controls and EM patients without rectorrhagia ($p = 0.006$). Interestingly, EM patients without rectorrhagia possessed even higher percentages of circulating CD8⁺ NK cells compared to controls.

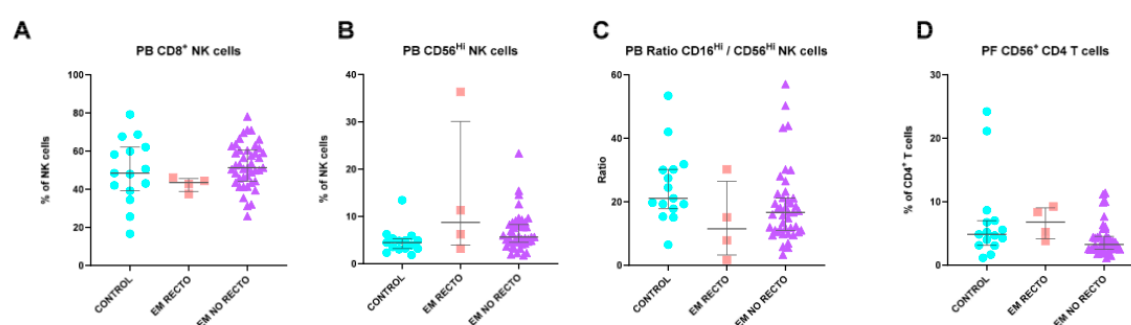


Figure 7. Differences in lymphocyte subsets of PB and PF between healthy controls and endometriosis (EM) patients divided according to the presence of rectorrhagia. (A–C) Peripheral blood subsets, CD8⁺ NK cells, CD56^{Hi} NK cells, and CD16^{Hi} NK cells/CD56^{Hi} NK cells ratios. (D) Peritoneal fluid CD56⁺ CD4 T cells. Graphs are presented as scatter dot plots with lines referring to medians and interquartile ranges. p -values obtained from KW—Kruskal–Wallis statistical analysis followed by multiple comparison tests. EM, endometriosis; PB, peripheral blood; PF, peritoneal fluid; RECTO, rectorrhagia.

Additionally, patients with rectorrhagia displayed increased levels of circulating CD56^{Hi}CD16^{dim} NK cells ($p = 0.046$), indicating significantly lower circulating CD56^{dim}CD16^{Hi} NK cells/CD56^{Hi}CD16^{dim} NK cells ratios ($p = 0.046$) than controls. On a local level, the PF samples of EM patients with rectorrhagia contained significantly higher levels of CD56⁺ CD4 T cells compared to controls ($p = 0.036$). Once more, EM patients without rectorrhagia also differed from controls by showing a decrease in CD56⁺ CD4 T cells.

2.11.5. Dyschezia

Finally, dyschezia, also known as obstructed defecation syndrome, was reported in some EM patients (34%). However, alterations in lymphoid populations seem to occur particularly in patients without this condition. As shown in Figure 8, EM patients without dyschezia exhibited increased CD4, CD8, and total T cell counts ($p = 0.048$; $p = 0.019$; and $p = 0.016$, respectively) in PB. Locally, PF analysis demonstrated lower CD56⁺ CD4 T cells in patients without dyschezia compared to both controls and EM patients with dyschezia ($p = 0.005$).

When examining dyschezia according to its severity, we noted that EM patients suffering from intense dyschezia demonstrated lower circulating CD8 T cell counts ($p = 0.021$). In contrast, patients reporting mild to moderate dyschezia exhibited higher total T cell ($p = 0.016$) and CD4 T cell counts ($p = 0.043$), along with lower NK cell percentages ($p = 0.026$). Similarly, in PF, patients with severe dyschezia also significantly showed lower percentages of CD8⁺ CD16⁺ CD56⁺ NK cells ($p = 0.038$).

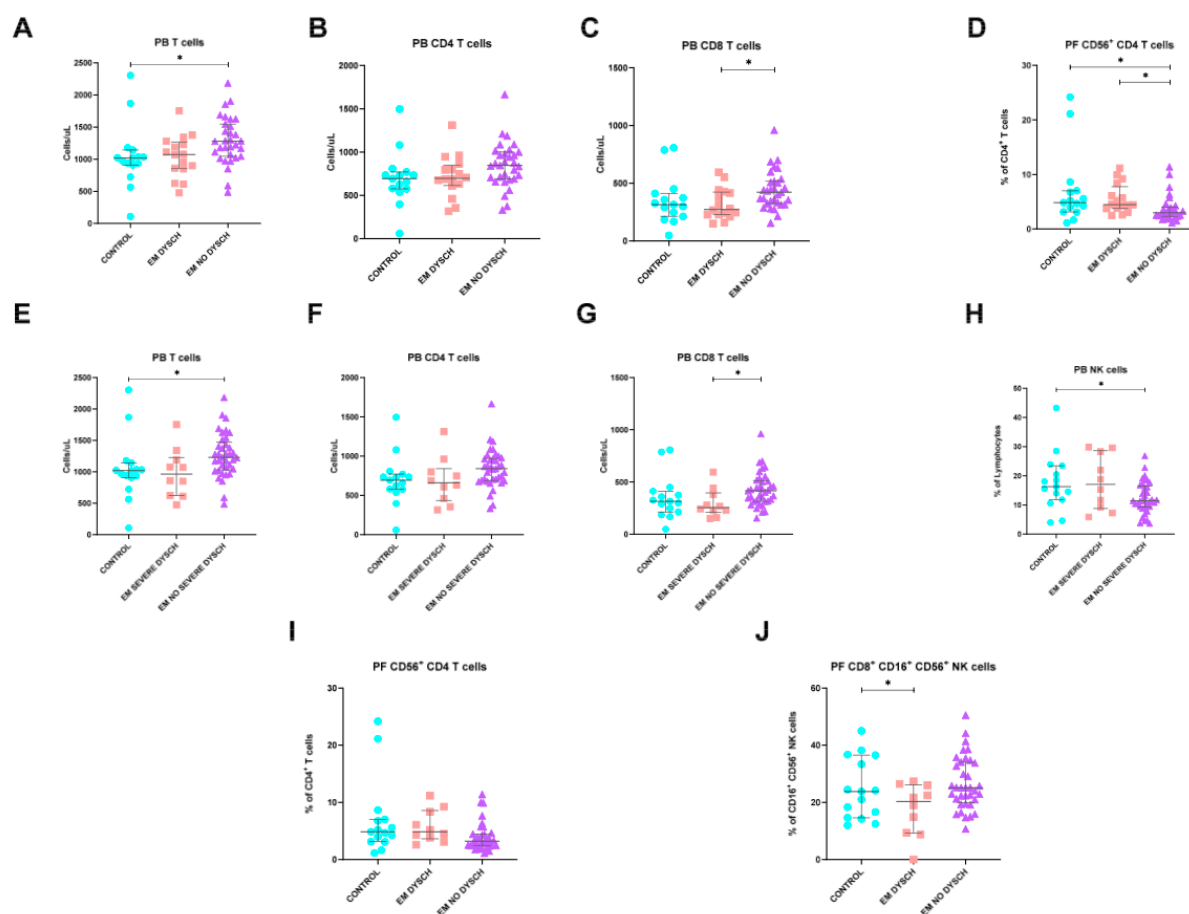


Figure 8. Differences in lymphocyte subsets of PB and PF between healthy controls and endometriosis (EM) patients divided according to the presence and severity of dyschezia symptoms. (A–C) Peripheral blood subsets with significant differences according to the presence of dyschezia, absolute counts of T cells, CD4 T cells, and CD8 T cells. (D) Peritoneal fluid CD56⁺ CD4 T cells according to the presence of dyschezia. (E–H) Peripheral blood subsets with significant differences according to the severity of dyschezia, absolute counts of T cells, CD4 T cells, CD8 T cells, and NK cell percentages. (I,J) Peritoneal fluid CD56⁺ CD4 T cells and CD8⁺ CD16⁺ CD56⁺ NK cells according to the severity of dyschezia. Graphs are presented as scatter dot plots with lines referring to medians and interquartile ranges. *p*-values obtained from KW—Kruskal–Wallis statistical analysis followed by multiple comparison tests. DYSCH, dyschezia; EM, endometriosis; PB, peripheral blood; PF, peritoneal fluid. * *p* < 0.05.

3. Discussion

In this study, we investigated the effects of EM on both the systemic (PB) and local (PF) immune profiles of patients. We specifically focused on the variations of NK and T cell populations in comparison to a control group. To our knowledge, 18 studies [3–20] have already investigated NK cell and T cell activity or the expression of specific NK cell subsets in the PB and PF of EM patients. However, our study introduces a unique clinical perspective, significantly diverging from the methodologies and parameters previously examined. First, our research deviates from the current literature by establishing a baseline, comparing specific T and NK cell populations in PB versus PF of control cohorts. Additionally, we characterized patterns of aberrant NK and T cell profiles according to clinical presentation (dysmenorrhea, dyspareunia, non-menstrual pelvic pain, rectorrhagia,

dyschezia), exploring the correlation between immune subsets and symptoms. Furthermore, we formally assessed the impact of demographic factors, such as race and smoking status, on systemic and local NK cell and T cell populations in control and EM patients. One notable divergence of our study is that the exclusion of EM in the control group was confirmed by laparoscopic surgery with direct visualization of the pelvis, thereby providing a fully reliable method for confirming or refuting the presence of EM rather than relying solely on clinical inquiry.

As expected, our comparative analysis revealed significant differences in the immune environments of PB and PF. A previous study [23] also investigated baseline immunological differences between PB and PF, but we chose to analyze additional NK and T cell subsets that were not covered in this study. Absolute cell counts, including total lymphocytes, were significantly lower in PF compared to PB. However, lymphocytes were present in a significantly greater proportion of total white cells in PF. PF also exhibited smaller CD4/CD8 T cell ratios, showing an increased abundance of CD8 T cells at the expense of CD4 T cells. Conversely, as expected, the opposite relationship was observed in PB, which was richer in CD4 T cells compared to CD8. This is in agreement with Guo et al. [23], who also found a relative abundance of CD8 T cells and increased global T cell activation in PF compared to PB via mass cytometry. The increased proportions of lymphocytes and lower CD4/CD8 ratios in PF may suggest enhanced cytotoxic responses in the peritoneal space, likely due to local immune adaptation relating to proximity to abdominal organs and possible exposure to gut microbial challenges. In essence, the peritoneal space seems to be a dynamic, pro-inflammatory immunological microenvironment [23,24], which is an important consideration when studying the pathogenesis of EM. Supporting this hypothesis, we found that CD16⁺ CD4 T cells, CD16⁺ CD56⁺ T cells, CD56⁺ T cells, and CD56⁺ T cells were proportionally more abundant in PF than PB.

Interestingly, total NK cells in our cohort did not significantly differ between PB and PF samples as compared to other studies [23]. Indeed, in the study by Guo et al. [23], the peritoneal compartment showed an increased NK cell presence compared to PB. Several causes can be noted for this discrepancy, such as the heterogeneity of PF introducing sample variation, the timing of sample collections, and the small sample size of our control group, which limits statistical power. However, a detailed analysis of NK cell subsets did reveal important differences. First, two predominant NK subsets were identified in PB (the abundantly present CD56^{dim}CD16^{Hi} and less prevalent CD56^{Hi}CD16^{dim}), while PF samples showed four identifiable NK subsets (in order of abundance: CD56⁺/CD16^{Hi}, CD56^{Hi}CD16⁺, CD56^{Hi}CD16⁺, and CD56^{dim}CD16⁺ NK cells). Moreover, CD8⁺ NK cells were also increased in PB compared to PF. Our data align with the established literature [21,25,26], which states that CD56^{Hi}CD16^{dim} NK cells are less cytotoxic and prominent cytokine producers and are abundantly found in secondary lymphoid tissues and among uterine cells [27]. The suggested mature NK cells CD56^{dim}CD16^{Hi}, with stronger cytotoxic capacities, are more abundant in PB, where protection against aggression requires more potent surveillants. These variations in local and systemic NK cell subsets may partially underpin the pathogenesis of EM, thus creating a local immune environment that promotes immune evasion, cytokine-mediated symptoms, and inflammatory changes [21].

After characterizing lymphoid subpopulations in PB and PF, we next assessed how the presence of EM disrupted immunological patterns. Hassa et al. [7] reported no significant differences in peritoneal major lymphoid populations (i.e., NK cells and CD4 and CD8 T cells) between controls and EM patients. Our results align with theirs for PF samples. However, our EM cohorts showed increased circulating CD56^{Hi}CD16^{dim} NK cells and lower CD56^{dim}CD16^{Hi}/CD56^{Hi}CD16^{dim} ratios, suggesting decreased NK cell cytotoxicity systemically compared to the general population. Additionally, our EM cohorts may be more susceptible to cytokine-mediated inflammation, leading to symptomatology, as corroborated by previous studies [20,28,29].

A more detailed analysis of the specific PF subsets allowed us to identify an increase in CD8⁺ CD56^{dim}CD16^{Hi} NK cells in EM patients, which may be significant given that the CD8

molecule typically enhances NK cell cytotoxicity [30]. Although Schmitz et al. [31] found reduced perforin-positive CD8 T cells in the menstrual effluent of EM patients—indicating a reduced cytotoxic potential—other authors assert that CD8 T cells are increased in endometriotic lesions [32,33]. This somewhat mirrors our observations on CD8⁺ NK cell subsets.

Therefore, we hypothesize that while there may be an increase in CD8⁺ NK cells in EM PF—likely as an adaptive response to the disease—they might function as compensation for altered T cells. Alternatively, these cells could carry alterations in their cytotoxic potential that allow the development and establishment of endometriotic lesions. However, these variations in local CD8⁺ cells might be a consequence, not a cause of EM, according to D'Hooghe et al. [32]. Therefore, we contend that the role of CD8⁺ lymphocytes in EM remains controversial and necessitates further investigation [33].

3.1. Endometriosis: Immunology of Demographic and Disease Factors

To further understand the immunological landscape of EM, we analyzed differences across various demographic parameters. Race appeared to influence EM's immunological profile. The differences identified, specifically higher percentages of circulating CD56^{Hi}CD16^{dim} NK cells and lower ratios of circulating CD56^{dim}CD16^{Hi} NK cells to CD56^{Hi}CD16^{dim} NK cells, along with a local increase in CD8⁺ CD56^{dim}CD16^{Hi} NK cells, were more pronounced in Black EM patients. In addition, Black EM patients exhibited higher overall PF lymphocyte and CD8⁺ CD56^{Hi}CD16^{dim} NK cell absolute counts.

This phenomenon may be due to several reasons. A meta-analysis highlights a troubling trend: Black patients with EM are 50% less likely to receive an accurate diagnosis compared to their White counterparts [34]. Coupled with the reported average duration of 5–10 years for an EM diagnosis [35], this suggests Black EM patients may have more severe, late-stage disease, contributing to heightened disparity in medical, societal, and socioeconomic factors, and access to treatment or healthcare [34].

Genetic variation and predisposition might also influence EM pathogenesis. Indeed, certain genetic polymorphisms have been linked to an increased risk of EM [21,36,37], although these have predominantly been studied in Asian populations. Further investigation is necessary to confirm these associations in Black populations.

Another demographic parameter assessed was age, which apparently had a lower impact than race/ethnicity. Indeed, differences were primarily observed when comparing younger cohorts (<37.5 years) (EM vs. controls). These cohorts showed greater absolute counts of circulating CD56^{Hi}CD16^{dim} NK cells and CD56⁺ T cell percentages in PF. The absence of significant differences in older cohorts aligns with the existing literature [38]. Thus, we hypothesize that as EM is an estrogen-dependent condition [38], disease severity and aberrant immune features are more pronounced during the hormonal and menstrual cycle and are alleviated by age-related estrogen decline and menopause. Interestingly, older EM patients (>37.5 years) exhibited higher percentages of CD8⁺ CD56^{dim}CD16^{Hi} NK cells in PF compared to younger controls, aligning with previous studies reporting increased CD56^{dim}CD16^{Hi} NK cells with aging [39,40]. This may be an adaptive function of the reproductive tract against possible pathogens, but its role in EM calls for further study.

Smoking status also affected the circulating immune profiles of EM patients. CD16⁺ CD4 T cells were significantly lower in EM patients who smoked compared to non-smokers. This suggests that smoking may impair T cell activation, promoting both immunosuppression and immune evasion in these patients, although the literature on this topic is controversial. Martos et al. found additional senescence-associated genes in CD4 T cells from smokers, leading to immune cell dysregulation [41], while Chapron et al. [42] found that smoking habits had no significant impact on EM risk or American Fertility Society stage/score. However, one significant limitation of our study is that all individuals in the control group were non-smokers; this precluded establishing a comprehensive baseline for comparison, making it difficult to discern the independent immunological effects of smoking, EM, and their relationship.

Women with extrauterine (intestinal) EM, often deemed the most aggressive form of EM, typically exhibit immune profiles similar to those lacking intestinal endometriotic lesions. Nonetheless, as confirmed earlier, patients with extrauterine EM reveal elevated percentages of CD56^{Hi}CD16^{dim} NK cells and reduced CD56^{dim}CD16^{Hi} NK cells/CD56^{Hi}CD16^{dim} NK cells compared to the control group. This hints at a pro-inflammatory environment, conducive to processes like angiogenesis, pivotal in establishing ectopic endometriotic lesions [43]. Also, a significant decline in circulating CD8⁺ CD56⁺ T cells suggests a systemic reduction in immunosurveillance and lesion apoptosis. It is crucial to discern whether these immune modifications are a cause or an effect of a modified immune profile. As far as we know, no other studies have immunologically characterized extrauterine EM, with most of the literature reviewing changes based on severity instead of lesion location. Despite this, considering current knowledge and assuming our moderate trends are valid, we propose that women with extra-pelvic EM exhibit a more distinct immune and inflammatory microenvironment that systemically encourages processes, such as angiogenesis, thus helping establish ectopic endometriotic lesions at more remote locations [43]. This, however, needs more exploration, as present study limitations, particularly the small number of participants, could obscure additional insights.

Hormonal contraceptives have long been recognized as a standard treatment for EM, primarily aimed at providing symptomatic relief due to the condition's dependence on estrogen. In our investigation, we evaluated whether hormonal contraception, a standard therapeutic approach, modifies immune profiles. Hormonal contraception reduced total peripheral lymphocyte levels in both control and EM groups. The literary findings on this topic are elusive. Though several studies have tried to characterize hormone-mediated immunological changes, they reported varied results based on the type of contraception, active ingredient, and lymphocyte subpopulation studied [44–46]. However, it is well-established that hormonal contraception is linked to changes in the frequency of immune cells in the genital mucosa [47] and a reduction in cytotoxicity and cytokine production [44]. Thus, this decrease in total lymphocyte count may substantiate the symptomatic relief provided by hormonal contraception owing to reduced cytokine production and inflammation. Upon investigating other subsets, hormonal contraception resulted in decreased counts of CD8-positive CD56⁺ T cells, CD56^{dim}CD16^{Hi} NK cells, and total NK cells in the control group, but not in EM patients, indicating a relatively lower impact on the immune system in the latter group. Notably, Waiyaput et al. [48] observed that the combined oral contraceptive (COC), containing ethinyl estradiol and desogestrel, significantly reduced the macrophage cell count while enhancing NK and regulatory T cell counts in women with ovarian EM [48]. Prathoomthong et al. [49] also noted that dienogest increased NK cell counts in the eutopic endometrium. However, it is crucial to highlight that these observations were identified within the context of adenomyosis, not EM [49]. These variations could result from several factors including methodological differences, type and duration of contraceptive use, distinct hormonal components, and individual hormonal level fluctuations. Consequently, future research should employ a more targeted and comprehensive approach to decipher the immunological impacts of hormonal contraception on EM.

Recently diagnosed EM (less than 2 years) appear to display more noticeable immune alterations, such as increases in CD16⁺ CD8 T cells and CD8⁺ CD56^{dim}CD16^{Hi} NK cells. This pattern stabilizes 2 years after diagnosis, leading to speculation about potential immune exhaustion. Several studies support this hypothesis, with cohorts with EM showing reduced T cell and NK cell effector function, suggesting a failure in immunosurveillance [3,50,51].

When assessing immune profiles based on EM severity, patients with EM showed progressively lower percentages of circulating CD56⁺ CD8 T cells with increasing disease severity. This decrease supports the hypothesis that ectopic endometrial cells evade the immune system. However, the literature presents conflicting viewpoints, with some studies suggesting a decrease in T cell populations in EM [12,17], while others report no significant differences [9,33,52], or even an increase [53,54]. It is pivotal to determine whether this

decrease is a causative factor, indicating a predisposition to more severe disease, or whether a persistently chronic inflammatory microenvironment induces apoptosis and thereby causes a decline in these populations. Notably, the percentages of CD16⁺ CD8 T cells in PB were lower in stages 1, 2, and 3 of EM compared to controls. In stark contrast, individuals with more severe disease (stage 4) exhibited an increase in CD16⁺ CD8 T cells compared to controls and other EM groups. One possible explanation could relate to the dynamic nature of the immune response at various stages of EM. The evolving pathology might interact with the immune system, causing fluctuations in T cell populations. The functional role of CD16⁺ CD8 T cells in severe disease remains unclear. They could signal a stronger or more specific immune response in serious cases. Conversely, they could be a byproduct of disease progression in response to a chronic inflammatory microenvironment. As previously mentioned, increased cell counts do not automatically mean an enhanced immune response or cytotoxicity since immune exhaustion and inhibitory mechanisms need to be taken into account [50,51]. The body of evidence on this topic is much more conflicted and ambiguous [9,12,17,33,52], which underscores the need for further research considering potential confounding variables. This factor was noted by Slabe and colleagues, who reported fluctuating regulatory T cell concentrations in response to altered immune responses, such as increased serum cortisol [52].

PF analysis revealed an increased percentage of CD8⁺ CD56^{dim}CD16^{Hi} NK cells in EM cohorts compared to controls. Notably, this increase was more pronounced in minimal/mild disease (stages 1 and 2), suggesting a heightened immune response in these patients, potentially reflecting the immune system's effort to control the disease process. Hassa et al. reported no significant changes in PF NK cell populations between control groups and early- and late-stage EM, despite the study's focus on the NK compartment, which was as in-depth as ours [7]. Thus, our study underscores the importance of extended immune characterizations to better understand the EM mechanisms.

Overall, our evaluation of immune profiles pertaining to EM progression reveals a complex interplay within the immune system. This contributes to the complexity of the existing literature on the subject matter that has yet to be entirely deciphered.

3.2. Endometriosis: The Relationship between Symptomatology and Immunological Profiles

To encompass the clinical aspects of EM, we investigated the relationship between immune profiles and symptomatic presentations, considering factors like dysmenorrhea, dyspareunia, non-menstrual pelvic pain, rectorrhagia, and dyschezia.

Dysmenorrhea is a prevalent symptom of EM, apparently demonstrating a complex interplay between immune cell populations and their pathogenesis. Notably, higher percentages of PF CD16⁺ CD8 T cells and CD56⁺ T cells were observed in patients without dysmenorrhea. Although there are no studies regarding EM-associated dysmenorrhea, other investigations have implicated CD8⁺ T cells in pain suppressive mechanisms. Increased levels of CD8⁺ T cells were found in sural nerve biopsies of murine subjects with diabetic peripheral neuropathy [55,56] and in the cerebrospinal fluid (CSF) of HIV patients with peripheral neuropathy [57]. This suggests that CD8⁺ T cells (and potentially other T cell subpopulations) provide a pain-suppressing or protective immunophenotype. However, it is crucial to note that increased CD8⁺ T cells have also been implicated in acute and chronic pain processes, such as Herpes Zoster neuralgia [56,58]. This dual role of CD8⁺ T cells in both pain suppression and exacerbation in different pain conditions illuminates the elusive nature of their role in pain modulation and emphasizes the need for future research on their relationships with dysmenorrhea.

The pathophysiology of dyspareunia is multifactorial, involving structural (e.g., pelvic anatomy), psychosocial, and inflammatory factors [59]. EM-associated dyspareunia is suggested to be due to the presence of endometriotic lesions in the pouch of Douglas [60]. For the immune profile of these patients, our results suggest that dyspareunia in EM might not be associated with systemic immune alterations. On the contrary, EM patients without reported dyspareunia displayed elevated levels of circulating CD56^{Hi}CD16^{dim} NK cells

and lower ratios of circulating $CD56^{\dim}CD16^{Hi}/CD56^{Hi}CD16^{\dim}$ NK cells compared to controls. Noticeably, a study by Guevoghlian-Silva et al. found increased Transforming Growth Factor Beta (TGF) expression in patients with EM who reported dyspareunia [60]. TGF is known to modulate immune responses, limiting NK cell expansion/proliferation by keeping them in a quiescent state [61,62], potentially modulating the predominance of some NK subsets, as we report here.

Our results indicate that EM patients with non-menstrual pelvic pain exhibit elevated circulating T cell counts, including both CD4 and CD8 T cell subsets. This increased presence of T cells suggests an active adaptive immune response, potentially indicative of an ongoing inflammatory process. Moreover, PF analysis of EM patients with pelvic pain characterized higher percentages of $CD16^{+}CD8$ T cells. This could imply the existence of a local cytotoxic response, either in response to painful stimuli or causing tissue damage and inflammation, thereby resulting in pelvic pain.

To our knowledge, the immunopathogenesis of pelvic pain in EM has only been explored in one previous study conducted by He et al. [63]. Our results partially support their findings, particularly in recognizing the association between pelvic pain and systemic immune dysregulation, but there are significant distinctions. In fact, while He et al. [63] reported higher circulating NK cells, our study only demonstrated differences in the absolute counts of T cells, which were higher in patients with this complaint. This variation in T cell count is supported by the broader literature, whereby regulatory T cell dysfunction has been associated with the exacerbation and severity of EM [53,54].

Rectal bleeding associated with EM is attributed to the implantation of deep ectopic endometrial lesions on the rectal wall. This manifestation of EM is sometimes referred to as Digestive System Infiltrating Endometriosis (DSIE) [64]. Given its relatively low incidence [64], the literature is scarce on this topic, which is why we aimed to clarify the immunological landscape of rectal bleeding.

Systematically, EM patients with rectal bleeding showed lower percentages of $CD8^{+}$ NK cells in PB compared to those without rectal bleeding and controls. These results suggest a potential immunoregulatory response or pathogenic role of NK cells in the context of rectal bleeding. Reduced NK activity in DSIE has also been observed in other studies [60,64,65], whereby an enhanced release of cytokines in EM impedes NK cell proliferation and activity [60,64,65]. Additionally, we observed that EM patients without rectal bleeding displayed a significantly larger proportion of $CD8^{+}$ NK cells compared to controls. A further analysis of PB revealed that EM patients with rectal bleeding had higher levels of $CD56^{Hi}CD16^{\dim}$ NK cells along with a significantly lower ratio of circulating $CD56^{\dim}CD16^{Hi}/CD56^{Hi}CD16^{\dim}$ NK cells compared to controls and EM patients without rectal bleeding. These findings further support the hypothesis of an immune microenvironment characterized by pro-inflammatory cells and reduced cytotoxicity, allowing immune evasion and the establishment of endometriotic lesions on the rectal wall.

Locally, EM patients with rectorrhagia displayed significantly higher levels of $CD56^{+}CD4$ T cells in PF compared to controls. Conversely, EM patients without rectorrhagia showed a decrease in these cells compared to controls. These results further underscore the presence of a distinct immune environment associated with the gastrointestinal symptoms of EM. We hypothesize that the $CD56^{+}CD4$ T cell population increases in response to rectal bleeding, thereby managing inflammation linked to gastrointestinal symptoms. These findings align with the current literature, which notes an elevation in T cell populations, specifically regulatory T cells, in DSIE [64,66,67].

It is important to acknowledge the limitations of our study regarding the investigation of rectorrhagia. A confounding factor emerges, as rectal bleeding primarily derives from gastrointestinal pathologies [68], like hemorrhoids, rather than EM. Furthermore, this study encounters limitations in statistical power due to the exceedingly small sample size of EM patients with rectorrhagia enrolled.

Dyschezia, characterized by difficulty and pain during bowel movements, is a prevalent clinical presentation associated with bowel EM. When stratified by severity, it was

observed that patients with severe dyschezia had lower circulating CD8 T cell counts, possibly indicating a compromised cytotoxic T cell response, which is crucial for immune surveillance of EM. Locally, patients with severe dyschezia exhibited reduced levels of CD16⁺ CD56⁺ CD8 NK cells, possibly pointing to potential NK cell exhaustion or diminished cytotoxic activity [3,50,51]. In simpler terms, prolonged or severe gastrointestinal symptoms may be contributing to immune exhaustion, affecting NK cell cytotoxicity. This variation could also result from increased expression of NK inhibitory receptors in severe EM, leading to localized immunosuppression [21].

The current literature concerning the immunological profile of bowel symptoms in EM is notably limited. However, the scarce available literature seems to contradict our research findings. According to Gueuvoghian-Silva et al., IL-15 expression in deep rectosigmoid lesions was significantly greater in EM patients with dyschezia compared to those without dyschezia [60]. IL-15 is known to enhance CD8 T cell and NK cell expansion and survival [69,70] which, in turn, is expected to result in increased levels of these subsets. We observed the contrary. Though heightened IL-15 levels should theoretically contribute to an increase in NK cell and T cell populations, other studies have not explicitly examined this aspect, overlooking potential interactions with other cytokines and the broader immune environment. Overall, our study suggests a complex interplay between the immune system and gastrointestinal symptoms in EM, highlighting potential NK cell protective/regulatory mechanisms and distinct T cell immune profiles.

