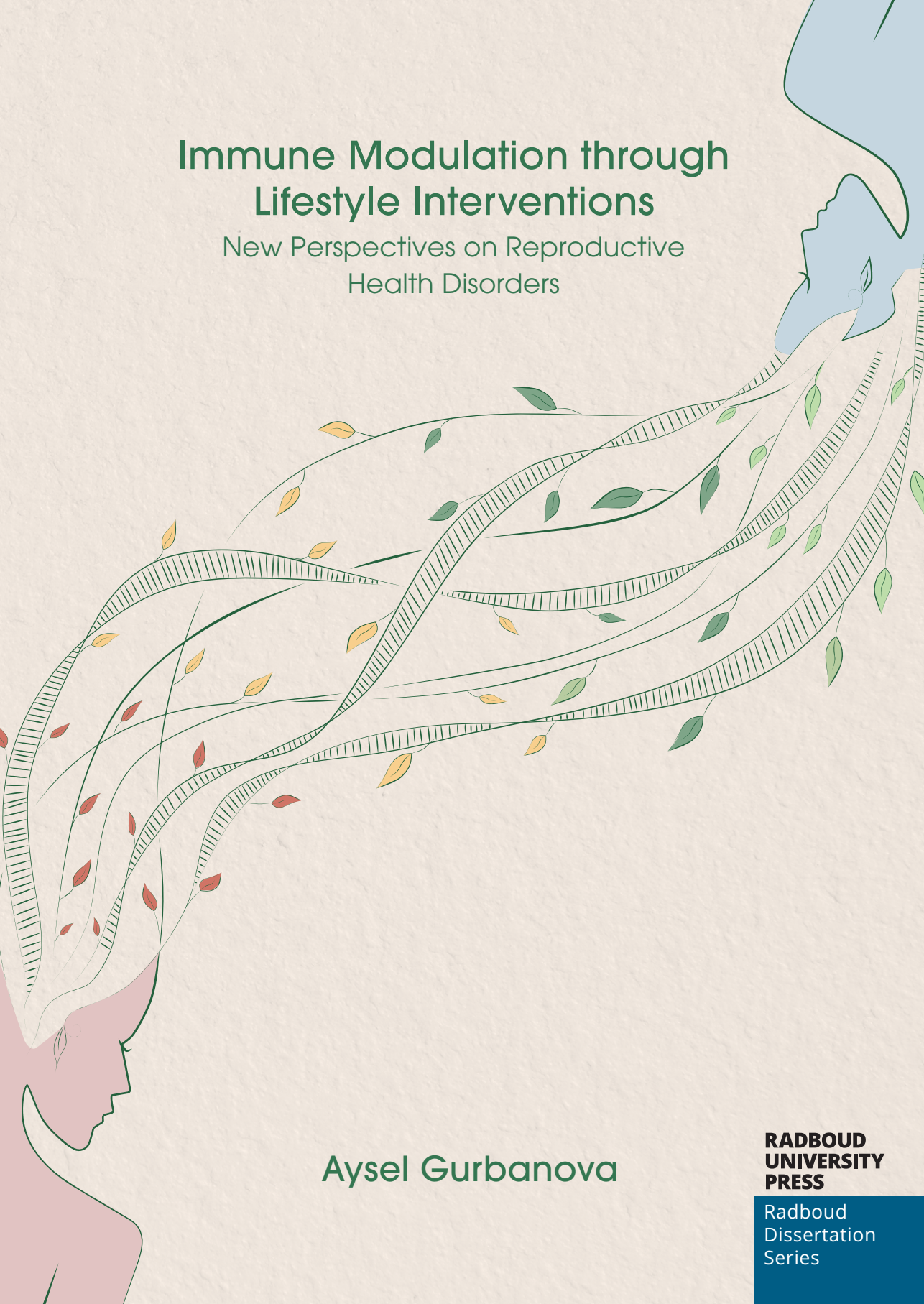


Immune Modulation through Lifestyle Interventions

New Perspectives on Reproductive
Health Disorders



Aysel Gurbanova

**RADBOD
UNIVERSITY
PRESS**

Radboud
Dissertation
Series

Immune Modulation through Lifestyle Interventions

**New Perspectives on Reproductive
Health Disorders**

Aysel Gurbanova

Immune Modulation through Lifestyle Interventions - New Perspectives on Reproductive Health Disorders

Aysel Gurbanova

Radboud Dissertation Series

ISSN: 2950-2772 (Online); 2950-2780 (Print)

Published by RADBOUD UNIVERSITY PRESS

Postbus 9100, 6500 HA Nijmegen, The Netherlands

www.radbouduniversitypress.nl

Design: Proefschrift AIO | Guus Gijben

Cover: Aarti Ramchandran / Proefschrift AIO | Guntra Laivacuma

Printing: DPN Rikken/Pumbo

ISBN: 9789465152479

DOI: 10.54195/9789465152479

Free download at: <https://doi.org/10.54195/9789465152479>

© 2026 Aysel Gurbanova

**RADBOUD
UNIVERSITY
PRESS**

This is an Open Access book published under the terms of Creative Commons Attribution-Noncommercial-NoDerivatives International license (CC BY-NC-ND 4.0). This license allows reusers to copy and distribute the material in any medium or format in unadapted form only, for noncommercial purposes only, and only so long as attribution is given to the creator, see <http://creativecommons.org/licenses/by-nc-nd/4.0/>.

Immune Modulation through Lifestyle Interventions: New Perspectives on Reproductive Health Disorders

Proefschrift ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. dr. J.M. Sanders,
volgens besluit van het college voor promoties
in het openbaar te verdedigen op

vrijdag 8 mei 2026
om 10.30 uur precies

door

Aysel Gurbanova
geboren op 22 november 1997
te Baku, Azerbeidjan

Promotor:

Prof. dr. M.I. de Jonge

Copromotoren:

Dr. R.G. van der Molen

Dr. T. Meuleman

Dr. L. Wieten (Maastricht Universitair Medisch Centrum)

Manuscriptcommissie:

Prof. dr. I.J.M. de Vries

Prof. dr. J. Garssen (Universiteit Utrecht)

Prof. dr. U. Markert (Universitätsklinikum Jena, Duitsland)

Immune Modulation through Lifestyle Interventions: New Perspectives on Reproductive Health Disorders

Dissertation to obtain the degree of doctor
from Radboud University Nijmegen
on the authority of the Rector Magnificus prof. dr. J.M. Sanders,
according to the decision of the Doctorate Board
to be defended in public on

Friday, May 8, 2026
at 10:30 am

by

Aysel Gurbanova
born on November 22, 1997
in Baku, Azerbaijan

PhD supervisor:

Prof. dr. M.I. de Jonge

PhD co-supervisors:

Dr. R.G. van der Molen

Dr. T. Meuleman

Dr. L. Wieten (Maastricht University Medical Center)

Manuscript Committee:

Prof. dr. I.J.M. de Vries

Prof. dr. J. Garssen (Utrecht University)

Prof. dr. U. Markert (University Hospital Jena, Germany)

Table of contents

CHAPTER 1

Introduction	9
--------------	---

CHAPTER 2

Menstrual effluent derived immune cell composition is distinct in women using contraceptives: a pilot study	29
---	----

CHAPTER 3

Profiling NK cell receptor repertoire of women with idiopathic recurrent pregnancy loss	49
---	----

CHAPTER 4

An aerobe exercise intervention for optimizing metabolic, cardiovascular and immune status: protocol of an intervention study with a multi-systemic approach for women with unexplained recurrent pregnancy loss	75
--	----

CHAPTER 5

Impact of a Moderate-Intensity Aerobic Exercise Intervention on Systemic and Uterine Natural Killer Cells in Women with Unexplained Recurrent Pregnancy Loss	93
--	----

CHAPTER 6

Cellular fingerprinting reveals a dominant role for the IL-6-JAK-STAT3 axis in relation to T-cell lineage commitment in endometriosis	123
---	-----

CHAPTER 7

Impact of an Anti-Inflammatory Diet on quality of life, pain, and inflammation in Endometriosis: AIDEN-study	149
--	-----

CHAPTER 8

Discussion	191
------------	-----

CHAPTER 9

Nederlandse Samenvatting	207
--------------------------	-----

Appendix

Research data management	214
PhD portfolio of Aysel Gurbanova	216
Acknowledgements	218



CHAPTER 1

Introduction

‘One is not born, but rather becomes, a woman’, wrote Simone de Beauvoir, challenging the notion of fixed identity and emphasizing the role of environment and experience in shaping who we are. While her statement specifically addresses gender and social roles, it echoes a broader question at the heart of both philosophy and biology: are we solely the product of our genetic blueprint, or are we continuously shaped by our environment and the choices we make?

This question is particularly relevant in the context of reproductive immunology, where biological complexity intersects with lifestyle choices. Every woman carries a unique, genetically determined immune system which can both support and interfere with reproductive success. In this thesis, I explore the immune system of women with reproductive health problems such as recurrent pregnancy loss and endometriosis, where impaired immune function is thought to play a key role. While genetic and hormonal factors are known to be foundational to immune function, it remains unclear to what extent lifestyle choices such as contraceptive use, physical activity, and dietary habits contribute to variation in immune responses. Understanding this interaction may offer new insights into both the prevention and treatment of reproductive health challenges.

In this work, I aim to explore the impact of lifestyle interventions on the immune system of women with reproductive health issues and identify whether these interventions can be used as new treatment strategies to improve quality of life and reproductive outcomes.

The dynamic uterine cycle in a glance

The uterus is a dynamic organ with a central role in reproduction, orchestrated by a complex network of cells that regulate key processes such as menstrual cycling, embryo implantation, and tissue remodeling. The inner epithelial lining of the uterus, called the **endometrium**, undergoes cyclical, structural and functional changes in response to fluctuating ovarian steroid hormones. Its primary functions are to prepare for embryo implantation, support pregnancy if implantation occurs, or undergo menstruation in the absence of implantation (1). The endometrium is composed of various cells types including stromal, epithelial, vascular, and immune cells. Among these immune cells are essential for proper breakdown and regeneration of endometrial tissue (2). Throughout each menstrual cycle, the endometrium transitions through two main phases: the **proliferative** and **secretory phase**. During the proliferative phase, rising estradiol levels stimulate rapid proliferation of vascular and endometrial cells, resulting in the formation of a thin, linear, echogenic layer. This is followed by the secretory phase, dominated by progesterone, during which

estradiol-primed stromal cells undergo **decidualization**, a transformation into rounded, epithelioid-like decidual cells (3). Decidualization of the endometrium takes place in each cycle in response to elevated levels of steroid hormones (4). If implantation occurs, the decidua plays a vital role in protecting the embryo from maternal immune rejection and supporting the development of the placenta (5, 6).

In the absence of implantation, declining ovarian hormone levels initiate a tightly regulated inflammatory process that leads to the breakdown and shedding of the decidua, resulting in menstruation. This process involves immune cell infiltration, including macrophages, neutrophils, and **natural killer (NK)** cells (2, 7, 8), as well as the release of inflammatory mediators such as prostaglandins, cytokines, and matrix metalloproteinases (9, 10). These factors drive tissue breakdown and vasoconstriction, facilitating menstrual shedding. However, menstruation is not only an inflammatory event but also a regenerative process. Immune cells play a key role in resolving inflammation and promoting endometrial repair (7). This cyclical process of tissue breakdown and repair occurs approximately 450 times in a woman's reproductive lifespan, highlighting the endometrium's remarkable regenerative capacity, which is unique among mucosal tissues (11).

Immune cell adaptations in the non-pregnant and pregnant uterus

Unlike other mucosal barriers, the function of the endometrium is not only to protect the uterus from pathogens, but also to accommodate enough tolerance to sustain a semi-allogeneic embryo. Peter Medawar, known for his work on graft rejection and the discovery of acquired immune tolerance, described **pregnancy** as "Nature's transplant," where two genetically distinct individuals coexist throughout gestation (12, 13). This perspective underscores the importance of immune cell adaptations in achieving maternal immune tolerance for a successful pregnancy.

Immune Cells in the Non-Pregnant Uterus (Endometrium)

In the **non-pregnant** endometrium, immune cells, including endometrial NK cells, T cells, B cells, and macrophages, fluctuate in composition throughout the menstrual cycle (14). During the early proliferative to the early secretory phase, CD45⁺ leukocytes are relatively scarce, but their numbers increase significantly towards the end of this phase (2, 15).

A distinctive feature of the non-pregnant endometrium is the fluctuation of NK cells. While only few NK cells are present in the proliferative phase, these cells increase drastically in the late secretory phase and constitute 70% of all leukocytes (16, 17). These cells, also termed **endometrial NK (eNK)** cells in the non-pregnant state, are

characterized as CD3⁺CD56^{bright}CD16⁺ and express the tissue-resident marker CD49a. Unlike cytotoxic peripheral NK (pNK) cells, eNK cells contain fewer but larger cytoplasmic granules, suggesting a primary role in cytokine production rather than cytolytic activity (16, 17). If pregnancy does not occur, eNK cells are shed during menstruation, making **menstrual blood (MB)** a valuable, non-invasive source for studying these cells (18). Immunophenotyping of MB-derived NK cells has shown that these cells possess a unique and stable repertoire of Killer Immunoglobulin Receptors (KIRs), and frequencies of KIR expressing NK cells and co-expression patterns with other receptors are distinct from those found in peripheral blood (19).

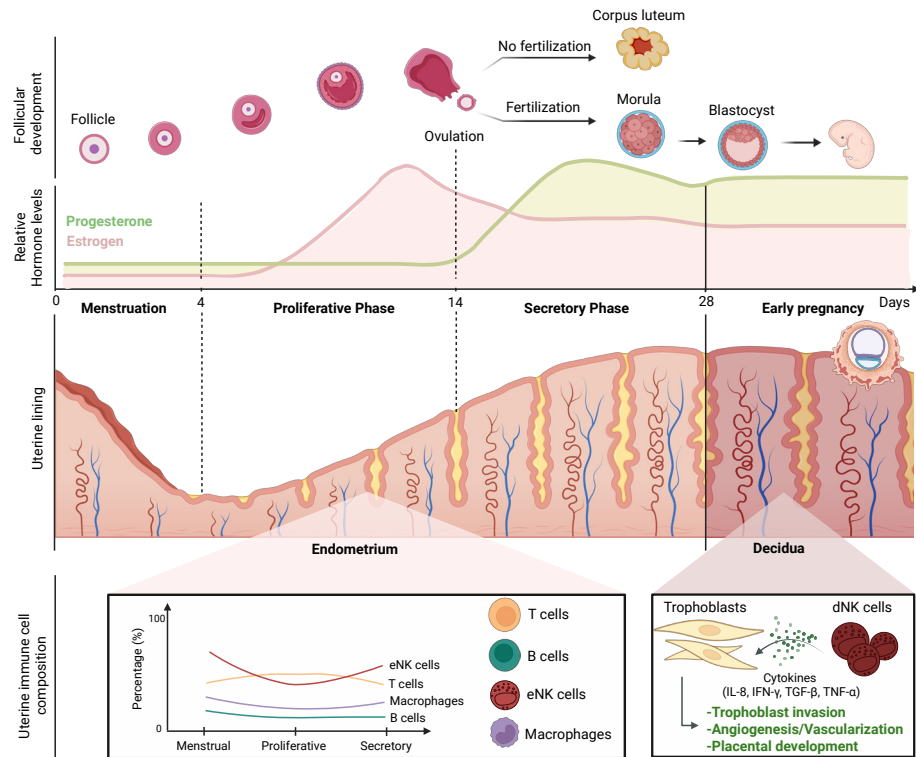


Figure 1. Overview of hormonal, endometrial, and immunological changes during the menstrual cycle and early pregnancy. The top panel illustrates follicular development, ovulation, and the potential outcomes of fertilization, alongside dynamic changes in relative estrogen and progesterone levels. The middle panel depicts corresponding morphological changes in the uterine lining (endometrium and decidua) across the menstrual cycle phases: menstruation, proliferative, and secretory, and during early pregnancy. The lower left graph shows the relative abundance of key uterine immune cell populations (T cells, B cells, endometrial NK (eNK) cells, and monocytes/macrophages) across cycle phases. The lower right schematic highlights the role of decidual NK (dNK) cells in supporting early pregnancy by producing cytokines that regulate trophoblast invasion, angiogenesis, and placental development.

Conflicting data exist regarding the abundance of **endometrial T cells** throughout the menstrual cycle. While some studies report a decrease in the percentage of T cells from the proliferative to the secretory phase, others suggest that the absolute numbers of these cells remain constant (15, 20). Unlike in peripheral blood, **endometrial T cells** obtained from biopsies predominantly consist of CD8+ T cells (2). These cells exhibit cytolytic activity during the proliferative phase, which diminishes in the secretory phase (2, 21). The reduced cytolytic may facilitate immune tolerance of the embryo during implantation. Additionally, the ratio of Th1/Th2 T cells also decreases over the course of menstrual cycle further supporting the establishment of a tolerant immune environment conducive to implantation (22).

Immune Cells in the Pregnant Uterus (Decidua)

Upon pregnancy, the composition and function of immune cells in the **decidua** changes to tolerate the semi-allogeneic fetus. The majority of the leukocytes present in the decidua early in pregnancy are **decidual NK (dNK)** cells and **decidual macrophages** comprising ~90% of leukocytes, whereas decidual T cells only contain ~10% leukocytes (23).

Macrophages, making up about 20% of leukocytes in the decidua (24). During pregnancy, they adopt an anti-inflammatory phenotype, facilitating placental development and immune tolerance by producing cytokines like interleukin-10 (IL-10) and transforming growth factor-beta (TGF- β) (25, 26). In addition, macrophages contribute to the formation of spiral arteries and angiogenesis by producing MMP-9 and VEGF (27, 28). It was suggested that macrophages might be involved in tissue remodeling by degradation of extracellular matrix and clearance of apoptotic cells (29). Decidual macrophages especially play a role in the onset of labor by secreting inflammatory cytokines while reducing IL-10 production (30, 31).

The proportion of dNK cells is initially high in early pregnancy but gradually declines as gestation progresses towards term (32, 33). Throughout pregnancy, dNK cells undergo dynamic changes in function, receptor repertoire, and cytokine production, which are essential for establishing immune tolerance and facilitating spiral artery modeling (34, 35). dNK cells express a wide range of both activating and inhibitory NK receptors (NKR), such as KIRs, leukocyte immunoglobulin-like receptor B1 (LILRB1), Natural Cytotoxicity Receptors (NCRs), and NKG2A/C/E/D. Inhibitory receptors, including KIRs and NKG2A, recognize human leukocyte antigen (HLA) molecules expressed by trophoblast cells, thereby suppressing dNK cellular cytotoxicity activation and promoting maternal-fetal immune tolerance (36, 37). In the first trimester decidua, three subsets of dNK cells, dNK1, dNK2, and dNK3 have

been identified (38). Beyond, their immunoregulatory role, dNK cells also contribute to placental development by secreting a diverse range of cytokines, chemokines, and growth factors, including VEGF, IP-10, IL-18, and interferon-gamma (IFN- γ). These factors play critical roles in supporting spiral artery remodeling and trophoblast invasion (39, 40). It is hypothesized that dNK1 cells mainly interact with trophoblasts due to their active glycolytic metabolism, elevated expression of KIR genes, LILRB1, and cytoplasmic granule proteins (38). The dual capacity of dNK cells for immune regulation and tissue remodeling highlights their importance in the successful establishment and maintenance of pregnancy.