The literature regarding the relationship between lymphocyte subpopulations and symptoms such as dysmenorrhea, dyspareunia, or pelvic pain remain scarce. Moreover, it remains imperative to determine whether these deviations in the immune profiles are causes or consequences of EM and whether the findings are supported by studies in larger cohorts, such as those with more severe presentations. Our research disclosed significant differences in the immunological profiles of EM, contributing new insights to an area that currently lacks in-depth exploration, especially concerning demographic factors and symptomatology. Variations in particular lymphocyte subsets, such as NK cells and T cells, indicate the existence of potential protective immunological mechanisms, particularly concerning gastrointestinal and pelvic symptoms. This knowledge could enable the development of symptom-centric targeted therapies, addressing a crucial gap in current treatment methods. Another strength of our study is its thorough examination of specific subsets within NK and T cell populations, enhancing understanding of the immunological landscape of EM on a cellular level, which may hold key implications for its pathophysiology.

However, these strengths also illuminate some limitations of this study. While our research efficiently detailed the immunological profile or composition of PB and PF, it revealed a complex interplay among each subpopulation. Our study highlighted the presence of diverse immune NK and T cell subpopulations across various demographic and clinical presentations. However, it is vital to note that presence does not necessarily equate to activity. To heighten the breadth of immunological understanding, future research could include functional tests, such as cytotoxicity assays, cytokine analysis, and surface receptor analysis, to supplement these findings. Additionally, even though our sample population was generally larger than those in prior investigations of this topic, it is still relatively small for drawing definitive conclusions. This reduced statistical power is particularly evident in the examination of smoking (no individuals in the control group were smokers), dysmenorrhea, and rectorrhagia, with some groups having less than five enrolled patients in some instances. Furthermore, the changes observed in Black women with endometriosis should be interpreted with caution, given the absence of Black controls, limiting a more comprehensive comparison.

Future research should aim to clarify whether variations in immunological profiles correspond to changes in cellular activity. It will also be crucial to investigate the potential impact of genetic and hormonal influences on these profiles, as well as understand the functional interplay of different cell subsets. Nevertheless, our study paves the way for

promising further research. The identification of specific immune cell subsets associated with demographic factors and symptomatology boosts our understanding of EM pathogenesis and promotes the development of more personalized and targeted treatments. By tailoring interventions based on individual clinical presentations, we can potentially amplify treatment efficacy and reduce adverse effects [71]. Notably, the observed variations in NK and T cell populations call for consideration of implementing NK and T cell therapies, similar to those used in cancer therapy, in the field of EM [72].

4. Materials and Methods

4.1. Study Design and Participants

This cross-sectional study, conducted from June 2021 to December 2022, involved 62 female patients, 47 of whom were diagnosed with EM, and 15 served as controls, without the disease. All participants were adult women of reproductive age treated at Hospital da Luz Lisboa and Hospital Beatriz Ângelo's outpatient clinics. The EM group underwent laparoscopic surgery for the condition, while the control group had surgery for other pathologies, all under general anesthesia. Diagnosis of EM initially relied on clinical and imaging findings, later confirmed histologically according to the Human Reproduction and Embryology (ESHRE) guidelines [73]. All the patients included in the endometriosis group had histopathological confirmation of the disease through material collected during laparoscopic surgery.

The exclusion criteria encompassed women who had been pregnant within the past year, as well as those with immunological, endocrine, neoplastic, or other chronic diseases. Individuals suspected of complications due to pelvic inflammatory disease were also excluded. This study received approval from the Ethics Committees of Beatriz Ângelo Hospital, Hospital da Luz Lisboa, and NOVA Medical School. We ensured all patients were comprehensively informed about the research and the use of their biological material for scientific purposes. All study participants provided written informed consent before their involvement in this study.

4.2. Demographic and Clinical Data

Data collected for all patients encompassed age, race, BMI, smoking status, hormone therapy, and the time elapsed between the diagnosis of EM and surgery. Clinical data were gathered to assess disease severity, including symptoms such as dysmenorrhea, dyspareunia, chronic pelvic pain, dyschezia, bowel issues (constipation, peri-menstrual diarrhea, and bloating), urinary tract symptoms (hematuria and dysuria), rectorrhagia, and the presence of extra-pelvic EM. Disease severity was classified based on the guidelines of the American Society for Reproductive Medicine [74].

4.3. Sample Collection

PB and PF samples were gathered from all patients during surgery. To lower the potential for blood contamination, PF was collected at the start of the procedure. A laparoscopic needle was used for fluid extraction from the pouch of Douglas. The samples were preserved in EDTA-coated tubes kept at room temperature following surgery and delivered to the Immunology laboratory at NOVA Medical School. All PF samples underwent processing on the day of their collection, while blood samples were dealt with within 24 h based on laboratory availability.

4.4. Flow Cytometry Immunophenotyping Analysis

Absolute counts of each cell subset were determined using a single-platform method with Trucount™ tubes (BD Biosciences, San Jose, CA, USA) for both samples. Specifically, we incubated a standardized sample volume with CD45 in a Trucount™ tube for 15 min, lysed it using a BD FACS lysing solution (BD Biosciences, San Jose, CA, USA), and collected the data. Afterward, we stained the PB and PF samples with a panel of monoclonal

antibodies, including CD45, CD3, CD8, CD16, CD56, and CD57. A comprehensive list of the antibodies utilized is provided in the Supplementary Materials (Table S1).

PF samples were centrifuged before immunophenotyping to concentrate the cells. A lyse-wash protocol was applied using a BD FACS lysing solution (BD Biosciences, San Jose, CA, USA). All samples were processed using a BD FACS Canto II (BD Biosciences, San Jose, CA, USA). Data analysis was conducted using Infinicyt™ (Cytognos, Salamanca, Spain) and FlowJo™ (BD Life Sciences, San Jose, CA, USA) software, both acquired from BD Biosciences (BD Life Sciences, San Jose, CA, USA). The gating strategy is illustrated in Figure 9.

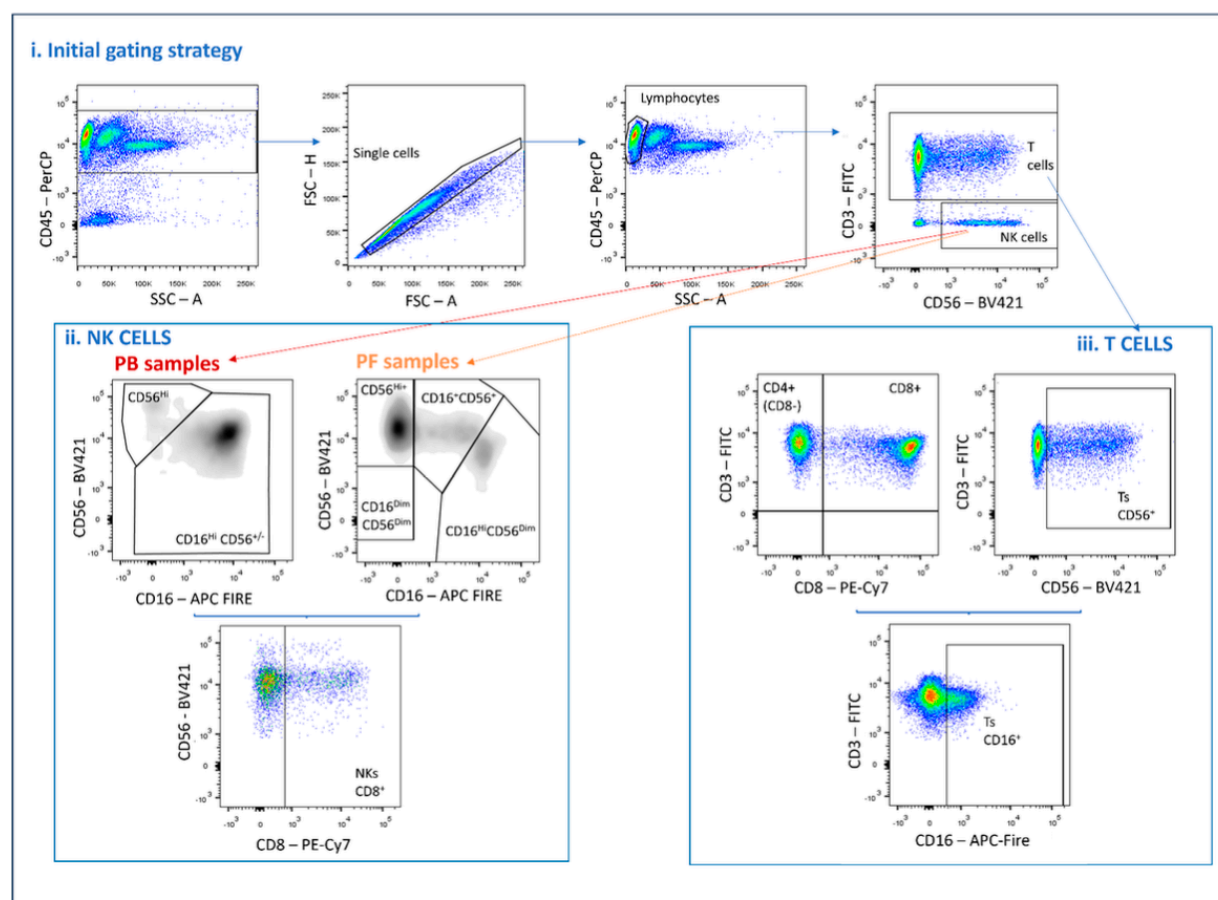


Figure 9. Gating strategy for lymphocyte subset characterization and marker expression in PB and PF samples. The maturation profile of NK cells was assessed according to the expression of NKG2A, KIR2DL1/CD158a, and CD57 as described elsewhere [75]. Fluorescence minus one (FMO)/fluorescence minus X (FMX) control strategies were used to properly set the threshold for positive staining for each marker in the respective populations of interest. All analyses were performed with FlowJo™ Software, version X 10.0.7.

4.5. Statistical Analysis

The demographic, clinical, and immunophenotypic data of the study participants were summarized using descriptive statistics. Categorical variables were reported as absolute and relative frequencies. Fisher's exact test was used for comparisons between two groups when contingency tables had over 20% of expected values less than 5; otherwise, the chi-squared test was used. Continuous variables were displayed as mean (SD) (minimum–maximum) or as median and first-to-third quartile (Q1–Q3), depending on the context. In

the Supplementary Tables, all continuous variables were represented as mean (SD) along with a 95% confidence interval [95% CI], as well as the median and Q1–Q3. The 95% CIs were computed using Student's t-distribution approximation for normally distributed continuous variables and bootstrap resampling for variables not normally distributed. Comparisons between two independent groups of normally distributed continuous data used Student's t-test, while comparisons involving two dependent groups applied the paired-samples t-test. The Wilcoxon rank-sum test and the Wilcoxon signed-rank test were used for non-parametric comparisons. For comparisons involving more than two groups, ANOVA with post hoc Tukey's HSD (honestly significant difference) test was used for normally distributed data, whereas the Kruskal–Wallis test with a post hoc Dunn's test was utilized for data not normally distributed. The Shapiro–Wilk test assessed the normality of distributions. As this study was exploratory, no formal sample size calculation was conducted. Statistical significance was defined as $p < 0.05$. All analyses were performed using SAS software (version 9.4).

5. Conclusions

Previous studies have suggested that deficiencies in immune surveillance create a favorable environment for ectopic endometrial cells to evade the immune system. Our study investigated the immunological impact of EM both locally and systemically, focusing on NK and T cell subpopulations. Our research revealed significant differences in the immunological profiles of EM, providing new insights into a field that currently lacks comprehensive investigation, particularly concerning demographic factors and symptomatology. Identifying specific immune cell subsets linked to these factors enhances our understanding of EM pathogenesis and potentiates the development of more personalized and targeted treatments.

Future research should clarify if variations in immunological profiles correspond to alterations in cellular activity. It should also explore the functional interplay between these factors and the potential impact of genetic and hormonal influences on these profiles.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/ijms251810141/s1>.

Author Contributions: Conceptualization, J.L.R., C.M., M.Â.-D., L.M.B. and J.L.; data curation, J.L.R.; formal analysis, J.L.R., C.M., M.Â.-D., L.M.B. and J.L.; funding acquisition, J.L.; methodology, J.L.R., N.N.R., C.M., M.Â.-D., L.M.B. and J.L.; project administration, J.L.; supervision, J.L.; writing—original draft, J.L.R., N.N.R., C.M., M.Â.-D. and J.L.; writing—review and editing, C.M., M.Â.-D., L.M.B. and J.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was partially funded by the Hospital da Luz Lisboa under the initiative “Luz Investigação”.

Institutional Review Board Statement: This study was conducted following the Declaration of Helsinki and was approved by the ethics committees of NOVA Medical School | Universidade NOVA de Lisboa (n°122/2020/CEFCM, approved on 24 February 2020), Beatriz Ângelo Hospital (3394/2020_MJH/MAB/NO, approved on 6 November 2020), and Luz Lisboa Hospital (CES/53/2021/ME, approved on 23 November 2021).

Informed Consent Statement: Informed consent was obtained from all subjects involved in this study.

Data Availability Statement: The data presented in this study are available upon request from the corresponding author. The data are not publicly available due to privacy restrictions.

Acknowledgments: The authors would like to acknowledge Jorge Gonçalves (Scientific ToolBox Consulting, Lisbon, Portugal) for statistical analysis and Sofia Nunes (Scientific ToolBox Consulting, Lisbon, Portugal), for providing medical writing assistance and technical editing.

Conflicts of Interest: Authors José Lourenço Reis, Luís Miguel Borrego and Jorge Lima were employed by the company Luz Saúde. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

- Shafirir, A.L.; Farland, L.V.; Shah, D.K.; Harris, H.R.; Kvaskoff, M.; Zondervan, K.; Missmer, S.A. Risk for and consequences of endometriosis: A critical epidemiologic review. *Best. Pract. Res. Clin. Obstet. Gynaecol.* **2018**, *51*, 1–15. [\[CrossRef\]](#) [\[PubMed\]](#)
- Sampson, J.A. Metastatic or Embolic Endometriosis, due to the Menstrual Dissemination of Endometrial Tissue into the Venous Circulation. *Am. J. Pathol.* **1927**, *3*, 93–110+143. [\[PubMed\]](#)
- Hosseinzadeh, R.; Moirani, A.; Hosseini, R.; Fatehnejad, M.; Yekaninejad, M.S.; Javidan, M.; Changaei, M.; Feizisani, F.; Rajaei, S. A higher number of exhausted local PD1+, but not TIM3+, NK cells in advanced endometriosis. *Heliyon* **2024**, *10*, e23294. [\[CrossRef\]](#)
- Olkowska-Truchanowicz, J.; Bialoszewska, A.; Zwierzchowska, A.; Sztokfisz-Ignasiak, A.; Janiuk, I.; Dabrowski, F.; Korczak-Kowalska, G.; Barcz, E.; Bocian, K.; Malejczyk, J. Peritoneal Fluid from Patients with Ovarian Endometriosis Displays Immunosuppressive Potential and Stimulates Th2 Response. *Int. J. Mol. Sci.* **2021**, *22*, 8134. [\[CrossRef\]](#)
- Meggyes, M.; Szereday, L.; Bohonyi, N.; Koppan, M.; Szegedi, S.; Marics-Kutas, A.; Marton, M.; Totsimon, A.; Polgar, B. Different Expression Pattern of TIM-3 and Galectin-9 Molecules by Peripheral and Peritoneal Lymphocytes in Women with and without Endometriosis. *Int. J. Mol. Sci.* **2020**, *21*, 2343. [\[CrossRef\]](#)
- Xu, H. Expressions of natural cytotoxicity receptor, NKG2D and NKG2D ligands in endometriosis. *J. Reprod. Immunol.* **2019**, *136*, 102615. [\[CrossRef\]](#)
- Hassa, H.; Tanir, H.M.; Tekin, B.; Kirilmaz, S.D.; Sahin Mutlu, F. Cytokine and immune cell levels in peritoneal fluid and peripheral blood of women with early- and late-staged endometriosis. *Arch. Gynecol. Obstet.* **2009**, *279*, 891–895. [\[CrossRef\]](#)
- Galandrini, R.; Porpora, M.G.; Stoppacciaro, A.; Micucci, F.; Capuano, C.; Tassi, I.; Di Felice, A.; Benedetti-Panici, P.; Santoni, A. Increased frequency of human leukocyte antigen-E inhibitory receptor CD94/NKG2A-expressing peritoneal natural killer cells in patients with endometriosis. *Fertil. Steril.* **2008**, *89*, 1490–1496. [\[CrossRef\]](#) [\[PubMed\]](#)
- Zhang, C.; Maeda, N.; Izumiya, C.; Yamamoto, Y.; Kusume, T.; Oguri, H.; Yamashita, C.; Nishimori, Y.; Hayashi, K.; Luo, J.; et al. Killer immunoglobulin-like receptor and human leukocyte antigen expression as immunodiagnostic parameters for pelvic endometriosis. *Am. J. Reprod. Immunol.* **2006**, *55*, 106–114. [\[CrossRef\]](#)
- Matsuoka, S.; Maeda, N.; Izumiya, C.; Yamashita, C.; Nishimori, Y.; Fukaya, T. Expression of inhibitory-motif killer immunoglobulin-like receptor, KIR2DL1, is increased in natural killer cells from women with pelvic endometriosis. *Am. J. Reprod. Immunol.* **2005**, *53*, 249–254. [\[CrossRef\]](#)
- Maeda, N.; Izumiya, C.; Kusum, T.; Masumoto, T.; Yamashita, C.; Yamamoto, Y.; Oguri, H.; Fukaya, T. Killer inhibitory receptor CD158a overexpression among natural killer cells in women with endometriosis is undiminished by laparoscopic surgery and gonadotropin releasing hormone agonist treatment. *Am. J. Reprod. Immunol.* **2004**, *51*, 364–372. [\[CrossRef\]](#) [\[PubMed\]](#)
- Szylo, K.; Tchorzewski, H.; Banasik, M.; Glowacka, E.; Lewkowicz, P.; Kamer-Bartosinska, A. The involvement of T lymphocytes in the pathogenesis of endometriotic tissues overgrowth in women with endometriosis. *Mediat. Inflamm.* **2003**, *12*, 131–138. [\[CrossRef\]](#) [\[PubMed\]](#)
- Maeda, N.; Izumiya, C.; Yamamoto, Y.; Oguri, H.; Kusume, T.; Fukaya, T. Increased killer inhibitory receptor KIR2DL1 expression among natural killer cells in women with pelvic endometriosis. *Fertil. Steril.* **2002**, *77*, 297–302. [\[CrossRef\]](#) [\[PubMed\]](#)
- Maeda, N.; Izumiya, C.; Oguri, H.; Kusume, T.; Yamamoto, Y.; Fukaya, T. Aberrant expression of intercellular adhesion molecule-1 and killer inhibitory receptors induces immune tolerance in women with pelvic endometriosis. *Fertil. Steril.* **2002**, *77*, 679–683. [\[CrossRef\]](#)
- Wu, M.Y.; Chao, K.H.; Chen, S.U.; Chen, H.F.; Yang, Y.S.; Huang, S.C.; Ho, H.N. The suppression of peritoneal cellular immunity in women with endometriosis could be restored after gonadotropin releasing hormone agonist treatment. *Am. J. Reprod. Immunol.* **1996**, *35*, 510–516. [\[CrossRef\]](#)
- Hua, L.; Xu, X.; Bian, D. The measurement of natural killer cell activity in peripheral blood and peritoneal fluid of patients with endometriosis. *Zhonghua Fu Chan Ke Za Zhi* **1996**, *31*, 586–589.
- Ho, H.N.; Chao, K.H.; Chen, H.F.; Wu, M.Y.; Yang, Y.S.; Lee, T.Y. Peritoneal natural killer cytotoxicity and CD25+ CD3+ lymphocyte subpopulation are decreased in women with stage III-IV endometriosis. *Hum. Reprod.* **1995**, *10*, 2671–2675. [\[CrossRef\]](#)
- Oosterlynck, D.J.; Meuleman, C.; Lacquet, F.A.; Waer, M.; Koninckx, P.R. Flow cytometry analysis of lymphocyte subpopulations in peritoneal fluid of women with endometriosis. *Am. J. Reprod. Immunol.* **1994**, *31*, 25–31. [\[CrossRef\]](#)
- Iwasaki, K.; Makino, T.; Maruyama, T.; Matsubayashi, H.; Nozawa, S.; Yokokura, T. Leukocyte subpopulations and natural killer activity in endometriosis. *Int. J. Fertil. Menopausal Stud.* **1993**, *38*, 229–234.
- Oosterlynck, D.J.; Meuleman, C.; Waer, M.; Vandeputte, M.; Koninckx, P.R. The natural killer activity of peritoneal fluid lymphocytes is decreased in women with endometriosis. *Fertil. Steril.* **1992**, *58*, 290–295. [\[CrossRef\]](#)
- Reis, J.L.; Rosa, N.N.; Angelo-Dias, M.; Martins, C.; Borrego, L.M.; Lima, J. Natural Killer Cell Receptors and Endometriosis: A Systematic Review. *Int. J. Mol. Sci.* **2022**, *24*, 331. [\[CrossRef\]](#) [\[PubMed\]](#)
- Lourenço Reis, J.; Martins, C.; Angelo-Dias, M.; Rosa, N.N.; Borrego, L.M.; Lima, J. TIM-3 Expression in Endometriosis. *Am. J. Reprod. Immunol.* **2024**, *91*, e13887. [\[CrossRef\]](#) [\[PubMed\]](#)

23. Guo, M.; Bafligil, C.; Tapmeier, T.; Hubbard, C.; Manek, S.; Shang, C.; Martinez, F.O.; Schmidt, N.; Obendorf, M.; Hess-Stumpp, H.; et al. Mass cytometry analysis reveals a distinct immune environment in peritoneal fluid in endometriosis: A characterisation study. *BMC Med.* **2020**, *18*, 3. [\[CrossRef\]](#)
24. Hill, J.A.; Anderson, D.J. Lymphocyte activity in the presence of peritoneal fluid from fertile women and infertile women with and without endometriosis. *Am. J. Obstet. Gynecol.* **1989**, *161*, 861–864. [\[CrossRef\]](#) [\[PubMed\]](#)
25. Shin, E.; Bak, S.H.; Park, T.; Kim, J.W.; Yoon, S.R.; Jung, H.; Noh, J.Y. Understanding NK cell biology for harnessing NK cell therapies: Targeting cancer and beyond. *Front. Immunol.* **2023**, *14*, 1192907. [\[CrossRef\]](#)
26. Poli, A.; Michel, T.; Theresine, M.; Andres, E.; Hentges, F.; Zimmer, J. CD56bright natural killer (NK) cells: An important NK cell subset. *Immunology* **2009**, *126*, 458–465. [\[CrossRef\]](#)
27. Sciezynska, A.; Komorowski, M.; Soszynska, M.; Malejczyk, J. NK Cells as Potential Targets for Immunotherapy in Endometriosis. *J. Clin. Med.* **2019**, *8*, 1468. [\[CrossRef\]](#)
28. Oosterlynck, D.J.; Cornillie, F.J.; Waer, M.; Vandeputte, M.; Koninckx, P.R. Women with endometriosis show a defect in natural killer activity resulting in a decreased cytotoxicity to autologous endometrium. *Fertil. Steril.* **1991**, *56*, 45–51. [\[CrossRef\]](#)
29. Viganò, P.; Vercellini, P.; Di Blasio, A.M.; Colombo, A.; Candiani, G.B.; Vignali, M. Deficient antiendometrium lymphocyte-mediated cytotoxicity in patients with endometriosis. *Fertil. Steril.* **1991**, *56*, 894–899. [\[CrossRef\]](#)
30. Addison, E.G.; North, J.; Bakhsh, I.; Marden, C.; Haq, S.; Al-Sarraj, S.; Malayeri, R.; Wickremasinghe, R.G.; Davies, J.K.; Lowdell, M.W. Ligation of CD8alpha on human natural killer cells prevents activation-induced apoptosis and enhances cytolytic activity. *Immunology* **2005**, *116*, 354–361. [\[CrossRef\]](#)
31. Schmitz, T.; Hoffmann, V.; Olliges, E.; Bobinger, A.; Popovici, R.; Nossner, E.; Meissner, K. Reduced frequency of perforin-positive CD8+ T cells in menstrual effluent of endometriosis patients. *J. Reprod. Immunol.* **2021**, *148*, 103424. [\[CrossRef\]](#) [\[PubMed\]](#)
32. Hooghe, T.M.D.; Hill, J.A.; Oosterlynck, D.J.; Koninckx, P.R.; Bamba, C.S. Endometriosis: Effect of endometriosis on white blood cell subpopulations in peripheral blood and peritoneal fluid of baboons. *Human. Reprod.* **1996**, *11*, 1736–1740. [\[CrossRef\]](#) [\[PubMed\]](#)
33. Kisovar, A.; Becker, C.M.; Granne, I.; Southcombe, J.H. The role of CD8+ T cells in endometriosis: A systematic review. *Front. Immunol.* **2023**, *14*, 1225639. [\[CrossRef\]](#) [\[PubMed\]](#)
34. Bougie, O.; Yap, M.I.; Sikora, L.; Flaxman, T.; Singh, S. Influence of race/ethnicity on prevalence and presentation of endometriosis: A systematic review and meta-analysis. *BJOG* **2019**, *126*, 1104–1115. [\[CrossRef\]](#)
35. Khashchenko, E.P.; Uvarova, E.V.; Fatkhudinov, T.K.; Chuprynin, V.D.; Asaturova, A.V.; Kulabukhova, E.A.; Vysokikh, M.Y.; Alakhverdiev, E.Z.; Alekseeva, M.N.; Adamyan, L.V.; et al. Endometriosis in Adolescents: Diagnostics, Clinical and Laparoscopic Features. *J. Clin. Med.* **2023**, *12*, 1678. [\[CrossRef\]](#)
36. Wu, Z.; Yuan, M.; Li, Y.; Fu, F.; Ma, W.; Li, H.; Wang, W.; Wang, S. Analysis of WNT4 polymorphism in Chinese Han women with endometriosis. *Reprod. Biomed. Online* **2015**, *30*, 415–420. [\[CrossRef\]](#) [\[PubMed\]](#)
37. Dai, Y.; Li, X.; Shi, J.; Leng, J. A review of the risk factors, genetics and treatment of endometriosis in Chinese women: A comparative update. *Reprod. Health* **2018**, *15*, 82. [\[CrossRef\]](#)
38. Dyson, M.T.; Bulun, S.E. Cutting SRC-1 down to size in endometriosis. *Nat. Med.* **2012**, *18*, 1016–1018. [\[CrossRef\]](#)
39. Solana, R.; Campos, C.; Pera, A.; Tarazona, R. Shaping of NK cell subsets by aging. *Curr. Opin. Immunol.* **2014**, *29*, 56–61. [\[CrossRef\]](#)
40. Rodriguez-Garcia, M.; Patel, M.V.; Shen, Z.; Wira, C.R. The impact of aging on innate and adaptive immunity in the human female genital tract. *Aging Cell* **2021**, *20*, e13361. [\[CrossRef\]](#)
41. Martos, S.N.; Campbell, M.R.; Lozoya, O.A.; Wang, X.; Bennett, B.D.; Thompson, I.J.B.; Wan, M.; Pittman, G.S.; Bell, D.A. Single-cell analyses identify dysfunctional CD16(+) CD8 T cells in smokers. *Cell Rep. Med.* **2020**, *1*, 100054. [\[CrossRef\]](#) [\[PubMed\]](#)
42. Chapron, C.; Souza, C.; de Ziegler, D.; Lafay-Pillet, M.C.; Ngo, C.; Bijaoui, G.; Goffinet, F.; Borghese, B. Smoking habits of 411 women with histologically proven endometriosis and 567 unaffected women. *Fertil. Steril.* **2010**, *94*, 2353–2355. [\[CrossRef\]](#) [\[PubMed\]](#)
43. Symons, L.K.; Miller, J.E.; Kay, V.R.; Marks, R.M.; Liblik, K.; Koti, M.; Tayade, C. The Immunopathophysiology of Endometriosis. *Trends Mol. Med.* **2018**, *24*, 748–762. [\[CrossRef\]](#) [\[PubMed\]](#)
44. Michel, K.G.; Huijbregts, R.P.; Gleason, J.L.; Richter, H.E.; Hel, Z. Effect of hormonal contraception on the function of plasmacytoid dendritic cells and distribution of immune cell populations in the female reproductive tract. *J. Acquir. Immune Defic. Syndr.* **2015**, *68*, 511–518. [\[CrossRef\]](#) [\[PubMed\]](#)
45. Fleming, D.C.; King, A.E.; Williams, A.R.; Critchley, H.O.; Kelly, R.W. Hormonal contraception can suppress natural antimicrobial gene transcription in human endometrium. *Fertil. Steril.* **2003**, *79*, 856–863. [\[CrossRef\]](#)
46. Huijbregts, R.P.; Helton, E.S.; Michel, K.G.; Sabbaj, S.; Richter, H.E.; Goepfert, P.A.; Hel, Z. Hormonal contraception and HIV-1 infection: Medroxyprogesterone acetate suppresses innate and adaptive immune mechanisms. *Endocrinology* **2013**, *154*, 1282–1295. [\[CrossRef\]](#)
47. Chandra, N.; Thurman, A.R.; Anderson, S.; Cunningham, T.D.; Yousefieh, N.; Mauck, C.; Doncel, G.F. Depot medroxyprogesterone acetate increases immune cell numbers and activation markers in human vaginal mucosal tissues. *AIDS Res. Hum. Retroviruses* **2013**, *29*, 592–601. [\[CrossRef\]](#)

48. Waiyaput, W.; Wattanakamolchai, K.; Tingthanatikul, Y.; Lertvikool, S.; Tantanavipas, S.; Dittharot, K.; Sroyraya, M.; Sophonsritsuk, A. Effect of combined contraceptive pill on immune cell of ovarian endometriotic tissue. *J. Ovarian Res.* **2021**, *14*, 66. [\[CrossRef\]](#)
49. Prathoomthong, S.; Tingthanatikul, Y.; Lertvikool, S.; Rodratn, N.; Waiyaput, W.; Dittharot, K.; Sroyraya, M.; Sophonsritsuk, A. The Effects of Dienogest on Macrophage and Natural Killer Cells in Adenomyosis: A Randomized Controlled Study. *Int. J. Fertil. Steril.* **2018**, *11*, 279–286. [\[CrossRef\]](#)
50. Xu, H.; Zhao, J.; Lu, J.; Sun, X. Ovarian endometrioma infiltrating neutrophils orchestrate immunosuppressive microenvironment. *J. Ovarian Res.* **2020**, *13*, 44. [\[CrossRef\]](#)
51. Walankiewicz, M.; Grywalska, E.; Polak, G.; Korona-Glowniak, I.; Witt, E.; Surdacka, A.; Kotarski, J.; Rolinski, J. The Increase of Circulating PD-1- and PD-L1-Expressing Lymphocytes in Endometriosis: Correlation with Clinical and Laboratory Parameters. *Mediat. Inflamm.* **2018**, *2018*, 7041342. [\[CrossRef\]](#) [\[PubMed\]](#)
52. Slabe, N.; Meden-Vrtovec, H.; Verdenik, I.; Kosir-Pogacnik, R.; Ihan, A. Cytotoxic T-Cells in Peripheral Blood in Women with Endometriosis. *Geburtshilfe Frauenheilkd.* **2013**, *73*, 1042–1048. [\[CrossRef\]](#) [\[PubMed\]](#)
53. Gogacz, M.; Winkler, I.; Bojarska-Junak, A.; Tabarkiewicz, J.; Semczuk, A.; Rechberger, T.; Adamiak, A. Increased percentage of Th17 cells in peritoneal fluid is associated with severity of endometriosis. *J. Reprod. Immunol.* **2016**, *117*, 39–44. [\[CrossRef\]](#)
54. Tanaka, Y.; Mori, T.; Ito, F.; Koshiba, A.; Takaoka, O.; Kataoka, H.; Maeda, E.; Okimura, H.; Mori, T.; Kitawaki, J. Exacerbation of Endometriosis Due To Regulatory T-Cell Dysfunction. *J. Clin. Endocrinol. Metab.* **2017**, *102*, 3206–3217. [\[CrossRef\]](#)
55. Agarwal, N.; Helmstadter, J.; Rojas, D.R.; Bali, K.K.; Gangadharan, V.; Kuner, R. Evoked hypoalgesia is accompanied by tonic pain and immune cell infiltration in the dorsal root ganglia at late stages of diabetic neuropathy in mice. *Mol. Pain.* **2018**, *14*, 1744806918817975. [\[CrossRef\]](#)
56. Galvin, D.A.; McCrory, C. The role of T-lymphocytes in neuropathic pain initiation, development of chronicity and treatment. *Brain Behav. Immun. Health* **2021**, *18*, 100371. [\[CrossRef\]](#) [\[PubMed\]](#)
57. Wang, S.X.; Ho, E.L.; Grill, M.; Lee, E.; Peterson, J.; Robertson, K.; Fuchs, D.; Sinclair, E.; Price, R.W.; Spudich, S. Peripheral neuropathy in primary HIV infection associates with systemic and central nervous system immune activation. *J. Acquir. Immune Defic. Syndr.* **2014**, *66*, 303–310. [\[CrossRef\]](#) [\[PubMed\]](#)
58. Lassen, J.; Stürner, K.H.; Gierthmühlen, J.; Dargviniene, J.; Kixmüller, D.; Leyboldt, F.; Baron, R.; Hüllemann, P. Protective role of natural killer cells in neuropathic pain conditions. *Pain* **2021**, *162*, 2366–2375. [\[CrossRef\]](#)
59. Tayyeb, M.; Gupta, V. Dyspareunia. In *StatPearls*; StatPearls: Treasure Island, FL, USA, 2024.
60. Gueuvoghlian-Silva, B.Y.; Bellelis, P.; Barbeiro, D.F.; Hernandez, C.; Podgaec, S. Treg and NK cells related cytokines are associated with deep rectosigmoid endometriosis and clinical symptoms related to the disease. *J. Reprod. Immunol.* **2018**, *126*, 32–38. [\[CrossRef\]](#)
61. Laouar, Y.; Sutterwala, F.S.; Gorelik, L.; Flavell, R.A. Transforming growth factor-beta controls T helper type 1 cell development through regulation of natural killer cell interferon-gamma. *Nat. Immunol.* **2005**, *6*, 600–607. [\[CrossRef\]](#)
62. Regis, S.; Dondero, A.; Caliendo, F.; Bottino, C.; Castriconi, R. NK Cell Function Regulation by TGF-beta-Induced Epigenetic Mechanisms. *Front. Immunol.* **2020**, *11*, 311. [\[CrossRef\]](#) [\[PubMed\]](#)
63. He, J.; Xu, Y.; Yi, M.; Gu, C.; Zhu, Y.; Hu, G. Involvement of natural killer cells in the pathogenesis of endometriosis in patients with pelvic pain. *J. Int. Med. Res.* **2020**, *48*, 300060519871407. [\[CrossRef\]](#) [\[PubMed\]](#)
64. Yin, W.; Li, X.; Liu, P.; Li, Y.; Liu, J.; Yu, S.; Tai, S. Digestive system deep infiltrating endometriosis: What do we know. *J. Cell Mol. Med.* **2023**, *27*, 3649–3661. [\[CrossRef\]](#) [\[PubMed\]](#)
65. Zhou, W.J.; Yang, H.L.; Shao, J.; Mei, J.; Chang, K.K.; Zhu, R.; Li, M.Q. Anti-inflammatory cytokines in endometriosis. *Cell Mol. Life Sci.* **2019**, *76*, 2111–2132. [\[CrossRef\]](#)
66. Riccio, L.; Santulli, P.; Marcellin, L.; Abrao, M.S.; Batteux, F.; Chapron, C. Immunology of endometriosis. *Best. Pract. Res. Clin. Obstet. Gynaecol.* **2018**, *50*, 39–49. [\[CrossRef\]](#)
67. Olkowska-Truchanowicz, J.; Sztokfisz-Ignasiak, A.; Zwierzchowska, A.; Janiuk, I.; Dabrowski, F.; Korczak-Kowalska, G.; Barcz, E.; Bocian, K.; Malejczyk, J. Endometriotic Peritoneal Fluid Stimulates Recruitment of CD4(+)CD25(high)FOXP3(+) Treg Cells. *J. Clin. Med.* **2021**, *10*, 3789. [\[CrossRef\]](#)
68. Sabry, A.O.; Sood, T. Rectal Bleeding. In *StatPearls*; StatPearls: Treasure Island, FL, USA, 2024.
69. Ma, S.; Caligiuri, M.A.; Yu, J. Harnessing IL-15 signaling to potentiate NK cell-mediated cancer immunotherapy. *Trends Immunol.* **2022**, *43*, 833–847. [\[CrossRef\]](#)
70. Brincks, E.L.; Woodland, D.L. Novel roles for IL-15 in T cell survival. *F1000 Biol. Rep.* **2010**, *2*, 67. [\[CrossRef\]](#)
71. Yao, J.; Zhang, L.J.; Zhou, Z.; Hua, M.F. NK-92 cells activated by IL-2 inhibit the progression of endometriosis in vitro. *J. Obstet. Gynaecol.* **2024**, *44*, 2372682. [\[CrossRef\]](#)
72. Hoogstad-van Evert, J.; Paap, R.; Nap, A.; van der Molen, R. The Promises of Natural Killer Cell Therapy in Endometriosis. *Int. J. Mol. Sci.* **2022**, *23*, 5539. [\[CrossRef\]](#)
73. Becker, C.M.; Bokor, A.; Heikinheimo, O.; Horne, A.; Jansen, F.; Kiesel, L.; King, K.; Kvaskoff, M.; Nap, A.; Petersen, K.; et al. ESHRE guideline: Endometriosis. *Hum. Reprod. Open* **2022**, *2022*, hoac009. [\[CrossRef\]](#) [\[PubMed\]](#)

74. Andrews, W.C.; Buttram, V.C., Jr.; Weed, J.C.; Hammond, C.B.; Thomas, H.H.; Behrman, S.J. Revised American Fertility Society classification of endometriosis: 1985. *Fertil. Steril.* **1985**, *43*, 351–352. [[CrossRef](#)]
75. Del Zotto, G.; Antonini, F.; Pesce, S.; Moretta, F.; Moretta, L.; Marcenaro, E. Comprehensive Phenotyping of Human PB NK Cells by Flow Cytometry. *Cytometry A* **2020**, *97*, 891–899. [[CrossRef](#)] [[PubMed](#)]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.

CHAPTER V

TIM-3 Expression in Endometriosis

ORIGINAL ARTICLE

TIM-3 Expression in Endometriosis

José Lourenço Reis¹  | Catarina Martins^{2,3} | Miguel Ângelo-Dias^{2,3} | Natacha Nurdine Rosa⁴ |
Luís Miguel Borrego^{2,3,5} | Jorge Lima^{1,2,3} 

¹Department of Obstetrics and Gynecology, LUZ SAÚDE, Hospital da Luz Lisboa, Lisboa, Portugal | ²CHRC, NOVA Medical School, Faculdade de Ciências Médicas, NMS/FCM, Universidade Nova de Lisboa, Lisboa, Portugal | ³Immunology Department, NOVA Medical School/Faculdade de Ciências Médicas, NMS/FCM, Universidade Nova de Lisboa, Lisboa, Portugal | ⁴UCD School of Medicine, University College Dublin, Dublin, Ireland | ⁵Department of Imunoallergy, LUZ SAÚDE, Hospital da Luz, Lisboa, Portugal

Correspondence: Jorge Lima (jorgeramoslima@sapo.pt)

Received: 8 January 2024 | **Revised:** 29 April 2024 | **Accepted:** 24 May 2024

Funding: This study was partially funded by the Hospital da Luz Lisboa under the initiative “Luz Investigação”, Gedeon Richter Portugal, and LifeWell Pharmaceutical & Healthcare.

Keywords: endometriosis | immune receptors | TIM-3

ABSTRACT

Problem: Endometriosis is a prevalent chronic gynecological disease linked to immune dysfunction. The protein T-cell immunoglobulin and mucin domain-containing protein 3 (TIM-3) plays a crucial role in immune system balance. Malfunction of TIM-3 may result in excessive immune activation and inflammatory tissue damage. Given TIM-3's established role in the development of cancer and autoimmune diseases, we decided to study its role in women suffering from endometriosis.

Study Method: We included a total of 62 female patients, all of whom had undergone laparoscopic surgery. Of these, 47 had endometriosis and 15 did not. During surgery, we collected peritoneal fluid (PF) and peripheral blood (PB) samples from every patient for analysis using flow cytometry. To mark the samples, we used a panel of monoclonal antibodies and examined TIM-3 expression in their immune cells.

Results: Endometriosis patients in PB demonstrated a significantly lower percentage of CD56+ T cells with TIM-3 expression. As endometriosis progressed through its stages, this expression lessened. This decrease was particularly notable in women with stage III/IV endometriosis. Additionally, both women diagnosed with intestinal endometriosis and those with recent endometriosis diagnoses showed a significantly reduced percentage of CD56+ T cells expressing TIM-3.

Conclusions: Patients with endometriosis exhibit diminished TIM-3 expression within circulating T cells. This warrants further investigation to discern whether it contributes to the progression of endometriosis, potentially through the amplification of autoreactive T cells and inflammation.

1 | Introduction

Endometriosis, a chronic gynecological disorder, is prevalent and distressing. This condition occurs when endometrial cells reside beyond the uterine cavity, majorly on the pelvic peritoneum or ovaries, leading to a wide range of symptoms, including dysmenorrhea, dyspareunia, infertility, and chronic pelvic pain [1]. Endometriosis significantly diminishes the quality of life of

one in ten women of reproductive age [2]. Currently, the exact cause of endometriosis is not known and effective methods for diagnosis and treatment are scant.

The retrograde menstruation theory is an accepted concept that explains the development of endometriosis [3]. However, since most healthy women experience retrograde menstruation without developing endometriosis, it becomes obvious that other

elements play a part. It is suggested that an immunologic malfunction could allow abnormal tissue to survive, potentially leading to the formation of ectopic endometrial implants in the peritoneal cavity [4–13]. The theory that the onset and growth of endometriosis are associated with immune dysfunction was first suggested in the 1990s [4]. Following this, numerous studies have supported this theory by illustrating the role of different immune cells at various stages in the immune response, resulting in compromised immune competence in women with endometriosis [1, 14]. Despite the numerous studies conducted thus far, the precise mechanism detailing the role of the immune system in the advancement of endometriosis remains largely unknown.

T-cell immunoglobulin and mucin domain-containing protein 3 (TIM-3) is part of the TIM immunoregulatory protein family. This protein consists of an extracellular glycosylated mucin domain, an immunoglobulin variable domain, a C-terminal cytoplasmic tail, and a single transmembrane domain. Even though they were first detected in T cells, studies show that myeloid and natural killer (NK) cells can also express TIM3 [15]. The human leukocyte antigen B-associated transcript 3 (BAT3) bonds with the cytoplasmic tail of TIM-3, absent ligand interaction. This helps maintain immune cells in an active condition.

TIM-3 reportedly has various ligands (galectin 9, phosphatidylserine (PtdSer), adhesion molecule carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), and high mobility group protein B1 (HMGB1)) which connect to different regions of the extracellular immunoglobulin domain. Upon acknowledging one of these ligands, BAT3 disassociates from TIM-3 and results in immune cell (T-cell, myeloid cell, or NK cell) anergy or apoptosis [15].

From prior research, we understand that the accurate control of T-cell activation is vital for reaching a healthy balance between immune response and self-tissue tolerance in the immune system. This balance facilitates efficient pathogen clearance instead of causing excessive inflammatory damage. The listed regulation process is effectuated by checkpoint protein receptors expressed on T cells when activated [16–19]. TIM-3 functions as a significant immune checkpoint receptor. The interaction between TIM-3 and its ligands is critical for inducing peripheral tolerance because it inhibits effector Th1 cells during regular immune responses [20–22]. If it malfunctions, it could lead to overproduction of effector T cells, which continuously intensify tissue inflammation [23].

TIM-3 serves different functions in different diseases, such as cancer and immune disorders. It has been discovered that TIM-3 is highly expressed in several types of tumors, including colon cancer [24], prostate cancer [25], cervical cancer [26], and stomach cancer [27]. In autoimmune diseases like systemic lupus erythematosus [28], multiple sclerosis [29–36], rheumatoid arthritis [37], and inflammatory bowel disease [38], altered TIM-3 expression has been linked to T-cell hyperactivity, which escalates disease progression and severity. One instance is multiple sclerosis, whose central nervous system plaques are distinguished by macrophage and T-cell infiltration. Here, TIM-3, which normally regulates T-cell function, is under expressed in the peripheral blood (PB) T cells of these patients. This under expression allows autoreactive and pathogenic T cells to operate without regulation [34].

In summary, in a healthy immune response, the TIM-3 ligand could play a key role in blocking effector T cells and inducing peripheral tolerance [20]. However, in some pathological conditions like cancer, a high expression of TIM-3 can lead to T-cell anergy, suppressing the immune response and enabling cancer cells to proliferate. In contrast, low expression of TIM-3 can empower autoreactive and pathogenic T cells to bypass negative regulation and perpetuate chronic inflammation, as seen in multiple sclerosis [34]. In light of these findings, we have chosen to study the behavior of TIM-3 in endometriosis patients. A deeper investigation into the expression of immune checkpoint proteins within this context could lead to the identification of a disease biomarker or inspire innovative therapeutic strategies. This suggests that immune modulation may serve as a potential targeted therapy for women suffering from endometriosis.

2 | Material and Methods

2.1 | Study Design and Participants

This cross-sectional study was conducted from June 2021 to December 2022 and included 62 female patients. Among them, 47 had endometriosis (EM), and 15 were controls without the disease. All participants were adult women of reproductive age treated at Hospital da Luz Lisboa and Hospital Beatriz Ângelo's outpatient clinics. Participants in the endometriosis group underwent laparoscopic surgery for the disease, while the control group had other pathologies treated. All surgical procedures were performed under general anesthesia. Endometriosis was initially diagnosed through clinical and imaging findings, followed by histological confirmation in line with the Human Reproduction and Embryology (ESHRE) guidelines [39].

All participating patients were adults of reproductive age. Women who had been pregnant in the last year or those suffering from immunologic, endocrine, neoplastic, or other chronic diseases were excluded. We also excluded those suspected of having complications from pelvic inflammatory disease.

The Ethics Committees of Beatriz Ângelo, Hospital da Luz Lisboa, and NOVA Medical School approved this study. We ensured all patients were adequately informed about the study and the use of their biological material for scientific analysis. Before participating in the study, all patients gave their written informed consent.

2.2 | Demographic and Clinical Data

The collected data for all patients included age, race, body mass index (BMI), smoking status, hormone therapy, and the duration between the diagnosis of endometriosis and surgery.

Clinical data were also collected to characterize the severity of the disease, including symptoms such as dysmenorrhea, dyspareunia, chronic pelvic pain, dyschesia, bowel symptoms (such as constipation, peri-menstrual diarrhea, and bloating), urinary tract symptoms (including hematuria and dysuria), rectorrhagia, and the presence of extra-pelvic endometriosis. The severity of

the disease was classified according to the American Society for Reproductive Medicine [40].

2.3 | Sample Collection

Peripheral blood (PB) and peritoneal fluid (PF) samples were gathered from all patients during surgery. To reduce the chance of blood contamination, PF was collected at the start of the operation. We employed a laparoscopic needle to draw fluid from the pouch of Douglas. The samples were collected into EDTA-coated tubes, which were stored at room temperature post-surgery and transported to the immunology laboratory at NOVA Medical School. All PF samples were processed on the collection day. Blood samples were dealt with either on the same day or the next morning, depending on laboratory availability.

2.4 | Flow Cytometry Immunophenotyping Analysis

The cell immunophenotyping procedure started with acquiring absolute counts of each cell subset. This was done using a single-platform technique with Trucount™ tubes (BD Biosciences) for both PB and PF samples. Briefly, we incubated a standardized sample volume with CD45 in a Trucount™ tube for 15 min, lysed it with BD FACS lysing solution, and collected the results. Next, we stained both PB and PF samples using a panel of monoclonal antibodies, including CD45, CD3, CD8, CD16, CD56, CD57, and TIM-3. The supplementary materials (Table S1) provide a comprehensive list of all the antibodies we used.

We centrifuged the PF samples before immunophenotyping to concentrate the cells. We then applied a lyse-wash protocol using BD FACS lysing solution as a lysing agent. We collected all samples in a BD FACS Canto II (BD Biosciences, San Jose, CA, USA). We then analyzed the data using Infinicyt™ (Cytognos, Salamanca, Spain) and FlowJo™ (BD Life Sciences) software, both procured from BD Biosciences. Additionally, we assessed TIM-3 expression levels in T cells and NK cells. To minimize the effect of daily variations, we used mean fluorescence intensity (MFI) indices and compared the MFIs between the target populations and the corresponding TIM-3-negative population. Figure 1 displays the gating strategy we adopted.

2.5 | Statistical Analysis

Study participants' demographic, clinical, and immunophenotyping data were summarized through descriptive statistics. Categorical variables were provided as absolute and relative frequencies. Comparisons between two groups for contingency tables with over 20% of expected values below five were made using Fisher's exact test, while the chi-squared test was used otherwise. Continuous variables were displayed as mean (standard deviation) (minimum–maximum) or median and first-to-third quartile (Q1–Q3), depending on the table. In the Supplementary tables, all continuous variables are displayed as mean (standard deviation) with a 95% confidence interval [95% CI], alongside the median and Q1–Q3. The 95% CIs were calculated using a

Student's *t* distribution approximation for normally distributed continuous variables and bootstrap resampling otherwise. For normally distributed continuous data, comparisons between two independent groups or two dependent groups were evaluated by the Student's *t*-test and the paired-samples *t*-test, respectively. Alternatively, the nonparametric Wilcoxon rank-sum test and the Wilcoxon signed-rank test were used. For comparisons involving more than two groups, ANOVA with post hoc Tukey's HSD test was employed for normally distributed data, and the Kruskal–Wallis test with post hoc Dunn's test was used for the paired comparisons otherwise.

The Shapiro–Wilk test was used to evaluate the normality of distributions. Given the exploratory nature of the study, no formal sample size calculation was performed. Statistical significance was considered at $p < 0.05$. All analyses were performed using SAS software (version 9.4).

3 | Results

3.1 | Demographic and Clinical Characteristics of Patients

Our study involved 62 female participants, including 47 diagnosed with endometriosis and 15 in a control group without the condition. Table 1 presents the demographic and clinical data of both groups.

In the control group, the average age was 38.5 years, with a standard deviation (SD) of 9.2, ranging from 20 to 55 years. For women with endometriosis, the average age was 36.2 years, with an SD of 6.34, and ages ranged from 31 to 42 years. Predominantly, patients from both groups were white.

The demographic and clinical characteristics of the two groups showed no noteworthy differences, except for the history of infertility. It was revealed that infertility was significantly common in women with endometriosis ($p = 0.003$).

In regards to hormone therapy, the difference in usage between 33% of the control group women and 66% of those with endometriosis was not statistically significant ($p = 0.09$). The control group's hormone regimen included combined oral estrogen–progesterone ($n = 2$), a progesterone-only pill ($n = 1$), a vaginal contraceptive ring ($n = 1$), and a levonorgestrel-releasing intrauterine system ($n = 1$). The hormone treatments for the endometriosis group included combined oral estrogen–progesterone ($n = 12$), a progesterone-only pill ($n = 6$), a vaginal contraceptive ring ($n = 1$), a levonorgestrel-releasing intrauterine system ($n = 4$), and dienogest ($n = 7$).

3.2 | Endometriosis Characteristics

Table 2 details the endometriosis characteristics of the participating patients. The average duration between endometriosis diagnosis and surgery was 2.8 years. A majority of endometriosis patients featured severe manifestations (78.7%), corresponding with stages III/IV. The most prevalent issue was dysmenorrhea, experienced by 93.6% of participants, with severe dysmenorrhea

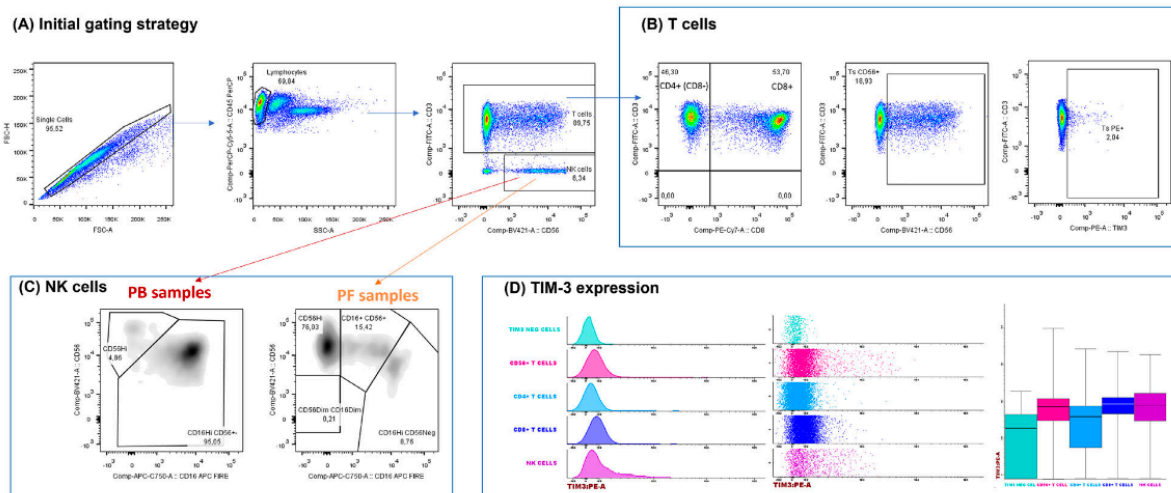


FIGURE 1 | Gating strategy used to characterize lymphocyte subsets in this study. (A) Gating strategy used to identify the major lymphocyte subsets (T cells and NK cells) in peritoneal fluid (PF) and peripheral blood (PB) samples. (B) Gating strategy used to identify CD4+, CD8+, and CD56+ T cells. All the subsets were further analyzed for the expression of TIM-3. (C) Gating strategy used to identify distinct NK cell subsets in PF and PB samples, which were further assessed for TIM-3 expression. (D) All the subsets mentioned above were further analyzed for the expression of TIM-3. The expression levels were normalized through experiments using an expression index obtained from the ratios between TIM-3 mean fluorescence intensity (MFI) in the population of interest and the negative population. The figure presents histograms and population band dot plots illustrative of the different levels of expression observed in CD4 T cells, CD8 T cells, CD56+ T cells, and NK cells of a peritoneal fluid (PF) sample, with the respective box plots diagrams. Analyses were performed with FlowJoTM software (panels A–C) and InfinicytTM software (panel D).

TABLE 1 | Demographic and clinical characteristics of control group and women with endometriosis.

Characteristics	Control (n = 15)	Endometriosis (n = 47)	p-value
Age ^a , mean (SD) (min–max), yr	38.5 (9.20) (20–55)	36.2 (6.34) (31–42)	0.434
Race ^b , n (%)			0.113
White	15 (100)	40 (85.1)	
Black	0 (0)	7 (14.9)	
BMI ^a , mean (SD) (min–max), kg/m ²	23.2 (2.97) (17–28)	25.2 (5.60) (20.8–28.0)	0.430
Fertile ^b , n (%)			0.003
Yes	14 (93.3)	24 (51.1)	
No	1 (6.7)	23 (48.9)	
Smoker ^b , n (%)			0.087
Yes	0 (0)	8 (17.0)	
No	15 (100)	39 (83.0)	
Hormonal therapy ^b , n (%)			0.090
Yes	5 (33.3)	31 (66.0)	
No	10 (66.7)	16 (34.0)	

Abbreviations: BMI, body mass index; max, maximum; min, minimum; n, number of patients; SD, standard deviation; yr, years.

Statistically significant results are indicated in bold.

^ap-values are based on the Wilcoxon rank-sum test.

^bp-values are based on the chi-squared test. Statistically significant results are indicated in bold.

(≥7) reported by 76.6%. Other symptoms included dyspareunia (59.6%), chronic pelvic pain (40.4%), dyschesia (34.0%), bowel symptoms (31.9%), urinary tract symptoms (10.6%), and rect-orrhagia (8.5%). About 4.3% of cases documented extra-pelvic endometriosis.

3.3 | Impact of Endometriosis on the Circulating and Local Immune Profiles

The main lymphoid subsets in circulating blood showed only minor differences between controls and endometriosis patients.

TABLE 2 | Endometriosis characteristics of the included patients.

Characteristics	Endometriosis (n = 47)
Endometriosis diagnosis, mean (SD) (min-max), yr	2.8 (2.52) (1-14)
Endometriosis severity, n (%)	
Stage I/II	10 (21.3)
Stage III/IV	37 (78.7)
Dysmenorrhea complaints, n (%)	44 (93.6)
Dysmenorrhea severity, n (%)	
None/Mild/Moderate (<7)	11 (23.4)
Severe (≥7)	36 (76.6)
Dyspareunia complaints, n (%)	28 (59.6)
Chronic pelvic pain, n (%)	19 (40.4)
Dyschesia complaints, n (%)	16 (34.0)
Bowel symptoms, n (%)	15 (31.9)
Urinary tract symptoms, n (%)	5 (10.6)
Rectorrhagia n (%)	4 (8.5)
Extra-pelvic endometriosis, n (%)	2 (4.3)

Abbreviations: max, maximum; min, minimum; n, number of patients; SD, standard deviation; yr, years.

We observed an increase in T-cell counts in endometriosis patients, while the proportion of circulating NK cells slightly decreased, but these differences lacked statistical significance ($p = 0.054$; $p = 0.081$). NK cells were the primary subset, and endometriosis patients had greater percentages ($p = 0.019$) and absolute numbers ($p = 0.047$) of CD56^{Hi}CD16^{dim} NK cells. Consequently, the ratios of CD56^{dim}CD16^{Hi} NK cells to CD56^{Hi}CD16^{dim} NK cells were distinctively lower in endometriosis patients ($p = 0.019$).

3.4 | Impact of Endometriosis on TIM-3 Expression in Immune Cells

Figure 2 illustrates the variation in TIM-3 expression across different T-cell subsets. The percentage of T cells expressing TIM-3 in the PB differed considerably between endometriosis patients and the control group. Endometriosis patients exhibited a substantially lower percentage of PB CD56+ T cells with TIM-3 expression, a difference that is statistically significant ($p = 0.009$). A decline in the proportion of TIM-3 expressing CD4+ and CD8+ T cells was also observed in endometriosis patients, but these differences did not reach statistical significance ($p = 0.055$; $p = 0.054$) (Figure 2).

No differences were observed in the proportion of T-cell subsets expressing TIM-3 in the PF between endometriosis patients and the control group. Similarly, there were no differences in the proportion of NK cells expressing TIM-3 in both the PB and PF between the endometriosis and control groups.

Considering the possible influence of hormone therapy on immune response, we categorized both the control and endometriosis groups based on whether participants were undergoing hormone therapy. The difference in the percentage of PB CD56+ T cells expressing TIM-3 remained statistically significant ($p = 0.04$) between control participants and endometriosis patients. Lower expression was observed specifically in the endometriosis patients who were receiving hormone therapy.

When comparing the two compartments, PB and PF, a higher percentage of CD8+ T cells are observed in the PF amongst both controls and patients ($p < 0.001$). In the PB, a greater proportion of CD8+ T cells express TIM-3 in both the endometriosis patients and the control group ($p < 0.001$; $p = 0.045$). Conversely, a lesser percentage of CD4+ T cells express TIM-3 in both groups ($p < 0.001$) compared to those in PF. For NK cells, a higher percentage of them in PB expressed CD8 ($p < 0.001$ in both groups) and TIM-3 ($p = 0.003$ for controls and $p < 0.001$ for endometriosis patients) compared to those in PF.