Regulatory T (Tregs) cells are also essential for immune tolerance during pregnancy. Their expansion within the decidua helps suppress pro-inflammatory Th1 and Th17 responses directed against the semi-allogeneic embryo, thereby supporting fetal tolerance (41). Evidence suggests that depletion of Tregs can lead to increased infiltration of effector T cells which may lead to inflammation and implantation failure (42).

Dysregulation of these immune mechanisms, including imbalances in dNK cell activity or Treg function, has been implicated in various reproductive disorders. The following section, will further elaborate on the contribution of such immune alterations on reproductive complications such as **recurrent pregnancy loss (RPL)** and **endometriosis**.

The immune dysregulation, inflammation and reproductive disorders

During the female reproductive cycle, immune cell adaptations occur to support many physiological events including menstruation, implantation, and labour onset/parturition which are highly inflammatory processes. Inflammation is tightly regulated by the immune system and involves release of cytokines, chemokines, and growth factors. Since initiation and maintenance of inflammatory pathways are essential for these processes, the dysregulated inflammatory responses may potentially contribute to various **reproductive disorders**.

Recurrent Pregnancy Loss (RPL)

Uterine inflammation during the implantation process has been hypothesized as a potential contributor to RPL (Figure 2). RPL is defined as the loss of 2 or more pregnancies before 24 weeks of gestation (43) and has a multifactorial etiology including parental chromosomal abnormalities, uterine anomalies, and antiphospholipid syndrome (APS) (44). However, in approximately 50% of the cases, no identifiable cause is found, leading to the diagnosis of **idiopathic RPL (iRPL)**.

Immune dysregulation and abnormal inflammatory responses have been increasingly recognized as potential contributors to iRPL (45).

Due to its role in implantation, the number and function of NK cells, both in peripheral blood and the uterus have been extensively studied to understand the etiology of iRPL. A meta-analysis reported higher numbers of pNK cells in women with iRPL compared to controls, however significant heterogeneity was observed across the studies (46). Moreover, pNK cell numbers did not predict miscarriage in subsequent pregnancies in these women with iRPL (47). Another meta-analysis in 2023 reported no significant differences in uNK cell abundance in women with iRPL compared to control subjects and found no significant difference in subsequent pregnancy outcome and uNK cell levels (48). The lack of consensus regarding the optimal range of NK cell levels necessary for a successful pregnancy complicates the interpretation and clinical utility of these findings.

Consequently, many studies have shifted their focus toward NK cell function, particularly cytokine production, in relation to iRPL. Fukui et al. reported that the eNK cells of women with iRPL exhibited reduced production of IFN- γ and TNF- α compared to controls (49), while pNK cells of iRPL women produced higher IFN- γ and TNF- α alongside reduced levels of IL-4 and IL-10 (50). Interestingly, Mishra et al. found no significant differences in serum levels of IFN- γ and TNF- α levels between iRPL patients and controls, however reported significant increase in C-reactive protein (CRP), IL-1 β , and IL-4 levels suggesting role of inflammatory cytokines (51). Further research is required to elucidate the role of immune dysregulation in the pathogenesis of iRPL and its impact on pregnancy outcomes.

Endometriosis

Endometriosis is a chronic, estrogen dominant disease which is defined by the presence of endometrium-like tissues (lesions) outside uterus, affecting ~10% of women of reproductive age (52). The diagnosis is often delayed taking up to 7 years on average (53), and the condition presents with a wide range of symptoms, including dysmenorrhea, dyspareunia, cyclic and non-cyclic **pelvic pain**, and dyschezia, all of which significantly **impair quality of life** (54).

Although the exact origin of endometriosis remains unclear, several hypotheses have been proposed. The most widely accepted hypothesis is Sampson's retrograde menstruation theory which proposes that during menstruation menstrual debris containing viable cells flows back into the peritoneal cavity through fallopian tubes where these cells later implant and grow outside the uterus causing inflammatory

response, fibrosis, and pain (55). Even though retrograde menstruation occurs in many women, only some women develop endometriosis indicating that additional factors play role in the disease etiology. Subsequent studies demonstrated that while endometrial stromal cells in endometriosis patients do not harbor genetic mutations, they display distinct epigenetic alterations and subtle molecular abnormalities (56).

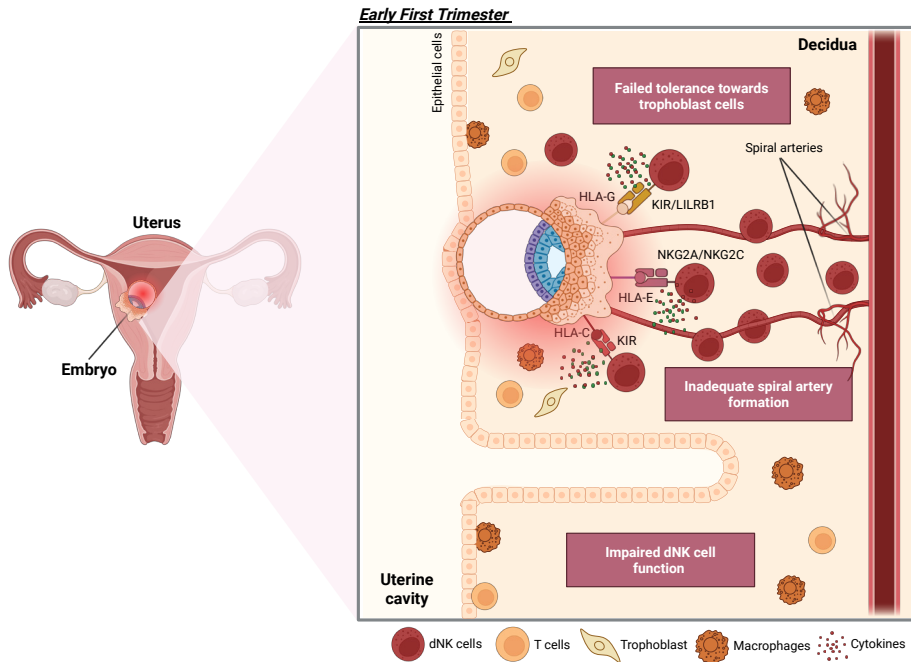


Figure 2. Disrupted maternal immune–trophoblast interactions in the decidua during early pregnancy. The left panel shows embryo implantation in the uterus. The right panel zooms into the decidual microenvironment, highlighting interactions between decidual NK (dNK) cells and invading trophoblasts. Trophoblast cells express HLA-C, HLA-E, and HLA-G molecules that interact with maternal immune receptors including KIR, NKG2A/C, and LILRB1 present on dNK cells. Impaired recognition or signaling through these pathways may result in defective dNK cell function, leading to inadequate spiral artery remodeling and poor placental development. The resulting immune imbalance characterized by altered cytokine secretion and disrupted tolerance can contribute to pathological pregnancy outcomes such as recurrent pregnancy loss. Key immune cells shown include dNK cells, T cells, and macrophages, as well as cytokine/chemokine signaling.

Another critical factor is the immune system’s ability, or failure, to recognize and clear the ectopic endometrial cells. It has been proposed that impaired immune surveillance allows endometrial cells to evade clearance, implant, and form persistent lesions. This impaired immune response contributes to a sustained inflammatory environment that not only supports lesion survival but also drives the

chronic pain and tissue remodeling characteristic of the disease (56, 57). Elevated levels of pro-inflammatory cytokines and chemokines such as IL-1 β , IL-6, IL-8, and TNF- α have been frequently observed in the peritoneal fluid of patients with endometriosis (56, 58). These cytokines facilitate recruitment of diverse immune cell populations into the peritoneal cavity, amplifying inflammation and promoting angiogenesis and lesion progression (Figure 3).

In individuals with endometriosis, macrophages are increased in number but display impaired phagocytic function in both the peritoneal fluid and eutopic endometrium (59, 60). The cytokines released by these dysfunctional macrophages promote the migration, invasion, and survival of endometrial cells, thereby facilitating lesion establishment and progression (59).

NK cells also play a critical role in the pathophysiology of endometriosis. In affected individuals, NK cells exhibit increased expression of inhibitory KIRs, leading to reduced cytotoxic activity and impaired clearance of ectopic endometrial tissue (61, 62). This compromised immune surveillance enables the implantation and proliferation of endometrial cells outside the uterine cavity.

Moreover, the immune-regulatory environment in endometriosis is altered, characterized by an increased accumulation of regulatory T cells (Tregs) in the peritoneal cavity and within endometrial lesions, further suppressing local immune responses (63). Furthermore, an imbalance between T-helper 1 (Th1) and Th2 immune responses has also been observed. Specifically, decreased Th1 cells and their associated cytokine IFN- γ , combined with elevated Th2 cytokines such as IL-4 and IL-10, contribute to an immunosuppressive and pro-growth microenvironment in endometriotic lesions (64).

The unmet clinical need for effective therapies in endometriosis and idiopathic RPL

Immune dysregulation and chronic inflammation significantly impact reproductive health in women, particularly in conditions like RPL and endometriosis. Despite growing recognition of the immune component in these disorders, there are currently no established clinical protocols that specifically target immune pathways. For women with iRPL, clinicians often cannot identify specific targets for therapy. As a result, patients receive empiric treatments with limited or unproven benefit (65). Commonly used treatment options for RPL are progesterone supplementation, low-dose aspirin, glucocorticoids (prednisone/prednisolone), and leukocyte immunotherapy. Unfortunately, these treatment options have not consistently led to improve-

ment in live birth rate or reduction in miscarriage rate in iRPL across controlled studies (66). This underscores an urgent need for new treatment strategies.

Current treatments for endometriosis largely manage symptoms rather than eradicate disease. First-line pharmacologic options for pain relief in endometriosis include nonsteroidal anti-inflammatory drugs (NSAIDs) and hormonal therapies (combined oral contraceptives, progestins, GnRH analogs/antagonists). However, efficacy for chronic pain is often lacking upon NSAIDs use (67); symptoms frequently recur after discontinuation (68), and response can be limited in advanced stages of the disease (69). When medical therapy fails or is not tolerated, surgery is considered. Even though surgery often provides positive outcomes in terms of pain relief, the recurrence rate remains a problem (70, 71).

These limitations have prompted interest in complementary strategies particularly lifestyle interventions such as regular **physical activity** and **dietary modification**. These approaches offer accessible, low-risk, non-medical means aimed at modulating inflammation and supporting immune homeostasis while enhancing agency in self-management.

Impact of lifestyle factors on the immune system and inflammation

Lifestyle factors, particularly **exercise and diet**, play a significant role in modulating immune function and inflammatory responses (Figure 4). Exercise, in particular, is hypothesized to reduce systemic inflammation and enhance immunosurveillance against pathogens and cancer cells (72). A growing body of evidence supports the association between regular physical activity and reduced levels of biomarkers indicative of **low-grade inflammation** (72, 73). Numerous studies have consistently reported that physically active individuals exhibit lower resting levels of pro-inflammatory biomarkers such as CRP, IL-6, and TNF- α . At the same time, they tend to have elevated levels of anti-inflammatory cytokines like IL-10 and IL-1 receptor antagonist (IL-1Ra), compared to their sedentary counterparts (74).

Acute bouts of exercise trigger the mobilization of various immune cells, particularly NK cells and CD8⁺ T cells (75). Interestingly, while lymphocyte counts initially rise during exercise, they typically decrease to below baseline level within 1-2 hours post-exercise before returning to normal within approximately 24 hours (76). Regarding NK cells, exercise predominantly redistributes mature CD56^{dim} NK cells characterized by lack of NKG2A expression and high expression of KIRs and CD57 (77). Similarly, exercise selectively mobilizes memory CD8⁺ T cells with effector functions to peripheral tissues, temporarily reducing their availability in peripheral blood, leading

to decreased IFN- γ production by these cells shortly after exercise (78). However, findings regarding lymphocyte dynamics post-exercise have been inconsistent, highlighting the importance of exercise intensity and duration in modulating immune responses. Notably, immune effects differ markedly between **acute high-intensity and chronic moderate-intensity exercise** (75).

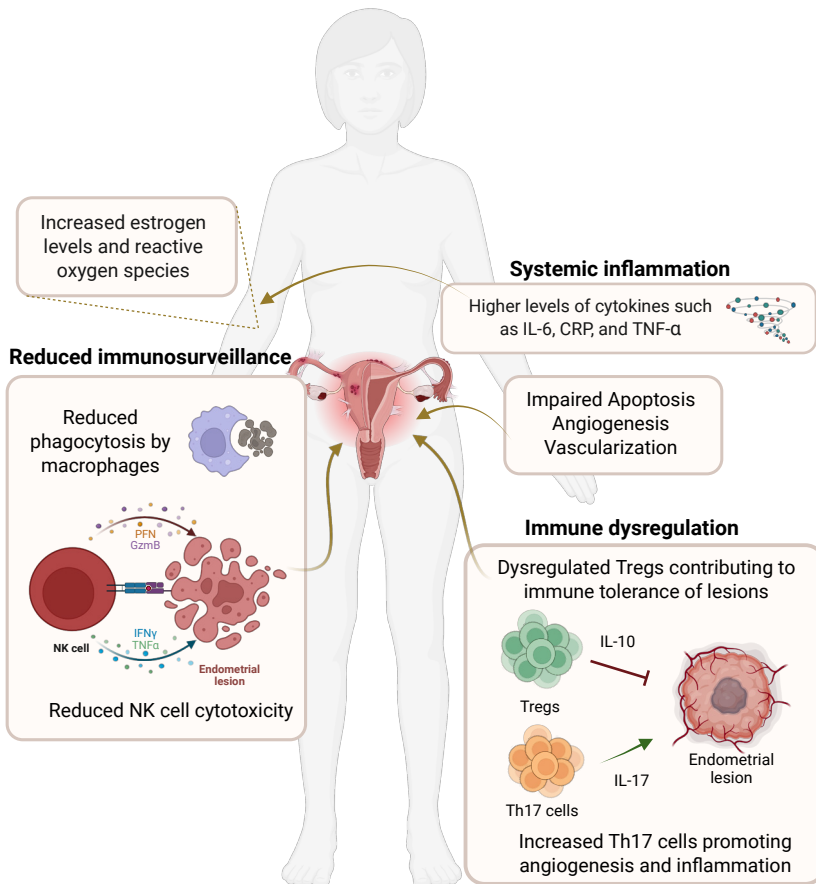


Figure 3. Immune mechanisms contributing to chronic inflammation and lesion growth in endometriosis. This figure illustrates key immune mechanisms contributing to the pathophysiology of endometriosis. Reduced immunosurveillance is observed due to diminished NK cell cytotoxicity (e.g., reduced secretion of perforin [PFN] and granzyme B [GzmB]) and impaired phagocytosis by macrophages, allowing endometriotic lesions to persist. The local microenvironment promotes immune dysregulation, characterized by an increase in Th17 cells and Tregs, which secrete IL-17 and IL-10 and contribute to both inflammation and aberrant angiogenesis. Estrogen (E2) stimulates the production of reactive oxygen species (ROS) and pro-inflammatory cytokines (e.g., IL-6, TNF- α , IFN- γ), contributing to a chronic inflammatory state. The resulting systemic inflammation is reflected by elevated levels of circulating inflammatory markers (e.g., CRP, IL-6, TNF- α) and impaired apoptosis, angiogenesis, and vascular remodeling.

Also dietary components play a crucial role in modulating inflammation. Polyunsaturated fatty acids (PUFAs), particularly omega-3 (*n*-3 PUFA) and omega-6 (*n*-6 PUFA), are essential macronutrients with distinct immunomodulatory properties. It was shown that inflammatory cells contain a high proportion of *n*-6 PUFAs and a low proportion of *n*-3 PUFAs (79). While *n*-6 PUFAs generally promote pro-inflammatory responses, *n*-3 PUFAs are associated with anti-inflammatory effects (80). *In vitro* studies have demonstrated that *n*-3 PUFAs can inhibit the production of IL-1 β and TNF- α by monocytes (81). Clinical studies investigating the supplementation of the diet with *n*-3 PUFAs containing food such as fish oil, have reported reduced production of TNF, IL-1 and IL-6 by immune cells in healthy individuals (82-84). However, despite evidence supporting the anti-inflammatory effects of omega-3 supplementation, definitive clinical recommendations for their routine use remain limited.

Dietary fiber represents another essential nutritional component with **anti-inflammatory** properties. Fiber-rich diets typically contain significant amounts of polyphenols and complex carbohydrates, both of which have been shown to reduce inflammation (85). Non-digestible carbohydrates are fermented by **gut microbiota** into short-chain fatty acids (SCFAs), which inhibit nuclear factor-kappa B (NF- κ B) activation and subsequently decrease the production of pro-inflammatory mediators like CRP, TNF- α , monocyte chemoattractant protein-1 (MCP-1), and IL-6 (86). Furthermore, fiber-rich foods increase antioxidant availability, contributing to improved immune function.

Polyphenols are especially noteworthy for their combined anti-inflammatory and antioxidant properties. They scavenge ROS and modulate key inflammatory signaling pathways, including NF- κ B, mitogen-activated protein kinase (MAPK), and cyclooxygenase (COX) pathways (87). Clinical trials have demonstrated that polyphenol supplementation can lower TNF- α and IL-6 levels, particularly in elderly individuals (88).

Given the complex interactions among various dietary components, evaluating overall dietary patterns, especially those rich in anti-inflammatory components including monounsaturated fats, flavonoids, vitamins C and E, and polyphenols might provide a more comprehensive understanding of the overall impact of the diet on immune regulation and inflammation (89, 90).

Despite growing evidence that dietary components and physical activity regulate inflammation and modulate immune function, these findings have yet to be effectively applied to inflammatory reproductive disorders such as endometriosis

and iRPL. In clinical practice, lifestyle-based interventions remain largely overlooked as therapeutic strategies in these contexts. This thesis aims to help bridge this gap by exploring whether lifestyle-based interventions can meaningfully influence immune cell profiles and improve clinical outcomes in affected women.

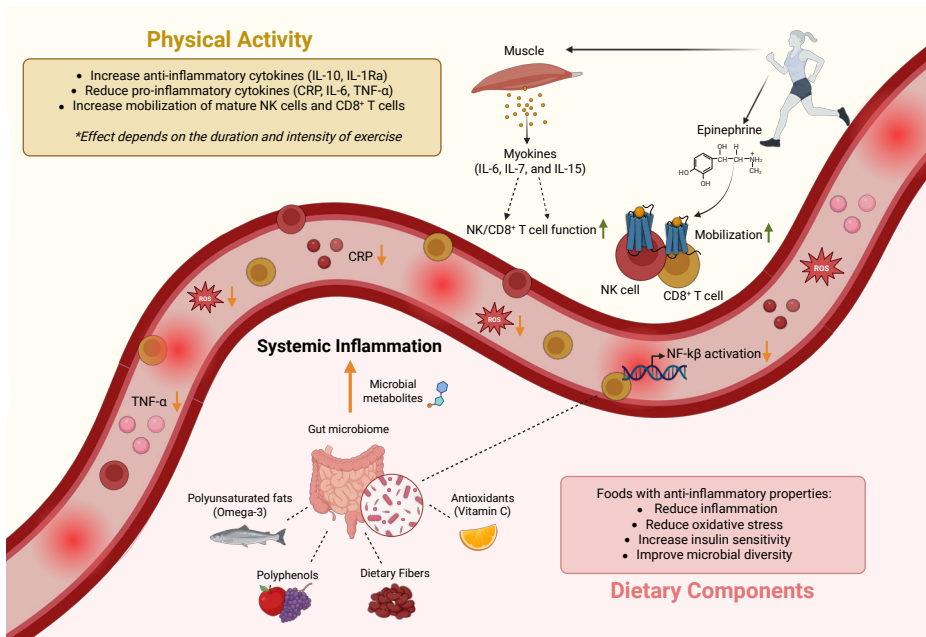


Figure 4. Modulation of systemic inflammation by physical activity and dietary components. Physical activity and specific dietary factors may reduce systemic inflammation through complementary pathways. Exercise promotes the release of myokines (e.g., IL-6, IL-7, IL-15) and epinephrine, which enhance mobilization and function of NK cells and CD8⁺ T cells, leading to increased immunosurveillance. Exercise may also reduce circulating pro-inflammatory cytokines (e.g., TNF-α, IL-6, CRP) and reactive oxygen species (ROS). Dietary components such as omega-3 polyunsaturated fats, polyphenols, dietary fibers, and antioxidants (e.g., vitamin C) modulate systemic inflammation by improving gut microbiome composition, increasing microbial metabolites, and suppressing oxidative stress while downregulating NF-κB activation, a key pro-inflammatory transcription factor. Collectively, these lifestyle interventions can support reduced inflammation, improve immune function, and metabolic health.