Table S2 shows the effects of smoking on the expression of TIM-3 in CD4+ T cells in both the control group and women diagnosed with endometriosis. In comparison, the eight patients with smoking habits had particularly lower percentages of CD4 T cells expressing TIM-3 than the control group ($p = 0.017$).

3.5 | Impact of Extrauterine Locations of Endometriosis on the Immune Profile and TIM-3 Expression

Table S3 compares circulating CD56 CD8+ T cells between the control group and women with bowel endometriosis. Figure 3A illustrates the difference in TIM-3 expression in CD56+ T cells between the same groups.

Surgically identified women with bowel endometriosis exhibited a reduced percentage of PB CD56 CD8+ T cells ($p = 0.033$) (Table S3). Additionally, fewer of these patients' total CD56+ T cells expressed TIM-3 compared to the control group ($p = 0.025$) (Figure 3A).

Figure 3B and C illustrates the change in TIM-3 expression in CD56+ T cells based on the stage of endometriosis and the years elapsed since the endometriosis diagnosis, respectively.

The proportion of circulating CD56+ T cells expressing TIM-3 significantly dropped as endometriosis progressed, particularly noticeable in women at stages III and IV ($p = 0.023$) (Figure 3B). Moreover, women diagnosed recently (under 2 years) showed a lesser proportion of PB CD56+ T cells expressing TIM-3 relative to the control group ($p = 0.029$) (Figure 3C).

3.6 | Impact of Dysmenorrhea on the Immune Profile and TIM-3 Expression

Table S4 demonstrates the changes in CD8 CD16+, and CD56 T cells associated with endometriosis dysmenorrhea. Figure 3D presents variations in the expression of TIM-3 in CD4+ and

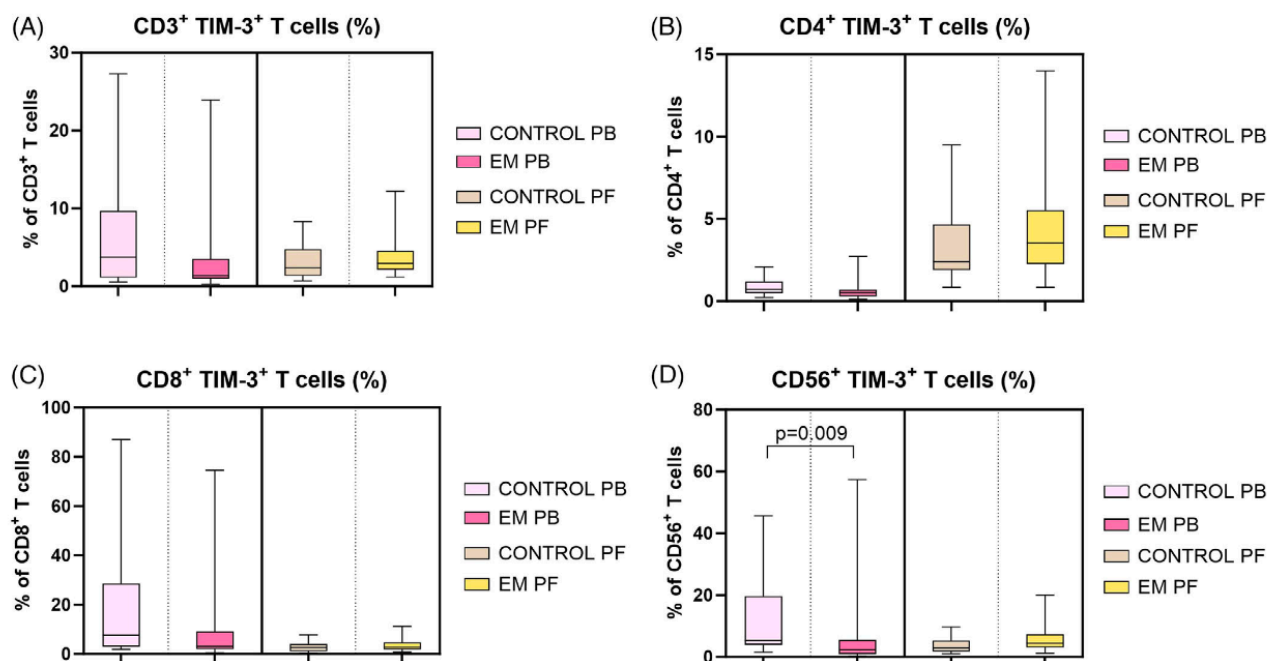


FIGURE 2 | TIM-3 expression in T-cell subsets, both in PB and PF samples, in the control group and women with endometriosis. (A) to (D) Box and whisker plots, representative of the percentages of T cells, CD4 T cells, CD8 T cells and CD56⁺ T cells expressing TIM-3. Graph lines refer to medians, interquartile ranges, and minimum and maximum values. P-values are based on the Wilcoxon rank-sum test. EM, endometriosis; PB, peripheral blood; PF, peritoneal fluid.

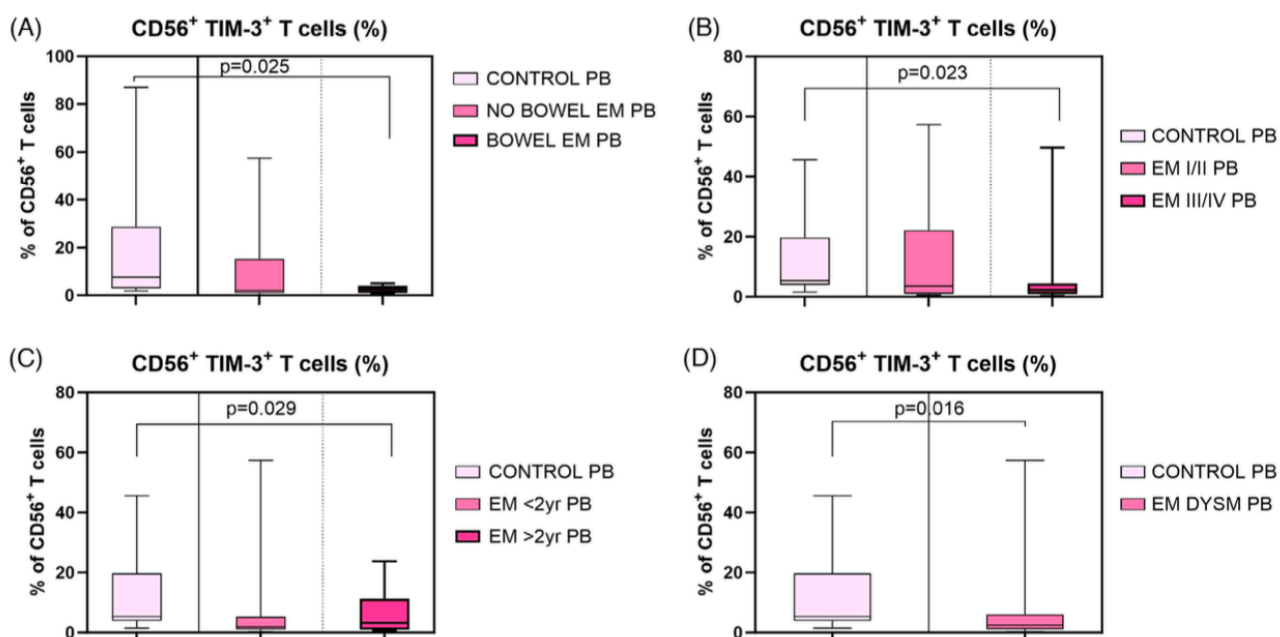


FIGURE 3 | Variation of TIM-3 expression in circulating CD56⁺ T-cell subsets, according to different clinical conditions. Box and whisker plots, representative of the percentages of CD56⁺ T cells expressing TIM-3. Graph lines refer to medians, interquartile ranges and minimum and maximum values. (A) Variation in TIM-3 expression between control group and women with and without bowel endometriosis. P-value is based on the Kruskal-Wallis test with post hoc Dunn's test for paired comparisons. (B) Variation in TIM-3 expression according to the stage of endometriosis. P-value is based on the Kruskal-Wallis test with post hoc Dunn's test for paired comparisons. (C) Variation in TIM-3 expression according to the years of endometriosis diagnosis. P-value is based on the Kruskal-Wallis test with post hoc Dunn's test for paired comparisons. (D) Variation in TIM-3 expression between control group and women with endometriosis dysmenorrhea. P-value is based on Wilcoxon rank-sum test. DYSM, dysmenorrhea; EM, endometriosis; PB, peripheral blood.

CD56+ T cells, comparing the control group to women with endometriosis dysmenorrhea.

Patients with endometriosis but without dysmenorrhea demonstrated increased proportions of both CD8 CD16+ T cells and CD56 T cells in their PF ($p = 0.033$ and $p = 0.037$, respectively), as documented in Table S4. Alternatively, those suffering from dysmenorrhea exhibited a reduced percentage of circulating CD56+ T cells that expressed TIM-3 when compared to the control group ($p = 0.016$) (Figure 3D).

4 | Discussion

In this study, we examined the expression of TIM-3 on T and NK cell subsets in the PF and PB of women with endometriosis and controls. Our findings revealed a significant decrease in the proportion of PB CD56+ T cells expressing TIM-3 in patients with endometriosis compared to the control group ($p = 0.009$). However, there were no observable differences in PF samples.

Only one prior study by Meggyes et al. [41] tackled this issue, though they used a fundamentally different methodology and included fewer patients. They also observed a reduced expression of TIM-3 in the PB of endometriosis patients, but solely in CD4+ and CD8+ T cells.

Other studies have demonstrated that immune responses are regulated through immunological checkpoint protein receptors located on activated T cells, with TIM-3 serving as a crucial receptor in this process. Dysfunction of TIM-3 can lead to an overabundance of effector T cells, contributing to persistent tissue inflammation and damage [23]. This mechanism, for example, has been demonstrated in the progression of multiple sclerosis [42]. It is conceivable that decreased TIM-3 expression on T cells in the PB of women with endometriosis could lead to T-cell dysfunction and increased immune system reactivity, thus triggering inflammation at sites of endometrial cell implantation. T cells lacking TIM-3 expression might lose their regulatory capabilities and, by releasing inflammatory molecules, contribute to an uncontrolled inflammatory environment in the implantation zones of endometriotic cells. These changes could facilitate the progression of the disease, which is characterized by recurrent episodes of inflammation and fibrosis.

Our research further strengthens the previously introduced concept, yielding some intriguing findings. Women with bowel endometriosis, representing the more severe form of the disease, exhibited lower percentages of total CD56+ T cells expressing TIM-3 compared to the control group ($p = 0.025$). Notably, as the stages of endometriosis advanced, the percentage of circulating CD56+ T cells expressing TIM-3 declined significantly. This discrepancy was most pronounced in women with stages III and IV of the disease ($p = 0.023$) and was not observed among other T-cell subtypes or NK cells. Also, women with recent endometriosis progression—measured from diagnosis to study evaluation—presented a diminutive percentage of circulating CD56+ T cells expressing TIM-3 relative to the controls ($p = 0.029$). Another determining factor was smoking habits, which resulted in patients with endometriosis having a lower percentage of CD4 T cells expressing TIM-3 compared to the

control group ($p = 0.017$). However, we advise caution with this outcome as the sample size of smoking endometriosis patients was small ($n = 8$).

The fact that TIM-3 expression is altered specifically in CD56+ T cells is noteworthy. CD56-expressing cell types have strong immunostimulatory effector capabilities, such as the generation of T helper 1 cytokines and an effective cytotoxic potential [43]. In inflammatory conditions, not only do CD56+ T cells exhibit a killing activity akin to NK cells, but they also show increased resistance to apoptosis [44–48].

We observed no variation between women with endometriosis and the control group in the percentage of NK cells expressing TIM-3. Prior research has established a role for TIM-3 in the optimal activation of innate immune cells through its diverse expression [47]. Different groups, when exposed to antigens specific to the disease condition or test, can alter their TIM-3 expression pattern. Notably, varying types of macrophages display unique TIM-3 expression behaviors post-stimulation [48]. This could imply that these groups might have different signaling pathways activated, leading to a range of TIM-3 expressions contingent upon the pathway. Besides, CD56+ T cells are potentially more like Natural killer T (NKT) populations. These NKT cells act as a bridge between innate and adaptive immunity. Decreased TIM-3 expression on NKT cells corresponded with elevated interferon- γ (IFN- γ) production in pre-eclamptic pregnant women [49], implying that TIM-3 controls NKT cell activation. Additionally, a negative correlation has been found between TIM-3 expression and tumor necrosis factor (TNF) production by NKT cells [50], suggesting the suppressive role of TIM-3 in NKT activities.

We found significant differences in the expression of TIM-3 in immune cells between PB and PF compartments. Both NK cells and CD8+ T cells showed a higher percentage of TIM-3 expression in PB than in PF. Interestingly, CD4+ T cells were the exception, with a higher percentage expressing TIM-3 in PF.

The role of PF in the immunopathogenesis of endometriosis is currently unclear. It is likely that immune cells in PB provide a more accurate representation of the immune system's response to endometriotic cells than those in PF. This is due to the greater external influence on PF cells, such as the link between the uterine cavity and the pelvis via the fallopian tubes.

Our research offers new insights into a field where extensive research is currently lacking. To our understanding, this is the inaugural study to simultaneously analyze TIM-3 expression in samples from both PF and PB in women with endometriosis and those without it. Only one previous study [41] has examined the expression of TIM-3/galectin-9 in the T and NK cells of women with endometriosis, but our approach differs significantly. Our study boasts a larger sample size and includes analysis of PF in both the endometriosis and control groups. Notably, we confirmed the absence of endometriosis in our control group through laparoscopic surgery – a definitive method for ruling out the condition. Our work also stands out because of the detailed examination of specific subsets within the NK and T-cell populations, improving our understanding of the cellular-level

immunological landscape in endometriosis and potentially shedding light on the disease's pathophysiology.

While this study effectively explores the immunologic profile of PB and PF in relation to TIM-3 expression, it also exposes certain limitations. Our findings show the complex interplay between each subpopulation and present TIM-3's presence in NK and T-cell subpopulations across a range of demographic and clinical scenarios. However, it is significant to note that presence does not automatically imply activity. Future research could bolster our immunologic comprehension through functional assays – including cytotoxicity assays and cytokine analysis – supplementing our current findings. The observed immune system changes could impact the implantation and growth of endometriotic cells, but they might also be a reaction or symptom of the disease. Another limitation is that we just observed altered TIM expression in a specific type of T cells in PB without corroborating this change in PF or other immune cell types, notably NK cells. Lastly, while our sample population is larger than that of previous study in this area, it is still too small to draw definitive conclusions.

Our findings on the TIM-3 receptor expression in T cells offer promising insights into its role in endometriosis pathology. However, more extensive studies reflecting similar outcomes are vital to further substantiate our deductions.

5 | Conclusion

We noted a decrease in TIM-3 expression in women diagnosed with endometriosis compared to those without the disease. The difference was especially noticeable in individuals with possibly more serious forms of the disease, especially those with stage III/IV endometriosis and bowel invasion. We propose that autoreactive T cells with lower surface TIM-3 expression might aid T-cell responses, as these cells are more easily activated and show resistance to tolerance development. Considering this, reduced TIM-3 levels on circulating T cells could play a role in endometriosis pathogenesis. Our findings need to be validated by larger subsequent studies.

Acknowledgments

The authors would like to acknowledge Jorge Gonçalves (Scientific ToolBox Consulting, Lisbon, Portugal) for statistical analysis and Sofia Nunes, Ph.D. (Scientific ToolBox Consulting, Lisbon, Portugal) for providing medical writing assistance and technical editing. This study was partially funded by the Hospital da Luz Lisboa under the initiative “Luz Investigação”, Gedeon Richter Portugal, and LifeWell Pharmaceutical & Healthcare.

Ethics Statement

The authors affirm adherence to the journal's ethical policies, as detailed in the author guidelines, and confirm they have obtained the necessary approval from the suitable ethical review committee.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The article contains all data relevant to the study. For additional information, the corresponding author can be contacted.

References

1. A. Fukui, C. Mai, S. Saeki, et al., “Pelvic Endometriosis and Natural Killer Cell Immunity,” *American Journal of Reproductive Immunology* 85, no. 4 (2021): e13342, <https://doi.org/10.1111/aji.13342>.
2. A. L. Shafrir, L. V. Farland, D. K. Shah, et al., “Risk for and Consequences of Endometriosis: A Critical Epidemiologic Review,” *Best Practice & Research Clinical Obstetrics & Gynaecology* 51 (2018): 1–15, <https://doi.org/10.1016/j.bpobgyn.2018.06.001>.
3. S. E. Bulun, “Endometriosis Caused by Retrograde Menstruation: Now Demonstrated by DNA Evidence,” *Fertility and Sterility* 118, no. 3 (2022): 535–536, <https://doi.org/10.1016/j.fertnstert.2022.07.012>.
4. D. J. Oosterlynck, F. J. Cornillie, M. Waer, M. Vandeputte, and P. R. Koninckx, “Women with Endometriosis Show a Defect in Natural Killer Activity Resulting in a Decreased Cytotoxicity to Autologous Endometrium,” *Fertility and Sterility* 56, no. 1 (1991): 45–51.
5. D. J. Oosterlynck, C. Meuleman, M. Waer, M. Vandeputte, and P. R. Koninckx, “The Natural Killer Activity of Peritoneal Fluid Lymphocytes Is Decreased in Women with Endometriosis,” *Fertility and Sterility* 58, no. 2 (1992): 290–295.
6. H. Kanzaki, H. S. Wang, M. Kariya, and T. Mori, “Suppression of Natural Killer Cell Activity by Sera from Patients with Endometriosis,” *American Journal of Obstetrics and Gynecology* 167, no. 1 (1992): 257–261, [https://doi.org/10.1016/s0002-9378\(11\)91670-6](https://doi.org/10.1016/s0002-9378(11)91670-6).
7. E. Tanaka, F. Sando, S. Kawagoe, and M. Hiroi, “Decreased Natural Killer Cell Activity in Women with Endometriosis,” *Gynecologic and Obstetric Investigation* 34, no. 1 (1992): 27–30, <https://doi.org/10.1159/000292720>.
8. G. G. Garzetti, A. Ciavattini, M. Provinciali, M. Muzzioli, G. Di Stefano, and N. Fabris, “Natural Killer Activity in Stage III and IV Endometriosis: Impaired Cytotoxicity Anti Retained Lymphokine Responsiveness of Natural Killer Cells,” *Gynecological Endocrinology* 9, no. 2 (1995): 125–130, <https://doi.org/10.3109/09513599509160201>.
9. L. Hua, X. Xu, and D. Bian, “The Measurement of Natural Killer Cell Activity in Peripheral Blood and Peritoneal Fluid of Patients with Endometriosis,” *Zhonghua Fu Chan Ke Za Zhi* 1, no. 10 (1996): 586–589, <https://www.ncbi.nlm.nih.gov/pubmed/9275450>.
10. D. Vinatier, P. Dufour, and D. Oosterlynck, “Immunological Aspects of Endometriosis,” *Human Reproduction Update* 2, no. 5 (1996): 371–384, <https://doi.org/10.1093/humupd/2.5.371>.
11. H. N. Ho, M. Y. Wu, and Y. S. Yang, “Peritoneal Cellular Immunity and Endometriosis,” *American Journal of Reproductive Immunology* 38, no. 6 (1997): 400–412.
12. D. Vinatier and P. Dufour, “Is Endometriosis an Immunological Disease?” *Contraception Fertilite Sexualite* 25, no. 3 (1997): 179–191, <https://www.scopus.com/inward/record.uri?eid=2-s2.0-0031085665&partnerID=40&md5=c581b4bd9c6cbb611f9235815401962c>.
13. P. R. Koninckx, S. H. Kennedy, and D. H. Barlow, “Endometriotic Disease: The Role of Peritoneal Fluid,” *Human Reproduction Update* 4, no. 5 (1998): 741–751, <https://doi.org/10.1093/humupd/4.5.741>.
14. J. L. Reis, N. N. Rosa, M. Angelo-Dias, C. Martins, L. M. Borrego, and J. Lima, “Natural Killer Cell Receptors and Endometriosis: A Systematic Review,” *International Journal of Molecular Sciences* 24, no. 1 (2022): 331, <https://doi.org/10.3390/ijms24010331>.
15. Y. Wolf, A. C. Anderson, and V. K. Kuchroo, “TIM3 Comes of Age as An Inhibitory Receptor,” *Nature Reviews Immunology* 20, no. 3 (2020): 173–185, <https://doi.org/10.1038/s41577-019-0224-6>.
16. Y. Zhang and J. Zheng, “Function of Immune Checkpoint Molecules Beyond Immune Evasion,” *Advanced Immunology* 1248 (2020): 201–226, https://doi.org/10.1007/978-981-15-3266-5_9.

17. S. Bagchi, R. Yuan, and E. G. Engleman, "Immune Checkpoint Inhibitors for the Treatment of Cancer: Clinical Impact and Mechanisms of Response and Resistance," *Annual Review of Pathology* 16 (2021): 223–249, <https://doi.org/10.1146/annurev-pathol-042020-042741>.
18. S. P. Kubli, T. Berger, D. V. Araujo, L. L. Siu, and T. W. Mak, "Beyond Immune Checkpoint Blockade: Emerging Immunological Strategies," *Nature Reviews Drug Discovery* 20, no. 12 (2021): 899–919, <https://doi.org/10.1038/s41573-021-00155-y>.
19. H. Chen, J. Zha, R. Tang, and G. Chen, "T-Cell Immunoglobulin and Mucin-Domain Containing-3 (TIM-3): Solving a Key Puzzle in Autoimmune Diseases," *International Immunopharmacology* 121 (2023): 110418, <https://doi.org/10.1016/j.intimp.2023.110418>.
20. C. Zhu, A. C. Anderson, A. Schubart, et al., "The Tim-3 Ligand Galectin-9 Negatively Regulates T Helper Type 1 Immunity," *Nature Immunology* 6, no. 12 (2005): 1245–1252, <https://doi.org/10.1038/ni1271>.
21. A. Sanchez-Fueyo, J. Tian, D. Picarella, et al., "Tim-3 Inhibits T Helper Type 1-Mediated Auto- and Alloimmune Responses and Promotes Immunological Tolerance," *Nature Immunology* 4, no. 11 (2003): 1093–1101, <https://doi.org/10.1038/ni987>.
22. C. A. Sabatos, S. Chakravarti, E. Cha, et al., "Interaction of Tim-3 and Tim-3 Ligand Regulates T Helper Type 1 Responses and Induction of Peripheral Tolerance," *Nature Immunology* 4, no. 11 (2003): 1102–1110, <https://doi.org/10.1038/ni988>.
23. A. C. Anderson, D. E. Anderson, L. Bregoli, et al., "Promotion of Tissue Inflammation by the Immune Receptor Tim-3 Expressed on Innate Immune Cells," *Science* 318, no. 5853 (2007): 1141–1143, <https://doi.org/10.1126/science.1148536>.
24. E. Zhou, Q. Huang, J. Wang, et al., "Up-Regulation of Tim-3 is Associated with Poor Prognosis of Patients with Colon Cancer," *International Journal of Clinical and Experimental Pathology* 8, no. 7 (2015): 8018–8027.
25. Y. R. Piao, L. Z. Piao, L. H. Zhu, Z. H. Jin, and X. Z. Dong, "Prognostic Value of T Cell Immunoglobulin Mucin-3 in Prostate Cancer," *Asian Pacific Journal of Cancer Prevention* 14, no. 6 (2013): 3897–3901, <https://doi.org/10.7314/apjcp.2013.14.6.3897>.
26. Y. Cao, X. Zhou, X. Huang, et al., "Correction: Tim-3 Expression in Cervical Cancer Promotes Tumor Metastasis," *PLoS ONE* 11, no. 3 (2016): e0152830, <https://doi.org/10.1371/journal.pone.0152830>.
27. B. Cao, L. Zhu, S. Zhu, et al., "Genetic Variations and Haplotypes in TIM-3 Gene and the Risk of Gastric Cancer," *Cancer Immunology, Immunotherapy* 59, no. 12 (2010): 1851–1857, <https://doi.org/10.1007/s00262-010-0910-5>.
28. Q. Luo, Y. Kong, B. Fu, et al., "Increased TIM-3(+)PD-1(+) NK Cells Are Associated with the Disease Activity and Severity of Systemic Lupus Erythematosus," *Clinical and Experimental Medicine* 22, no. 1 (2022): 47–56, <https://doi.org/10.1007/s10238-021-00726-8>.
29. S. Alvermann, C. Hennig, O. Stüve, H. Wiendl, and M. Stangel, "Immunophenotyping of Cerebrospinal Fluid Cells in Multiple Sclerosis: in Search of Biomarkers," *JAMA Neurology* 71, no. 7 (2014): 905–912, <https://doi.org/10.1001/jamaneurol.2014.395>.
30. I. Lavon, C. Heli, L. Brill, H. Charbit, and A. Vaknin-Dembinsky, "Blood Levels of Co-inhibitory-Receptors: A Biomarker of Disease Prognosis in Multiple Sclerosis," *Frontiers in Immunology* 10 (2019): 835. (In eng), <https://doi.org/10.3389/fimmu.2019.00835>.
31. M. Khademi, Z. Illés, A. W. Gielen, et al. "T Cell Ig- and Mucin-Domain-Containing Molecule-3 (TIM-3) and TIM-1 Molecules Are Differentially Expressed on Human Th1 and Th2 Cells and in Cerebrospinal Fluid-Derived Mononuclear Cells in Multiple Sclerosis," *Journal of Immunology* 172, no. 11 (2004): 7169–7176, <https://doi.org/10.4049/jimmunol.172.11.7169>.
32. B. Afshar, Z. Khalifehzadeh-Esfahani, N. Seyfizadeh, G. Rezaei Danbarani, M. Hemmatzadeh, and H. Mohammadi, "The Role of Immune Regulatory Molecules in Multiple Sclerosis," *Journal of Neuroimmunology* 337 (2019): 577061, <https://doi.org/10.1016/j.jneuroim.2019.577061>.
33. K. Koguchi, D. E. Anderson, L. Yang, K. C. O'Connor, V. K. Kuchroo, and D. A. Hafler, "Dysregulated T Cell Expression of TIM3 in Multiple Sclerosis," *Journal of Experimental Medicine* 203, no. 6 (2006): 1413–1418, <https://doi.org/10.1084/jem.20060210>.
34. L. Yang, D. E. Anderson, J. Kuchroo, and D. A. Hafler, "Lack of TIM-3 Immunoregulation in Multiple Sclerosis," *Journal of Immunology* 180, no. 7 (2008): 4409–4414.
35. M. Saresella, F. Piancone, I. Marventano, et al., "A Role for the TIM-3/GAL-9/BAT3 Pathway in Determining the Clinical Phenotype of Multiple Sclerosis," *Faseb Journal* 28, no. 11 (2014): 5000–5009, <https://doi.org/10.1096/fj.14-258194>.
36. L. Tang, G. Li, Y. Zheng, et al., "Tim-3 Relieves Experimental Autoimmune Encephalomyelitis by Suppressing MHC-II," *Frontiers in Immunology* 12 (2021): 770402, <https://doi.org/10.3389/fimmu.2021.770402>.
37. C. M. Weyand, and J. J. Goronzy, "The Immunology of Rheumatoid Arthritis," *Nature Immunology* 22, no. 1 (2021): 10–18, <https://doi.org/10.1038/s41590-020-00816-x>.
38. X. Jiang, J. Yu, Q. Shi, et al., "Tim-3 Promotes Intestinal Homeostasis in DSS Colitis by Inhibiting M1 Polarization of Macrophages," *Clinical Immunology* 160, no. 2 (2015): 328–335, <https://doi.org/10.1016/j.clim.2015.07.008>.
39. C. M. Becker, A. Bokor, O. Heikinheimo, et al., "ESHRE Guideline: Endometriosis," *Hum Reprod Open* 2022, no. 2 (2022): hoac009, <https://doi.org/10.1093/hropen/hoac009>.
40. W. C. Andrews, V. C. Buttram Jr., J. C. Weed, C. B. Hammond, H. H. Thomas, and S. J. Behrman, "Revised American Fertility Society Classification of Endometriosis: 1985," *Fertility and Sterility* 43, no. 3 (1985): 351–352, [https://doi.org/10.1016/s0015-0282\(16\)48430-x](https://doi.org/10.1016/s0015-0282(16)48430-x).
41. M. Meggyes, L. Szereday, N. Bohonyi, et al., "Different Expression Pattern of TIM-3 and Galectin-9 Molecules by Peripheral and Peritoneal Lymphocytes in Women With and Without Endometriosis," *International Journal of Molecular Sciences* 21, no. 7 (2020): 2343, <https://doi.org/10.3390/ijms21072343>.
42. L. Yang, D. E. Anderson, J. Kuchroo, and D. A. Hafler, "Lack of TIM-3 Immunoregulation in Multiple Sclerosis," *Journal of Immunology* 180, no. 7 (2008): 4409–4414, <https://doi.org/10.4049/jimmunol.180.7.4409>.
43. H. H. Van Acker, A. Capsomidis, E. L. Smits, and V. F. Van Tendeloo, "CD56 in the Immune System: More Than a Marker for Cytotoxicity?," *Frontiers in Immunology* 8 (2017): 892, <https://doi.org/10.3389/fimmu.2017.00892>.
44. W. K. Chan, P. Rujkijyanont, G. Neale, et al., "Multiplex and Genome-Wide Analyses Reveal Distinctive Properties of KIR+ and CD56+ T Cells in Human Blood," *Journal of Immunology* 191, no. 4 (2013): 1625–1636, <https://doi.org/10.4049/jimmunol.1300111>.
45. M. P. Correia, A. V. Costa, M. Uhrberg, E. M. Cardoso, and F. A. Arosa, "IL-15 Induces CD8+ T Cells to Acquire Functional NK Receptors Capable of Modulating Cytotoxicity and Cytokine Secretion," *Immunobiology* 216, no. 5 (2011): 604–612, <https://doi.org/10.1016/j.imbio.2010.09.012>.
46. S. Guia, C. Cognet, L. de Beaucoudrey, et al., "A Role for Interleukin-12/23 in the Maturation of Human Natural Killer and CD56+ T Cells in Vivo," *Blood* 111, no. 10 (2008): 5008–5016, <https://doi.org/10.1182/blood-2007-11-122259>.
47. G. Han, G. Chen, B. Shen, and Y. Li, "Tim-3: An Activation Marker and Activation Limiter of Innate Immune Cells," *Frontiers in Immunology* 4 (2013): 449, <https://doi.org/10.3389/fimmu.2013.00449>.
48. R. Ocaña-Guzman, L. A. Ramon-Luing, L. A. Vazquez-Bolaños, et al., "Tim-3 Is Differentially Expressed During Cell Activation and Interacts with the LSP-1 Protein in Human Macrophages," *Journal of Immunology Research* 2023 (2023): 3577334, <https://doi.org/10.1155/2023/3577334>.
49. E. Miko, L. Szereday, A. Barakonyi, A. Jarkovich, P. Varga, and J. Szekeres-Bartho, "Immunoactivation in Preeclampsia: Vdelta2+ and Regulatory T Cells During the Inflammatory Stage of Disease," *Journal*

of *Reproductive Immunology* 80, no. 1-2 (2009): 100–108, <https://doi.org/10.1016/j.jri.2009.01.003>.