Scope and outline of the thesis

This thesis investigates the role of the systemic and uterine immune system in reproductive disorders, with a particular focus on endometriosis and iRPL. It begins by characterizing the immune landscape of both blood and the uterus in affected women, aiming to identify immunological mechanisms that may contribute to disease etiology. Building on these insights, the thesis examines whether

lifestyle interventions including moderate-intensity aerobic exercise and anti-inflammatory diet can modulate immune cell composition, inflammatory status, and clinical outcomes.

In **Chapter 2**, the impact of contraceptive use on the composition of uterine immune cells in healthy women is explored. The findings reveal differences in the immune cell composition between contraceptive users and non-users. In **Chapter 3**, the NK cell receptor (NKR) repertoire in women with iRPL is compared to that of control fertile women. This chapter shows distinct NKR repertoire in iRPL and highlights the importance of accounting for both previous live birth and cytomegalovirus (CMV) serostatus when profiling NK cells. **Chapter 4** outlines the study protocol investigating the multisystemic benefits of moderate-intensity aerobic exercise in women with iRPL. In **Chapter 5**, the impact of a 12-week moderate-intensity aerobic exercise intervention on the abundance, phenotype, and function of pNK and eNK cells in women with iRPL is presented. The findings underscore the distinct effects of exercise on the systemic versus uterine immune compartments. In **Chapter 6**, the role of JAK-STAT pathway in the inflammation and immune cell function of individuals with endometriosis is explored. We demonstrate that individuals with endometriosis have significantly higher STAT3 phosphorylation in their T cells upon stimulation with inflammatory cytokines. In **Chapter 7**, the impact of a 12-week anti-inflammatory diet intervention (AIDEN-intervention) on pain, inflammatory markers, gut and vaginal microbiome composition and both systemic and uterine immune cell composition in individuals with endometriosis is described. The results show that the intervention reduces pain and improves quality of life, while also altering the frequencies of unconventional T cells in the uterine microenvironment. Finally, **Chapter 8** provides an integrative discussion, highlighting overarching themes and offering concluding remarks.

Overall, this thesis demonstrates that lifestyle interventions such as diet and exercise can modulate immune function and inflammatory responses in women with reproductive disorders. By identifying accessible and effective strategies for immune modulation, this work contributes to the development of non-invasive approaches for improving reproductive outcomes in women with iRPL and enhancing the quality of life in individuals with endometriosis.

References

1. Critchley HOD, Maybin JA, Armstrong GM, Williams ARW. Physiology of the Endometrium and Regulation of Menstruation. *Physiol Rev.* 2020;100(3):1149-79.
2. Salamonsen LA, Lathbury LJ. Endometrial leukocytes and menstruation. *Hum Reprod Update.* 2000;6(1):16-27.
3. Okada H, Tsuzuki T, Murata H. Decidualization of the human endometrium. *Reprod Med Biol.* 2018;17(3):220-7.
4. Wetendorf M, DeMayo FJ. The progesterone receptor regulates implantation, decidualization, and glandular development via a complex paracrine signaling network. *Mol Cell Endocrinol.* 2012;357(1-2):108-18.
5. Vinketova K, Mourdjeva M, Oreshkova T. Human Decidual Stromal Cells as a Component of the Implantation Niche and a Modulator of Maternal Immunity. *J Pregnancy.* 2016;2016:8689436.
6. Mori M, Bogdan A, Balassa T, Csabai T, Szekeres-Bartho J. The decidua-the maternal bed embracing the embryo-maintains the pregnancy. *Semin Immunopathol.* 2016;38(6):635-49.
7. Salamonsen LA, Zhang J, Brasted M. Leukocyte networks and human endometrial remodelling. *J Reprod Immunol.* 2002;57(1-2):95-108.
8. Jones RL, Hannan NJ, Kaitu'u TJ, Zhang J, Salamonsen LA. Identification of chemokines important for leukocyte recruitment to the human endometrium at the times of embryo implantation and menstruation. *J Clin Endocrinol Metab.* 2004;89(12):6155-67.
9. Salamonsen LA, Woolley DE. Menstruation: induction by matrix metalloproteinases and inflammatory cells. *J Reprod Immunol.* 1999;44(1-2):1-27.
10. Finn CA. Implantation, menstruation and inflammation. *Biol Rev Camb Philos Soc.* 1986;61(4):313-28.
11. Seli E, Senturk LM, Bahtiyar OM, Kayisli UA, Arici A. Expression of aminopeptidase N in human endometrium and regulation of its activity by estrogen. *Fertil Steril.* 2001;75(6):1172-6.
12. Medawar PB. Some Immunological and Endocrinological Problems Raised by the Evolution of Viviparity in Vertebrates. *Sym Soc Exp Biol.* 1953;7:320-38.
13. Male V. Medawar and the immunological paradox of pregnancy: in context. *Oxf Open Immunol.* 2021;2(1):iqaa006.
14. Lee SK, Kim CJ, Kim DJ, Kang JH. Immune cells in the female reproductive tract. *Immune Netw.* 2015;15(1):16-26.
15. Flynn L, Byrne B, Carton J, Kelehan P, O'Herlihy C, O'Farrelly C. Menstrual cycle dependent fluctuations in NK and T-lymphocyte subsets from non-pregnant human endometrium. *Am J Reprod Immunol.* 2000;43(4):209-17.
16. Lee JY, Lee M, Lee SK. Role of endometrial immune cells in implantation. *Clin Exp Reprod Med.* 2011;38(3):119-25.
17. King A. Uterine leukocytes and decidualization. *Hum Reprod Update.* 2000;6(1):28-36.
18. van der Molen RG, Schutten JH, van Cranenbroek B, ter Meer M, Donckers J, Scholten RR, et al. Menstrual blood closely resembles the uterine immune micro-environment and is clearly distinct from peripheral blood. *Hum Reprod.* 2014;29(2):303-14.
19. Ivarsson MA, Stiglund N, Marquardt N, Westgren M, Gidlof S, Bjorkstrom NK. Composition and dynamics of the uterine NK cell KIR repertoire in menstrual blood. *Mucosal Immunol.* 2017;10(2):322-31.

20. Manaster I, Mizrahi S, Goldman-Wohl D, Sela HY, Stern-Ginossar N, Lankry D, et al. Endometrial NK cells are special immature cells that await pregnancy. *J Immunol*. 2008;181(3):1869-76.
21. Wira CR, Fahey JV, Rodriguez-Garcia M, Shen Z, Patel MV. Regulation of Mucosal Immunity in the Female Reproductive Tract: The Role of Sex Hormones in Immune Protection Against Sexually Transmitted Pathogens. *American Journal of Reproductive Immunology*. 2014;72(2):236-58.
22. Feyaerts D, Benner M, van Cranenbroek B, van der Heijden OWH, Joosten I, van der Molen RG. Human uterine lymphocytes acquire a more experienced and tolerogenic phenotype during pregnancy. *Scientific Reports*. 2017;7.
23. King A, Balendran N, Wooding P, Carter NP, Loke YW. CD3- leukocytes present in the human uterus during early placentation: phenotypic and morphologic characterization of the CD56++ population. *Dev Immunol*. 1991;1(3):169-90.
24. Vento-Tormo R, Efremova M, Botting RA, Turco MY, Vento-Tormo M, Meyer KB, et al. Single-cell reconstruction of the early maternal-fetal interface in humans. *Nature*. 2018;563(7731):347-+.
25. Heikkinen J, Mottonen M, Komi J, Alanen A, Lassila O. Phenotypic characterization of human decidual macrophages. *Clin Exp Immunol*. 2003;131(3):498-505.
26. McIntire RH, Hunt JS. Antigen presenting cells and HLA-G--a review. *Placenta*. 2005;26 Suppl A:S104-9.
27. Gustafsson C, Mjosberg J, Matussek A, Geffers R, Matthiesen L, Berg G, et al. Gene expression profiling of human decidual macrophages: evidence for immunosuppressive phenotype. *PLoS One*. 2008;3(4):e2078.
28. Engert S, Rieger L, Kapp M, Becker JC, Dietl J, Kammerer U. Profiling chemokines, cytokines and growth factors in human early pregnancy decidua by protein array. *Am J Reprod Immunol*. 2007;58(2):129-37.
29. Abrahams VM, Kim YM, Straszewski SL, Romero R, Mor G. Macrophages and apoptotic cell clearance during pregnancy. *Am J Reprod Immunol*. 2004;51(4):275-82.
30. Hamilton S, Oomomian Y, Stephen G, Shynlova O, Tower CL, Garrod A, et al. Macrophages infiltrate the human and rat decidua during term and preterm labor: evidence that decidual inflammation precedes labor. *Biol Reprod*. 2012;86(2):39.
31. Gomez-Lopez N, StLouis D, Lehr MA, Sanchez-Rodriguez EN, Arenas-Hernandez M. Immune cells in term and preterm labor. *Cell Mol Immunol*. 2014;11(6):571-81.
32. Moffett-King A. Natural killer cells and pregnancy. *Nat Rev Immunol*. 2002;2(9):656-63.
33. Bartmann C, Segerer SE, Rieger L, Kapp M, Sutterlin M, Kammerer U. Quantification of the predominant immune cell populations in decidua throughout human pregnancy. *Am J Reprod Immunol*. 2014;71(2):109-19.
34. Zhang J, Dunk CE, Kwan M, Jones RL, Harris LK, Keating S, et al. Human dNK cell function is differentially regulated by extrinsic cellular engagement and intrinsic activating receptors in first and second trimester pregnancy. *Cell Mol Immunol*. 2017;14(2):203-13.
35. Zhang JH, Lye SJ. The immune potential of decidua-resident CD16-CD56+ NK cells in human pregnancy. *Hum Immunol*. 2021;82(5):332-9.
36. Williams PJ, Searle RF, Robson SC, Innes BA, Bulmer JN. Decidual leucocyte populations in early to late gestation normal human pregnancy. *J Reprod Immunol*. 2009;82(1):24-31.
37. Tilburgs T, Evans JH, Crespo AC, Strominger JL. The HLA-G cycle provides for both NK tolerance and immunity at the maternal-fetal interface. *Proc Natl Acad Sci U S A*. 2015;112(43):13312-7.
38. Vento-Tormo R, Efremova M, Botting RA, Turco MY, Vento-Tormo M, Meyer KB, et al. Single-cell reconstruction of the early maternal-fetal interface in humans. *Nature*. 2018;563(7731):347-53.

39. Hanna J, Goldman-Wohl D, Hamani Y, Avraham I, Greenfield C, Natanson-Yaron S, et al. Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. *Nat Med.* 2006;12(9):1065-74.
40. Fraser R, Whitley GS, Johnstone AP, Host AJ, Sebire NJ, Thilaganathan B, et al. Impaired decidual natural killer cell regulation of vascular remodelling in early human pregnancies with high uterine artery resistance. *J Pathol.* 2012;228(3):322-32.
41. Saito S, Nakashima A, Shima T, Ito M. Th1/Th2/Th17 and regulatory T-cell paradigm in pregnancy. *Am J Reprod Immunol.* 2010;63(6):601-10.
42. Wang WJ, Liu FJ, Xin L, Hao CF, Bao HC, Qu QL, et al. Adoptive transfer of pregnancy-induced CD4+CD25+ regulatory T cells reverses the increase in abortion rate caused by interleukin 17 in the CBA/JxBALB/c mouse model. *Hum Reprod.* 2014;29(5):946-52.
43. RPL EGGo, Bender Atik R, Christiansen OB, Elson J, Kolte AM, Lewis S, et al. ESHRE guideline: recurrent pregnancy loss. *Hum Reprod Open.* 2018;2018(2):hoy004.
44. Turesheva A, Aimagambetova G, Ukybassova T, Marat A, Kanabekova P, Kaldygulova L, et al. Recurrent Pregnancy Loss Etiology, Risk Factors, Diagnosis, and Management. *Fresh Look into a Full Box. J Clin Med.* 2023;12(12).
45. Bagkou Dimakou D, Lissauer D, Tamblyn J, Coomarasamy A, Richter A. Understanding human immunity in idiopathic recurrent pregnancy loss. *Eur J Obstet Gynecol Reprod Biol.* 2022;270:17-29.
46. Seshadri S, Sunkara SK. Natural killer cells in female infertility and recurrent miscarriage: a systematic review and meta-analysis. *Hum Reprod Update.* 2014;20(3):429-38.
47. Tang AW, Alfirevic Z, Quenby S. Natural killer cells and pregnancy outcomes in women with recurrent miscarriage and infertility: a systematic review. *Hum Reprod.* 2011;26(8):1971-80.
48. Von Woon E, Greer O, Shah N, Nikolaou D, Johnson M, Male V. Number and function of uterine natural killer cells in recurrent miscarriage and implantation failure: a systematic review and meta-analysis. *Hum Reprod Update.* 2022;28(4):548-82.
49. Fukui A, Funamizu A, Fukuhara R, Shibahara H. Expression of natural cytotoxicity receptors and cytokine production on endometrial natural killer cells in women with recurrent pregnancy loss or implantation failure, and the expression of natural cytotoxicity receptors on peripheral blood natural killer cells in pregnant women with a history of recurrent pregnancy loss. *J Obstet Gynaecol Res.* 2017;43(11):1678-86.
50. Fukui A, Kwak-Kim J, Ntrivalas E, Gilman-Sachs A, Lee SK, Beaman K. Intracellular cytokine expression of peripheral blood natural killer cell subsets in women with recurrent spontaneous abortions and implantation failures. *Fertil Steril.* 2008;89(1):157-65.
51. Mishra S, Ashish A, Rai S, Sahni C, Tiwari S, Kumar B, et al. The Impact of Inflammatory Cytokines on Recurrent Pregnancy Loss: A Preliminary Investigation. *Reprod Sci.* 2025;32(3):804-14.
52. Giudice LC, Kao LC. Endometriosis. *Lancet.* 2004;364(9447):1789-99.
53. Hadfield R, Mardon H, Barlow D, Kennedy S. Delay in the diagnosis of endometriosis: a survey of women from the USA and the UK. *Hum Reprod.* 1996;11(4):878-80.
54. Rolla E. Endometriosis: advances and controversies in classification, pathogenesis, diagnosis, and treatment. *F1000Res.* 2019;8.
55. Sampson JA. Metastatic or Embolic Endometriosis, due to the Menstrual Dissemination of Endometrial Tissue into the Venous Circulation. *Am J Pathol.* 1927;3(2):93-110 43.
56. Bulun SE, Yilmaz BD, Sison C, Miyazaki K, Bernardi L, Liu S, et al. Endometriosis. *Endocr Rev.* 2019;40(4):1048-79.
57. Zondervan KT, Becker CM, Missmer SA. Endometriosis. *N Engl J Med.* 2020;382(13):1244-56.
58. Harada T, Iwabe T, Terakawa N. Role of cytokines in endometriosis. *Fertil Steril.* 2001;76(1):1-10.

59. Capobianco A, Rovere-Querini P. Endometriosis, a disease of the macrophage. *Front Immunol.* 2013;4:9.
60. Bacci M, Capobianco A, Monno A, Cottone L, Di Puppo F, Camisa B, et al. Macrophages are alternatively activated in patients with endometriosis and required for growth and vascularization of lesions in a mouse model of disease. *Am J Pathol.* 2009;175(2):547-56.
61. 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(10).
62. Vallve-Juanico J, Houshdaran S, Giudice LC. The endometrial immune environment of women with endometriosis. *Hum Reprod Update.* 2019;25(5):564-91.
63. Tanaka Y, Mori T, Ito F, Koshiba A, Takaoka O, Kataoka H, et al. Exacerbation of Endometriosis Due To Regulatory T-Cell Dysfunction. *J Clin Endocrinol Metab.* 2017;102(9):3206-17.
64. Podgaec S, Abrao MS, Dias JA, Jr., Rizzo LV, de Oliveira RM, Barakat EC. Endometriosis: an inflammatory disease with a Th2 immune response component. *Hum Reprod.* 2007;22(5):1373-9.
65. Matthiesen L, Kalkunte S, Sharma S. Multiple pregnancy failures: an immunological paradigm. *Am J Reprod Immunol.* 2012;67(4):334-40.
66. Lima J, Guerreiro J, Angelo-Dias M, Serra SS, Costa T, Marto N, et al. Efficacy of therapeutic interventions for idiopathic recurrent pregnancy loss: a systematic review and network meta-analysis. *Front Med (Lausanne).* 2025;12:1569819.
67. Brown J, Crawford TJ, Allen C, Hopewell S, Prentice A. Nonsteroidal anti-inflammatory drugs for pain in women with endometriosis. *Cochrane Database Syst Rev.* 2017;1(1):CD004753.
68. Becker CM, Gattrell WT, Gude K, Singh SS. Reevaluating response and failure of medical treatment of endometriosis: a systematic review. *Fertil Steril.* 2017;108(1):125-36.
69. Szubert M, Zietara M, Suzin J. Conservative treatment of deep infiltrating endometriosis: review of existing options. *Gynecol Endocrinol.* 2018;34(1):10-4.
70. Nirgianakis K, Ma L, McKinnon B, Mueller MD. Recurrence Patterns after Surgery in Patients with Different Endometriosis Subtypes: A Long-Term Hospital-Based Cohort Study. *J Clin Med.* 2020;9(2).
71. Guo SW. Recurrence of endometriosis and its control. *Hum Reprod Update.* 2009;15(4):441-61.
72. Gleeson M, Bishop NC, Stensel DJ, Lindley MR, Mastana SS, Nimmo MA. The anti-inflammatory effects of exercise: mechanisms and implications for the prevention and treatment of disease. *Nat Rev Immunol.* 2011;11(9):607-15.
73. Beavers KM, Brinkley TE, Nicklas BJ. Effect of exercise training on chronic inflammation. *Clin Chim Acta.* 2010;411(11-12):785-93.
74. Petersen AM, Pedersen BK. The anti-inflammatory effect of exercise. *J Appl Physiol (1985).* 2005;98(4):1154-62.
75. Walsh NP, Gleeson M, Shephard RJ, Gleeson M, Woods JA, Bishop NC, et al. Position statement. Part one: Immune function and exercise. *Exerc Immunol Rev.* 2011;17:6-63.
76. Pedersen BK, Hoffman-Goetz L. Exercise and the immune system: regulation, integration, and adaptation. *Physiol Rev.* 2000;80(3):1055-81.
77. Bigley AB, Rezvani K, Chew C, Sekine T, Pistillo M, Crucian B, et al. Acute exercise preferentially redeploys NK-cells with a highly-differentiated phenotype and augments cytotoxicity against lymphoma and multiple myeloma target cells. *Brain Behav Immun.* 2014;39:160-71.
78. Kruger K, Lechtermann A, Fobker M, Volker K, Mooren FC. Exercise-induced redistribution of T lymphocytes is regulated by adrenergic mechanisms. *Brain Behav Immun.* 2008;22(3):324-38.
79. Calder PC. n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. *Am J Clin Nutr.* 2006;83(6 Suppl):1505S-19S.

80. Calder PC. Omega-3 fatty acids and inflammatory processes: from molecules to man. *Biochem Soc Trans.* 2017;45(5):1105-15.
81. Chu AJ, Walton MA, Prasad JK, Seto A. Blockade by polyunsaturated n-3 fatty acids of endotoxin-induced monocytic tissue factor activation is mediated by the depressed receptor expression in THP-1 cells. *J Surg Res.* 1999;87(2):217-24.
82. Endres S, Ghorbani R, Kelley VE, Georgilis K, Lonnemann G, van der Meer JW, et al. The effect of dietary supplementation with n-3 polyunsaturated fatty acids on the synthesis of interleukin-1 and tumor necrosis factor by mononuclear cells. *N Engl J Med.* 1989;320(5):265-71.
83. Caughey GE, Mantzioris E, Gibson RA, Cleland LG, James MJ. The effect on human tumor necrosis factor alpha and interleukin 1 beta production of diets enriched in n-3 fatty acids from vegetable oil or fish oil. *Am J Clin Nutr.* 1996;63(1):116-22.
84. Trebble T, Arden NK, Stroud MA, Wootton SA, Burdge GC, Miles EA, et al. Inhibition of tumour necrosis factor-alpha and interleukin 6 production by mononuclear cells following dietary fish-oil supplementation in healthy men and response to antioxidant co-supplementation. *Br J Nutr.* 2003;90(2):405-12.
85. King DE, Egan BM, Woolson RF, Mainous AG, 3rd, Al-Solaiman Y, Jesri A. Effect of a high-fiber diet vs a fiber-supplemented diet on C-reactive protein level. *Arch Intern Med.* 2007;167(5):502-6.
86. Maslowski KM, Mackay CR. Diet, gut microbiota and immune responses. *Nat Immunol.* 2011;12(1):5-9.
87. Zhang H, Tsao R. Dietary polyphenols, oxidative stress and antioxidant and anti-inflammatory effects. *Curr Opin Food Sci.* 2016;8:33-42.
88. Yahfoufi N, Alsadi N, Jambi M, Matar C. The Immunomodulatory and Anti-Inflammatory Role of Polyphenols. *Nutrients.* 2018;10(11).
89. Giugliano D, Ceriello A, Esposito K. The effects of diet on inflammation: emphasis on the metabolic syndrome. *J Am Coll Cardiol.* 2006;48(4):677-85.
90. Schwingshackl L, Hoffmann G. Mediterranean dietary pattern, inflammation and endothelial function: a systematic review and meta-analysis of intervention trials. *Nutr Metab Cardiovasc Dis.* 2014;24(9):929-39.



Menstrual effluent derived immune cell composition is distinct in women using contraceptives: a pilot study

Aysel Gurbanova¹, Anne Stoverink², Imke M.B van Wandeloo¹, Annemiek Nap³, Nicole M. de Roos², Marien I. de Jonge¹, Janneke Hoogstad- van Evert⁴ and Renate G. van der Molen¹

¹ Department of Laboratory Medicine, Laboratory of Medical Immunology, Radboud University Medical Center, Nijmegen, The Netherlands

² Division of Human Nutrition and Health, Wageningen University, Wageningen, The Netherlands

³ Department of Obstetrics and Gynecology, Radboud University Medical Center, Nijmegen, The Netherlands

⁴ Department of Obstetrics and Gynecology, Amphia, Breda, The Netherlands

American Journal of Reproductive Immunology 94, no. 2 (2025): 94, e70145.

<https://doi.org/10.1111/aji.70145>

Abstract

Problem

Menstrual effluent (ME) is widely used to study immune-related reproductive disorders. However, women using contraceptives are often excluded from such studies due to limited knowledge of their impact on the endometrial immune cell composition. This gap in knowledge restricts our ability to include women using contraceptives in the scientific investigations and account for potential immunological variations associated with contraceptive use. Therefore, we aim to investigate whether contraceptive use is associated with differences on the uterine immune system.

Method of study

ME was collected from women using combined oral contraceptives (COC, n=5), a copper intrauterine device (IUD, n=3), or no contraceptives (n=5). Immune cells were isolated from ME and analysed using flow-cytometry. Data were analyzed using unpaired t-test, followed by multiple testing correction using the false discovery rate (FDR) method.

Results

Women using COCs exhibited a marked reduction in the frequency of the immunoregulatory CD56^{bright}CD16⁻ endometrial Natural Killer (eNK) cells compared to women using no contraceptives (30.1% $\pm$ 5.99 and 66.0% $\pm$ 10.6; p=0.001). Additionally, women using copper IUD showed elevated frequencies of proliferating Ki-67⁺CD8⁺ T cells compared to COC users and controls (19.8% $\pm$ 4.3, 2.69% $\pm$ 1.23, and 3.77% $\pm$ 4.4).

Conclusions

This pilot study highlights the importance of considering contraceptive use when studying immune-related reproductive problems.

Keywords

Menstrual effluent, endometrial immune cells, combined oral contraception, copper intra-uterine device, uterine immune system

Introduction

Menstrual effluent (ME) is a non-invasive source of endometrium-derived immune cells which closely resembles the immune cell composition and phenotypic characteristics of endometrial biopsies (1-3). Several studies have used ME-derived immune cells as a proxy for endometrial immune cell profiling to investigate reproductive problems such as recurrent pregnancy loss and endometriosis (2, 4). Notably, ME like endometrial biopsies contains a high proportion of CD56^{bright}CD16⁻ endometrial Natural Killer (NK) cells that are important for mucosal integrity, endometrial angiogenesis and endometrial shedding (5). Until now most studies using ME focused exclusively on women not using hormonal contraceptives or an intrauterine device (IUD). This is mainly due to the limited knowledge on how contraceptive use may influence the composition of immune cells in the endometrium. However, contraceptive use, especially combined oral contraceptives (COC), is widespread, particularly among women with endometriosis, for whom COC is often prescribed as first-line treatment (6).

COCs include a combination of artificial progesterone and estrogen which inhibit ovulation by preventing production and secretion of follicle stimulating hormone and luteinizing hormone from pituitary gland (7). When COCs are paused during hormone-free interval, withdrawal bleeding occurs as a response to the sudden drop in hormone levels. Estrogen and progesterone are known to have immunomodulatory functions playing a role in inflammation and immune response to infections (8-10). Previously, it was already shown that endometrial immune cells are responsive to hormonal changes (11). Additionally, endometrial transcriptional profiling of women using hormonal IUD was shown to differentially express genes related to inflammation and immune activation compared to women not using any contraceptives (12). These findings suggest that ME collected during a withdrawal bleeding from women using contraceptives and ME collected from women using a copper IUD may differ in immune composition from ME collected during natural cycles.

Therefore, the aim of this pilot study was to investigate the composition of ME-derived immune cells in healthy volunteers using COC, or copper IUD compared to controls not using any contraceptives. This will help determine whether ME remains a reliable proxy for endometrial immune assessment in women using contraceptives and inform future studies investigating endometrial immune cells using ME.

Materials & Methods

Study participants

In this study, women with a regular menstrual cycle, aged 18 until maximum reproductive age of 45 with no known fertility issues, immunological diseases or infectious diseases, using COC, a copper IUD or no medical contraception were compared. Women were excluded when they met one or more of the following exclusion criteria: active smoker, diagnosed with a gynecological condition, use of other hormonal medication or use of immunomodulating drugs. Women included in the control group, not using contraceptives, were only included if they had not been using COC or IUD for the last 6 months. The participants did not report any heavy menstrual bleeding or menstrual pain. COC use was stopped for 7 days to induce withdrawal bleeding. For clarity and consistency, we refer to this withdrawal bleeding as ME throughout the manuscript. This study was approved by the medical research ethics committee (committee on research involving human subjects) of the region of Arnhem-Nijmegen (file number CMO: NL8065209122). Informed consent was obtained from study participants before inclusion in the study. The cohort information including age, BMI, duration of contraceptive use, and number of previous pregnancies can be found in Table 1.

Table 1. Cohort characteristics

Donor characteristics	Control (n=5)	Oral contraceptive (n=5)	Copper IUD (n=3)
Type of contraceptive	NA	0,15 mg levonorgestrel and 0,03 mg ethinylestradiol (4/5)	NA
Age as median [range]	24 [24-40]	24 [22-27]	24 [23-34]
BMI (kg/m²) as median [range]	24.7 [19.8-28.7]	23.9 [20.5-29.4]	22.0 [18.7-23.9]
Duration of contraceptive use (in months) as median [range]	NA	84 [60-120]	11 [4-29]
Previous pregnancies	0/5	0/5	1/3

Sample collection

ME was collected from study participants over a period of 36 hours, in three 12-hour intervals, by means of a menstrual cup (AllMatters ApS, Copenhagen, Denmark) (1, 3, 4). Each 12 hour sample was transferred to ME collection tubes containing RPMI 1640 medium supplemented with sodium pyruvate [1 mM], GlutaMAX [2 mM], penicillin [100 U/mL], streptomycin [100 µg/mL] (all Thermo Fisher

Scientific, Waltham, MA, USA), 10% human pooled serum (HPS) (manufactured in-house) and 0.3% sodium citrate (Merck, Darmstadt, Germany).

Isolation of ME-derived mononuclear cells

ME-derived mononuclear cells (MMCs) were isolated from ME as previously described (3, 4). The volume of ME and the total number of cells isolated from ME were recorded. In brief, ME was filtered (70 μ m) to remove mucus, followed by granulocyte depletion using RosetteSep (StemCell technologies Inc, Vancouver, Canada) and density gradient centrifugation (Lymphoprep).

Flow cytometry

Immunophenotyping of MMCs was conducted with the Navios EX (Beckman Coulter, Fullerton, CA, USA) flow cytometry system. A minimum of 500,000 MMCs were stained using fluorochrome-conjugated monoclonal antibodies (moABs) for 30 minutes at 4°C in the dark. Immunophenotyping of cells was done for general immune cell lineage markers, cytokine expression and markers associated with NK cells, B cells, T cells (including regulatory T cells) and monocytes. An overview of used moABs can be found in Supplementary Table 1. Fixation and permeabilization of cells was performed according to manufacturer's instructions (eBioscience, San Diego, CA, USA). For intracellular cytokine detection MMCs were stimulated with phorbol 12-myristate 13-acetate (PMA) [12,5 ng/mL], Ionomycin [500 ng/mL] and Brefeldin-A [5 μ g/mL] for 4 hours at 37°C and subsequently intra-cellularly stained for IFN- γ .

Different flow cytometry panels were used to measure NK cells, T cells, B cells, monocytes and intracellular cytokines.

Data analysis

Flow cytometry data were analyzed and compensated using *Kaluza* software (Beckman Coulter Inc., Brea, CA). Immune cell subsets were manually gated, and their relative frequencies were extracted for statistical analysis. Gating strategies for immune cell subsets are described in Supplementary Figure 1 and 2. Data visualization and statistical testing were performed using the *ggplot2* package in RStudio. The percentages of NK, T, B cells and monocytes were derived from CD45⁺ lymphocytes. Group comparisons were conducted using unpaired *t*-tests, with multiple testing correction applied using the false discovery rate (FDR) method. Results were considered statistically significant at an adjusted *p*-value (FDR) < 0.05. Data are presented as percentages $\pm$ standard deviation (SD).

Results

The immune cell composition of ME is distinct in women using COC and IUD compared to women who do not use contraception

We first observed a trend toward a lower volume of ME collected in the COC group compared to the IUD and control groups. However, the total cell count per milliliter of ME was not reduced and even tended to be higher in the COC group (Figure 1A–B). Next, we examined the frequencies of CD45⁺ lymphocytes which did not differ between the three groups (Figure 1C). The distribution of immune cell subsets per sample was also comparable across groups (Supplementary Figure 3). A trend toward lower frequencies of CD14⁺HLA-DR⁺ monocytes was noted in IUD users compared to controls (Figure 1D). The frequency of CD19⁺CD20⁺ B cells was significantly higher in COC group compared to copper IUD users and controls ($14.1\% \pm 4.0$, $4.1\% \pm 1.3$ and $6.6\% \pm 3.0$; $p=0.032$ and $p=0.035$, respectively) (Figure 1E). Moreover, we observed a trend towards a reduced total CD56⁺CD3⁻ NK cell frequency in the COC group; however, this decrease was not significant (Figure 1F). We did not find any significant changes in CD56⁻CD3⁺ T cell frequencies (Figure 1G).

The frequency of CD56^{Bright}CD16⁻ endometrial NK cells is significantly decreased in COC users

Next, we analyzed specific subsets of ME-derived NK cells (Figure 2A). It is known that ME-derived NK cells mainly contain CD56^{bright}CD16⁻ endometrial NK (eNK) cells with immunoregulatory functions (5). The analysis of the NK cell subsets showed significantly low abundance of the CD56^{bright}CD16⁻ eNK cells in ME of COC users compared to controls ($30.1\% \pm 5.99$ and $66.0\% \pm 10.6$; $p=0.001$) (Figure 2B). In contrast, these women had higher frequencies of CD56^{dim}CD16⁺ NK cells (Figure 2C). For IUD users a similar trend was observed. Additionally, CD56^{bright}CD16⁻:CD56^{dim}CD16⁺ ratios were significantly lower in COC and IUD users compared to controls (0.81 ± 0.34 , 0.88 ± 0.28 and 5.6 ± 2.64 respectively).

The frequency of KI-67⁺CD8⁺ cytotoxic T cells is elevated in IUD users

No significant differences in the frequency of endometrial T cell subsets among the three groups were observed (Supplementary Figure 4A). However, analysis of the proliferation status of endometrial T cells with proliferation marker Ki-67 (Figure 2D), showed increased percentage of Ki-67⁺ cytotoxic CD8⁺ T cells in IUD users compared to COC users and controls ($19.8\% \pm 4.3$, $2.69\% \pm 1.23$, and $3.77\% \pm 4.4$ respectively) (Figure 2E). We did not observe this increase for Ki-67⁺ helper CD4⁺ T cells (Figure 2F).

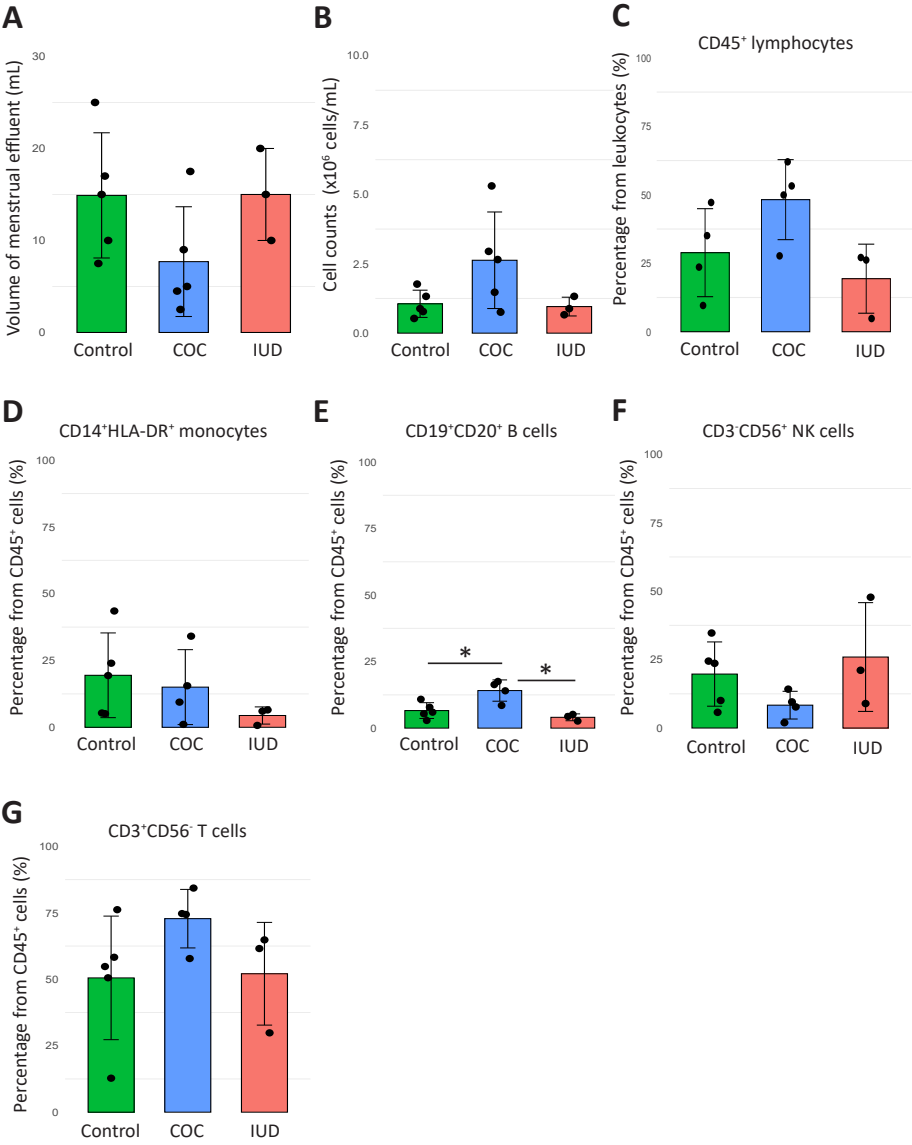


Figure 1. The immune cell composition of menstrual effluent in women using either combined oral contraceptive (COC) or a copper intra-uterine device (IUD), and controls. A) Bar plots indicating volume of ME in controls (n=5, green), combined oral contraceptive (COC, n=5, blue), and copper intrauterine device users (IUD, n=3, red). B) Bar plots indicating total cell counts isolated from ME indicated as $\times 10^6$ cells/mL of ME. C) The bar plot represent the percentages of CD45⁺ lymphocytes in controls (green), COC (blue), and copper IUD (red) users. D) Bar plots indicating frequencies of monocytes, E) B cells, F) NK cells, and G) T cells. For statistical analysis, unpaired t-test and FDR correction have been performed (* <0.05 , ** <0.005).