50. Y. Liu, Q. Shu, L. Gao, et al., “Increased Tim-3 Expression on Peripheral Lymphocytes from Patients with Rheumatoid Arthritis Negatively Correlates with Disease Activity,” *Clinical Immunology* 137, no. 2 (2010): 288–295, <https://doi.org/10.1016/j.clim.2010.07.012>.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.

CHAPTER VI

PD-1 in Endometriosis: Uncovering Potential Links to Cancer Pathways

PD-1 in Endometriosis: Uncovering Potential Links to Cancer Pathways

José Lourenço Reis ¹, Catarina Martins ^{2,3}, Miguel Ângelo-Dias ^{2,3}, Natacha Nurdine Rosa ⁴, Luís Miguel Borrego ^{2,3,5} and Jorge Lima ^{1,2,3*}

¹Department of Obstetrics and Gynecology, Hospital da Luz Lisboa, Luz Saúde, Portugal.

²CHRC, NOVA Medical School|Faculdade de Ciências Médicas, NMS|FCM, Universidade NOVA de Lisboa; Lisboa, Portugal;

³Immunology Department, NOVA Medical School|Faculdade de Ciências Médicas, NMS|FCM, Universidade NOVA de Lisboa; Lisboa, Portugal.

⁴UCD School of Medicine, University College Dublin, Dublin, Ireland

⁵Department of Imunoallergy, Hospital da Luz Lisboa, Luz Saúde, Portugal.

* Correspondence: Jorge Lima, MD, PhD; jorgeramoslima@sapo.pt

Abstract: Endometriosis, believed by many to be rooted in immunology, is a chronic disease. Natural killer (NK) cells play a crucial role in immune defense, but their efficacy against cancer cells is diminished by the upregulation of programmed cell death protein 1 (PD-1). This research aims to investigate the potential for a similar mechanism in endometriosis. A total of 62 patients who underwent laparoscopic surgery were analyzed, with 47 diagnosed with endometriosis and 15 serving as controls. We collected peritoneal fluid and peripheral blood samples during surgery and examined them using flow cytometry. Using a panel of monoclonal antibodies, the samples were stained and the expression of PD-1 in immune cells was evaluated. We observed a statistically significant rise in the percentage of the CD56+CD16+ NK cell subset expressing PD-1 within the peritoneal fluid of endometriosis patients compared to the control group ($p = 0.021$). Similarly, we found that PD-1 expression on immune cells significantly differed based on factors such as body mass index and smoking habits. Moreover, peritoneal subsets of PD-1+ T and NK cells showed an increase in patients presenting symptomatic endometriosis and those with more widespread disease. Our evaluation of NK cells and their inhibitory PD-1 receptor has strengthened the potential connection between immune escape mechanisms often seen in cancer cells and those in endometriotic cells. This concept could pave the way for future research in the field of immunotherapy and endometriosis.

Keywords: NK cells; T cells; PD-1; immune receptors; endometriosis

1. Introduction

Endometriosis is a common chronic gynecological condition, often associated with symptoms like painful menstruation, persistent pelvic pain and infertility. It is characterized by the abnormal presence of endometrial cells, typically located on the ovaries or the pelvic lining (Fukui et al., 2021). Despite its long history and prevalence amongst women of reproductive age (one in ten), the condition's cause remains a mystery (Shafrir et al., 2018). Presently, treatment predominantly involves hormone suppression, which unfortunately proves ineffective for numerous patients (Capezzuoli et al., 2022).

Since the 1990s, the role of the immune system in the development and progression of endometriosis has been conjectured (Tanaka et al., 1992; Vinatier et al., 1996). Although the precise mechanism remains unclear, recent emphasis on the study of natural killer (NK) cells and their receptors, spurred by advances in flow cytometry, has made this theory increasingly significant. A comprehensive recent review has further explored this subject (Reis et al., 2022). Endometriosis development shares several characteristics with cancer growth, including immune system evasion, pelvic dissemination, and mutagenesis (Quatrini et al., 2020; Wu et al., 2019; Zhang & Zheng, 2020).

PD-1, a crucial inhibitory checkpoint, was first identified on T, B, and myeloid cells, but has also been recently found on NK cells (Pesce et al., 2017). Independent studies on diverse cancer types, including ovarian cancer, multiple myeloma, Kaposi sarcoma, and lung and digestive cancers, have revealed that tumor-infiltrating PD-1+ NK cells produce significantly fewer cytokines upon interaction with the tumor (Pesce et al., 2017). Ahmadzadeh et al. revealed that most tumor-infiltrating T lymphocytes primarily express PD-1, a trait linked to an exhausted phenotype and diminished effector function (Ahmadzadeh et al., 2009). These are referred to as “immune checkpoints” due to their role in regulating immunological responses, observed in both NK and cytotoxic T cells (Mariotti et al., 2020). Increased expression of inhibitory receptors in NK cells has been correlated with decreased cell functionality in the tumor environment (Moretta et al., 2014; Quatrini et al., 2020).

Given that tumor cells often use the PD-1 axis to avoid the immune system, it is logical that this pathway has been explored extensively to devise new treatments intended to augment the anti-tumor immune response. The activity of these immune checkpoints can be curtailed by using blocking antibodies that hinder ligand-receptor interactions, both in the lab and the body. This, in turn, improves NK cell reactivity by inhibiting the PD-1/PD-L1 pathway (Concha-Benavente et al., 2018; Liu et al., 2017). In fact, anti-PD-1/PD-L1 therapy is now approved for treating a wide variety of cancers (Liu et al., 2021; Tang et al., 2022). It can be said that the study of immune escape mechanisms in oncology has successfully paved the way for the development of immunotherapy, which has improved cancer treatment and is now a widely used therapeutic approach for various types of cancer. (Alsaab et al., 2017; Makuku et al., 2021; Ribas, 2012).

NK cells, recognized for their ability to swiftly respond and eradicate cancerous or virally infected cells, were initially found to distinguish themselves from “non-self” cells by identifying MHC class I molecules (Ljunggren & Kärre, 1990; Moretta et al., 1996). However, a broader range of functions and a more powerful molecular toolset is now attributed to these versatile lymphoid cells. One such function includes the interaction of NK cells with additional inhibitory checkpoints like PD-1, primarily concerned with maintaining immune cell homeostasis. Additionally, these cells may engage inhibitory receptors specific for HLA-I molecules. They typically do not express these inhibitory checkpoints when at rest. In pathological situations, expression of such receptors may be newly generated through interaction with select ligands prevalent on tumor cells surface (Pesce et al., 2017). This scenario can prompt checkpoint receptors to hinder NK cell activity, thereby facilitating immune escape for the tumor (Pesce et al., 2019).

This study aims to examine the mechanisms by which endometriotic cells may evade the immune system, with a focus on PD-1 expression in immune cells from both peripheral blood and peritoneal fluid in women with endometriosis. Drawing parallels between the immune evasion strategies of cancer and endometriotic cells could provide valuable insights for developing future immunomodulatory therapies for endometriosis.

2. Materials and Methods

2.1. Study design and subjects

This cross-sectional study, conducted from June 2021 to December 2022, included 62 female patients. Among these, 47 had endometriosis (EM) and 15 served as controls without endometriosis. All participants are adult women of reproductive age, selected from the hospital's outpatient clinic and who have undergone laparoscopic surgery at the Hospital da Luz Lisboa and Hospital Beatriz Ângelo, those in the endometriosis group to treat this disease and those in the control group to treat pathologies other than endometriosis. Their gynecological issues were treated surgically under general anesthesia.

Endometriosis was initially diagnosed in accordance with the ESHRE criteria based on clinical and imaging findings, and subsequently confirmed through histology (Becker et al., 2022). We excluded women who were pregnant within the last year, those suspected of complications from pelvic inflammatory disease, and those with immunologic, endocrine, cancer, or other chronic disorders.

The Ethics Committees at NOVA Medical School, Hospital da Luz Lisboa, and Beatriz Ângelo approved this study. We provided each patient with essential information about the research, including the use of their biological materials for scientific analysis. Every patient granted their explicit written consent before participating in the study.

2.2. Clinical and demographic information

We recorded each patient's age, race, body mass index (BMI), smoking status, hormonal medication use, and the time span between endometriosis diagnosis and surgery. In terms of BMI, normal weight was defined as a BMI between 18.5 and 24.9, overweight as a BMI between 25 and 29.9, and obesity as a BMI of 30 or above.

Clinical data on various symptoms was gathered to assess the severity of the disease. This included dysmenorrhea, dyspareunia, chronic pelvic pain, dyschesia, bowel issues such as constipation or perimenstrual diarrhea and bloating, urinary tract symptoms like hematuria and dysuria, rectorrhagia, and the presence of extra-pelvic endometriosis. Pain was assessed by the Numerical Rating Scale. The American Society for Reproductive Medicine's established criteria was employed to categorize the severity of the disease (Andrews et al., 1985).

2.3. Sample collection

During the procedure, peritoneal fluid (PF) and peripheral blood (PB) samples were collected from each patient. To minimize blood contamination, PF was procured at the surgery's outset using EDTA-coated tubes. After surgery, these samples were stored and transported at room temperature to NOVA Medical School's immunology laboratory. All PF samples were processed on the day of collection, while blood samples were processed on the day of the collection or kept at room temperature and processed in the following morning, as per the laboratory availability.

2.4. Analysis of immunophenotyping using flow cytometry

A single-platform method with Trucount™ tubes (BD Biosciences) was utilized to acquire the absolute counts of major leucocyte populations and discern the lymphoid compartment in each PB and PF sample. In this process, each sample was stained with CD45 for 15 min in a Trucount™ tube, followed by lysis using a BD FACS lysing solution. The PB and PF samples were then stained with an additional set of monoclonal antibodies, including CD45, CD3, CD8, CD16, CD56, CD57, and PD-1. A thorough inventory of all antibodies employed is available in the Supplementary Materials (Table S1). To intensify the cell concentration in the sample, PF samples underwent centrifugation preceding immunophenotyping. A lyse-wash method was performed, entailing a 20-min incubation with mAbs and a 15-min lysis utilizing BD FACS lysing solution. Subsequently, the cells were cleaned using BD FACS Flow Solution (BD Biosciences) and fixed using BD Cell Fix solution (BD Biosciences). All samples were processed in a BD FACS Canto II (BD Biosciences, San Jose, CA, USA). Data analysis was conducted using Infinicyt™ (Cytognos, Salamanca, Spain) and FlowJo™ (BD Life Sciences) software (both from BD Biosciences). Figure 1 depicts the gating strategy, which includes the evaluation of PD-1 and CD57 in T and NK cell subsets.

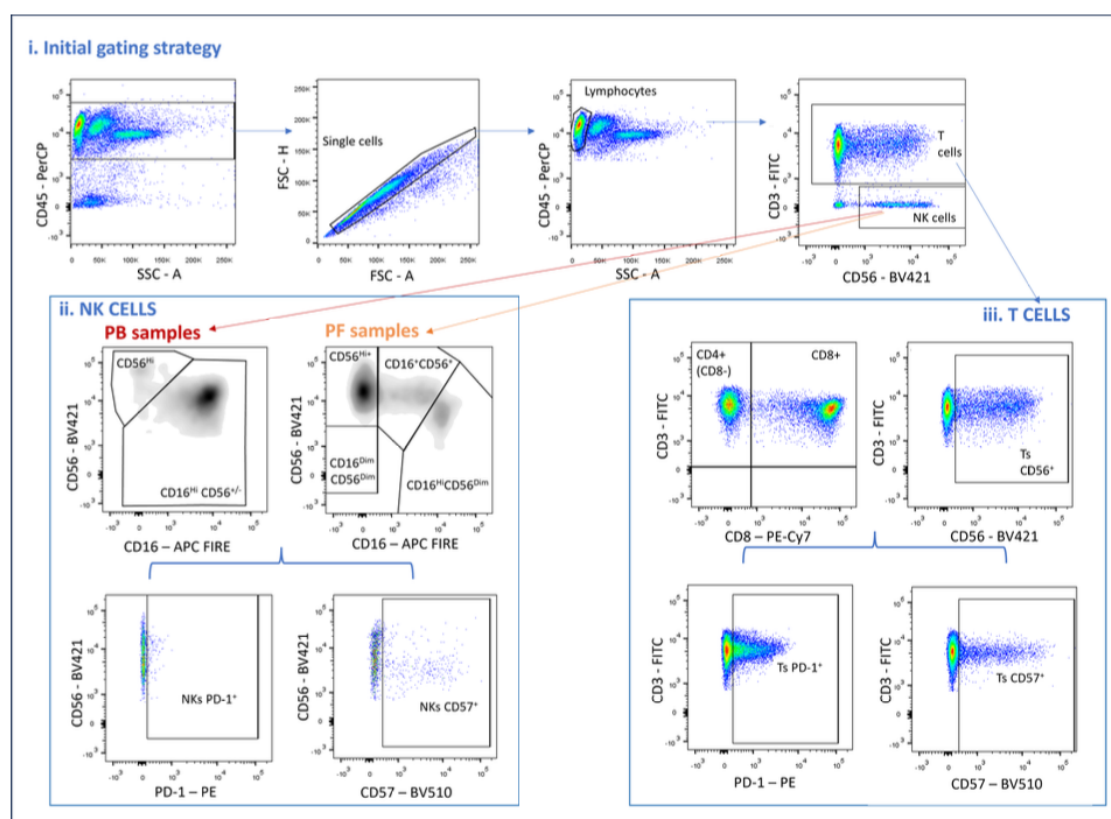


Figure 1. Gating strategy used to characterize PD-1 in lymphocyte subsets of peripheral blood and peritoneal fluid samples. i: Gating Strategy used for the identification of major lymphocyte subsets in Peritoneal Fluid (PF) and Peripheral blood (PB) samples, i.e., T cells (CD45^{hi} SSC^{Lo} CD3⁺) and NK cells (CD45^{hi} SSC^{Lo} CD3⁻ CD56⁺); ii: Gating strategy used for the identification of distinct NK cell subsets in PF and PB samples, along with the subsequent analysis of PD-1 and CD57 in these populations; iii: Gating strategy used for the identification of CD4 and CD8 T cells, along with CD56⁺ T cells. The percentage of cells expressing PD-1 and CD57 were further analyzed in each subset.

2.5. Statistical analysis

The study participants' characteristics were summarized using descriptive statistics, which included demographic, clinical, and immunophenotyping data. We represented categorical variables as absolute and relative frequencies. If over 20% of expected values were less than 5 in contingency tables, we used Fisher's exact test to compare the two groups and utilized the chi-squared test otherwise. The presentation of continuous variables was done either by indicating the mean (standard deviation) (minimum-maximum) or the median along with the first-to-third quartile (Q1-Q3). In the Supplementary Tables, continuous variables are shown as average (standard deviation) [95% confidence interval (95% CI)], median, and quartiles (Q1-Q3). We obtained 95% CIs for normally distributed variables using a student's t distribution approximation, using bootstrap resampling for others. To compare two separate groups, we used student's t-test for normally distributed continuous data and applied the Wilcoxon rank-sum test otherwise. For comparing two dependent groups, we employed a paired-samples t-test if data distributed normally, and the Wilcoxon signed-rank test when it did not. For more than two groups, we used ANOVA with a follow-up Tukey's HSD paired-comparison test for normal distributions, and the Kruskal-Wallis test with a follow-up Dunn's paired-comparison test otherwise. We assessed distribution normality with the Shapiro-Wilk test. Because of the study's

exploratory nature, we did not calculate a formal sample size. We considered a p-value less than 0.05 statistically significant. We executed all analyses with SAS software (Version 9.4).

3. Results

3.1. Demographic and clinical characteristics of patients

The tables 1 and 2 display the demographic and clinical attributes of the control group (n = 15) and women suffering from endometriosis (n = 47). No significant age differences were observed between the groups, with most women being Caucasian. As anticipated, the only noteworthy disparity between the groups was in relation to infertility history, where it was significantly more common in women with endometriosis (p = 0.003) (Table 1).

The clinical data from women with endometriosis highlights several important points. On average, there was a 2.8±2.52 (1-14) year gap between the diagnosis of endometriosis and surgery. The majority of patients (78.7%) were classified in stages III or IV. A high percentage of these patients reported experiencing dysmenorrhea (93.6%), with the majority (76.6%) describing the pain as severe. Other commonly reported symptoms included dyspareunia (59.6%), chronic pelvic pain (40.4%), dyschesia (34.0%), bowel issues (31.9%), urinary tract problems (10.6%), and rectorrhagia (8.5%) (Table 2). Lastly, 33% of women in the control group and 66% of women with endometriosis were receiving hormone therapy.

Table 1. Demographic and clinical characteristics of control group and women with endometriosis.

Characteristics	Control (n = 15)	Endometriosis (n = 47)	p-value
Age , mean ±SD (min-max), yr	38.5±9.20 (20-55)	36.2±6.3 (31-42)	0.434a
Race , n (%)			0.113b
White	15 (100)	40 (85.1)	
Black	0 (0)	7 (14.9)	
BMI , mean ±SD (min-max), kg/m ²	23.2±3.0 (17-28)	25.2±5.6 (20.8-28.0)	0.430a
Fertile , n (%)			0.003b
Yes	14 (93.3)	24 (51.1)	
No	1 (6.7)	23 (48.9)	
Any Pregnancy , n (%)			0.087b
Yes	7 (46.7)	19 (40.4)	
No	8 (53.3)	28 (59.6)	
Smoker , n (%)			0.087b
Yes	0 (0)	8 (17.0)	
No	15 (100)	39 (83.0)	
Hormonal therapy , n (%)			0.090b
Yes	5 (33.3)	31 (66.0)	
No	10 (66.7)	16 (34.0)	

BMI, body mass index; max, maximum; min, minimum; n, number of patients; SD, standard deviation; yr, years. aWilcoxon rank-sum test. bChi-squared test. Statistically significant results are indicated in bold.

Table 2. Endometriosis characteristics of the included patients.

Characteristics	Endometriosis (n = 47)
Endometriosis diagnosis, mean±SD (min-max), yr	2.8±2.5 (1-14)
Endometriosis severity, n (%)	
Stage I/II	10 (21.3)
Stage III/IV	37 (78.7)
Dysmenorrhea complaints, n (%)	44 (93.6)
Dysmenorrhea severity, n (%)	
None/Mild/Moderate (< 7/10)	11 (23.4)
Severe (≥ 7/10)	36 (76.6)
Dyspareunia complaints, n (%)	28 (59.6)
Chronic pelvic pain, n (%)	19 (40.4)
Dyschesia complaints, n (%)	16 (34.0)
Bowel symptoms, n (%)	15 (31.9)
Urinary tract symptoms, n (%)	5 (10.6)
Rectorrhagia, n (%)	4 (8.5)
Extra-pelvic endometriosis, n (%)	2 (4.3)

max, maximum; min, minimum; n, number of patients; SD, standard deviation; yr, years.

3.2. PD-1 in endometriosis: expression patterns in PB and PF

Table S2 in the Supplementary Materials showcases a comprehensive characterization of primary circulating and peritoneal lymphoid subsets in both the control group and women diagnosed with endometriosis. We focused on analyzing the expression of programmed cell death protein 1 (PD-1) within these cells. Generally, we observed higher percentages of T cells expressing PD-1 ($p \leq 0.024$) in PF samples relative to PB. This was the case for both endometriosis patients and the control group (Figure 2).

In this study, we identified a higher percentage of a particular type of peritoneal NK cells, namely, PD-1+ CD16+ CD56+ NK cells, in women with endometriosis ($p = 0.021$) (Figure 3A). No additional differences were found in other NK or T cell subsets across both groups and compartments.

Patients' clinical conditions and lifestyles can influence the expression of this marker. Notably, smoking habits showed a significant impact, displaying differences in the groups for peritoneal PD-1+ total T cells ($p = 0.021$) and PD-1+ CD8+ T cells ($p = 0.040$). Lower levels of both subsets were found in non-smoking EM women (Figure 3B and C). For the PD-1+ CD16+ CD56+ NK cells, the overall comparison indicated differences among the groups ($p = 0.050$), with EM women having a higher percentage of this subset. This higher percentage was also notably present in the subgroup of EM women who smoked. All the control participants in the study were non-smokers.

After comparing women with and without endometriosis, it was observed that the overweight group showed differences in the expression of PD-1 by peritoneal T cells ($p \leq 0.041$) (Figure 3D, E and F). Despite the small sample size of this subgroup ($n = 4$), overweight control women displayed a potential upward trend in the percentages of PD-1+ cells across all T cell subgroups evaluated (Total T, CD4+ T, CD8+ T, and CD56+ T cells; Figure 3D-F). However, women with endometriosis presented declining values of all PD-1+ subsets, moving from normal weight down to overweight and obese subgroups. Moreover, when considering all groups, women with endometriosis and obesity demonstrated the lowest levels, specifically for PD-1+

CD8⁺ T cells and PD-1⁺ total T cells, when compared to overweight controls, and for PD-1⁺ CD4⁺ T cells when compared to normal-weight women with endometriosis (Figure 3D, E and F).

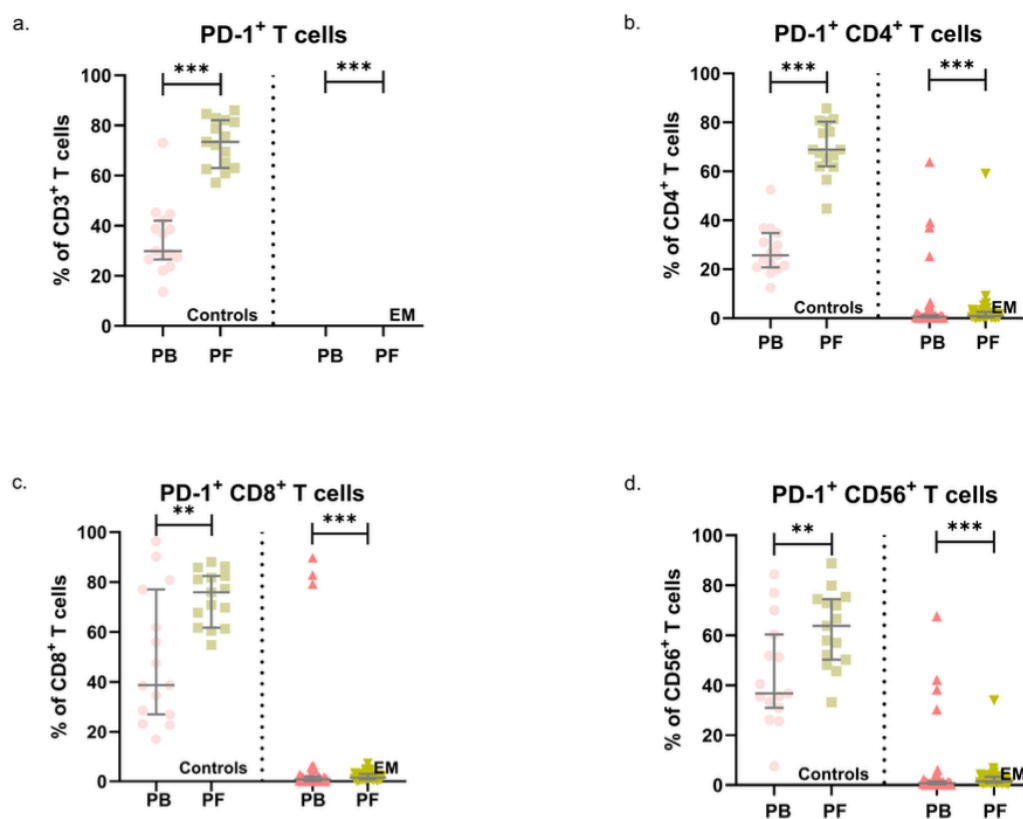


Figure 2. PD-1 expressing T cells in PF and PB samples between EM and controls. (A-D) % of PD-1⁺ cells in (A) total T cells, (B) CD4⁺ T cells, (C) CD8⁺ T cells, and (D) CD56⁺ T cells. Differences were tested with paired-samples t-test (A and C) and Wilcoxon rank-sum test (B and D). Scatter plots are presented with individual values and the lines representing median with IQR. The control group considers 15 peripheral blood and peritoneal fluid samples, and the EM group, 47 peripheral blood and peritoneal fluid samples. **p<0.01; ***p<0.001. EM, endometriosis; IQR, interquartile range; PB, peripheral blood; PF, peritoneal fluid.

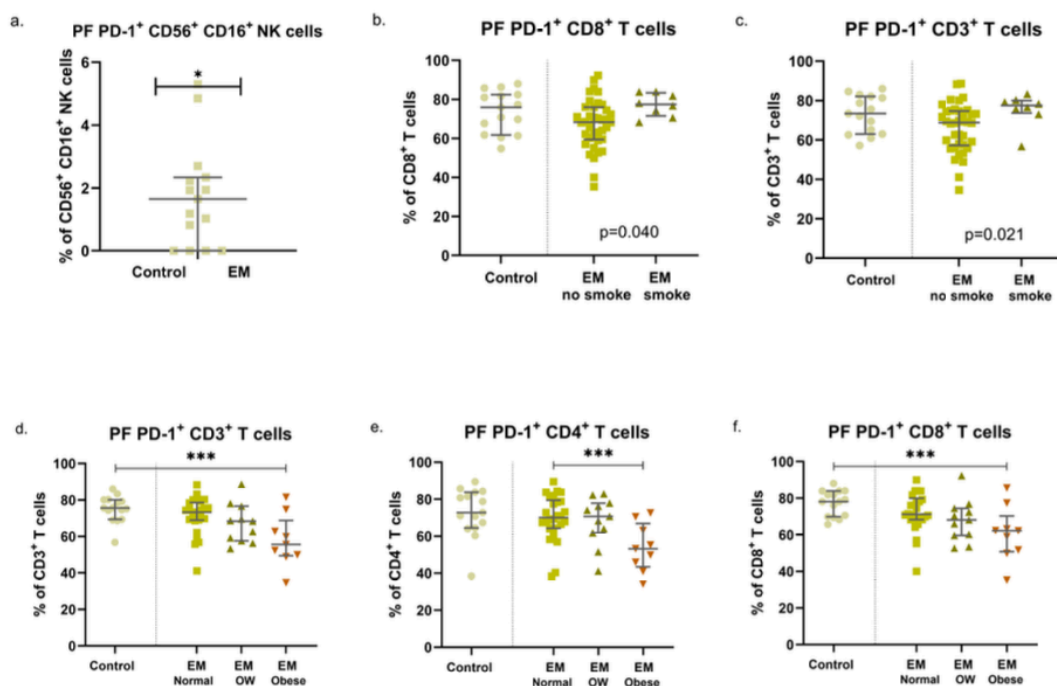


Figure 3. PD-1 expression in peritoneal cells according to weight subgroups and smoking habits. (A) % of PD-1+ CD56+CD16+ NK cells in controls and women with endometriosis; (B and C) % of PD-1+ (B) CD8+ T cells and (C) total T cells in controls and EM according to smoking habits; and (D-F) % of PD-1+ (D) total T cells, (E) CD4+ T cells, and (F) CD8+ T cells in controls and EM according to weight. Differences were tested with Wilcoxon rank-sum test (A), ANOVA with post-hoc Tukey HSD test for paired comparison (B and F) and Kruskal-Wallis with post-hoc Dunn's test for paired comparison (C, D and E). Test. The control group considers 15 peripheral blood and peritoneal fluid samples, and the EM group, 47 peripheral blood and peritoneal fluid samples. Considering the EM subgroups, the EM smoke subgroup had n=8; considering the BMI, the normal BMI subgroup had n=27, the overweight n=11, and the obese n=9). Scatter plots are presented with individual values and the lines representing median with IQR. EM, endometriosis; IQR, interquartile range; OW, overweight; PB, peripheral blood; PF, peritoneal fluid.

3.3. Endometriosis, previous pregnancies, and fertility status: modulation of PD-1 expression in circulating and peritoneal immune cells

We also identified varying patterns of PD-1 expression in women who have and have not been pregnant before. Looking at circulating T cells, we found that non-pregnant EM women exhibited lower levels of PD-1+ CD4+ T cells ($p = 0.041$, Figure 4A). Furthermore, in EM women dealing with fertility issues, there were reduced levels of both PD-1+ CD56+ T cells ($p = 0.039$, Figure 4B) and PD-1+ total T cells ($p = 0.005$, Figure 4C) in peripheral blood samples specifically when compared to EM women and controls without fertility concerns.