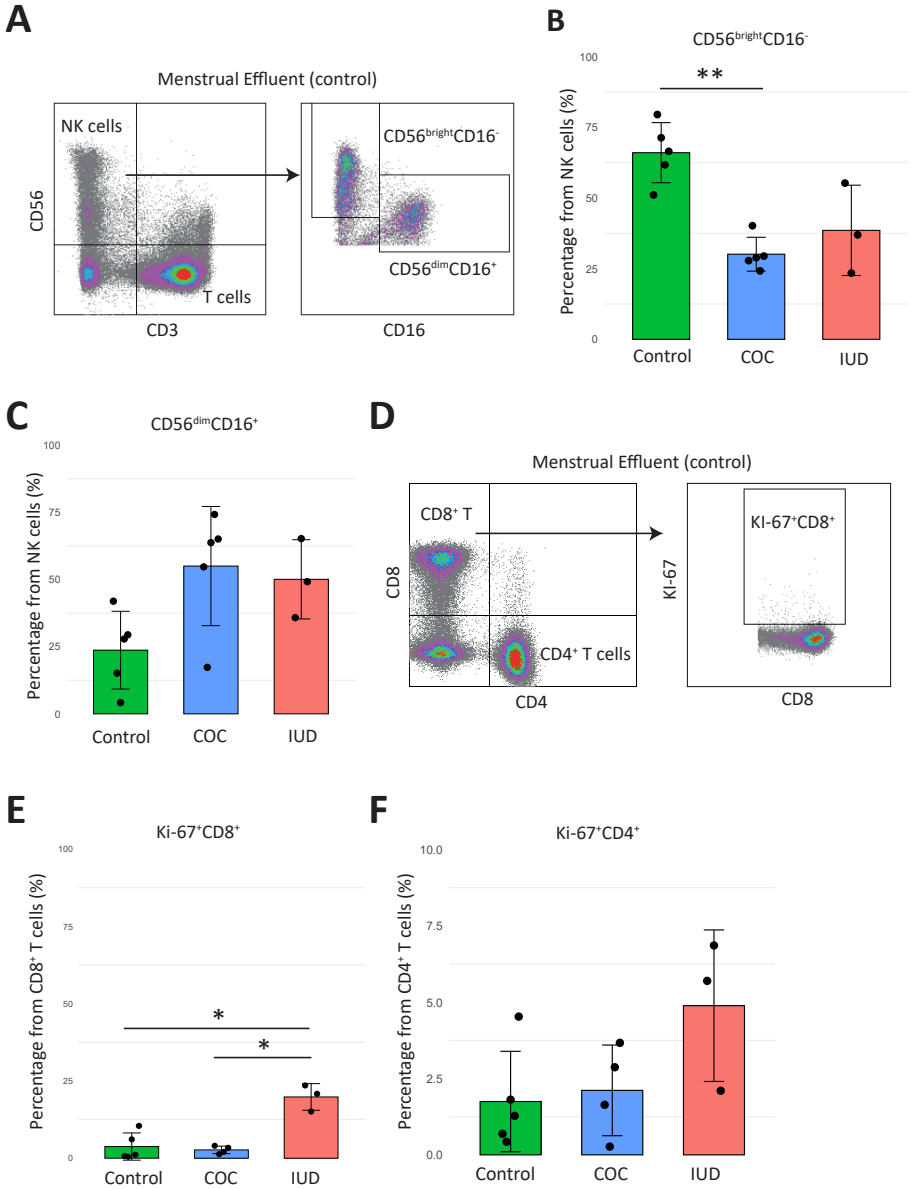


Figure 2. The frequency of endometrial NK cells of menstrual effluent decreases significantly in combined oral contraceptive (COC) users. A) Dot plot representing gating strategy for NK cell subsets in ME of control. B) Bar plot of endometrial NK cell percentages, also defined as CD56^{bright}CD16⁻, and C) CD56^{dim}CD16⁺ NK cells in controls (n=5, green), combined oral contraceptive (COC, n=5, blue), and copper intrauterine device (IUD, n=3, red). D) Dot plot representing gating strategy for proliferating Ki-67⁺CD8⁺ T cells in ME of control. E) Bar plots of Ki67⁺CD8⁺ cytotoxic T cell and F) Ki67⁺CD4⁺ effector T cell percentages in each group. For statistical analysis, unpaired t-test and FDR correction have been performed (*<0.05, **<0.005).

Discussion

In this pilot study, we explored the association between contraceptive use and endometrial immune cell composition by analyzing ME from women using COCs, copper IUD, and those not using any contraception. We observed differences in immune cell subsets across all groups, including both hormonal and non-hormonal contraceptive users. These preliminary findings suggest that contraceptive use and the method of contraception may differently influence the endometrial immune cell composition.

The human menstrual cycle is tightly regulated by ovarian sex hormones, primarily estrogen and progesterone (13). During the proliferative phase, estradiol promotes the cellular proliferation of endometrial tissue (14). In the subsequent secretory phase, progesterone induces the transformation of both endometrial stromal and immune cells into special decidual cells creating a receptive environment for embryo implantation and placentation (15). In the absence of pregnancy, the withdrawal of estrogen and progesterone triggers menstruation (16). This physiological process is altered in women using COCs due to the continuous presence of synthetic estrogen and progesterone. The bleeding is only induced upon cessation of COC, known as withdrawal bleeding. The impact of these synthetic hormones on the immune cells and immune cell infiltration during endometrial shedding remains poorly understood, making it challenging to interpret the ME from COC users as a reliable surrogate for endometrial immune cell composition.

Progesterone, plays a critical role in regulating immune cell infiltration in the uterus, among which the immunoregulatory CD56^{bright}CD16⁻ eNK cells are the most abundant (11, 17). eNK cells sequentially express surface markers representing recurrent cycle of differentiation in response to progesterone-mediated release of stromal IL-15 (11). The frequencies of eNK cells fluctuate throughout the menstrual cycle, peaking during the late secretory phase and persisting throughout pregnancy (18). Moreover, they are known to be involved in the establishment and maintenance of a successful pregnancy (19, 20). In our study, we observed a significant reduction in the frequency of CD56^{bright}CD16⁻ eNK cells in the ME of women using COCs. Bister et al. also showed significantly reduced frequencies of eNK cells expressing CD9 in the ME of COC users (21). This reduction may be either due to the downstream effects of synthetic hormones on the endometrial tissue development or the altered hormone withdrawal pattern affecting the immune cell recruitment to the uterus during endometrial shedding.

Previously, analysis of endometrial transcriptomes of endometrial biopsies showed significant differences upon hormonal IUD use, while endometrial transcriptomes of women using copper IUD did not show significant differences (12). We found increased percentage of proliferating cytotoxic Ki-67⁺CD8⁺ T cells in ME samples of copper IUD users, likely due to the influence of copper ions on the function and proliferation of immune cells (22). The frequency of Ki-67⁺CD8⁺ T cells was found to be higher in inflamed tissues (23). Since this increase was not observed in COC users, our findings suggest that the copper IUD exerts a distinct and potentially more inflammatory effect on endometrial immune cells compared to COCs, indicating that hormonal and non-hormonal contraceptive methods may differentially influence the endometrial immune cells.

The main limitation of our study is the small number of ME samples obtained across the three groups. Recruitment of COC users proved challenging, as participation required discontinuation of COC use for at least one week to allow withdrawal bleeding. Inclusion of IUD users was also limited due to the potential risk of IUD expulsion associated with ME collection (24). Despite the small sample size, our data revealed clear group-specific differences in immune cell composition. A second limitation was the low cell yield in ME samples, particularly from COC users, likely due to reduced bleeding volume. This limited the statistical power to detect differences in less abundant immune cell subsets. Additionally, a longitudinal approach such as following women before and after initiating contraception would have been more informative. However, this design proved difficult to implement, as participants using contraception were often reluctant to discontinue it, while those not using contraception were generally unwilling to start. Lastly, we did not assess the presence of infections in participants, which could have influenced the immune cell composition. Future studies should account for potential underlying infections when interpreting immune profiles in ME. Despite these limitations, our findings suggest that contraceptive use is associated with changes in endometrial immune cell composition and this influence varies depending on the type of contraception.

In conclusion, our study highlights the importance of considering contraceptive use when investigating ME for immune-related reproductive health problems. ME may not be a reliable surrogate for assessing endometrial immune composition in contraceptive users. Further research is needed to better understand the underlying mechanisms of these contraceptive-related immune changes and their potential implications for women's overall health and fertility.

Conflict of Interest

The 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.

Author contributions

AG was involved in the data collection, data analysis, and manuscript writing. AS was involved in the inclusion of the study participants and manuscript writing. IW was involved in the sample processing, data analysis and manuscript writing. JHvE, AN, NR, MIdJ and RGvdM were involved in providing critical feedback in the study design, data analysis and the manuscript preparation.

Funding

No funding was obtained for this study.

Acknowledgements

I would like to thank to all the women who participated in the study and Janeri Fröberg, for help with visualization of the data.

Data Availability Statement

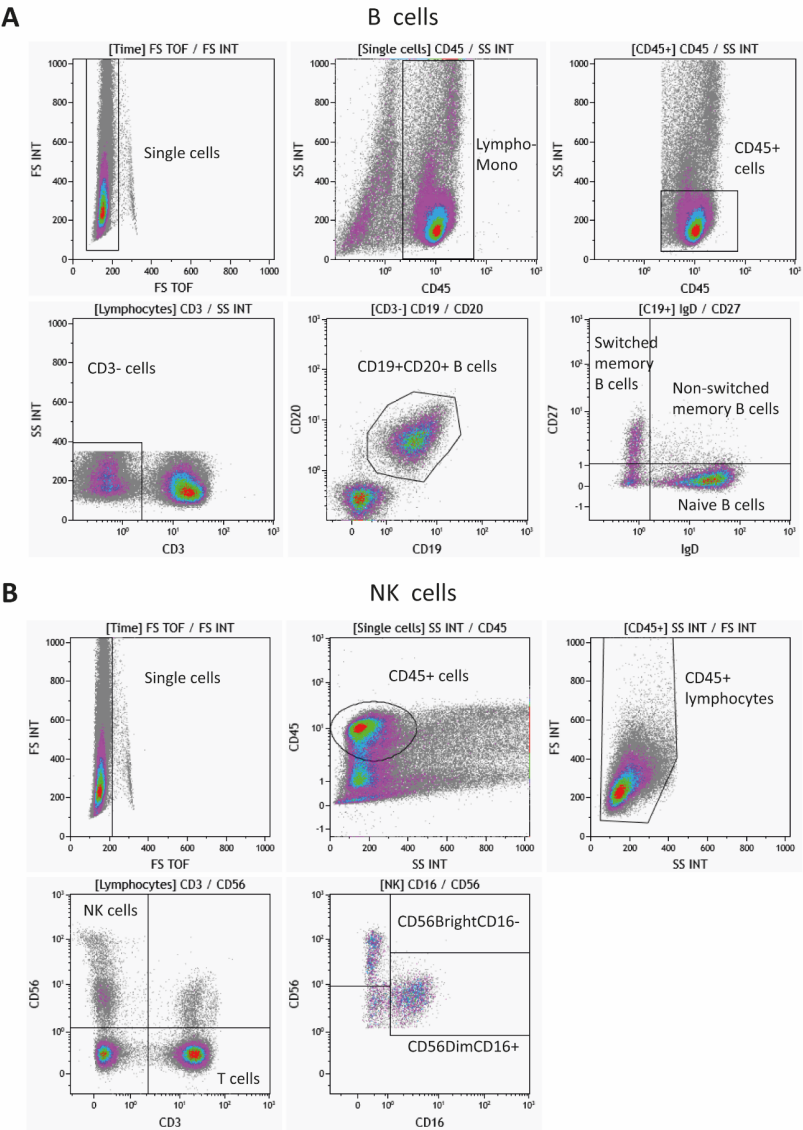
The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

References

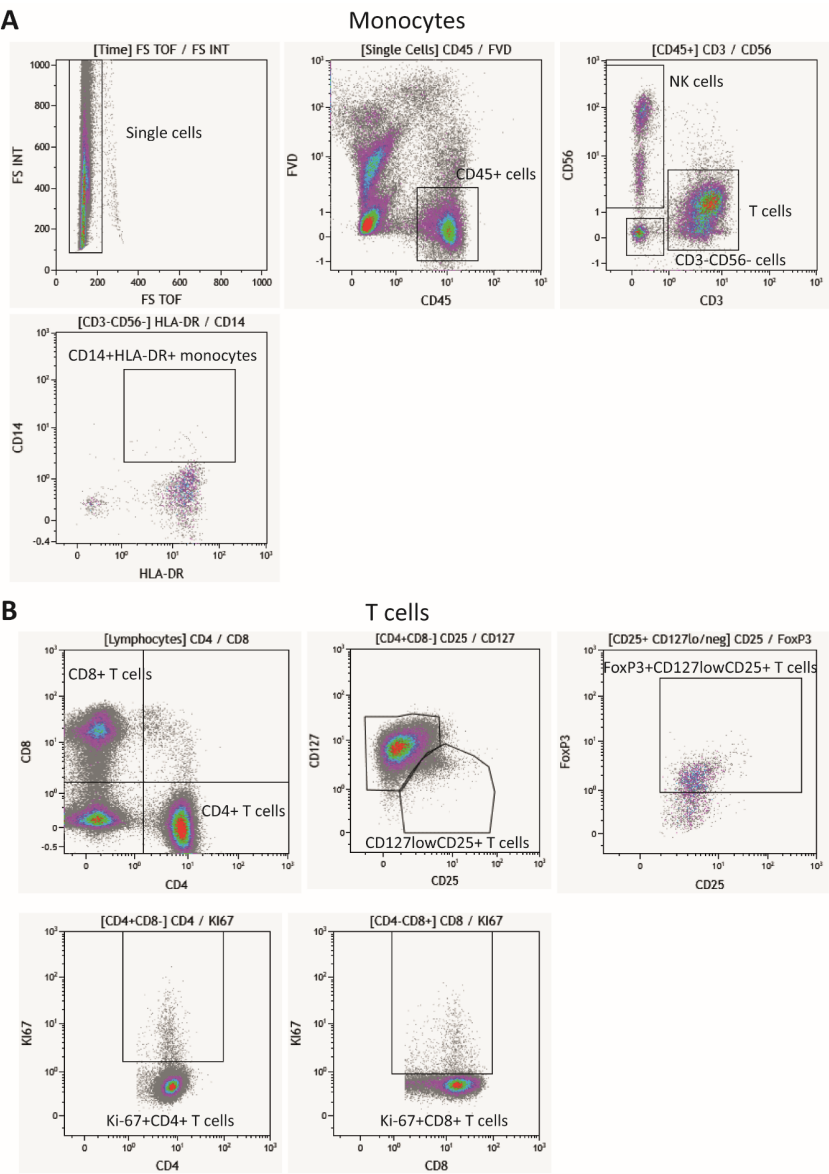
1. van der Molen RG, Schutten JH, van Cranenbroek B, ter Meer M, Donckers J, Scholten RR, et al. Menstrual blood closely resembles the uterine immune micro-environment and is clearly distinct from peripheral blood. *Hum Reprod.* 2014;29(2):303-14.
2. Vomstein K, Egerup P, Kolte AM, Behrendt-Moller I, Boje AD, Bertelsen ML, et al. Biopsy-free profiling of the uterine immune system in patients with recurrent pregnancy loss and unexplained infertility. *Reprod Biomed Online.* 2023;47(2).
3. Feyaerts D, Kuret T, van Cranenbroek B, van der Zeeuw-Hingrez S, van der Heijden OWH, van der Meer A, et al. Endometrial natural killer (NK) cells reveal a tissue-specific receptor repertoire. *Hum Reprod.* 2018;33(3):441-51.
4. Benner M, Feyaerts D, Lopez-Rincon A, van der Heijden OWH, van der Hoorn ML, Joosten I, et al. A combination of immune cell types identified through ensemble machine learning strategy detects altered profile in recurrent pregnancy loss: a pilot study. *F S Sci.* 2022;3(2):166-73.
5. Gaynor LM, Colucci F. Uterine Natural Killer Cells: Functional Distinctions and influence on Pregnancy in Humans and Mice. *Frontiers in Immunology.* 2017;8.
6. Maia HJ, Casoy J. Non-contraceptive health benefits of oral contraceptives. *Eur J Contracept Reprod Health Care.* 2008;13(1):17-24.
7. Rivera R, Yacobson I, Grimes D. The mechanism of action of hormonal contraceptives and intrauterine contraceptive devices. *Am J Obstet Gynecol.* 1999;181(5 Pt 1):1263-9.
8. Hoffmann JP, Liu JA, Seddu K, Klein SL. Sex hormone signaling and regulation of immune function. *Immunity.* 2023;56(11):2472-91.
9. Harding AT, Heaton NS. The Impact of Estrogens and Their Receptors on Immunity and Inflammation during Infection. *Cancers (Basel).* 2022;14(4).
10. Hall OJ, Klein SL. Progesterone-based compounds affect immune responses and susceptibility to infections at diverse mucosal sites. *Mucosal Immunol.* 2017;10(5):1097-107.
11. Strunz B, Bister J, Jonsson H, Filipovic I, Crona-Guterstam Y, Kvedaraite E, et al. Continuous human uterine NK cell differentiation in response to endometrial regeneration and pregnancy. *Sci Immunol.* 2021;6(56).
12. Smith-McCune K, Thomas R, Averbach S, Seidman D, Takeda M, Houshdaran S, et al. Differential Effects of the Hormonal and Copper Intrauterine Device on the Endometrial Transcriptome. *Scientific Reports.* 2020;10(1):6888.
13. Critchley HOD, Maybin JA, Armstrong GM, Williams ARW. Physiology of the Endometrium and Regulation of Menstruation. *Physiol Rev.* 2020;100(3):1149-79.
14. Maybin JA, Critchley HO. Menstrual physiology: implications for endometrial pathology and beyond. *Hum Reprod Update.* 2015;21(6):748-61.
15. Gellersen B, Brosens JJ. Cyclic decidualization of the human endometrium in reproductive health and failure. *Endocr Rev.* 2014;35(6):851-905.
16. Salamonsen LA. Tissue injury and repair in the female human reproductive tract. *Reproduction.* 2003;125(3):301-11.
17. Motomura K, Miller D, Galaz J, Liu TN, Romero R, Gomez-Lopez N. The effects of progesterone on immune cellular function at the maternal-fetal interface and in maternal circulation. *J Steroid Biochem.* 2023;229.
18. Kanter JR, Mani S, Gordon SM, Mainigi M. Uterine natural killer cell biology and role in early pregnancy establishment and outcomes. *F S Rev.* 2021;2(4):265-86.
19. Xie M, Li Y, Meng YZ, Xu P, Yang YG, Dong S, et al. Uterine Natural Killer Cells: A Rising Star in Human Pregnancy Regulation. *Front Immunol.* 2022;13:918550.
20. Sfakianoudis K, Rapani A, Grigoriadis S, Pantou A, Maziotis E, Kokkini G, et al. The Role of Uterine Natural Killer Cells on Recurrent Miscarriage and Recurrent Implantation Failure: From Pathophysiology to Treatment. *Biomedicines.* 2021;9(10).

21. Bister J, Filipovic I, Sun D, Crona-Guterstam Y, Cornillet M, Ponzetta A, et al. Tissue-specific nonheritable influences drive endometrial immune system variation. *Sci Immunol*. 2024;9(94):eadj7168.
22. He F, Chang C, Liu B, Li Z, Li H, Cai N, et al. Copper (II) Ions Activate Ligand-Independent Receptor Tyrosine Kinase (RTK) Signaling Pathway. *Biomed Res Int*. 2019;2019:4158415.
23. Blessin NC, Abu-Hashem R, Mandelkow T, Li W, Simon R, Hube-Magg C, et al. Prevalence of proliferating CD8(+) cells in normal lymphatic tissues, inflammation and cancer. *Aging (Albany NY)*. 2021;13(11):14590-603.
24. Bowman N, Thwaites A. Menstrual cup and risk of IUD expulsion - a systematic review(vol 8,15,2023). *Contracep Reprod Med*. 2023;8(1).

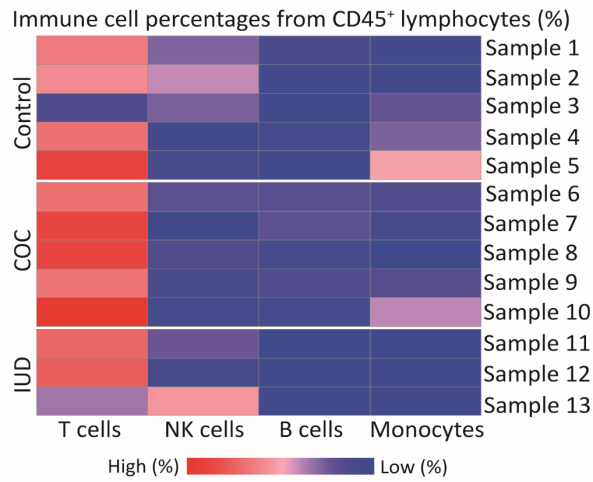
Supplementary Material



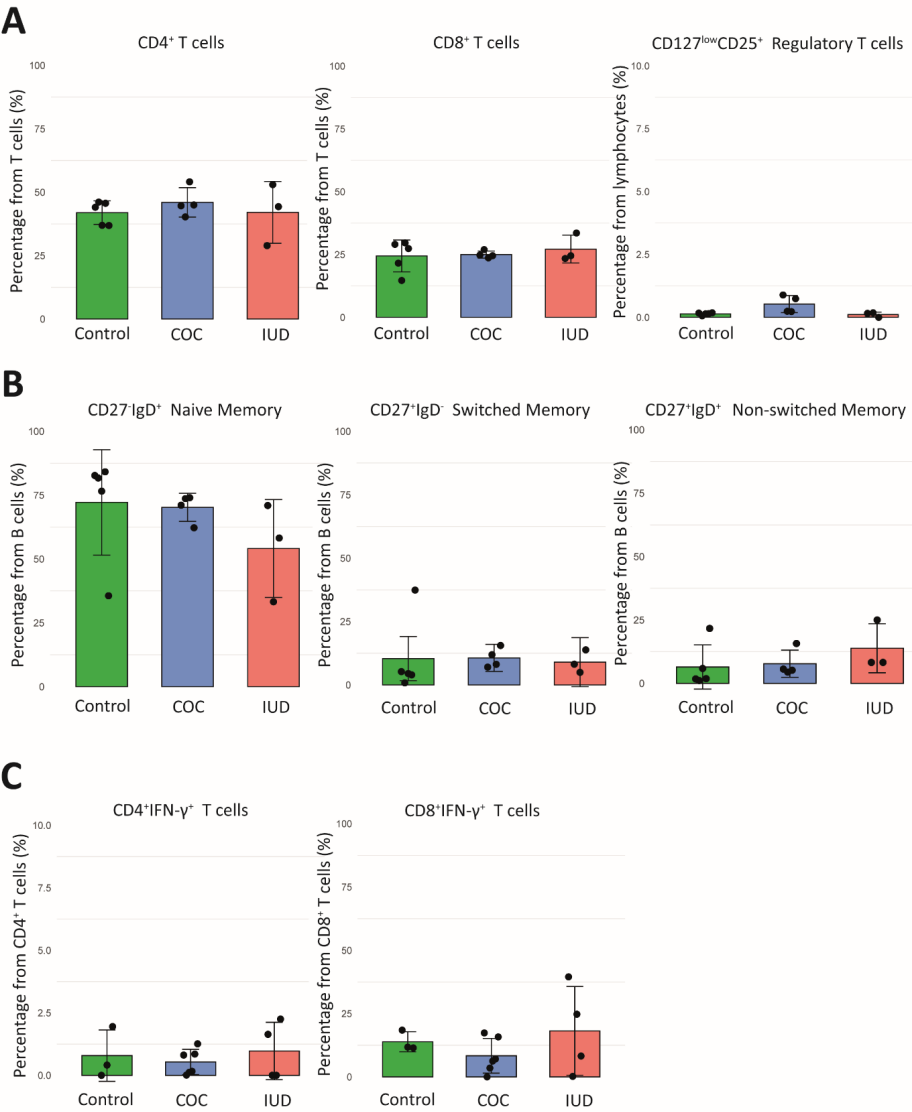
Supplementary Figure 1. Gating strategies for B cells and NK cells in menstrual effluent.



Supplementary Figure 2. Gating strategies for Monocytes and T cells in menstrual effluent.



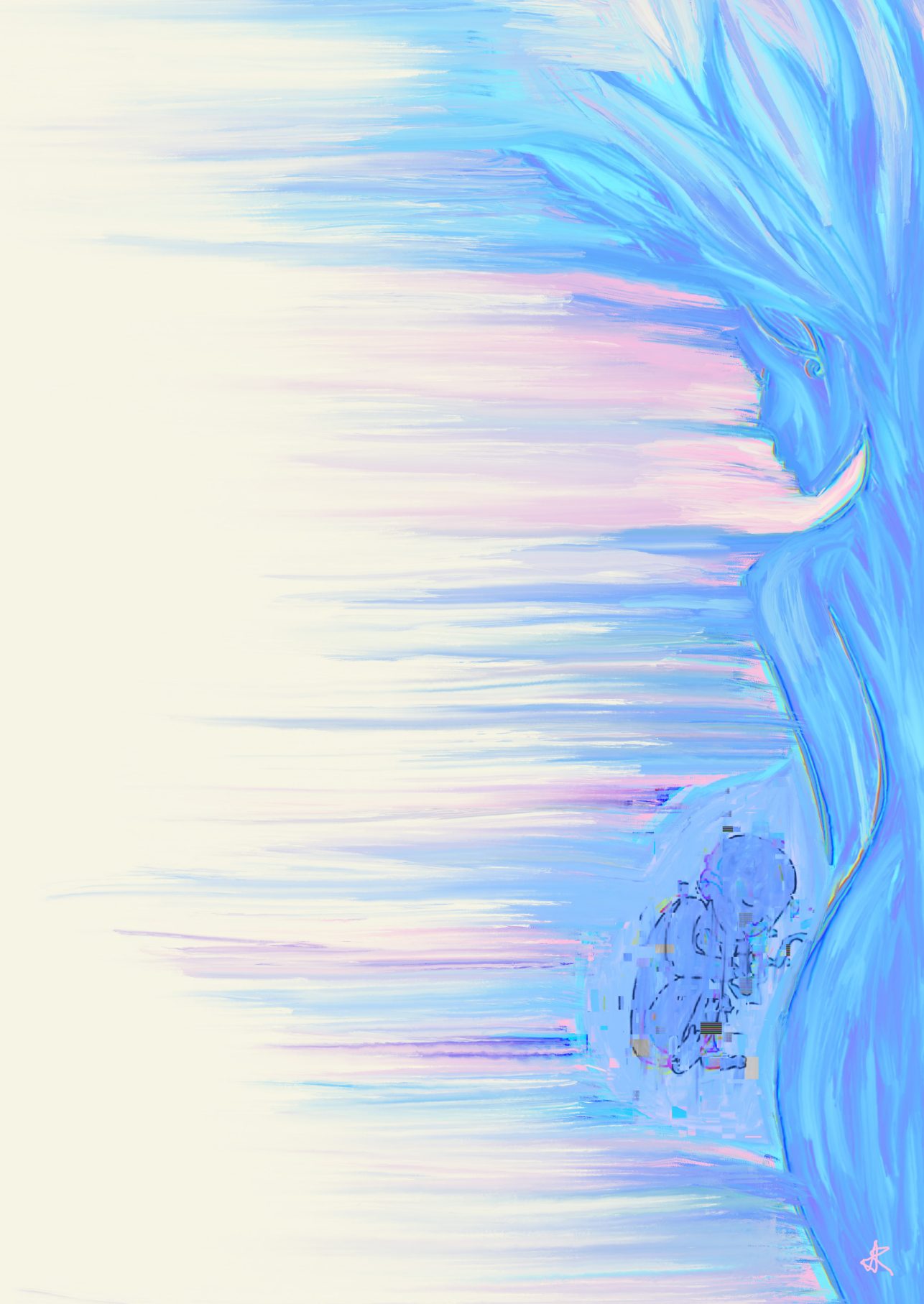
Supplementary Figure 3. Heatmap representing percentages of immune cell subsets from CD45⁺ lymphocytes for each donor (controls, n=5; COC, n=5; copper IUD, n=3) (red-high percentage, blue-low percentage).



Supplementary Figure 4. The immune cell composition of menstrual effluent in women using either combined oral contraceptive (COC) or intra-uterine device (IUD), and controls. A) Bar plots indicating percentages of helper (CD3⁺CD8⁺CD4⁺), cytotoxic (CD3⁺CD8⁺CD4⁺) and regulatory (CD3⁺CD4⁺CD127^{low}CD25^{high}FoxP3⁺) T cells in controls (n=5, green), combined oral contraceptive (COC, n=5, blue), and copper intrauterine device (IUD, n=3, red). B) Bar plots indicating percentages of naïve (CD19⁺CD27⁺IgD⁺), switched-memory (CD19⁺CD27⁺IgD⁺) and non-switched memory (CD19⁺CD27⁺IgD⁺) B cells. C) Bar plots of IFN- γ producing CD4⁺ helper T cell and CD8⁺ cytotoxic T cell percentages in each group. For statistical analysis, unpaired t-test and FDR correction have been performed (*<0.05, **<0.005).

Supplementary Table 1. List of monoclonal antibodies used for flow cytometry.

Fluorochrome	Antigen	Company	Catalog number
FITC	CD16	Beckman Coulter	B49215
PE	HLA-DR	Beckman Coulter	IM1639
ECD	CD14	Beckman Coulter	B92391
PE-Cy5.5	CD4	Beckman Coulter	B16491
PC7	CD25	BD Biosciences	557741
APC	CD56	Beckman Coulter	IM2474
APC-AF700	CD8	Beckman Coulter	B49181
APC-AF750	CD19	Beckman Coulter	A94681
PB	CD3	Beckman Coulter	B49204
KO	CD45	Beckman Coulter	B36294
FITC	IgD	Southern Biotec	2032-02
PE	IgM	Beckman Coulter	B30657
ECD	CD3	Beckman Coulter	A07748
PE-Cy5.5	CD27	Beckman Coulter	B21444
PC7	CD38	Beckman Coulter	B49198
APC	CD24	Beckman Coulter	A87785
APC-AF700	CD5	Beckman Coulter	A78835
APC-AF750	CD19	Beckman Coulter	A94681
PB	CD20	Beckman Coulter	B49208
KO	CD45	Beckman Coulter	B36294
PE	HLA-DR	Beckman Coulter	IM1639
ECD	CD45RA	Beckman Coulter	B49193
PE-Cy5.5	CD4	Beckman Coulter	B16491
PC7	CD25	BD Biosciences	557741
APC-AF700	CD127	Beckman Coulter	A71116
APC-AF750	CD8	Beckman Coulter	A94683
KO	CD45	Beckman Coulter	B36294
AF488	KI67 (ic)	BD Biosciences	561165
AF647	Helios (ic)	Biolegend	137218
e450	FoxP3 (ic)	eBioscience	48-4776-42
ECD	CD3	Beckman Coulter	A07748
PE-Cy5.5	CD4	Beckman Coulter	B16491
APC	CD161	Miltenyi	130-092-678
APC-AF700	CD8	Beckman Coulter	B49181
KO	CD45	Beckman Coulter	B36294
PC7	IFN- γ (ic)	eBioscience	25-7319-82
FITC	CD16	Beckman Coulter	IM0814U
ECD	CD56	Beckman Coulter	A82943
APC-AF750	CD3	Beckman Coulter	A94680



Profiling NK cell receptor repertoire of women with idiopathic recurrent pregnancy loss

Aysel Gurbanova^{1#}, Dorien Feyaerts^{1,2#}, Marilen Benner¹, Tess Meuleman³, Marie-Louise van der Hoorn⁴, Irma Joosten¹, Lotte Wieten⁵, Marien I. de Jonge¹ and Renate G van der Molen¹

¹ Department of Laboratory Medicine, Laboratory of Medical Immunology, Radboud University Medical Center, Nijmegen, The Netherlands

² Department of Anesthesiology, Perioperative and Pain Medicine, Stanford University School of Medicine, Stanford, CA, USA

³ Department of Obstetrics and Gynaecology, Radboud University Medical Center, Nijmegen, The Netherlands

⁴ Department of Obstetrics and Gynaecology, Leiden University Medical Center, Leiden, The Netherlands

⁵ Department of Transplantation Immunology, Maastricht University Medical Center+, Maastricht, The Netherlands

These authors contributed equally

Published in Journal of Reproductive Immunology, 2025 Nov 19;173:104803

<https://doi.org/10.1016/j.jri.2025.104803>

Abstract

Natural killer (NK) cells have been extensively studied to understand the pathophysiology of idiopathic recurrent pregnancy loss (iRPL), however a clear association between NK cell frequency and iRPL has yet to be established. Most studies have focused on peripheral NK (pNK) cells, which may not accurately reflect the uterine immune environment. This study aimed to investigate the receptor repertoire of uterine NK (uNK) cells derived from menstrual blood in women with iRPL (n=20) and compare it to controls with uncomplicated pregnancies (n=16). The uNK cell receptor analysis revealed a significant reduction in the expression intensities of 2B4 and NKp44 on several uNK cell clusters in women with iRPL. In addition, stratification by cytomegalovirus (CMV) seropositivity revealed that CMV-positive women had significantly higher NKp44 expression on uNK cells and frequencies of NKG2A⁺KIRs⁺LILRB1⁺NKG2C⁻ uNK cells (10.3% vs. 4.2%, p=0.025) compared to CMV-negative women. Surprisingly, this population was lowest in women with primary iRPL compared to secondary iRPL and control subjects (2.6% vs. 8.6%, 12.8%, p=0.005). These findings highlight differences in uNK cell receptor repertoire of women with iRPL and indicate that live birth history and CMV seropositivity influence uNK cells.

Keywords

Idiopathic recurrent pregnancy loss, menstrual blood, Natural killer (NK) cells, NK cell receptors, CMV, primary iRPL, secondary iRPL, live birth

Introduction

Recurrent pregnancy loss (RPL) is an early pregnancy complication that affects more than 2-5% of couples trying to conceive (1). It is defined as the loss of more than two consecutive pregnancies before 24 weeks of gestation (2). Although multiple factors have been identified that contribute to the occurrence of RPL, the etiology of the disease remains unknown in over 50% of the cases (1, 3). It is hypothesized that an inadequate maternal immune response during key processes at the maternal-fetal interface, such as decidualization, implantation, and placentation, might contribute to the occurrence of idiopathic RPL (iRPL) (4).

Natural Killer (NK) cells are the most abundant immune cell subset at the maternal-fetal interface and are mainly involved in regulating trophoblast invasion and vascular remodeling of the spiral arteries during placentation (5, 6). The phenotype and the function of NK cells in the uterine environment (uterine (u)NK) differ significantly from those of circulating peripheral (p)NK cells (7-9). NK cells have traditionally been categorized into 2 distinct populations based on relative CD16 and CD56 expression (10): the cytotoxic, mostly circulating, CD56^{dim}CD16⁺ NK cells and the immunoregulatory CD56^{bright}CD16^{+/-} NK cells, which predominate in tissues such as the uterus (11). Recent advances in the determination of the NK cell receptor (NKR) repertoire have revealed that NK cells in peripheral blood (PB) and other tissues are much more diverse than this 2-population paradigm (10-12). NK cell function is regulated by the expression of both activating and inhibitory receptors such as natural cytotoxicity receptors (NCRs: NKp30, NKp44, NKp46), C-type lectin heterodimer family (NKG2; NKG2A, NKG2C, NKG2D), Killer immunoglobulin receptors (KIRs), immunoglobulin-like transcripts (LILRB1), and co-receptors such as 2B4 (CD244) and CD161 (13). Stimulation of NKG2D, NKp30, NKp46 and CD244 on decidual NK cells increases protein production to promote vascular growth and trophoblast invasion (14-16). Interaction of KIRs, LILRB1, and NKG2A with their ligands HLA-C, HLA-G, and HLA-E on trophoblasts, respectively, supports maternal-fetal tolerance (17, 18).

Deviations in abundance and function of both pNK and uNK cells have been associated with iRPL (19-21). A meta-analysis demonstrated a higher number of pNK cells in RPL compared to controls, but significant heterogeneity was observed across the studies (22). Additionally, pNK cell numbers did not predict miscarriage in a subsequent pregnancy in women with RPL (23). A 2023 meta-analysis found no significant difference in uNK cell abundance between women with RPL and control subjects, nor any association with subsequent pregnancy

outcome and uNK cell levels (24). While the studies in this 2023 meta-analysis used either decidual tissue or endometrial tissue derived through biopsy, menstrual blood (MB) has emerged as a non-invasive and well-established biospecimen to assess uNK cells (8, 25, 26). However, the few studies investigating uNK cell levels in MB of iRPL women present conflicting results, from either no differences in total uNK cell levels (27, 28), to decreased (29, 30) or increased uNK cell levels (31) compared to control subjects. While we found no significant difference in total uNK cell levels between iRPL and control subjects, sub-setting uNK cells, demonstrated increased percentages of CD56^{dim}CD16⁺ uNK cells and decreased CD56^{bright}CD16^{+/-} uNK cells in iRPL women compared to control subjects (28). Specifically, the percentage of CD56^{bright}CD16^{+/-} uNK cells contributed to the classification features to distinguish iRPL cases from control subjects. Moreover, reduced Nkp46 expression on uterine NK cells and reduced KIR2DS2 gene expression has been observed in iRPL (32, 33). As such, expanding these investigations to capture a broader diversity of uNK cells, extending beyond total uNK cell numbers or percentages, could aid in identifying the association of uNK cells with iRPL.

Based on this rationale, we evaluated the receptor repertoire of uNK cells in women with a history of iRPL using an unsupervised analysis approach and compared it to women who had successful uncomplicated pregnancies.

Materials & Methods

Human samples

MB and PB samples were collected from iRPL and control women with regular menstrual cycles. PB samples were collected either a few days prior to the onset of menstruation or during menstruation. We included only women with at least three instead of two consecutive idiopathic miscarriages to decrease the risk of inclusion of patients with possible aneuploid-related miscarriages. Participants were excluded if a known cause of miscarriage was identified, including the presence of antithyroid, antiphospholipid, or antinuclear autoantibodies; endocrine disorders; uterine malformations; hemostatic abnormalities; or abnormal parental or fetal karyotypes (2). Control participants had uncomplicated pregnancies and previous successful live birth. Additional exclusion criteria for both groups included autoimmune diseases, smoking, and current use of intra-uterine devices or hormonal contraceptives. Cohort characteristics are presented in Table 1. From each woman, 10 ml of peripheral blood was collected in ACD-A tubes. MB was collected during 3 12h intervals during the first 36h of menstruation. MB was transferred into collection

tubes containing Dutch modified RPMI 1640 medium supplemented with pyruvate (1 mM), glutamax (2 mM), penicillin (100 U/ml), streptomycin (100 ug/ml) (all Thermo Fisher Scientific, Waltham, USA), 10% human pooled serum (manufactured in-house), and 0.3% sodium citrate (Merck, Darmstadt, Germany)) (8). Samples were stored at room temperature (RT) and processed within 24h. All participants gave written informed consent conforming to the Dutch Medical Research involving Human Subject Art (Commissie Mensgebonden Onderzoek Arnhem-Nijmegen, Nr 2017-3256).

Table 1. Cohort characteristics

	Control (n=16)	iRPL (n=20)
Age (mean $\pm$ SEM)	34.7 $\pm$ 1.3	32.7 $\pm$ 0.8
Number of women with previous live birth	15/16	6/20
Number of live births (mean $\pm$ SEM)	1.8 $\pm$ 0.2	0.4 $\pm$ 0.1
Number of miscarriages (mean $\pm$ SEM)	0.75 $\pm$ 0.3	4.4 $\pm$ 0.5
CMV seropositivity: positive	7/15*	5/14**
Maternal KIR and HLA-C typing available	13/16	15/20
HLA-C typing partner available	8/16	12/20
Peripheral blood collected	13/16	11/20
Menstrual blood collected	14/16	18/20

*CMV status unknown in n=1, **CMV status unknown n=3 primary iRPL and n=3 secondary iRPL

Lymphocyte isolation

Mononuclear cells (PBMCs) were isolated from PB using density gradient centrifugation (Lymphoprep, Axis Shield PoC AS, Oslo, Norway). Mononuclear cells were isolated from MB (MMCs) as previously described (8, 28).