Upon examining peritoneal populations, we noticed a rise in several PD-1+ NK subsets in endometriosis (EM) women who had previously been pregnant (PD-1+ total NK cells: $p = 0.017$; PD-1+ CD56+CD16+ NK cells: $p = 0.027$; PD-1+ CD56Hi NK cells: $p = 0.002$) (Figure 4 D-F). On the other hand, there was a decrease in these subsets in EM women who had not been pregnant when compared to their EM counterparts with past pregnancies. It was also observed that EM women without a history of infertility exhibited a significant increase in PD-1+ CD56+CD16+ NK cells, not only compared to fertile controls, but also compared to infertile EM women ($p = 0.031$).

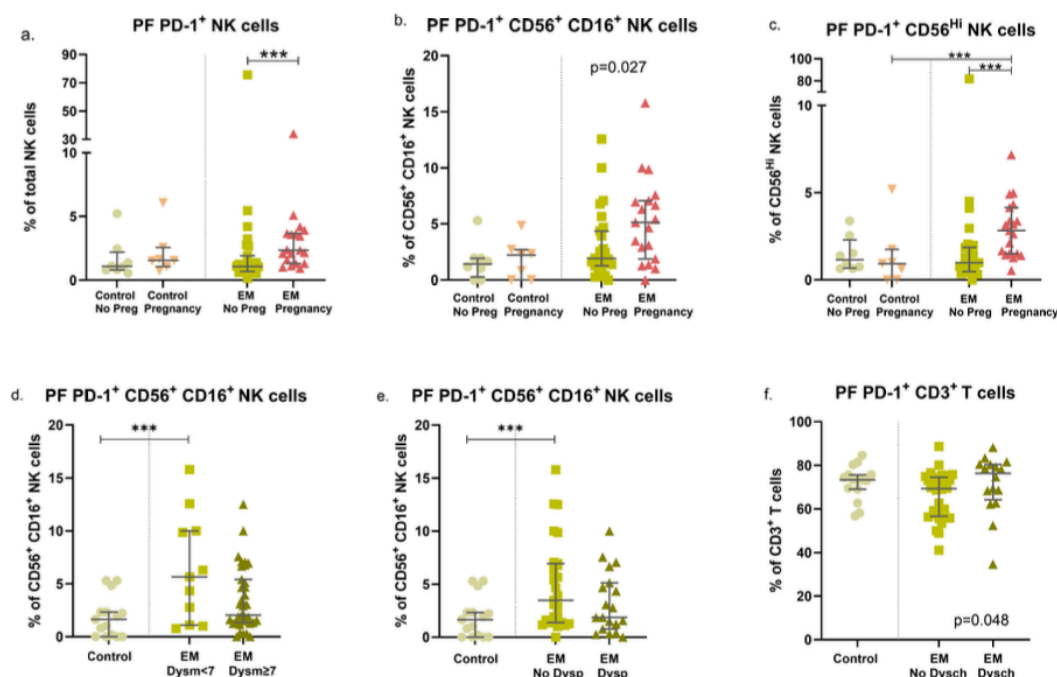


Figure 4. PD-1 expression in peritoneal cells according to fertility status. (A-C) Percentages of circulating PD-1+ CD4+ T cells (A) in controls and EM women according to pregnancy history, and PD-1+ CD56+ T cells and PD-1+ total T cells (B and C) in controls and EM women with and without fertility issues. (D-E) % of PD-1+ total NK cells (D), PD-1+ CD56+CD16+ NK cells (E), and PD-1+ CD56^{Hi} NK cells (F) in controls and EM women according to pregnancy history. Non-Pregnant controls n=8, Pregnant controls, n= 7; non-Pregnant EM, n=28; Pregnant EM, n=19. Controls without fertility issues, n=14; EM without fertility issues, n=24; EM with fertility issues, n=23. All differences were tested with Kruskal-Wallis with post-hoc Dunn's test for paired comparison. Scatter plots are presented with individual values and the lines representing median with IQR. *p-value<0.05; **p-value<0.01; ***p-value<0.001. EM, endometriosis; IQR, interquartile range; PF, peritoneal fluid.

3.4. EM symptoms associate with different expression patterns of PD-1 in NK and T cell subsets

Our results further revealed that in endometriosis, particularly peritoneal populations, the expression of PD-1 can vary significantly among EM patients with diverse symptoms. Complete data are presented in Supplementary Materials (Table S3).

We observed a trend toward higher percentages of peritoneal PD-1+ CD56+CD16+ NK cells in women with endometriosis who experienced dysmenorrhea, although this trend was not statistically significant ($p = 0.069$). Upon further separation of the EM women based on the severity of dysmenorrhea, we found higher levels of this cell subset in women with mild to moderate symptoms ($p=0.028$).

In patients with endometriosis experiencing dyspareunia, we noted higher percentages of peritoneal PD-1+ total NK cells as well as PD-1+ CD56+CD16+ NK cells ($p = 0.035$ and $p = 0.029$, respectively). Decreased levels of PD-1+ total T cells ($p = 0.021$) and PD-1+ CD4+ T cells ($p = 0.049$) were found in the peripheral blood of women not experiencing this symptom. There was also a noticeable increase, although not statistically substantial, in PD-1+ total NK cells in EM patients with dyspareunia ($p = 0.052$), especially in severe cases (T cells: $p = 0.038$; total NK cells: $p = 0.074$).

Dyschesia is a common symptom in EM patients. In our group, a trend was found associating higher levels of PF PD-1+ CD8+ T cells ($p = 0.052$) and PD-1+ total T cells ($p = 0.048$)

with this symptom. However, no significant variations were observed when comparing patients with mild, moderate, or severe symptoms.

Similarly, an increase in peritoneal PD-1+ CD56+CD16+ NK cells was observed in endometriosis patients without rectal bleeding ($p = 0.021$). This trend was also present in patients without chronic pelvic pain ($p = 0.057$) and those without bowel endometriosis ($p = 0.056$). Lastly, endometriosis patients with urinary tract infiltration demonstrated increased percentages of PD-1+ total NK cells ($p = 0.042$). These patients likewise exhibited higher percentages of circulating PD-1+ CD8+ T cells ($p = 0.037$), PD-1+ CD56+ T cells ($p = 0.036$), and PD-1+ total T cells ($p = 0.029$).

4. Discussion

In our study, we analyzed PD-1 expression in NK and T cell subsets from the PF and PB of women, both with and without endometriosis. To the best of our knowledge, this study utilizes the largest patient sample to assess PD-1 expression in both compartments.

We observed a statistically significant increase in the percentage of CD56+CD16+ NK cells expressing PD-1 in the PF of patients with endometriosis compared to the control group. This difference was not detected in PB. Likewise, no significant differences were observed in PD-1 expression on T cells in either the PF or PB.

NK cells, a vital component of the innate immune system, play an essential role in early defense mechanisms against various types of aggression through their natural cytotoxicity activation, which is not antibody-dependent (Bryceson et al., 2006; Moretta et al., 2014; Vivier et al., 2008). The increase in PD-1-expressing NK cells in peritoneal fluid is particularly important to emphasize, as these cells play a crucial role in the initial defense within this microenvironment. This is the same environment where endometriotic cells attempt to implant and spread, especially within the pelvic cavity. Interestingly, this only impacts CD16+CD56+ NK cells. NK cells expressing CD16 serve as potent mediators of antibody-dependent cellular cytotoxicity, in addition to their inherent cytotoxicity (Bryceson et al., 2006; Perussia, 1998). On the other hand, CD56-expressing cells possess strong immunostimulatory effector capabilities, such as producing T helper 1 cytokines and having effective cytotoxic potential (Van Acker et al., 2017). Elevated PD-1 expression may adversely affect the protective function of NK cells in controlling the spread of ectopic endometrial cells, a pattern that has been demonstrated in oncology studies involving cancer cells (Pesce et al., 2017).

When we analyzed the variations based on demographic and clinical factors, we uncovered some interesting findings. In our study BMI significantly impacts the expression of the PD-1 marker on T cells in patients with endometriosis, specifically within the peritoneal cavity. We noticed the lowest levels of PD-1+ CD8+ T cells and total PD-1+ T cells in obese women with endometriosis compared to overweight controls. Additionally, these women exhibited lower PD-1+ CD4+ T cell values than endometriosis patients with a normal weight. Although the influence of BMI on the immune system, including various aspects of the immune response, has long been recognized (Ilavská et al., 2012), its specific effect on PD-1 expression remains unexplored in past research. When examining smoking habits, we found an increased presence of PD-1+ CD16+CD56+ NK cells in the PF of endometriosis patients who smoke, suggesting a potential connection to disease progression in these individuals.

When considering obstetric history, we observed an increase in several PD-1+ NK cell subsets within the peritoneal populations of women with endometriosis who had previous pregnancies. Notably, our findings revealed a significant rise in PD-1+ CD56+ CD16+ NK cells in the peritoneal fluid of women without a history of infertility compared to those who were infertile. Although no current studies examine the relationship between PD-1 and fertility, our results suggest a potential link between this marker and a woman's fecundity, particularly in patients with endometriosis. This hypothesis certainly merits further investigation.

When we categorized the patients based on the typical symptoms of endometriosis, we observed distinct patterns of PD-1 marker expression, with a higher prevalence in those with widespread disease, particularly within the peritoneal cell population. Specifically, we found increased levels of PD-1+ CD56+CD16+ NK cells and PD-1+ total NK cells in patients suffering from dyspareunia, with the count of PD-1+ total NK cells significantly higher in those experiencing severe dyspareunia compared to those with milder symptoms. Dyschesia, a symptom often associated with bowel involvement, was linked to elevated levels of PD-1+ total T cells in the peritoneal fluid of our cohort. In endometriosis patients with urinary tract involvement, there was a substantial increase in PD-1+ total NK cells in the peritoneal cavity, alongside higher percentages of circulating PD-1+ CD8+ T cells, PD-1+ CD56+ T cells, and PD-1+ total T cells. Overall, these findings suggest an increase in PD-1 expression in the immune cells of patients with more severe or symptomatic endometriosis.

Looking at published studies evaluating the PD-1 marker in women with endometriosis, the available data is scarce. Sobstyl, M. et al. examined the expression of PD-1 and PD-L1 on T and B lymphocytes, but their analysis was limited to the peripheral blood of patients with endometriosis. They found a significantly higher expression of both PD-1 and PD-L1 on T and B lymphocytes in endometriosis patients compared to the control group (Sobstyl et al., 2024). Other recent study (Hosseinzadeh et al., 2024) examined the PD-1 expression on NK cells in PF and PB. Although limited to 11 controls and 11 women with endometriosis, the study found a higher percentage of PD-1+ NK cells in the PF of patients with stage IV endometriosis compared to controls (Hosseinzadeh et al., 2024). Wu et al. (Wu et al., 2019) conducted a study on eutopic and ectopic endometria and blood samples from 15 women with endometriosis and 15 healthy controls. They utilized immunohistochemistry and western blot analysis to detect PD-1/PD-L1 expression in endometrial tissues. Flow cytometry was used for blood analysis. Their research revealed increased PD-1/PD-L1 expression in both eutopic and ectopic endometria of patients with endometriosis. Additionally, they reported a heightened PD-1 expression in CD41/CD81 T cells in PB (Wu et al., 2019). Walankiewicz and colleagues (Walankiewicz et al., 2018) examined PB samples from patients with endometriosis and a control group using flow cytometry. They found a significantly higher percentage of PD-1 and PD-L1-positive CD4+ T cells, CD8+ T cells, and CD19+ B cells in the patients compared to the control group (Walankiewicz et al., 2018). Suszczyk et al. (Suszczyk et al., 2022) used flow cytometry to study expressions of PD-L1/PD-L2 on myeloid (m-) and plasmacytoid (p-) dendritic cells (DCs) in the PB and PF of both endometriosis patients and healthy individuals. They found a higher proportion of mDCs and pDCs expressing PD-L1 or PD-L2 in the PF of endometriosis patients compared to their plasma, indicating the possible influence of the peritoneal cavity environment on immune response modulation and the development or progression of endometriosis. However, this study only included PB samples from the control group and the exclusion of endometriosis in this group was based solely on clinical inquiries, not direct pelvic observation through laparoscopy (Suszczyk et al., 2022). Okşaşoğlu et al. (Okşaşoğlu et al., 2021) collected PB samples from women with endometriosis during surgery or examination, as well as from healthy individuals. Using an ELISA platform, they found elevated serum levels of PD-1 in women suffering from endometriosis (Okşaşoğlu et al., 2021). Nero et al. (Nero et al., 2022) used immunohistochemistry assays on formalin-fixed paraffin-embedded tissue samples and found a higher PD-1/PD-L1 expression in endometriosis-associated ovarian cancer than in benign endometriosis-related diseases (Nero et al., 2022).

Our study offers a distinct clinical perspective, differing significantly from previously examined methodologies and parameters. We identified patterns of abnormal PD-1 expression in relation to specific clinical symptoms such as dysmenorrhea, dyspareunia, and dyschezia, investigating the correlation between immune cell subsets and these symptoms. Additionally, we systematically evaluated the influence of demographic factors, including BMI and smoking status, on both systemic and local PD-1 expression in control and endometriosis patients. A key

distinction of our study is that the exclusion of endometriosis in the control group was confirmed through laparoscopic surgery with direct pelvic visualization, providing a more reliable method for verifying the absence of the disease, rather than relying solely on clinical evaluation. A key strength of our study is its detailed examination of specific NK and T cell subsets and immune receptors, deepening the understanding of the immunological landscape of endometriosis at the cellular level, which may have important implications for its pathophysiology.

This study provides valuable insights into the immunologic profile of PB and PF concerning PD-1 expression, though it acknowledges certain limitations. Despite our sample size being larger than previous studies in this field, it remains insufficient for drawing definitive conclusions. The findings demonstrate complex interactions within each subpopulation, with PD-1 expression observed in NK and T-cell subpopulations across various demographic and clinical contexts. However, it is important to recognize that the presence of PD-1 does not necessarily indicate functional activity. Future research incorporating functional assays, such as cytotoxicity and cytokine analyses, could further enhance our understanding. Finally, while the immune changes observed may contribute to the implantation and growth of endometriotic cells, they could also reflect a response to the disease itself.

5. Conclusions

Our findings suggest that the immune system may be a key factor in the spread of endometriotic cells, uncovering potential abnormalities in primary defense mechanisms driven by NK and T cell dysfunction within the pelvic microenvironment. This dysfunction may be associated with elevated PD-1 inhibitory receptor expression, which appears more prominent in patients with severe symptoms and disease extending beyond the pelvis. A common pathway for PD-1 modulation may exist between endometriosis and cancer. Further research is required to confirm these findings and investigate new immunomodulatory treatment options for endometriosis, similar to the use of checkpoint inhibitors in cancer therapy. This is particularly important given the limitations of current hormone-based therapies, which not only reduce their effectiveness but also cause side effects that hinder long-term use.

Disclosure of ethical statements: Informed consent was obtained from all subjects involved in the study. The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics committees of NOVA Medical School | Universidade NOVA de Lisboa (nº122/2020/CEFCM approved on february 24th, 2020), Beatriz Ângelo Hospital (3394/2020_MJH/MAB/NO approved on november 6th, 2020) and Luz Lisboa Hospital (CES/53/2021/ME approved on november 23th, 2021).

Funding: This research was partially funded by the Hospital da Luz Lisboa under the initiative “Luz Investigação”, Gedeon Richter Portugal, and LifeWell Pharmaceutical & Healthcare.

CRedit authorship contribution statement: Study conceptualization: José Lourenço Reis, Catarina Martins, Miguel Ângelo-Dias, Luís Miguel Borrego, and Jorge Lima; Methodology: José Lourenço Reis, Catarina Martins, Miguel Ângelo-Dias, Natacha Nurdine Rosa, Luís Miguel Borrego, and Jorge Lima; Data curation: José Lourenço Reis; Formal analysis: José Lourenço Reis, Catarina Martins, Miguel Ângelo-Dias, Jorge Lima, Luís Miguel Borrego; Writing—Original Draft: José Lourenço Reis, Catarina Martins, Miguel Ângelo-Dias, and Jorge Lima; Writing—Review and Editing: Catarina Martins, Natacha Nurdine Rosa, Luís Miguel Borrego, and Jorge Lima; Supervision, Jorge Lima; Project administration, Jorge Lima; Funding acquisition, Jorge Lima. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Acknowledgments: The authors would like to acknowledge Jorge Gonçalves (Scientific ToolBox Consulting, Lisbon, Portugal) for statistical analysis and Sofia Nunes, Ph.D. (Scientific ToolBox Consulting, Lisbon, Portugal), for providing medical writing assistance and technical editing.

Appendix A. Supplementary Materials

Supplementary data associated with this article can be found in the online version at...

Table S1: List of antibodies used in the immunophenotyping panel; Table S2: Summary of major circulating and peritoneal lymphoid subsets in control group and in women with endometriosis; Table S3: Characterization of circulating and peritoneal lymphoid subsets in control group and in EM women with different clinical manifestations.

References

- Ahmadzadeh, M., Johnson, L. A., Heemskerk, B., Wunderlich, J. R., Dudley, M. E., White, D. E., & Rosenberg, S. A. (2009). Tumor antigen-specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. *Blood*, 114(8), 1537-1544. <https://doi.org/10.1182/blood-2008-12-195792>
- Alsaab, H. O., Sau, S., Alzhrani, R., Tatiparti, K., Bhise, K., Kashaw, S. K., & Iyer, A. K. (2017). PD-1 and PD-L1 Checkpoint Signaling Inhibition for Cancer Immunotherapy: Mechanism, Combinations, and Clinical Outcome. *Front Pharmacol*, 8, 561. <https://doi.org/10.3389/fphar.2017.00561>
- Andrews, W. C., Buttram, V. C., Jr.; Weed, J. C., Hammond, C. B., Thomas, H. H., & Behrman, S. J. (1985). Revised American Fertility Society classification of endometriosis: 1985. *Fertil Steril*, 43(3), 351-352. [https://doi.org/10.1016/s0015-0282\(16\)48430-x](https://doi.org/10.1016/s0015-0282(16)48430-x)
- Becker, C. M., Bokor, A., Heikinheimo, O., Horne, A., Jansen, F., Kiesel, L., King, K., Kvaskoff, M., Nap, A., Petersen, K., Saridogan, E., Tomassetti, C., van Hanegem, N., Vulliamoz, N., & Vermeulen, N. (2022). ESHRE guideline: endometriosis. *Hum Reprod Open*, 2022(2), hoac009. <https://doi.org/10.1093/hropen/hoac009>
- Bryceson, Y. T., March, M. E., Ljunggren, H. G., & Long, E. O. (2006). Activation, coactivation, and costimulation of resting human natural killer cells. *Immunol Rev*, 214, 73-91. <https://doi.org/10.1111/j.1600-065X.2006.00457.x>
- Capezzuoli, T., Rossi, M., La Torre, F., Vannuccini, S., & Petraglia, F. (2022). Hormonal drugs for the treatment of endometriosis. *Curr Opin Pharmacol*, 67, 102311. <https://doi.org/10.1016/j.coph.2022.102311>
- Concha-Benavente, F., Kansy, B., Moskovitz, J., Moy, J., Chandran, U., & Ferris, R. L. (2018). PD-L1 Mediates Dysfunction in Activated PD-1(+) NK Cells in Head and Neck Cancer Patients. *Cancer Immunol Res*, 6(12), 1548-1560. <https://doi.org/10.1158/2326-6066.Cir-18-0062>
- Fukui, A., Mai, C., Saeki, S., Yamamoto, M., Takeyama, R., Kato, T., Ukita, Y., Wakimoto, Y., Yamaya, A., & Shibahara, H. (2021). Pelvic endometriosis and natural killer cell immunity. *Am J Reprod Immunol*, 85(4), e13342. <https://doi.org/10.1111/aji.13342>
- Hosseinzadeh, R., Moini, A., Hosseini, R., Fatehnejad, M., Yekaninejad, M. S., Javidan, M., Changaei, M., Feizisani, F., & Rajaei, S. (2024). A higher number of exhausted local PD1+, but not TIM3+, NK cells in advanced endometriosis. *Heliyon*, 10(1), e23294. <https://doi.org/10.1016/j.heliyon.2023.e23294>
- Ilavská, S., Horváthová, M., Szabová, M., Nemessányi, T., Jahnová, E., Tulinská, J., Líšková, A., Wsolová, L., Staruchová, M., & Volkovová, K. (2012). Association between the human

- immune response and body mass index. *Hum Immunol*, 73(5), 480-485. <https://doi.org/10.1016/j.humimm.2012.02.023>
- Liu, J., Chen, Z., Li, Y., Zhao, W., Wu, J., & Zhang, Z. (2021). PD-1/PD-L1 Checkpoint Inhibitors in Tumor Immunotherapy. *Front Pharmacol*, 12, 731798. <https://doi.org/10.3389/fphar.2021.731798>
- Liu, Y., Cheng, Y., Xu, Y., Wang, Z., Du, X., Li, C., Peng, J., Gao, L., Liang, X., & Ma, C. (2017). Increased expression of programmed cell death protein 1 on NK cells inhibits NK-cell-mediated anti-tumor function and indicates poor prognosis in digestive cancers. *Oncogene*, 36(44), 6143-6153. <https://doi.org/10.1038/onc.2017.209>
- Ljunggren, H. G., & Kärre, K. (1990). In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol Today*, 11(7), 237-244. [https://doi.org/10.1016/0167-5699\(90\)90097-s](https://doi.org/10.1016/0167-5699(90)90097-s)
- Makuku, R., Khalili, N., Razi, S., Keshavarz-Fathi, M., & Rezaei, N. (2021). Current and Future Perspectives of PD-1/PDL-1 Blockade in Cancer Immunotherapy. *J Immunol Res*, 2021, 6661406. <https://doi.org/10.1155/2021/6661406>
- Mariotti, F. R., Quatrini, L., Munari, E., Vacca, P., Tumino, N., Pietra, G., Mingari, M. C., & Moretta, L. (2020). Inhibitory checkpoints in human natural killer cells: IUPHAR Review 28. *Br J Pharmacol*, 177(13), 2889-2903. <https://doi.org/10.1111/bph.15081>
- Moretta, A., Bottino, C., Vitale, M., Pende, D., Biassoni, R., Mingari, M. C., & Moretta, L. (1996). Receptors for HLA class-I molecules in human natural killer cells. *Annu Rev Immunol*, 14, 619-648. <https://doi.org/10.1146/annurev.immunol.14.1.619>
- Moretta, L., Montaldo, E., Vacca, P., Del Zotto, G., Moretta, F., Merli, P., Locatelli, F., & Mingari, M. C. (2014). Human natural killer cells: origin, receptors, function, and clinical applications. *Int Arch Allergy Immunol*, 164(4), 253-264. <https://doi.org/10.1159/000365632>
- Nero, C., Romito, I., Spadola, S., Romito, A., Turco, L. C., Cosentino, F., De Ninno, M., Catena, U., De Cicco Nardone, A., Moroni, R., Zannoni, G., Fagotti, A., & Scambia, G. (2022). Infiltrating T lymphocytes and programmed cell death protein-1/programmed death-ligand 1 expression in endometriosis-associated ovarian cancer. *Fertil Steril*, 117(1), 160-168. <https://doi.org/10.1016/j.fertnstert.2021.08.032>
- Okşaşoğlu, B., Hepokur, C., Misir, S., Yildiz, Ç., Sönmez, G., & Yanik, A. (2021). Determination of PD-1 expression in peripheral blood cells in patients with endometriosis. *Gynecol Endocrinol*, 37(2), 157-161. <https://doi.org/10.1080/09513590.2020.1821358>
- Perussia, B. (1998). Fc receptors on natural killer cells. *Curr Top Microbiol Immunol*, 230, 63-88. https://doi.org/10.1007/978-3-642-46859-9_6
- Pesce, S., Greppi, M., Grossi, F., Del Zotto, G., Moretta, L., Sivori, S., Genova, C., & Marcenaro, E. (2019). PD-1/PD-Ls Checkpoint: Insight on the Potential Role of NK Cells. *Front Immunol*, 10, 1242. <https://doi.org/10.3389/fimmu.2019.01242>
- Pesce, S., Greppi, M., Tabellini, G., Rampinelli, F., Parolini, S., Olive, D., Moretta, L., Moretta, A., & Marcenaro, E. (2017). Identification of a subset of human natural killer cells expressing high levels of programmed death 1: A phenotypic and functional characterization. *J Allergy Clin Immunol*, 139(1), 335-346.e333. <https://doi.org/10.1016/j.jaci.2016.04.025>
- Quatrini, L., Mariotti, F. R., Munari, E., Tumino, N., Vacca, P., & Moretta, L. (2020). The Immune Checkpoint PD-1 in Natural Killer Cells: Expression, Function and Targeting in Tumour Immunotherapy. *Cancers (Basel)*, 6(11), 3285. <https://doi.org/10.3390/cancers12113285>
- Reis, J. L., Rosa, N. N., Angelo-Dias, M., Martins, C., Borrego, L. M., & Lima, J. (2022). Natural Killer Cell Receptors and Endometriosis: A Systematic Review. *Int J Mol Sci*, 24(1), 331. <https://doi.org/10.3390/ijms24010331>
- Ribas, A. (2012). Tumor immunotherapy directed at PD-1. *N Engl J Med*, 366(26), 2517-2519. <https://doi.org/10.1056/NEJMe1205943>
- Shafir, A. L., Farland, L. V., Shah, D. K., Harris, H. R., Kvaskoff, M., Zondervan, K., & Missmer, S. A. (2018). Risk for and consequences of endometriosis: A critical epidemiologic review. *Best Pract Res Clin Obstet Gynaecol*, 51, 1-15. <https://doi.org/10.1016/j.bpobgyn.2018.06.001>

- Sobstyl, M., Mertowska, P., Mertowski, S., Zaborek-Łyczba, M., Dudziński, D., Polak, G., & Grywalska, E. (2024). The PD-1/PD-L1 Gateway: Peripheral Immune Regulation in the Pathogenesis of Endometriosis. *International Journal of Molecular Sciences*, 25(12). <https://doi.org/10.3390/ijms25126775>
- Suszczyk, D., Skiba, W., Zardzewiały, W., Pawłowska, A., Włodarczyk, K., Polak, G., Tarkowski, R., & Wertel, I. (2022). Clinical Value of the PD-1/PD-L1/PD-L2 Pathway in Patients Suffering from Endometriosis. *Int J Mol Sci*, 23(19), 11607. <https://doi.org/10.3390/ijms231911607>
- Tanaka, E., Sendo, F., Kawagoe, S., & Hiroi, M. (1992). Decreased natural killer cell activity in women with endometriosis [Article]. *Gynecologic and Obstetric Investigation*, 34(1), 27-30. <https://www.embase.com/search/results?subaction=viewrecord&id=L22243387&from=export>
- Tang, Q., Chen, Y., Li, X., Long, S., Shi, Y., Yu, Y., Wu, W., Han, L., & Wang, S. (2022). The role of PD-1/PD-L1 and application of immune-checkpoint inhibitors in human cancers. *Front Immunol*, 13, 964442. <https://doi.org/10.3389/fimmu.2022.964442>
- Van Acker, H. H., Capsomidis, A., Smits, E. L., & Van Tendeloo, V. F. (2017). CD56 in the Immune System: More Than a Marker for Cytotoxicity? *Front Immunol*, 8, 892. <https://doi.org/10.3389/fimmu.2017.00892>
- Vinatier, D., Dufour, P., & Oosterlynck, D. (1996). Immunological aspects of endometriosis [Review]. *Human Reproduction Update*, 2(5), 371-384. <https://doi.org/10.1093/humupd/2.5.371>
- Vivier, E., Tomasello, E., Baratin, M., Walzer, T., & Ugolini, S. (2008). Functions of natural killer cells. *Nat Immunol*, 9(5), 503-510. <https://doi.org/10.1038/ni1582>
- Walankiewicz, M., Grywalska, E., Polak, G., Korona-Glowniak, I., Witt, E., Surdacka, A., Kotarski, J., & Rolinski, J. (2018). The Increase of Circulating PD-1- and PD-L1-Expressing Lymphocytes in Endometriosis: Correlation with Clinical and Laboratory Parameters. *Mediators Inflamm*, 2018, 7041342. <https://doi.org/10.1155/2018/7041342>
- Wu, L., Lv, C., Su, Y., Li, C., Zhang, H., Zhao, X., & Li, M. (2019). Expression of programmed death-1 (PD-1) and its ligand PD-L1 is upregulated in endometriosis and promoted by 17beta-estradiol. *Gynecol Endocrinol*, 35(3), 251-256. <https://doi.org/10.1080/09513590.2018.1519787>
- Zhang, Y., & Zheng, J. (2020). Functions of Immune Checkpoint Molecules Beyond Immune Evasion. *Adv Exp Med Biol*, 1248, 201-226. https://doi.org/10.1007/978-981-15-3266-5_9

CHAPTER VII

General Discussion

Increasing evidence suggests immunological dysfunction in the pathophysiology of endometriosis. Researchers are exploring “innate-adaptive immune pathways” that contribute to the formation and persistence of endometriotic lesions within the peritoneal cavity^{72,103-119}. Normally, when endometrial cells enter the pelvic cavity through retrograde menstruation, immune cells strive to eliminate these cells, preventing their implantation. However, studies suggest women with endometriosis have weakened immune surveillance, which allows ectopic cells to evade destruction and establish implants on peritoneal surfaces^{64,65,72,103-119}.

Further research is vital for enhancing our understanding of the immune aspects of endometriosis. In this study, we assess the immunological impact of endometriosis both locally (PF) and systemically (PB), placing a special emphasis on NK and T-cell subpopulations along with their inhibitory receptors, namely TIM-3 and PD-1. By establishing a baseline comparison between the control and endometriosis groups, we offer a clinical perspective that contrasts with most previous studies. Specifically, we investigate abnormal immune profiles within NK and T-cell populations in connection with demographic factors, clinical manifestations, and symptom severity, analyzing their influence both systemically and locally in control and endometriosis patients.