Flow Cytometry

Staining of cellular surface proteins with monoclonal antibodies (Supplementary Table 1) was performed on 250,000 MMCs and PBMCs at RT for 20 min in the dark. Cells were washed by centrifugation for 2.30 min at 1800 rpm at RT. We used two NK cell panels to investigate different NK cell receptors. Data was acquired using the Navios EX (Beckman Coulter) flow cytometer and analyzed with Kaluza (Beckman Coulter, v2.1). Gate settings were based on a fluorescence minus one strategy.

KIR and HLA genotyping

Genomic DNA was isolated from the remaining cell pellet after MMC isolation using silica gel membrane technology for *KIR* and *HLA-C* genotyping (Qiagen, Hilden, Germany). Buccal swab was collected from the partners of the participants

for HLA-C typing. *KIR* genotype and *HLA-C* allelic variants were typed by PCR-SSOP, using Luminex 100 (Sanbio, Uden, Netherlands) for the following genes: *KIR2DL1/S1*, *KIR2DL1/L2/L3/L4/L5*, *KIR3DL1/L2/L2*, *KIR2DS1/S2/S3/S4/S5*, *KIR3DS1*, *KIR2DP1*, *KIR3DP1*, *HLA-C1* and *HLA-C2*.

Anti-CMV IgG seropositivity

Anti-CMV IgG antibodies were determined in plasma samples by chemiluminescence immunoassay (CMV IgG II, Liaison XL, DiaSorin, Saluggia, Italy).

Data Analysis Approach

Unsupervised Analysis

After acquisition, data was pre-processed using Kaluza to exclude doublets and to analyze either CD45⁺ or CD45⁺CD3⁺CD56⁺ lymphocytes. The data for these CD45⁺ and CD45⁺CD56⁺ cell subsets were exported into the Cytobank platform (Beckman Coulter) using the Kaluza Cytobank plugin embedded in Kaluza for further analysis. The clustering parameters differed for each research question and are provided in the respective figure legend. The number of clusters was determined by the dimensionality of the staining panel and the distribution of events across clusters. The phenotypic characteristics of each cluster were determined using heatmaps of marker expression intensity.

Statistical analysis

Statistical analyses were performed using RStudio ggpubr package ([CRAN: Package ggpubr \(r-project.org\)](https://cran.r-project.org/web/packages/ggpubr/index.html)). For comparison of unpaired observations non-parametric Mann-Whitney U test and Kruskal Wallis tests were performed. We reported unadjusted p-values as the analyses performed here are hypothesis-generating. A Tukey's range test was used to identify outliers in NK cell populations based on two criteria: outliers should represent at least 5% of the total NK cell population and at least 20% of NKG2A⁺NKG2C⁻, NKG2A⁻NKG2C⁻, or NKG2A⁺NKG2C⁺ expressing cells (34).

Results

Overall systemic and uterine immune cell composition of women with iRPL does not differ from control subjects

Previously, we demonstrated that immune cell proportions are distinct between PB and MB, and distinct sets of immune cells in MB and PB classifies iRPL from

control (25, 28). Visualization with viSNE dimensionality reduction clearly highlights differences between systemic and uterine immune cells (Figure 1 A-B). FlowSOM-based clustering created thirteen meta-clusters (Figure 1C-D), of which eight were distinct between PB and MB (Figure 1C and E). Two subsets of CD14⁺CD16⁺HLA-DR⁺ classical monocytes were observed, with cluster 1 mainly present in PB and cluster 2 in MB. Moreover, T cell subsets (cluster 9, 11, and 13) were mainly present in PB. The biggest difference could be observed for NK cell subsets (cluster 3, 4, 7 and 8), which were predominant in MB. In addition, only subtle differences in immune cell distribution could be observed between iRPL and control (Figure 1B-E). Classical monocyte subsets (cluster 1 in PB and cluster 2 in MB) were more abundant in iRPL compared to control (Figure 1E), while NK cell cluster 8 showed decreased presence in MB of iRPL. Nevertheless, the abundance of NK cells in MB, the observed differences in NK cell subsets in our previous work (28), and the broad receptor diversity of uNK cells warrants a deeper investigation of NK cell receptor diversity in MB of iRPL and control women.

NKp44 and 2B4 expression intensity on uNK cells is lower in iRPL compared to control

FlowSOM-based clustering using specific NK cell receptors identified 8 different phenotypic clusters (Figure 2A, B). Three clusters represented CD56^{dim}CD16⁺ uNK cells (cluster 6, 7, and 8), while the other 5 clusters represented CD56^{bright}CD16⁻ uNK cells, highlighting predominance of CD56^{bright}CD16⁻ uNK cells in the uterine environment (Figure 2C). None of the clusters were specific to either iRPL or control and no significant difference in cell abundance could be observed for any of the clusters besides a trend towards decreased cell abundance of cluster 1 and cluster 2 uNK cells for iRPL (Figure 2D). While cell cluster frequency did not differ between iRPL and control, significant differences in expression levels could be observed. Although expression levels of most receptors appeared to be lower in iRPL on all identified uNK cell subsets (Supplementary Figure 1A-B), only the expression levels of NKp44 and 2B4 showed to be significantly decreased on iRPL uNK cells compared to control. The mean fluorescence intensity (MFI) of NKp44 was significantly lower in iRPL for cluster 1 (472.3 vs 347.8, $p=0.004$), cluster 2 (385.8 vs 310.3, $p=0.026$), and cluster 7 (474.7 vs 319.7, $p=0.010$) (Figure 2 E). Similarly, the MFI of 2B4 was significantly lower in iRPL for cluster 1 (3349 vs 2562, $p=0.006$) and cluster 2 (2242 vs 2043, $p=0.023$) (Figure 2 F). We did not observe any changes in the expression intensities of NKp30, NKp46, CD161, and NKG2D (Supplementary Figure 1A-D).

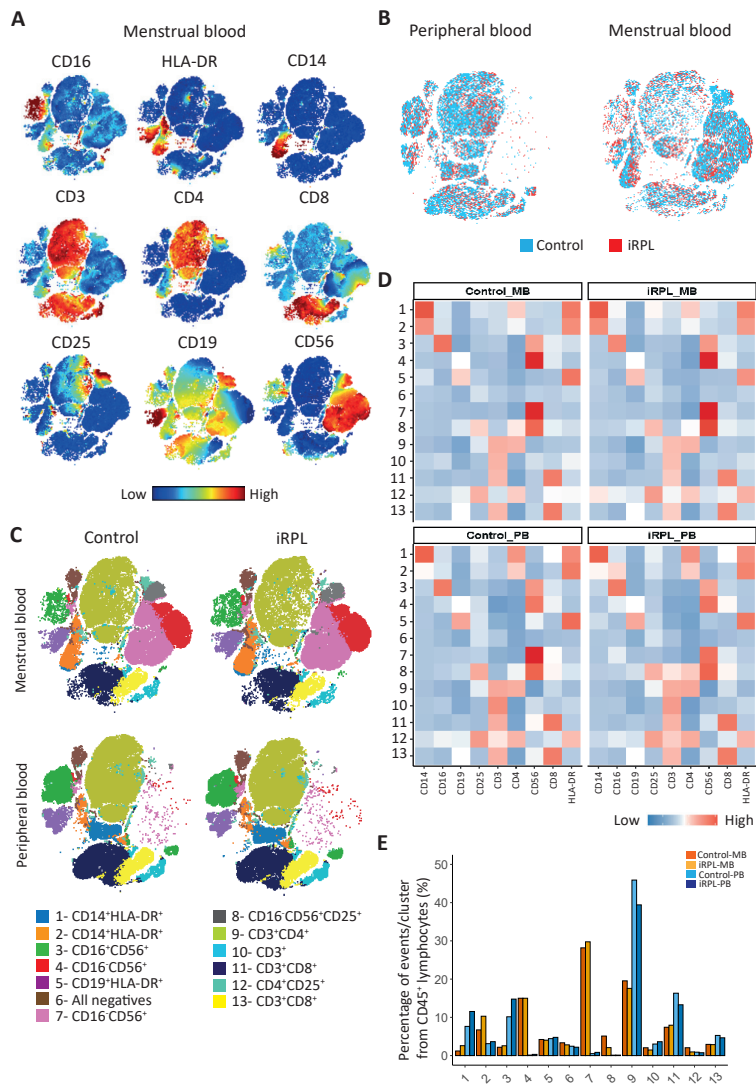


Figure 1. Unsupervised analysis approach reveals distinct immune cell populations in menstrual blood compared to peripheral blood. Dimensionality reduction using the viSNE algorithm was performed on merged CD45⁺ cells with default settings (Control MB=14, PB=13; iRPL MB=18, PB=11). 50,000 events were selected for each group. **A)** vi-SNE projections showing immune landscape of MB in control. vi-SNE algorithm was applied to merged CD45⁺ cells within each group. **B)** vi-SNE plots overlaid for control and iRPL group, separated based on MB or PB. The FlowSOM plugin was applied for further phenotyping and identified thirteen clusters. FlowSOM clusters were created based on nine parameters: CD3, CD4, CD8, CD25, CD56, CD16, CD14, CD19, and HLA-DR and embedded on the previously established vi-SNE plots. **C)** FlowSOM generated immune cell clusters in MB and PB of iRPL and control women. The identity of each cluster was determined by the expression intensity of the markers used for analysis. **D)** Heatmap showing the median expression intensity of each marker within a cluster. **E)** Bar plot representing the percentages of events from CD45⁺ cells for each cluster (MB-Control-red, MB-iRPL-orange, PB-Control-blue, PB-iRPL-dark blue).

NKR co-expression patterns are comparable between iRPL and control

To determine whether women with iRPL exhibit different NKR co-expression patterns for receptors that can recognize different HLA molecules, such as HLA-C, HLA-G and HLA-E, we assessed the co-expression of KIR2DL2/L3/S2, KIR2DL1/S1, KIR3DL1/S1, NKG2C, and LILRB1 in both NKG2A⁺ and NKG2A⁻ uNK cells. Presence of KIRs was confirmed with KIR genotyping (Supplementary Table 2). In NKG2A⁻ uNK cells, the frequency of KIRs⁺NKG2C⁻LILRB1⁺ (0.4% vs 0.1%, $p=0.045$) cells was significantly reduced in iRPL women (Supplementary Figure 2B), while no major differences were observed in the NKG2A⁺ uNK cell populations (Supplementary Figure 2A). An outlier analysis identified seven subpopulations of NKG2A⁺ uNK cells in women with iRPL and four in controls (Table 2). Based on HLA-C typing we determined that the marker expression of these outliers was mostly specific for both self-HLA-C and HLA-C of the male partner, especially in iRPL (Table 2).

The frequency of the NKG2A⁺KIRs⁺ LILRB1⁺NKG2C⁻ subset is decreased in women with ipRPL compared to isRPL and control

To investigate whether live birth alters the composition of uNK cells in MB of women with iRPL, we divided the iRPL group into those with primary iRPL, defined as no previous live birth (ipRPL, $n=9$), and those with secondary iRPL, defined as at least one previous live birth (isRPL, $n=5$), and compared them to the controls, all of whom had previous live births ($n=12$). The analysis of Natural Cytotoxicity Receptors (NCR), 2B4, CD161, and NKG2D expression showed a trend in uNK cell composition changes among control, ipRPL, and isRPL for clusters 1, 2, 4, and 8 (Figure 3A). Even though significant differences were observed in the expression intensities of the analyzed markers towards controls, there was no significant difference between ipRPL and isRPL women (Figure 3 B-G). In addition, co-expression patterns of KIRs, LILRB1, and NKG2C in NKG2A^{+/+} uNK cells revealed significant difference in frequency of the NKG2A⁺KIRs⁺LILRB1⁺NKG2C⁻ (8.6%, 2.6%, and 12.8%, $p=0.005$; control, ipRPL, and isRPL respectively; KIR⁺=KIR2DL1/S1⁺KIR2DL2/L3/S2⁺KIR3DL1/S1⁺) uNK cell populations, showing a major decrease in frequency in women with ipRPL compared to isRPL (Supplementary Figure 3A).

The frequency of the NKG2A⁺KIRs⁺ LILRB1⁺NKG2C⁻ subset is significantly higher in CMV⁺ women

To assess the influence of CMV seropositivity, we performed a sub-analysis of Figures 2 and 3 by stratifying the data into CMV⁺ ($n=9$) and CMV⁻ ($n=10$) women, regardless of iRPL or control status. Significant differences were observed for the expression intensities of NKp44 and CD161 (Figure 4A, B), while NKp30, NKp46,

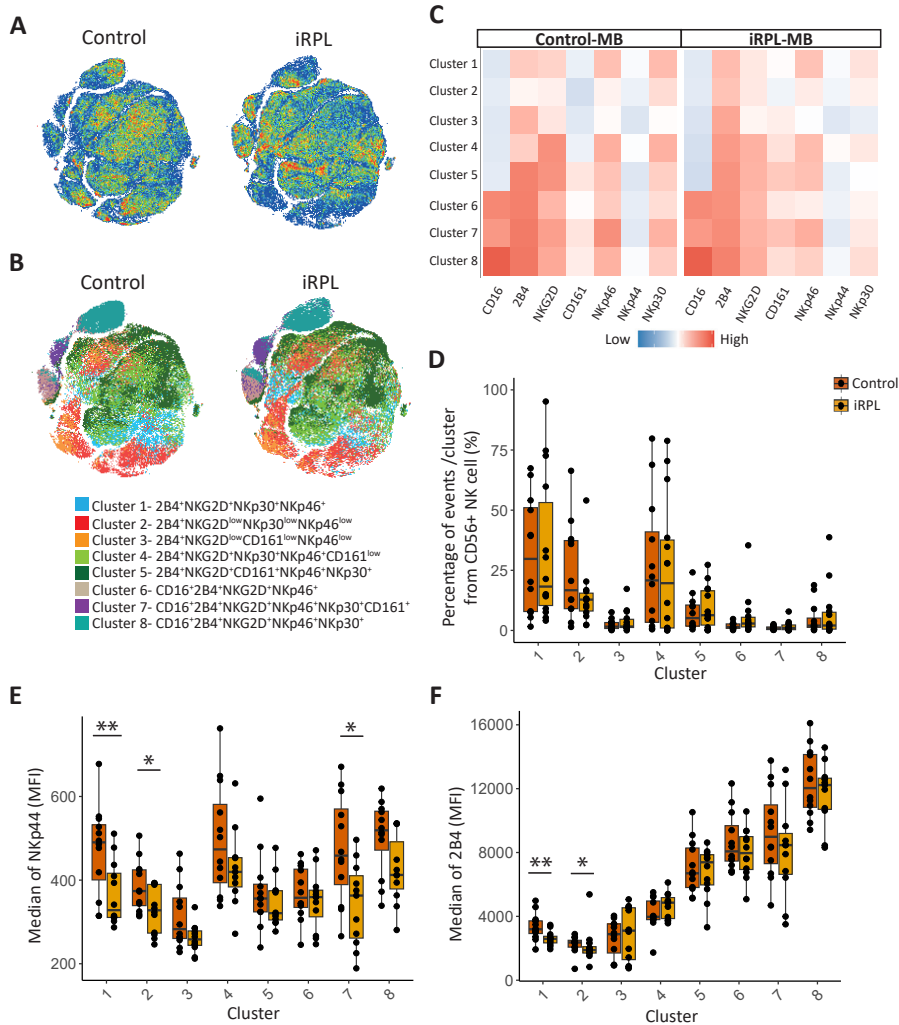


Figure 2. The expression intensities of 2B4 and Nkp44 are altered on uNK cells of iRPL women. NK cell marker analysis was performed on CD45⁺CD3⁺CD56⁺ NK cells from MB samples of both control and iRPL women (Control MB=12; iRPL MB=14). **A**) The viSNE plots representing immune landscape of aggregated uNK cells for healthy controls and iRPL women. **B**) Eight FlowSOM clusters embedded on viSNE projections. Clusters were generated based on the parameters: CD336/Nkp44, CD335/Nkp46, CD336/Nkp44, CD337/Nkp30, CD314/NKG2D, CD244/2B4, CD161, and CD16. Three clusters (6,7,8) had CD56^{Dim}CD16⁺ phenotype. **C**) Heatmap showing median expression intensities of each marker for iRPL and control. **D**) Boxplot showing frequencies of each cluster for control and iRPL women. **E-F**) Boxplots comparing median expression intensities of **E**) Nkp44 and **F**) 2B4. For statistical analysis Mann-Whitney U test was performed (*<0.05; **< 0.01).

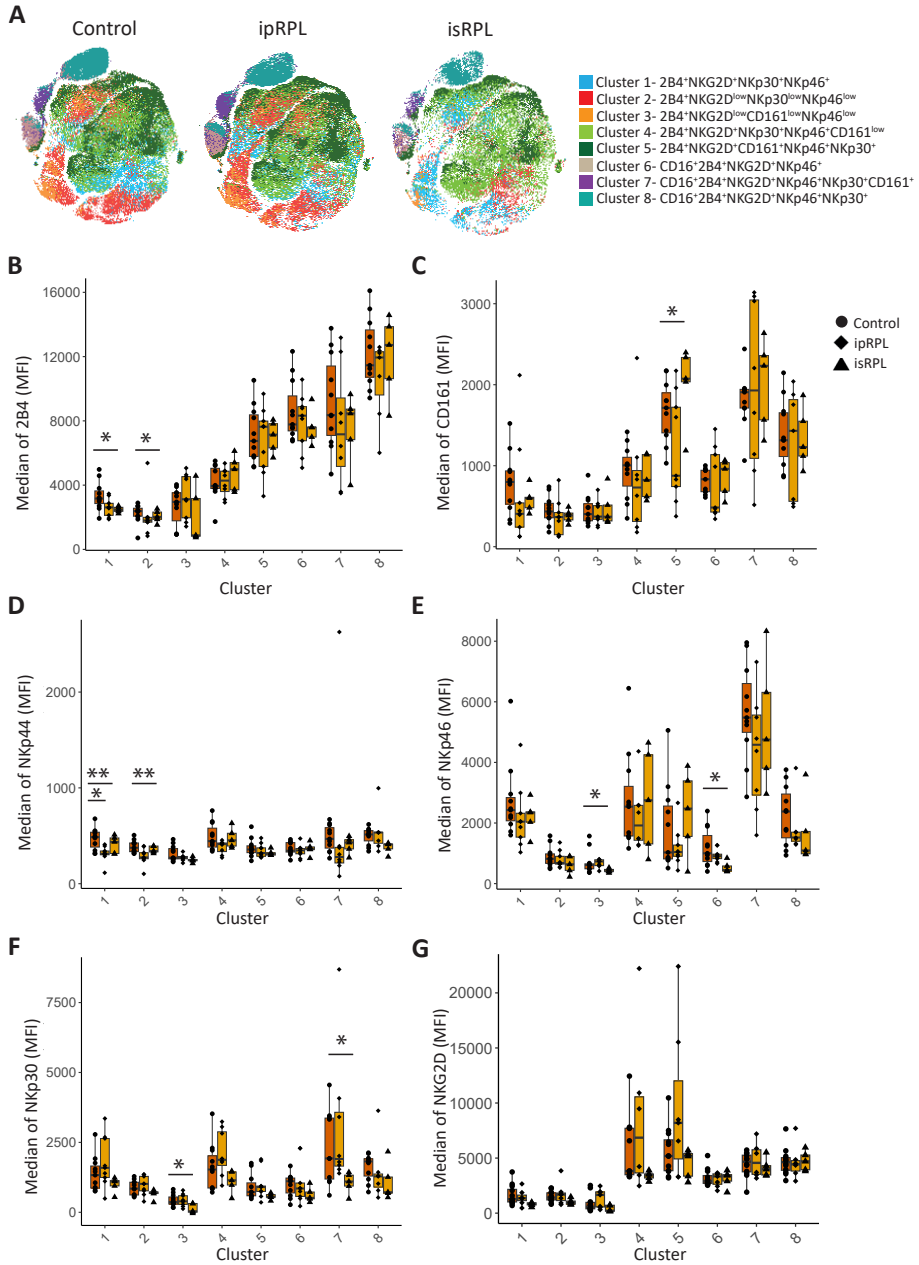


Figure 3. uNK cell composition changes depending on live birth status. A) vi-SNE plots showing immune composition of uNK cells for control, ipRPL, and isRPL (Control-red round, ipRPL-orange diamond, isRPL- orange triangle). Boxplots representing the median fluorescence intensities (MFIs) of **B)** 2B4, **C)** 161, **D)** NKp44, **E)** NKp46, **F)** NKp30, and **G)** NGK2D. For statistical analysis Kruskal Wallis test was performed (* <0.05 ; ** <0.01).