We conducted a cross-sectional study over 2 years, recruiting 62 female participants, including 47 diagnosed with endometriosis and 15 serving as controls without the condition. The endometriosis group underwent laparoscopic surgery for their condition, while the control group had surgery for other unrelated pathologies. The initial diagnosis of endometriosis was based on clinical and imaging findings and was subsequently confirmed histologically in accordance with Human Reproduction and Embryology (ESHRE) guidelines¹²⁰. Data collection for all patients included age, race, BMI, smoking status, hormone therapy use, and the interval between endometriosis diagnosis and surgery. We gathered clinical data to assess disease severity, which covered symptoms such as dysmenorrhea, dyspareunia, chronic pelvic pain, bowel symptoms, urinary symptoms, and the presence of extra-pelvic endometriosis. We classified disease severity following the guidelines of the American Society for Reproductive Medicine¹²¹.

PB and PF samples were collected from all patients during surgery. For a detailed characterization of PB and PF immune cell populations, these samples were stained with a comprehensive panel of monoclonal antibodies such as CD3, CD8, CD16, CD45, CD56, CD57, TIM-3, and PD-1. The samples were then analyzed using flow cytometry. Concurrently, the absolute count of each cell subset was ascertained during the immunophenotyping procedures.

The research detailed in Chapter IV focused on the analysis of the impact of endometriosis on PB and PF immune profiles, paying particular attention to variations in NK and T cells in comparison to a control group. A baseline was established through the comparison of immune cell populations in

control cohorts. We identified abnormal NK and T-cell patterns that were associated with symptoms such as dysmenorrhea, dyspareunia, pelvic pain, rectorrhagia, and dyschezia. Additionally, we examined the influence of demographic factors.

In our endometriosis cohorts, we noted an increase in circulating CD56^{Hi}CD16^{dim} NK cells and a decrease in the CD56^{dim}CD16^{Hi}/CD56^{Hi}CD16^{dim} ratio, indicating reduced systemic NK cell cytotoxicity compared to the general population. This finding is consistent with the understanding that CD56^{dim} NK cells have a significantly higher cytotoxic activity than CD56^{Hi} cells, with the CD56^{Hi}CD16^{dim} population being the least cytotoxic¹²².

Further analysis of specific PF subsets revealed an increase in CD8⁺ CD56^{dim}CD16^{Hi} NK cells in patients with endometriosis. Previous studies suggest that the CD8 molecule generally enhances NK cell cytotoxicity¹²³. Thus, we hypothesize that the increase in CD8⁺ NK cells in endometriosis PF might be an adaptive response to the disease – potentially compensating for altered T cells. Alternatively, these cells could have a modified cytotoxic potential, permitting the development of endometriotic lesions. However, as suggested by D’Hooghe et al.^{124, 125}, these variations in local CD8⁺ cells may be a consequence, rather than a cause, of endometriosis.

The influence of smoking status on circulating immune profiles in patients with endometriosis is noteworthy. Our findings indicated that smokers had significantly lower levels of CD16⁺ CD4 T cells than non-smokers. This suggests that smoking may hinder T-cell activation, potentially fostering immunosuppression and immune evasion in these patients. However, literature on this subject presents variations in findings. For example, Martos et al. identified additional senescence-associated genes in CD4 T cells from smokers, leading to immune dysregulation¹²⁶. On the other hand, Chapron et al. found no significant impact of smoking on the risk of endometriosis or the American Fertility Society stage/score¹²⁷.

Additionally, percentages of CD16⁺ CD8 T cells in PB were lower in endometriosis stages 1, 2, and 3 compared to controls. In individuals with more severe disease (stage 4), however, there was an increase in CD16⁺ CD8 T cells compared to controls and other endometriosis groups. This might reflect the dynamic nature of immune responses across different endometriosis stages, with the evolving pathology potentially interacting with the immune system and causing fluctuations in T-cell populations. The exact role of CD16⁺ CD8 T cells in severe endometriosis remains unclear; they might indicate a stronger or more specific immune response in advanced cases, or they could merely be a byproduct of disease progression within a chronic inflammatory environment. As mentioned earlier, increased cell counts do not necessarily indicate heightened immune response or cytotoxicity, as immune exhaustion and inhibitory mechanisms must also be taken into consideration^{128,129}. The existing evidence surrounding this topic remains conflicted and ambiguous^{72,109,112,125,130}, highlighting

the need for more comprehensive research that takes potential confounding factors into account. This point was further stressed by Slabe and colleagues, who reported fluctuating regulatory T-cell concentrations in response to altered immune states such as increased serum cortisol¹³⁰.

Our findings reveal that endometriosis patients exhibiting non-menstrual pelvic pain have elevated circulating T-cell counts, which include both CD4 and CD8 T-cell subsets. This suggests an active adaptive immune response, which could potentially indicate ongoing inflammation. Furthermore, PF analysis of endometriosis patients suffering from pelvic pain presents higher percentages of CD16⁺ CD8 T cells. This could suggest a local cytotoxic response, which might be either neural stimulus-triggered or contribute to tissue damage and inflammation – both of which could underpin the experience of pelvic pain.

In Chapter V, we investigated the significant immune checkpoint inhibitor, TIM-3, in patients with endometriosis. TIM-3 is extensively studied in oncology and holds relevance in autoimmune disease research. The interaction between TIM-3 and its ligands is instrumental in inducing peripheral tolerance. In a healthy immune response, the TIM-3 ligand likely plays an essential role in blocking effector T cells and promoting peripheral tolerance¹³¹. However, in pathological conditions such as cancer, raised TIM-3 expression may induce T-cell anergy, which suppresses immune activity, allowing cancer cells to proliferate¹³²⁻¹³⁵. Conversely, low TIM-3 expression might enable autoreactive and pathogenic T cells to evade negative regulation, thereby fueling chronic inflammation, as seen in autoimmune diseases like systemic lupus erythematosus¹³⁶ or multiple sclerosis¹³⁷⁻¹⁴⁴. Specifically, multiple sclerosis, characterized by plaques in the central nervous system with macrophage and T-cell infiltration, presents under expression of TIM-3 in PB T cells, allowing autoreactive and pathogenic T cells to evade regulation¹⁴².

Our findings revealed a significant decrease in the proportion of PB CD56⁺ T cells expressing TIM-3 in patients with endometriosis compared to controls. Specifically, women with bowel endometriosis, a more severe form of the disease, had distinctly lower percentages of CD56⁺ T cells expressing TIM-3 compared to the control group. Importantly, as endometriosis advanced in stages, the percentage of circulating CD56⁺ T cells expressing TIM-3 declined significantly, especially in women with stages III and IV. This trend was confined to CD56⁺ T cells and was not observed in other T-cell subtypes or NK cells.

The reduced expression of TIM-3 in the circulating T cells of women with endometriosis may contribute to T-cell dysfunction. In the absence of TIM-3, T cells could lose their regulatory function, leading to the release of inflammatory molecules that endorse an unregulated inflammatory milieu at endometriotic cell implantation sites. This shift in immune activity could potentially drive disease progression, which is characterized by recurring inflammation and fibrosis.

The specific change in TIM-3 expression on CD56⁺ T cells is particularly significant. Cells expressing CD56 possess robust immunostimulatory effector functions, such as Th1 cytokine production and a potent cytotoxic potential¹⁴⁵. In inflammatory conditions, CD56⁺ T cells not only show killing activity analogous to NK cells but also exhibit increased resistance to apoptosis¹⁴⁶⁻¹⁵⁰.

In Chapter VI, we recognize that tumor cells often exploit the PD-1 pathway to evade the immune system¹⁵¹⁻¹⁵⁵. Thus, we investigated PD-1 expression in the immune cells of both endometriosis patients and controls. We aimed to identify potential similarities between the immune evasion strategies of cancer and endometriotic cells.

Endometriosis and cancer share prominent similarities such as immune evasion, the ability to avoid apoptosis, invasive growth, angiogenesis, and genetic and epigenetic modifications, despite being separate diseases. Both conditions can spread in a metastasis-like manner, with endometriotic tissue implanting in distant areas and, in rare instances, undergoing malignant transformation, particularly in the ovaries. These parallels suggest that immune dysregulation and abnormal blood vessel construction play significant roles in both diseases^{156,157}. Previous studies have shown that an increased expression of inhibitory immune checkpoints is a crucial factor in tumor immune evasion and suppression, with PD-1¹⁵⁵ being one of the main focal points that reduces the effector functions of T cells and NK cells¹⁵⁸. Although the immune system can eliminate tumor cells in the early stages, in advanced stages, immune checkpoints may instead promote tumor growth and progression¹⁵⁹. Consequently, blocking immune checkpoints has emerged as a promising therapeutic strategy, and immunotherapy is now widely recognized as an effective treatment for many cancer patients¹⁶⁰⁻¹⁶².

In our research, we noted a statistically significant increase in the percentage of CD56⁺ CD16⁺ NK cells expressing PD-1 in the PF of patients with endometriosis compared to those in the control group. This increase in PD-1-expressing NK cells in the PF is particularly significant, as these cells play a vital role in the initial immune defense within this microenvironment, where endometriotic cells frequently seek to implant and proliferate, especially within the pelvic cavity. Interestingly, this increase specifically affects CD56⁺ CD16⁺ NK cells. NK cells that express CD16 are potent mediators of antibody-dependent cellular cytotoxicity, aside from their inherent cytotoxic functions^{163,164}. In contrast, cells expressing CD56 have robust immunostimulatory effects such as producing Th1 cytokines and demonstrating effective cytotoxic potential¹⁴⁵. An upward trend in PD-1 expression may undermine the ability of NK cells to curb the proliferation of ectopic endometrial cells, a scenario mirrored in cancer studies¹⁵¹.

In our study, we found that body mass index (BMI) significantly influences the expression of the PD-1 marker on T cells in endometriosis patients, particularly in the peritoneal cavity. Compared to overweight controls, obese women with endometriosis had the fewest PD-1⁺ CD8⁺ T cells and total

PD-1⁺ T cells. Additionally, their PD-1⁺ CD4⁺ T-cell levels were lower than those of endometriosis patients of normal weight. Although the impact of BMI on immune function has long been established¹⁶⁵, the specifics of its influence on PD-1 expression have not been explored in previous research. Additionally, in examining smoking habits, we discovered a greater presence of PD-1⁺ CD56⁺ CD16⁺ NK cells in the PF of endometriosis patients who smoke, hinting at a possible difficulty in controlling disease progression in these patients.

In categorizing patients by prevalent endometriosis symptoms, we observed distinct patterns of PD-1 marker expression. These were particularly higher in patients with more advanced disease, primarily within the peritoneal cell population. Specifically, patients with dyspareunia exhibited elevated levels of both PD-1⁺ CD56⁺ CD16⁺ NK cells and PD-1⁺ total NK cells. Furthermore, those experiencing severe dyspareunia had notably higher counts of PD-1⁺ total NK cells compared to those with milder conditions. Dyschezia, frequently associated with bowel involvement, correlated with an increase in PD-1⁺ total T cells in the PF. In endometriosis patients presenting urinary tract involvement, we noted a considerable rise in PD-1⁺ total NK cells within the peritoneal cavity, complemented by higher levels of circulating PD-1⁺ CD8⁺ T cells, PD-1⁺ CD56⁺ T cells, and PD-1⁺ total T cells. Collectively, these findings suggest an elevation in PD-1 expression in the immune cells of patients suffering from more severe or symptomatic endometriosis.

1. Strengths and limitations

Our research has several key strengths. We executed a comprehensive analysis of specific NK and T-cell types and immune receptors, thereby enriching our knowledge of endometriosis at the cellular level and potentially elucidating aspects of its development. Our study uncovers pronounced differences in the immune profiles of women with endometriosis, particularly in relation to symptoms and demographic factors. Such insights could guide symptom-specific treatments and usher in novel avenues for immunomodulation within this disease. To safeguard accuracy, we confirmed the absence of endometriosis in the control group through laparoscopic surgery - a technique more reliable than sole clinical evaluation. Moreover, our study provides a clinical perspective by associating aberrant PD-1 and TIM-3 expression with precise symptoms, disease severity, and demographic factors. Notably, this is the inaugural study to investigate TIM-3 expression in both PF and PB in women both with and without endometriosis, considerably broadening the scope of research in this sphere.

While this research offers valuable insights, it is important to acknowledge that it also possesses limitations. Although it provides a detailed exploration of the immunologic profiles of PB and PF, relative to PD-1 and TIM-3 expression, the constraint of a relatively small sample size

remains. While the sample size is larger than in previous studies, it still falls short of providing definitive conclusions. Particularly, certain variables such as smoking, dysmenorrhea, and rectorrhagia are not sufficiently represented. This limited sample size consequently curtails the statistical power, impacting the reliability of the findings in these subgroups. Furthermore, the limited body of existing literature on the connection between lymphocyte subpopulations and symptoms like dysmenorrhea, dyspareunia, and pelvic pain curtails comparative analysis. Lastly, it is uncertain whether the noted immune deviations are causes or consequences of endometriosis; they may be mechanisms that aid cell implantation or merely responses to the disease. Indeed, despite our study underscoring a complex interaction between NK and T-cell subpopulations across various demographic and clinical contexts, it is vital to understand that the presence of PD-1 and TIM-3 on immune cells does not invariably signify functional activity. To sum up, while this study significantly contributes to our understanding of immunologic profiles in endometriosis, more comprehensive research involving larger, diverse cohorts and additional functional assays is a must to confirm and build upon these findings.

2. Final remarks and future perspectives

Previous studies propose that deficiencies in immune surveillance may cause an environment conducive for ectopic endometrial cells to bypass the immune system. We examined the immunological impact of endometriosis at both local and systemic levels, focusing on NK and T-cell subpopulations. We found significant differences in the immunological profiles of patients with endometriosis. This provides fresh insights into an area that is currently deficient in comprehensive research, especially when it comes to demographic factors and symptomatology.

We observed a decrease in TIM-3 expression in women with endometriosis compared to those without the disease. The reduction was particularly pronounced in individuals with advanced stages (III/IV) and bowel involvement. The reduction in TIM-3 expression could lead to circulating T cells losing their regulatory function and releasing inflammatory molecules. This can foster an uncontrolled inflammatory environment at the endometriotic cell implantation sites, leading to persistent inflammation and fibrosis – characteristic features of endometriosis. Therefore, reduced TIM-3 levels in circulating T cells could have a role in the pathogenesis of endometriosis and it could be considered in future research as a biomarker of the disease.

We also observed that a deficiency in immune surveillance might be associated with an increased expression of the PD-1 inhibitory receptor. This appears more pronounced in patients with

severe symptoms and diseases that extend beyond the pelvis. This suggests a potential shared pathway for PD-1 modulation in both endometriosis and cancer.

Future research should aim to clarify whether variations in immunological profiles correspond to changes in cellular activity. To enhance understanding, future studies should incorporate functional tests such as cytotoxicity assays, cytokine analysis, and surface receptor analysis. This approach would enable a more accurate evaluation of cell and receptor activity. Additionally, understanding the potential impacts of genetic and hormonal factors on these profiles will be important. The functional interactions among different cell subsets must also be explored.

Understanding the observed variations in NK and T-cell populations, especially in the expression of PD-1 and TIM-3 receptors, could usher in new possibilities for immunomodulation in treating endometriosis by potentially targeting specific immune checkpoints. A significant challenge with the current medical therapies for endometriosis is their hormone-based and contraceptive nature, rendering them unsuitable for women who wish to conceive. This issue is crucial because endometriosis is highly prevalent among women with infertility, the majority of whom are diagnosed during their reproductive years. Additionally, some women cannot tolerate hormone therapy, have contraindications, or develop resistance, resulting in disease progression despite treatment. These factors highlight the need for non-hormonal therapeutic options. Immunomodulation presents a promising pathway, and research in this domain has already begun²⁴⁻³⁰.

REFERENCES

***Note:* additional references for each study were placed
in their respective chapter**

1. Klemmt PAB, Starzinski-Powitz A. Molecular and Cellular Pathogenesis of Endometriosis. *Curr Womens Health Rev* 2018;14(2):106-116. (In eng). DOI: 10.2174/1573404813666170306163448.
2. Signorile PG, Baldi A, Viceconte R, Ronchi A, Montella M. Pathogenesis of Endometriosis: Focus on Adenogenesis-related Factors. *In Vivo* 2023;37(5):1922-1930. (In eng). DOI: 10.21873/invivo.13288.
3. Imperiale L, Nisolle M, Noël JC, Fastrez M. Three Types of Endometriosis: Pathogenesis, Diagnosis and Treatment. State of the Art. *J Clin Med* 2023;12(3) (In eng). DOI: 10.3390/jcm12030994.
4. Brosens IA. Endometriosis - a disease because it is characterized by bleeding. *Am J Obstet Gynecol* 1997;176(2):263-7. (In eng). DOI: 10.1016/s0002-9378(97)70482-4.
5. Guo SW. Fibrogenesis resulting from cyclic bleeding: the Holy Grail of the natural history of ectopic endometrium. *Hum Reprod* 2018;33(3):353-356. (In eng). DOI: 10.1093/humrep/dey015.
6. Eisenberg VH, Weil C, Chodick G, Shalev V. Epidemiology of endometriosis: a large population-based database study from a healthcare provider with 2 million members. *Bjog* 2018;125(1):55-62. (In eng). DOI: 10.1111/1471-0528.14711.
7. Meuleman C, Vandenabeele B, Fieuws S, Spiessens C, Timmerman D, D'Hooghe T. High prevalence of endometriosis in infertile women with normal ovulation and normospermic partners. *Fertil Steril* 2009;92(1):68-74. (In eng). DOI: 10.1016/j.fertnstert.2008.04.056.
8. Bellelis P, Dias JA, Jr., Podgaec S, Gonzales M, Baracat EC, Abrão MS. Epidemiological and clinical aspects of pelvic endometriosis—a case series. *Rev Assoc Med Bras (1992)* 2010;56(4):467-71. (In eng). DOI: 10.1590/s0104-42302010000400022.
9. Wrobel M, Wielgos M, Laudanski P. Diagnostic delay of endometriosis in adults and adolescence-current stage of knowledge. *Adv Med Sci* 2022;67(1):148-153. DOI: 10.1016/j.advms.2022.02.003.
10. Zondervan KT, Becker CM, Missmer SA. Endometriosis. *N Engl J Med* 2020;382(13):1244-1256. (In eng). DOI: 10.1056/NEJMra1810764.
11. Dior UP, Reddington C, Cheng C, Levin G, Healey M. Sexual Function of Women With Deep Endometriosis Before and After Surgery: A Prospective Study. *J Sex Med* 2021. DOI: 10.1016/j.jsxm.2021.11.009.
12. Legendri S, Carbonnel M, Feki A, et al. Improvement of Post-Operative Quality of Life in Patients 2 Years after Minimally Invasive Surgery for Pain and Deep Infiltrating Endometriosis. *J Clin Med* 2022;11(20). DOI: 10.3390/jcm11206132.
13. Wuest A, Limacher JM, Dingeldein I, et al. Pain Levels of Women Diagnosed with Endometriosis: Is There a Difference in Younger Women? *J Pediatr Adolesc Gynecol* 2022. DOI: 10.1016/j.jpag.2022.10.011.
14. Knapp VJ. How old is endometriosis? Late 17th- and 18th-century European descriptions of the disease. *Fertil Steril* 1999;72(1):10-4. (In eng). DOI: 10.1016/s0015-0282(99)00196-x.
15. Sampson JA. Peritoneal endometriosis due to the menstrual dissemination of endometrial tissue into the peritoneal cavity. *American Journal of Obstetrics and Gynecology* 1927;14(4):422-469. DOI: [https://doi.org/10.1016/S0002-9378\(15\)30003-X](https://doi.org/10.1016/S0002-9378(15)30003-X).
16. Liu DTY, Hitchcock A. Endometriosis: its association with retrograde menstruation, dysmenorrhoea and tubal pathology. *BJOG: An International Journal of Obstetrics & Gynaecology* 1986;93(8):859-862. DOI: 10.1111/j.1471-0528.1986.tb07995.x.
17. Bulun SE. Endometriosis caused by retrograde menstruation: now demonstrated by DNA evidence. *Fertil Steril* 2022;118(3):535-536. (In eng). DOI: 10.1016/j.fertnstert.2022.07.012.
18. Jerman LF, Hey-Cunningham AJ. The role of the lymphatic system in endometriosis: a comprehensive review of the literature. *Biol Reprod* 2015;92(3):64. (In eng). DOI: 10.1095/biolreprod.114.124313.

19. Seli E, Berkkanoglu M, Arici A. Pathogenesis of endometriosis. *Obstet Gynecol Clin North Am* 2003;30(1):41-61. (In eng). DOI: 10.1016/s0889-8545(02)00052-9.
20. Cheng Y, Li L, Wang D, et al. Characteristics of Human Endometrium-Derived Mesenchymal Stem Cells and Their Tropism to Endometriosis. *Stem Cells Int* 2017;2017:4794827. (In eng). DOI: 10.1155/2017/4794827.
21. Maruyama T, Yoshimura Y. Stem cell theory for the pathogenesis of endometriosis. *Front Biosci (Elite Ed)* 2012;4(8):2754-63. (In eng). DOI: 10.2741/e589.
22. Koninckx PR, Ussia A, Adamyan L, Wattiez A, Gomel V, Martin DC. Pathogenesis of endometriosis: the genetic/epigenetic theory. *Fertil Steril* 2019;111(2):327-340. (In eng). DOI: 10.1016/j.fertnstert.2018.10.013.
23. Koninckx PR, Barlow D, Kennedy S. Implantation versus infiltration: the Sampson versus the endometriotic disease theory. *Gynecol Obstet Invest* 1999;47 Suppl 1:3-9; discussion 9-10. (In eng). DOI: 10.1159/000052853.
24. Kolanska K, Alijotas-Reig J, Cohen J, et al. Endometriosis with infertility: A comprehensive review on the role of immune deregulation and immunomodulation therapy. *Am J Reprod Immunol* 2021;85(3):e13384. (In eng). DOI: 10.1111/aji.13384.
25. Dera-Szymanowska A, Chmaj-Wierzchowska K, Horst N, et al. Immunomodulation inhibits the development of endometriosis in rats. *J Physiol Pharmacol* 2020;71(1) (In eng). DOI: 10.26402/jpp.2020.1.14.
26. Ścieżyńska A, Komorowski M, Soszyńska M, Malejczyk J. NK Cells as Potential Targets for Immunotherapy in Endometriosis. *J Clin Med* 2019;8(9) (In eng). DOI: 10.3390/jcm8091468.
27. He Y, Hung SW, Liang B, et al. Receptor Tyrosine Kinase Inhibitor Sunitinib as Novel Immunotherapy to Inhibit Myeloid-Derived Suppressor Cells for Treatment of Endometriosis. *Front Immunol* 2021;12:641206. (In eng). DOI: 10.3389/fimmu.2021.641206.
28. González-Cantó E, Mari-Alexandre J, Gilabert-Estellés J. Exploring the feasibility of anti-PD-1/PD-L1 immunotherapy in endometriosis-associated ovarian cancer. *Fertil Steril* 2022;117(1):169-170. (In eng). DOI: 10.1016/j.fertnstert.2021.11.007.
29. Maksym RB, Hoffmann-Młodzianowska M, Skibińska M, Rabijewski M, Mackiewicz A, Kieda C. Immunology and Immunotherapy of Endometriosis. *J Clin Med* 2021;10(24) (In eng). DOI: 10.3390/jcm10245879.
30. Li W, Lin A, Qi L, et al. Immunotherapy: A promising novel endometriosis therapy. *Front Immunol* 2023;14:1128301. (In eng). DOI: 10.3389/fimmu.2023.1128301.
31. Arosa FA, Pacheco FC, Cardoso EMC. *Fundamentos de Imunologia (2ª Edição)*: Lidel, 2012.
32. Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets. *Nat Rev Immunol* 2011;11(11):723-37. (In eng). DOI: 10.1038/nri3073.
33. Gordon S. The macrophage: past, present and future. *Eur J Immunol* 2007;37 Suppl 1:S9-17. (In eng). DOI: 10.1002/eji.200737638.
34. Borregaard N. Neutrophils, from marrow to microbes. *Immunity* 2010;33(5):657-70. (In eng). DOI: 10.1016/j.immuni.2010.11.011.
35. Brinkmann V, Zychlinsky A. Neutrophil extracellular traps: is immunity the second function of chromatin? *J Cell Biol* 2012;198(5):773-83. (In eng). DOI: 10.1083/jcb.201203170.
36. Nathan C. Neutrophils and immunity: challenges and opportunities. *Nat Rev Immunol* 2006;6(3):173-82. (In eng). DOI: 10.1038/nri1785.
37. Pahl J, Cerwenka A. Tricking the balance: NK cells in anti-cancer immunity. *Immunobiology* 2017;222(1):11-20. (In eng). DOI: 10.1016/j.imbio.2015.07.012.
38. Orange JS. Natural killer cell deficiency. *J Allergy Clin Immunol* 2013;132(3):515-525. (In eng). DOI: 10.1016/j.jaci.2013.07.020.
39. Antonangeli F, Zingoni A, Soriani A, Santoni A. Senescent cells: Living or dying is a matter of NK cells. *J Leukoc Biol* 2019;105(6):1275-1283. (In eng). DOI: 10.1002/jlb.Mr0718-299r.

40. Steinman RM, Banchereau J. Taking dendritic cells into medicine. *Nature* 2007;449(7161):419-26. (In eng). DOI: 10.1038/nature06175.
41. Rothenberg ME, Hogan SP. The eosinophil. *Annu Rev Immunol* 2006;24:147-74. (In eng). DOI: 10.1146/annurev.immunol.24.021605.090720.
42. Nguyen SMT, Rupprecht CP, Haque A, Pattanaik D, Yusin J, Krishnaswamy G. Mechanisms Governing Anaphylaxis: Inflammatory Cells, Mediators, Endothelial Gap Junctions and Beyond. *Int J Mol Sci* 2021;22(15) (In eng). DOI: 10.3390/ijms22157785.
43. Galli SJ, Tsai M. IgE and mast cells in allergic disease. *Nat Med* 2012;18(5):693-704. (In eng). DOI: 10.1038/nm.2755.
44. Galli SJ, Gaudenzio N, Tsai M. Mast Cells in Inflammation and Disease: Recent Progress and Ongoing Concerns. *Annu Rev Immunol* 2020;38:49-77. (In eng). DOI: 10.1146/annurev-immunol-071719-094903.
45. Vikstrom I, Tarlinton DM. B cell memory and the role of apoptosis in its formation. *Mol Immunol* 2011;48(11):1301-6. (In eng). DOI: 10.1016/j.molimm.2010.10.026.
46. Kapingidza AB, Kowal K, Chruszcz M. Antigen-Antibody Complexes. *Subcell Biochem* 2020;94:465-497. (In eng). DOI: 10.1007/978-3-030-41769-7_19.
47. Akkaya M, Kwak K, Pierce SK. B cell memory: building two walls of protection against pathogens. *Nat Rev Immunol* 2020;20(4):229-238. (In eng). DOI: 10.1038/s41577-019-0244-2.
48. Sun L, Su Y, Jiao A, Wang X, Zhang B. T cells in health and disease. *Signal Transduct Target Ther* 2023;8(1):235. (In eng). DOI: 10.1038/s41392-023-01471-y.
49. Sallusto F, Lanzavecchia A. Heterogeneity of CD4⁺ memory T cells: functional modules for tailored immunity. *Eur J Immunol* 2009;39(8):2076-82. (In eng). DOI: 10.1002/eji.200939722.
50. Zhu X, Zhu J. CD4 T Helper Cell Subsets and Related Human Immunological Disorders. *Int J Mol Sci* 2020;21(21) (In eng). DOI: 10.3390/ijms21218011.
51. Pishesha N, Harmand TJ, Ploegh HL. A guide to antigen processing and presentation. *Nat Rev Immunol* 2022;22(12):751-764. (In eng). DOI: 10.1038/s41577-022-00707-2.
52. Martínez-Lostao L, Anel A, Pardo J. How Do Cytotoxic Lymphocytes Kill Cancer Cells? *Clin Cancer Res* 2015;21(22):5047-56. (In eng). DOI: 10.1158/1078-0432.Ccr-15-0685.
53. Cancro MP, Tomayko MM. Memory B cells and plasma cells: The differentiative continuum of humoral immunity. *Immunol Rev* 2021;303(1):72-82. (In eng). DOI: 10.1111/imr.13016.
54. Mueller SN, Gebhardt T, Carbone FR, Heath WR. Memory T cell subsets, migration patterns, and tissue residence. *Annu Rev Immunol* 2013;31:137-61. (In eng). DOI: 10.1146/annurev-immunol-032712-095954.
55. Göschl L, Scheinecker C, Bonelli M. Treg cells in autoimmunity: from identification to Treg-based therapies. *Semin Immunopathol* 2019;41(3):301-314. (In eng). DOI: 10.1007/s00281-019-00741-8.
56. Goldmann O, Nwofor OV, Chen Q, Medina E. Mechanisms underlying immunosuppression by regulatory cells. *Front Immunol* 2024;15:1328193. (In eng). DOI: 10.3389/fimmu.2024.1328193.
57. Mempel TR, Lill JK, Altenburger LM. How chemokines organize the tumour microenvironment. *Nat Rev Cancer* 2024;24(1):28-50. (In eng). DOI: 10.1038/s41568-023-00635-w.
58. Ishina IA, Zakharova MY, Kurbatskaia IN, Mamedov AE, Belogurov AA, Jr., Gabibov AG. MHC Class II Presentation in Autoimmunity. *Cells* 2023;12(2) (In eng). DOI: 10.3390/cells12020314.
59. Pabón-Porras MA, Molina-Ríos S, Flórez-Suárez JB, Coral-Alvarado PX, Méndez-Patarroyo P, Quintana-López G. Rheumatoid arthritis and systemic lupus erythematosus:

- Pathophysiological mechanisms related to innate immune system. *SAGE Open Med* 2019;7:2050312119876146. (In eng). DOI: 10.1177/2050312119876146.
60. Galassi C, Chan TA, Vitale I, Galluzzi L. The hallmarks of cancer immune evasion. *Cancer Cell* 2024 (In eng). DOI: 10.1016/j.ccell.2024.09.010.
 61. Halme J, Hammond MG, Hulka JF, Raj SG, Talbert LM. Retrograde menstruation in healthy women and in patients with endometriosis. *Obstetrics and Gynecology* 1984;64(2):151-154. (Article) (<https://www.scopus.com/inward/record.uri?eid=2-s2.0-0021282396&partnerID=40&md5=aa11f74b312b675f994c00e271bf868b>).
 62. Sourial S, Tempest N, Hapangama DK. Theories on the pathogenesis of endometriosis. *Int J Reprod Med* 2014;2014:179515. (In eng). DOI: 10.1155/2014/179515.
 63. Paul Dmowski W, Braun DP. Immunology of endometriosis. *Best Pract Res Clin Obstet Gynaecol* 2004;18(2):245-63. (In eng). DOI: 10.1016/j.bpobgyn.2004.02.001.
 64. Symons LK, Miller JE, Kay VR, et al. The Immunopathophysiology of Endometriosis. *Trends Mol Med* 2018;24(9):748-762. (In eng). DOI: 10.1016/j.molmed.2018.07.004.
 65. Abramiuk M, Grywalska E, Małkowska P, Sierawska O, Hryniewicz R, Niedźwiedzka-Rystwej P. The Role of the Immune System in the Development of Endometriosis. *Cells* 2022;11(13). DOI: 10.3390/cells11132028.
 66. Podgaec S, Abrao MS, Dias JA, Jr., Rizzo LV, de Oliveira RM, Baracat EC. Endometriosis: an inflammatory disease with a Th2 immune response component. *Hum Reprod* 2007;22(5):1373-9. (In eng). DOI: 10.1093/humrep/del516.
 67. Milewski Ł, Dziunycz P, Barcz E, et al. Increased levels of human neutrophil peptides 1, 2, and 3 in peritoneal fluid of patients with endometriosis: association with neutrophils, T cells and IL-8. *J Reprod Immunol* 2011;91(1-2):64-70. (In eng). DOI: 10.1016/j.jri.2011.05.008.
 68. Lukács L, Kovács AR, Pál L, Szűcs S, Kövér Á, Lampé R. Phagocyte function of peripheral neutrophil granulocytes and monocytes in endometriosis before and after surgery. *J Gynecol Obstet Hum Reprod* 2021;50(4):101796. (In eng). DOI: 10.1016/j.jogoh.2020.101796.
 69. Izumi G, Koga K, Takamura M, et al. Involvement of immune cells in the pathogenesis of endometriosis. *J Obstet Gynaecol Res* 2018;44(2):191-198. (In eng). DOI: 10.1111/jog.13559.
 70. Shi FD, Ljunggren HG, La Cava A, Van Kaer L. Organ-specific features of natural killer cells. *Nature Reviews Immunology* 2011;11(10):658-671. DOI: 10.1038/nri3065.
 71. Oosterlynck DJ, Meuleman C, Waer M, Koninckx PR, Vandeputte M. Immunosuppressive activity of peritoneal fluid in women with endometriosis. *Obstet Gynecol* 1993;82(2):206-12. (In eng).
 72. Ho HN, Chao KH, Chen HF, Wu MY, Yang YS, Lee TY. Peritoneal natural killer cytotoxicity and CD25+ CD3+ lymphocyte subpopulation are decreased in women with stage III-IV endometriosis. *Hum Reprod* 1995;10(10):2671-5. DOI: 10.1093/oxfordjournals.humrep.a135765.
 73. Saeiki S, Fukui A, Mai C, Takeyama R, Yamaya A, Shibahara H. Co-expression of activating and inhibitory receptors on peritoneal fluid NK cells in women with endometriosis. *J Reprod Immunol* 2022;155:103765. DOI: 10.1016/j.jri.2022.103765.
 74. Reis JL, Rosa NN, Angelo-Dias M, Martins C, Borrego LM, Lima J. Natural Killer Cell Receptors and Endometriosis: A Systematic Review. *Int J Mol Sci* 2022;24(1):331. DOI: 10.3390/ijms24010331.
 75. Randall GW, Gantt PA, Poe-Zeigler RL, et al. Serum antiendometrial antibodies and diagnosis of endometriosis. *Am J Reprod Immunol* 2007;58(4):374-82. (In eng). DOI: 10.1111/j.1600-0897.2007.00523.x.
 76. Parazzini F, Esposito G, Tozzi L, Noli S, Bianchi S. Epidemiology of endometriosis and its comorbidities. *European Journal of Obstetrics and Gynecology and Reproductive Biology* 2017;209:3-7. (Article). DOI: 10.1016/j.ejogrb.2016.04.021.

77. Riccio LGC, Baracat EC, Chapron C, Batteux F, Abrão MS. The role of the B lymphocytes in endometriosis: A systematic review. *Journal of Reproductive Immunology* 2017;123:29-34. DOI: 10.1016/j.jri.2017.09.001.
78. Schmitz T, Hoffmann V, Olliges E, et al. Reduced frequency of perforin-positive CD8⁺ T cells in menstrual effluent of endometriosis patients. *J Reprod Immunol* 2021;148:103424. (In eng). DOI: 10.1016/j.jri.2021.103424.
79. Hanada T, Tsuji S, Nakayama M, et al. Suppressive regulatory T cells and latent transforming growth factor- β -expressing macrophages are altered in the peritoneal fluid of patients with endometriosis. *Reprod Biol Endocrinol* 2018;16(1):9. (In eng). DOI: 10.1186/s12958-018-0325-2.
80. Gogacz M, Winkler I, Bojarska-Junak A, et al. Increased percentage of Th17 cells in peritoneal fluid is associated with severity of endometriosis. *J Reprod Immunol* 2016;117:39-44. (In eng). DOI: 10.1016/j.jri.2016.04.289.
81. Takamura M, Koga K, Izumi G, et al. Simultaneous Detection and Evaluation of Four Subsets of CD4⁺ T Lymphocyte in Lesions and Peripheral Blood in Endometriosis. *Am J Reprod Immunol* 2015;74(6):480-6. (In eng). DOI: 10.1111/aji.12426.
82. Olkowska-Truchanowicz J, Bocian K, Maksym RB, et al. CD4⁺ CD25⁺ FOXP3⁺ regulatory T cells in peripheral blood and peritoneal fluid of patients with endometriosis. *Hum Reprod* 2013;28(1):119-24. (In eng). DOI: 10.1093/humrep/des346.
83. Oală IE, Mitranovici MI, Chiorean DM, et al. Endometriosis and the Role of Pro-Inflammatory and Anti-Inflammatory Cytokines in Pathophysiology: A Narrative Review of the Literature. *Diagnostics (Basel)* 2024;14(3) (In eng). DOI: 10.3390/diagnostics14030312.
84. Nishida M, Nasu K, Ueda T, Fukuda J, Takai N, Miyakawa I. Endometriotic cells are resistant to interferon-gamma-induced cell growth inhibition and apoptosis: a possible mechanism involved in the pathogenesis of endometriosis. *Mol Hum Reprod* 2005;11(1):29-34. (In eng). DOI: 10.1093/molehr/gah133.
85. Sikora J, Ferrero S, Mielczarek-Palacz A, Kondera-Anasz Z. The Delicate Balance between the Good and the Bad IL-1 Proinflammatory Effects in Endometriosis. *Curr Med Chem* 2018;25(18):2105-2121. (In eng). DOI: 10.2174/0929867325666180111093547.
86. Tanaka T, Narazaki M, Kishimoto T. IL-6 in inflammation, immunity, and disease. *Cold Spring Harb Perspect Biol* 2014;6(10):a016295. (In eng). DOI: 10.1101/cshperspect.a016295.
87. Harada T, Iwabe T, Terakawa N. Role of cytokines in endometriosis. *Fertil Steril* 2001;76(1):1-10. (In eng). DOI: 10.1016/s0015-0282(01)01816-7.
88. Kang YJ, Jeung IC, Park A, et al. An increased level of IL-6 suppresses NK cell activity in peritoneal fluid of patients with endometriosis via regulation of SHP-2 expression. *Hum Reprod* 2014;29(10):2176-89. (In eng). DOI: 10.1093/humrep/deu172.
89. Wu MY, Ho HN, Chen SU, Chao KH, Chen CD, Yang YS. Increase in the production of interleukin-6, interleukin-10, and interleukin-12 by lipopolysaccharide-stimulated peritoneal macrophages from women with endometriosis. *Am J Reprod Immunol* 1999;41(1):106-11. (In eng). DOI: 10.1111/j.1600-0897.1999.tb00082.x.
90. Iwabe T, Harada T, Tsudo T, Tanikawa M, Onohara Y, Terakawa N. Pathogenetic significance of increased levels of interleukin-8 in the peritoneal fluid of patients with endometriosis. *Fertil Steril* 1998;69(5):924-30. (In eng). DOI: 10.1016/s0015-0282(98)00049-1.
91. Iwabe T, Harada T, Tsudo T, et al. Tumor necrosis factor-alpha promotes proliferation of endometriotic stromal cells by inducing interleukin-8 gene and protein expression. *J Clin Endocrinol Metab* 2000;85(2):824-9. (In eng). DOI: 10.1210/jcem.85.2.6335.
92. Sabat R, Grütz G, Warszawska K, et al. Biology of interleukin-10. *Cytokine Growth Factor Rev* 2010;21(5):331-44. (In eng). DOI: 10.1016/j.cytogfr.2010.09.002.
93. Hsieh CS, Heimberger AB, Gold JS, O'Garra A, Murphy KM. Differential regulation of T helper phenotype development by interleukins 4 and 10 in an alpha beta T-cell-receptor

- transgenic system. *Proc Natl Acad Sci U S A* 1992;89(13):6065-9. (In eng). DOI: 10.1073/pnas.89.13.6065.
94. Antsiferova YS, Sotnikova NY, Posiseeva LV, Shor AL. Changes in the T-helper cytokine profile and in lymphocyte activation at the systemic and local levels in women with endometriosis. *Fertil Steril* 2005;84(6):1705-11. (In eng). DOI: 10.1016/j.fertnstert.2005.05.066.
 95. Chang LY, Shan J, Hou XX, Li DJ, Wang XQ. Synergy between Th1 and Th2 responses during endometriosis: A review of current understanding. *J Reprod Immunol* 2023;158:103975. (In eng). DOI: 10.1016/j.jri.2023.103975.
 96. Suen JL, Chang Y, Chiu PR, et al. Serum level of IL-10 is increased in patients with endometriosis, and IL-10 promotes the growth of lesions in a murine model. *Am J Pathol* 2014;184(2):464-71. (In eng). DOI: 10.1016/j.ajpath.2013.10.023.
 97. Nati ID, Malutan A, Ciortea R, et al. Exploring the Influence of IL-8, IL-10, Patient-Reported Pain, and Physical Activity on Endometriosis Severity. *Diagnostics (Basel)* 2024;14(16) (In eng). DOI: 10.3390/diagnostics14161822.
 98. Oosterlynck DJ, Meuleman C, Waer M, Koninckx PR. Transforming growth factor-beta activity is increased in peritoneal fluid from women with endometriosis. *Obstet Gynecol* 1994;83(2):287-92. (In eng).
 99. Bo C, Wang Y. Angiogenesis signaling in endometriosis: Molecules, diagnosis and treatment (Review). *Mol Med Rep* 2024;29(3) (In eng). DOI: 10.3892/mmr.2024.13167.
 100. McLaren J, Prentice A, Charnock-Jones DS, et al. Vascular endothelial growth factor is produced by peritoneal fluid macrophages in endometriosis and is regulated by ovarian steroids. *J Clin Invest* 1996;98(2):482-9. (In eng). DOI: 10.1172/jci118815.
 101. Cho S, Choi YS, Jeon YE, et al. Expression of vascular endothelial growth factor (VEGF) and its soluble receptor-1 in endometriosis. *Microvasc Res* 2012;83(2):237-42. (In eng). DOI: 10.1016/j.mvr.2011.12.004.
 102. Griffiths MJ, Horne AW, Gibson DA, Roberts N, Saunders PTK. Endometriosis: recent advances that could accelerate diagnosis and improve care. *Trends Mol Med* 2024;30(9):875-889. (In eng). DOI: 10.1016/j.molmed.2024.06.008.
 103. Hosseinzadeh R, Moini A, Hosseini R, et al. A higher number of exhausted local PD1+, but not TIM3+, NK cells in advanced endometriosis. *Heliyon* 2024;10(1):e23294. (In eng). DOI: 10.1016/j.heliyon.2023.e23294.
 104. Olkowska-Truchanowicz J, Bialoszewska A, Zwierzchowska A, et al. Peritoneal Fluid from Patients with Ovarian Endometriosis Displays Immunosuppressive Potential and Stimulates Th2 Response. *Int J Mol Sci* 2021;22(15). DOI: 10.3390/ijms22158134.
 105. Meggyes M, Szereday L, Bohonyi N, et al. Different Expression Pattern of TIM-3 and Galectin-9 Molecules by Peripheral and Peritoneal Lymphocytes in Women with and without Endometriosis. *Int J Mol Sci* 2020;21(7). DOI: 10.3390/ijms21072343.
 106. Xu H. Expressions of natural cytotoxicity receptor, NKG2D and NKG2D ligands in endometriosis. *J Reprod Immunol* 2019;136:102615. DOI: 10.1016/j.jri.2019.102615.
 107. Hassa H, Tanir HM, Tekin B, Kirilmaz SD, Sahin Mutlu F. Cytokine and immune cell levels in peritoneal fluid and peripheral blood of women with early- and late-staged endometriosis. *Arch Gynecol Obstet* 2009;279(6):891-5. DOI: 10.1007/s00404-008-0844-8.
 108. Galandrini R, Porpora MG, Stoppacciaro A, et al. Increased frequency of human leukocyte antigen-E inhibitory receptor CD94/NKG2A-expressing peritoneal natural killer cells in patients with endometriosis. *Fertil Steril* 2008;89(5 Suppl):1490-6. DOI: 10.1016/j.fertnstert.2007.05.018.
 109. Zhang C, Maeda N, Izumiya C, et al. Killer immunoglobulin-like receptor and human leukocyte antigen expression as immunodiagnostic parameters for pelvic endometriosis. *Am J Reprod Immunol* 2006;55(2):106-14. DOI: 10.1111/j.1600-0897.2005.00332.x.

110. Matsuoka S, Maeda N, Izumiya C, Yamashita C, Nishimori Y, Fukaya T. Expression of inhibitory-motif killer immunoglobulin-like receptor, KIR2DL1, is increased in natural killer cells from women with pelvic endometriosis. *Am J Reprod Immunol* 2005;53(5):249-54. DOI: 10.1111/j.1600-0897.2005.00271.x.
111. Maeda N, Izumiya C, Kusum T, et al. Killer inhibitory receptor CD158a overexpression among natural killer cells in women with endometriosis is undiminished by laparoscopic surgery and gonadotropin releasing hormone agonist treatment. *Am J Reprod Immunol* 2004;51(5):364-72. DOI: 10.1111/j.1600-0897.2004.00170.x.
112. Szylo K, Tchorzewski H, Banasik M, Glowacka E, Lewkowicz P, Kamer-Bartosinska A. The involvement of T lymphocytes in the pathogenesis of endometriotic tissues overgrowth in women with endometriosis. *Mediators Inflamm* 2003;12(3):131-8. DOI: 10.1080/0962935031000134842.
113. Maeda N, Izumiya C, Yamamoto Y, Oguri H, Kusume T, Fukaya T. Increased killer inhibitory receptor KIR2DL1 expression among natural killer cells in women with pelvic endometriosis. *Fertil Steril* 2002;77(2):297-302. DOI: 10.1016/s0015-0282(01)02964-8.
114. Maeda N, Izumiya C, Oguri H, Kusume T, Yamamoto Y, Fukaya T. Aberrant expression of intercellular adhesion molecule-1 and killer inhibitory receptors induces immune tolerance in women with pelvic endometriosis. *Fertil Steril* 2002;77(4):679-83. DOI: 10.1016/s0015-0282(01)03249-6.
115. Wu MY, Chao KH, Chen SU, et al. The suppression of peritoneal cellular immunity in women with endometriosis could be restored after gonadotropin releasing hormone agonist treatment. *Am J Reprod Immunol* 1996;35(6):510-6. DOI: 10.1111/j.1600-0897.1996.tb00050.x.
116. Hua L, Xu X, Bian D. The measurement of natural killer cell activity in peripheral blood and peritoneal fluid of patients with endometriosis. *Zhonghua Fu Chan Ke Za Zhi* 1996;31(10):586-9. (<https://www.ncbi.nlm.nih.gov/pubmed/9275450>).
117. Oosterlynck DJ, Meuleman C, Lacquet FA, Waer M, Koninckx PR. Flow cytometry analysis of lymphocyte subpopulations in peritoneal fluid of women with endometriosis. *Am J Reprod Immunol* 1994;31(1):25-31. DOI: 10.1111/j.1600-0897.1994.tb00843.x.
118. Iwasaki K, Makino T, Maruyama T, Matsubayashi H, Nozawa S, Yokokura T. Leukocyte subpopulations and natural killer activity in endometriosis. *Int J Fertil Menopausal Stud* 1993;38(4):229-34. (<https://www.ncbi.nlm.nih.gov/pubmed/8401682>).
119. Oosterlynck DJ, Meuleman C, Waer M, Vandeputte M, Koninckx PR. The natural killer activity of peritoneal fluid lymphocytes is decreased in women with endometriosis. *Fertil Steril* 1992;58(2):290-5. DOI: 10.1016/s0015-0282(16)55224-8.
120. Becker CM, Bokor A, Heikinheimo O, et al. ESHRE guideline: endometriosis. *Hum Reprod Open* 2022;2022(2):hoac009. (In eng). DOI: 10.1093/hropen/hoac009.
121. Andrews WC, Buttram VC, Jr.; Weed JC, Hammond CB, Thomas HH, Behrman SJ. Revised American Fertility Society classification of endometriosis: 1985. *Fertil Steril* 1985;43(3):351-2. DOI: 10.1016/s0015-0282(16)48430-x.
122. Jacobs R, Hintzen G, Kemper A, et al. CD56bright cells differ in their KIR repertoire and cytotoxic features from CD56dim NK cells. *Eur J Immunol* 2001;31(10):3121-7. (In eng). DOI: 10.1002/1521-4141(2001010)31:10<3121::aid-immu3121>3.0.co;2-4.
123. Addison EG, North J, Bakhsh I, et al. Ligation of CD8alpha on human natural killer cells prevents activation-induced apoptosis and enhances cytolytic activity. *Immunology* 2005;116(3):354-61. DOI: 10.1111/j.1365-2567.2005.02235.x.
124. Hooghe TMD, Hill JA, Oosterlynck DJ, Koninckx PR, Bamba CS. Endometriosis: Effect of endometriosis on white blood cell subpopulations in peripheral blood and peritoneal fluid of baboons. *Human Reproduction* 1996;11(8):1736-1740. DOI: 10.1093/oxfordjournals.humrep.a019478.

125. Kisovar A, Becker CM, Granne I, Southcombe JH. The role of CD8+ T cells in endometriosis: a systematic review. *Front Immunol* 2023;14:1225639. DOI: 10.3389/fimmu.2023.1225639.
126. Martos SN, Campbell MR, Lozoya OA, et al. Single-cell analyses identify dysfunctional CD16(+) CD8 T cells in smokers. *Cell Rep Med* 2020;1(4). DOI: 10.1016/j.xcrm.2020.100054.
127. Chapron C, Souza C, de Ziegler D, et al. Smoking habits of 411 women with histologically proven endometriosis and 567 unaffected women. *Fertil Steril* 2010;94(6):2353-5. DOI: 10.1016/j.fertnstert.2010.04.020.
128. Walankiewicz M, Grywalska E, Polak G, et al. The Increase of Circulating PD-1- and PD-L1-Expressing Lymphocytes in Endometriosis: Correlation with Clinical and Laboratory Parameters. *Mediators Inflamm* 2018;2018:7041342. DOI: 10.1155/2018/7041342.
129. Xu H, Zhao J, Lu J, Sun X. Ovarian endometrioma infiltrating neutrophils orchestrate immunosuppressive microenvironment. *J Ovarian Res* 2020;13(1):44. DOI: 10.1186/s13048-020-00642-7.
130. Slabe N, Meden-Vrtovec H, Verdenik I, Kosir-Pogacnik R, Ihan A. Cytotoxic T-Cells in Peripheral Blood in Women with Endometriosis. *Geburtshilfe Frauenheilkd* 2013;73(10):1042-1048. DOI: 10.1055/s-0033-1350702.
131. Zhu C, Anderson AC, Schubart A, et al. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat Immunol* 2005;6(12):1245-52. (In eng). DOI: 10.1038/ni1271.
132. Zhou E, Huang Q, Wang J, et al. Up-regulation of Tim-3 is associated with poor prognosis of patients with colon cancer. *Int J Clin Exp Pathol* 2015;8(7):8018-27. (In eng).
133. Piao YR, Piao LZ, Zhu LH, Jin ZH, Dong XZ. Prognostic value of T cell immunoglobulin mucin-3 in prostate cancer. *Asian Pac J Cancer Prev* 2013;14(6):3897-901. (In eng). DOI: 10.7314/apjcp.2013.14.6.3897.
134. Cao Y, Zhou X, Huang X, et al. Correction: Tim-3 Expression in Cervical Cancer Promotes Tumor Metastasis. *PLoS One* 2016;11(3):e0152830. (In eng). DOI: 10.1371/journal.pone.0152830.
135. Cao B, Zhu L, Zhu S, et al. Genetic variations and haplotypes in TIM-3 gene and the risk of gastric cancer. *Cancer Immunol Immunother* 2010;59(12):1851-7. (In eng). DOI: 10.1007/s00262-010-0910-5.
136. Luo Q, Kong Y, Fu B, et al. Increased TIM-3(+)PD-1(+) NK cells are associated with the disease activity and severity of systemic lupus erythematosus. *Clin Exp Med* 2022;22(1):47-56. (In eng). DOI: 10.1007/s10238-021-00726-8.
137. Alvermann S, Hennig C, Stüve O, Wiendl H, Stangel M. Immunophenotyping of cerebrospinal fluid cells in multiple sclerosis: in search of biomarkers. *JAMA Neurol* 2014;71(7):905-12. (In eng). DOI: 10.1001/jamaneurol.2014.395.
138. Lavon I, Heli C, Brill L, Charbit H, Vaknin-Dembinsky A. Blood Levels of Co-inhibitory-Receptors: A Biomarker of Disease Prognosis in Multiple Sclerosis. *Front Immunol* 2019;10:835. (In eng). DOI: 10.3389/fimmu.2019.00835.
139. Khademi M, Illés Z, Gielen AW, et al. T Cell Ig- and mucin-domain-containing molecule-3 (TIM-3) and TIM-1 molecules are differentially expressed on human Th1 and Th2 cells and in cerebrospinal fluid-derived mononuclear cells in multiple sclerosis. *J Immunol* 2004;172(11):7169-76. (In eng). DOI: 10.4049/jimmunol.172.11.7169.
140. Afshar B, Khalifehzadeh-Esfahani Z, Seyfizadeh N, Rezaei Danbaran G, Hemmatzadeh M, Mohammadi H. The role of immune regulatory molecules in multiple sclerosis. *J Neuroimmunol* 2019;337:577061. (In eng). DOI: 10.1016/j.jneuroim.2019.577061.
141. Koguchi K, Anderson DE, Yang L, O'Connor KC, Kuchroo VK, Hafler DA. Dysregulated T cell expression of TIM3 in multiple sclerosis. *J Exp Med* 2006;203(6):1413-8. (In eng). DOI: 10.1084/jem.20060210.

142. Yang L, Anderson DE, Kuchroo J, Hafler DA. Lack of TIM-3 immunoregulation in multiple sclerosis. *J Immunol* 2008;180(7):4409-4414.
143. Saresella M, Piancone F, Marventano I, et al. A role for the TIM-3/GAL-9/BAT3 pathway in determining the clinical phenotype of multiple sclerosis. *Faseb j* 2014;28(11):5000-9. (In eng). DOI: 10.1096/fj.14-258194.
144. Tang L, Li G, Zheng Y, et al. Tim-3 Relieves Experimental Autoimmune Encephalomyelitis by Suppressing MHC-II. *Front Immunol* 2021;12:770402. (In eng). DOI: 10.3389/fimmu.2021.770402.
145. Van Acker HH, Capsomidis A, Smits EL, Van Tendeloo VF. CD56 in the Immune System: More Than a Marker for Cytotoxicity? *Front Immunol* 2017;8:892. DOI: 10.3389/fimmu.2017.00892.
146. Chan WK, Rujkijyanont P, Neale G, et al. Multiplex and genome-wide analyses reveal distinctive properties of KIR⁺ and CD56⁺ T cells in human blood. *J Immunol* 2013;191(4):1625-36. (In eng). DOI: 10.4049/jimmunol.1300111.
147. Correia MP, Costa AV, Uhrberg M, Cardoso EM, Arosa FA. IL-15 induces CD8⁺ T cells to acquire functional NK receptors capable of modulating cytotoxicity and cytokine secretion. *Immunobiology* 2011;216(5):604-12. (In eng). DOI: 10.1016/j.imbio.2010.09.012.
148. Guia S, Cognet C, de Beaucoudrey L, et al. A role for interleukin-12/23 in the maturation of human natural killer and CD56⁺ T cells in vivo. *Blood* 2008;111(10):5008-16. (In eng). DOI: 10.1182/blood-2007-11-122259.
149. Han G, Chen G, Shen B, Li Y. Tim-3: an activation marker and activation limiter of innate immune cells. *Front Immunol* 2013;4:449. DOI: 10.3389/fimmu.2013.00449.
150. Ocaña-Guzman R, Ramon-Luing LA, Vazquez-Bolaños LA, et al. Tim-3 Is Differentially Expressed during Cell Activation and Interacts with the LSP-1 Protein in Human Macrophages. *J Immunol Res* 2023;2023:3577334. (In eng). DOI: 10.1155/2023/3577334.
151. Pesce S, Greppi M, Tabellini G, et al. Identification of a subset of human natural killer cells expressing high levels of programmed death 1: A phenotypic and functional characterization. *J Allergy Clin Immunol* 2017;139(1):335-346.e3. (In eng). DOI: 10.1016/j.jaci.2016.04.025.
152. Ahmadzadeh M, Johnson LA, Heemskerk B, et al. Tumor antigen-specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. *Blood* 2009;114(8):1537-44. (In eng). DOI: 10.1182/blood-2008-12-195792.
153. Moretta L, Montaldo E, Vacca P, et al. Human natural killer cells: origin, receptors, function, and clinical applications. *Int Arch Allergy Immunol* 2014;164(4):253-64. DOI: 10.1159/000365632.
154. Quatrini L, Mariotti FR, Munari E, Tumino N, Vacca P, Moretta L. The Immune Checkpoint PD-1 in Natural Killer Cells: Expression, Function and Targeting in Tumour Immunotherapy. *Cancers (Basel)* 2020;6(11):3285. DOI: 10.3390/cancers12113285.
155. Wang W, Lau R, Yu D, Zhu W, Korman A, Weber J. PD1 blockade reverses the suppression of melanoma antigen-specific CTL by CD4⁺CD25^{hi} regulatory T cells. *International Immunology* 2009;21(9):1065-1077. (Article). DOI: 10.1093/intimm/dxp072.
156. Torng PL. Clinical implication for endometriosis associated with ovarian cancer. *Gynecol Minim Invasive Ther* 2017;6(4):152-156. (In eng). DOI: 10.1016/j.gmit.2017.08.003.
157. Suszczyk D, Skiba W, Jakubowicz-Gil J, Kotarski J, Wertel I. The Role of Myeloid-Derived Suppressor Cells (MDSCs) in the Development and/or Progression of Endometriosis-State of the Art. *Cells* 2021;10(3) (In eng). DOI: 10.3390/cells10030677.
158. Han Y, Liu D, Li L. PD-1/PD-L1 pathway: current researches in cancer. *Am J Cancer Res* 2020;10(3):727-742. (In eng).
159. Yang Y. Cancer immunotherapy: Harnessing the immune system to battle cancer. *Journal of Clinical Investigation* 2015;125(9):3335-3337. (Review). DOI: 10.1172/JCI83871.

160. Ribas A, Wolchok JD. Cancer immunotherapy using checkpoint blockade. *Science* 2018;359(6382):1350-1355. (Review). DOI: 10.1126/science.aar4060.
161. Webb ES, Liu P, Baleeiro R, Lemoine NR, Yuan M, Wang Y. Immune checkpoint inhibitors in cancer therapy. *Journal of Biomedical Research* 2018;32(5):317-326. (Article). DOI: 10.7555/JBR.31.20160168.
162. Sadreddini S, Baradaran B, Aghebati-Maleki A, et al. Immune checkpoint blockade opens a new way to cancer immunotherapy. *Journal of Cellular Physiology* 2019;234(6):8541-8549. (Review). DOI: 10.1002/jcp.27816.
163. Bryceson YT, March ME, Ljunggren HG, Long EO. Activation, coactivation, and costimulation of resting human natural killer cells. *Immunol Rev* 2006;214:73-91. (In eng). DOI: 10.1111/j.1600-065X.2006.00457.x.
164. Perussia B. Fc receptors on natural killer cells. *Curr Top Microbiol Immunol* 1998;230:63-88. (In eng). DOI: 10.1007/978-3-642-46859-9_6.
165. Ilavská S, Horváthová M, Szabová M, et al. Association between the human immune response and body mass index. *Hum Immunol* 2012;73(5):480-5. (In eng). DOI: 10.1016/j.humimm.2012.02.023.