Table 2. Characteristics of NK cell subset outliers within with iRPL and controls

Donor	Expansion	CMV- positivity	Previous Live Birth	HLA-C	Partner's HLA-C	Self-specific KIR expansion?	Partner-specific KIR expansion?
<i>Control women (n=12)</i>							
HC23	NKG2A ⁺	No	No	C1C1	NA	No	No
HC29	NKG2A ⁺ KIR2DL2/L3/S2 ⁺ KIR2DL1/ S1 ⁺ KIR3DL1 ⁺ LILRB1 ⁺	Yes	Yes	NA	NA	?	?
	NKG2A ⁺ KIR2DL2/L3/S2 ⁺ KIR2DL1/S1 ⁺ LILRB1 ⁺						
HC31	NKG2A ⁺ KIR2DL2/L3/S2 ⁺ KIR2DL1/S1 ⁺ LILRB1 ⁺	No	Yes	C1C2	NA	Yes	?
HC54	NKG2A ⁺ KIR2DL2/L3/S2 ⁺	No	Yes	C1C2	C1C1	Yes	yes
<i>iRPL women (n=14)</i>							
iRPL6	NKG2A ⁺ KIR2DL2/L3/S2 ⁺	No	No	C1C1	C1C1	Yes	yes
iRPL7	NKG2A ⁺ KIR2DL2/L3/S2 ⁺ KIR2DL1/S1 ⁺	No	No	C1C1	C1C1	Yes	yes
iRPL8	NKG2A ⁺ KIR2DL2/L3/S2 ⁺ KIR2DL1/ S1 ⁺ KIR3DL1 ⁺ LILRB1 ⁺	NA	Yes	C1C2	C1C2	Yes	yes
iRPL9	NKG2A ⁺ KIR2DL2/L3/S2 ⁺ KIR3DL1 ⁺	Yes	No	NA	NA	?	?
iRPL11	NKG2A ⁺ KIR2DL2/L3/S2 ⁺ NKG2C ⁺ LILRB1 ⁺	NA	No	C1C2	C1C1	Yes	yes
iRPL13	NKG2A ⁺ KIR2DL2/L3/S2 ⁺	NA	No	C1C2	NA	Yes	?
iRPL14	NKG2A ⁺	No	No	NA	NA	No	?

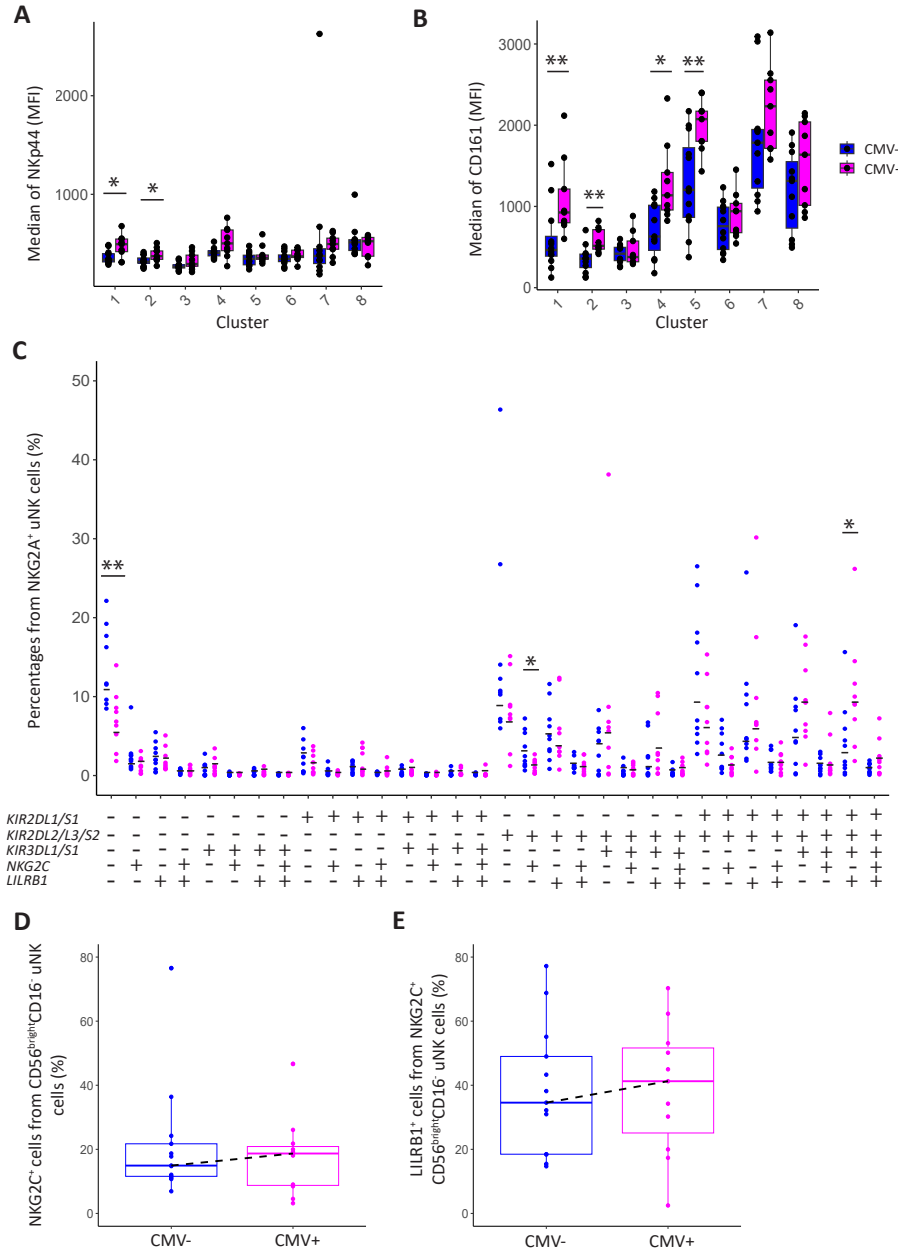


Figure 4. uNK cell composition changes depending on CMV seropositivity. Boxplots representing the mean fluorescence intensities (MFIs) of **A**) NKp44 and **B**) 161 (CMV⁻-blue, CMV⁺-magenta). NKR co-expression patterns were calculated with the 'Tree' function in Kaluza software. **C**) Tree plot indicating frequencies of NKG2A⁺ uNK cell clusters between CMV⁻ and CMV⁺ women. **D**) Boxplot indicating percentages of NKG2C⁺ cells from CD56^{Bright}CD16⁻ uNK cells. **E**) Boxplot indicating percentages of LILRB1⁺ cells from NKG2C⁺CD56^{Bright}CD16⁻ uNK cells. For statistical analysis Mann-Whitney U test was performed (* <0.05 ; ** <0.01).

NKG2D, and 2B4 expression was not different (data not shown). CMV⁺ women had significantly higher expression of NKp44 on cluster 1 (359.9 vs 473.6, $p=0.034$) and cluster 2 (325.2 vs 387.4, $p=0.028$) uNK cells (Figure 4A). Four clusters had significantly higher expression intensities of CD161 in CMV⁺ women: cluster 1 (590.0 vs 1089, $p=0.007$), cluster 2 (348.2 vs 573.7, $p=0.002$), cluster 4 (709.6 vs 1293, $p=0.013$), and cluster 5 (1288 vs 1996, $p=0.006$) (Figure 4B). Furthermore, we found significant differences in percentages of NKG2A⁺ uNK subsets upon stratification by CMV seropositivity in all MB samples, regardless of iRPL or control status. We found significantly lower percentages in CMV⁺ women of the subset expressing NKG2A⁺ without additional KIRs (7.0 to 14.0, $p=0.006$) and the uNK subset co-expressing NKG2A⁺ and NKG2C⁺ including KIR2DL2/L3/S2⁺ (1.2 to 3.1, $p=0.045$) (Figure 4C). We also observed that CMV⁺ women had substantially higher frequencies of NKG2A⁺KIRs⁺LILRB1⁺NKG2C⁻ uNK cells (10.3 to 4.2, $p=0.025$) (Figure 4C). Additionally, we observed a trend towards an increased percentage of cells expressing NKG2C⁺ within the CD56^{bright}CD16⁻ uNK subset in CMV⁺ women (Figure 4D) and a similar increased trend of LILRB1⁺ cells within NKG2C⁺CD56^{bright}CD16⁻ uNK cells in CMV⁺ women (Figure 4E).

Discussion

This study explored the diversity of uNK cell subsets by comparing the NK cell receptor repertoire of women with iRPL to those with a successful pregnancy. Our analysis revealed uNK cell clusters with significantly reduced expression of 2B4 and NKp44 in women with iRPL. 2B4 is widely recognized as a co-stimulatory receptor for NCRs, such as NKp44, where it enhances NK cell activation and promotes cytokine production including IL-8 and VEGF, which are necessary for trophoblast invasion, decidualization and angiogenesis (15, 35, 36). Notably, the reduction in 2B4 expression was present in the same clusters where NKp44 expression was diminished, suggesting a potential co-regulation or functional interplay between these two receptors. Although 2B4 is primarily considered a co-stimulatory receptor in mature pNK cells (35, 37), it also exerts inhibitory effects depending on its interaction with specific ligands (38). On decidual NK (dNK) cells 2B4 activation leads to the inhibition of NK cell activity, which is thought to contribute to maternal-fetal tolerance by limiting excessive immune activation at the maternal-fetal interface (39). While the expression of 2B4 is lower on uNK cells compared to dNK cells (40), the functional role of 2B4 in uNK versus dNK cells remains poorly understood. Nonetheless, it is plausible that reduced 2B4 expression in iRPL women could impair trophoblast tolerance, particularly given its potential inhibitory role.

To draw more definitive conclusions, further investigation into the functional outcomes of 2B4-NKp44 signaling in uNK cells of women with iRPL is needed.

Previously, we demonstrated that uNK cells from MB have a distinct NKR co-expression pattern compared to PB or decidua (40). In this study, NKR co-expression patterns showed only minor differences between women with iRPL and controls. Outlier analysis suggests a potential expansion of uNK subpopulations recognizing HLA-C from both self and partner in especially iRPL, which was not observed in our previous study consisting of only controls (8). Due to the relatively small sample size and the inability to perform HLA-C genotyping on all mother-partner pairs, we can only speculate that an increased presence of uNK cells recognizing HLA-C from both self and partner may influence the recognition and function of uNK cells towards fetal trophoblast cells. Further investigation is needed to determine whether this self-recognizing has a beneficial or detrimental impact on uNK cell function.

CMV infection is known to influence the frequency and function of pNK cells (41), inducing a population characterized by high NKG2C expression, co-expression of CD57 (42) and killer cell immunoglobulin-like receptors (KIRs), and reduced expression of NKG2A, NCRs (41), and CD161 (43). In concordance with Bister *et al.*, we did not find a significant difference in NKG2C⁺ and NKG2C⁺LILRB1⁺ uNK cells between CMV⁺ and CMV⁻ individuals (44). We previously observed an increased presence of NKG2C⁺LILRB1⁺ uNK cells in previously pregnant women who are CMV⁺ compared to CMV⁺ without previous pregnancies (45). However, the sample size in this study was too small to compare between CMV⁺ iRPL and isRPL. Interestingly, we found that CMV⁺ women, irrespective of obstetric history, had higher NKp44 and CD161 expression on uNK cells than CMV⁻ women. Moreover, we observed a significant increase in the expansion of NKG2A⁺KIRs⁺ LILRB1⁺NKG2C⁻ uNK cells when assessing NKR co-expression. These results indicate that CMV serostatus can alter the immune composition and phenotype of uNK cells and highlights the importance of considering CMV serostatus when comparing NK cell profiles between control subjects and women with iRPL.

Pregnancy induces the expansion of trained uNK cells and those with KIR specific for self-HLA-C (45-47). These phenotypic changes can persist post-pregnancy, potentially enhancing NK cell function in subsequent pregnancies (46). Despite this, some women experience iRPL even after a successful pregnancy. Although there is no standardized reference, uNK cell numbers tend to be higher in women with isRPL compared to those with iRPL (48). While our study was not specifically designed to stratify iRPL patients by live birth history, we observed reduced expression of NKp44

and 2B4 in ipRPL compared to both isRPL and controls. Furthermore, women with ipRPL exhibited significantly lower frequencies of a distinct uNK cell subpopulation marked by $\text{NKG2A}^+\text{KIRs}^+\text{LILRB1}^+\text{NKG2C}^-$ expression. These findings underscore the importance of considering live birth status as a potential confounder when studying the uterine immune microenvironment in iRPL.

Our study offers valuable insights into the immunological landscape of iRPL, it has however several limitations. The relatively small sample size, particularly for ipRPL versus isRPL comparisons, prevented analyzing CMV^- and CMV^+ iRPL subgroups, limiting our ability to assess the CMV's influence on uNK cell profiles. In addition, the small sample size limits the ability to draw definitive conclusions and underscores the need for further validation of the observed results in a larger independent cohort. Furthermore, the absence of KIR genotyping, HLA-C typing, and complete CMV status for all control and iRPL participants made it challenging to interpret the significance of uNK cell expansions. Additionally, we studied menstrual blood derived uNK cells, which do not directly represent the uNK cell populations present in the endometrium during the window of implantation.

In conclusion, our study reveals distinct uNK cell receptor repertoire differences in women with iRPL, including reduced expression levels of 2B4 and NKp44, and decreased frequency of $\text{NKG2A}^+\text{KIR}^+\text{LILRB1}^+\text{NKG2C}^-$ uNK cells. Moreover, we observe an influence of live birth history and CMV seropositivity on uNK cell profiles. Future studies should explore the functional role of receptor-specific uNK cell subsets and stratify patients based on previous live birth and CMV status.

Acknowledgments

We would like to thank to all the women who participated in the study and Bram van Cranenbroek for his help with data analysis.

CRediT authorship contribution statement

AG: Conceptualization, Data curation, Formal analysis, Software, Visualization, Writing-original draft, Writing-review and editing. DF: Conceptualization, Data curation, Writing-original draft, Writing-review and editing. MB: Data curation. TM: Supervision, Writing-review and editing. MH: Resources, Investigation. IJ: Funding acquisition, Supervision. LW: Supervision, Writing-review and editing. MJ: Supervision, Resources, Writing-review and editing. RM: Funding acquisition, Supervision, Resources, Writing-review and editing.

Funding

No funding was obtained for this study.

Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

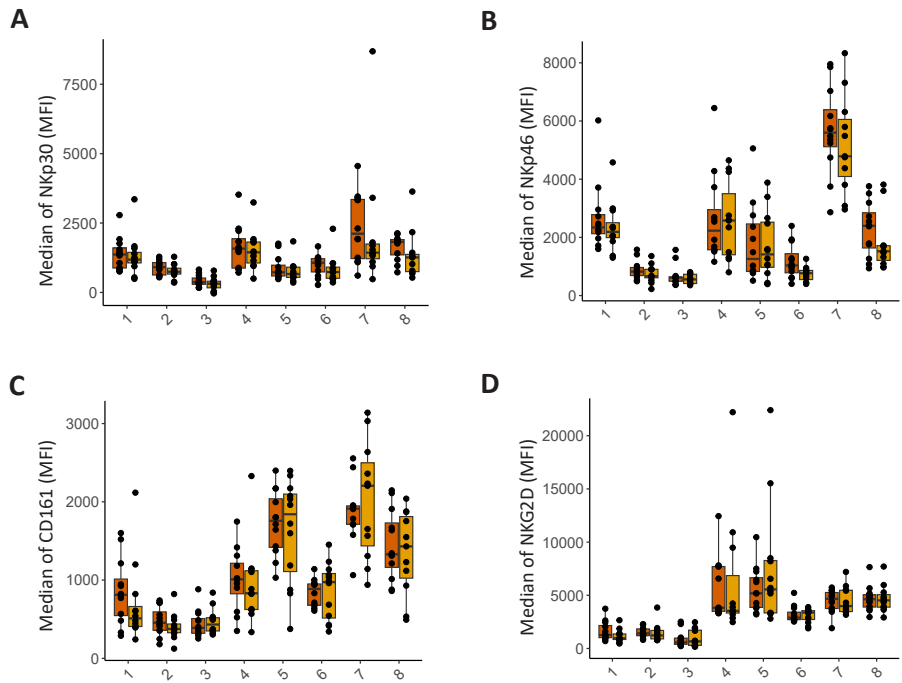
References

1. El Hachem H, Crepaux V, May-Panloup P, Descamps P, Legendre G, Bouet PE. Recurrent pregnancy loss: current perspectives. *Int J Womens Health*. 2017;9:331-45.
2. RPL EGGo, Bender Atik R, Christiansen OB, Elson J, Kolte AM, Lewis S, et al. ESHRE guideline: recurrent pregnancy loss. *Hum Reprod Open*. 2018;2018(2):hoy004.
3. Kutteh WH. Novel strategies for the management of recurrent pregnancy loss. *Semin Reprod Med*. 2015;33(3):161-8.
4. Ticconi C, Pietropolli A, Di Simone N, Piccione E, Fazleabas A. Endometrial Immune Dysfunction in Recurrent Pregnancy Loss. *Int J Mol Sci*. 2019;20(21).
5. King A, Balendran N, Wooding P, Carter NP, Loke YW. CD3- leukocytes present in the human uterus during early placentation: phenotypic and morphologic characterization of the CD56++ population. *Dev Immunol*. 1991;1(3):169-90.
6. King A, Wellings V, Gardner L, Loke YW. Immunocytochemical characterization of the unusual large granular lymphocytes in human endometrium throughout the menstrual cycle. *Hum Immunol*. 1989;24(3):195-205.
7. Manaster I, Mizrahi S, Goldman-Wohl D, Sela HY, Stern-Ginossar N, Lankry D, et al. Endometrial NK cells are special immature cells that await pregnancy. *J Immunol*. 2008;181(3):1869-76.
8. Feyaerts D, Kuret T, van Cranenbroek B, van der Zeeuw-Hingrez S, van der Heijden OWH, van der Meer A, et al. Endometrial natural killer (NK) cells reveal a tissue-specific receptor repertoire. *Hum Reprod*. 2018;33(3):441-51.
9. King A, Wooding P, Gardner L, Loke YW. Expression of perforin, granzyme A and TIA-1 by human uterine CD56+ NK cells implies they are activated and capable of effector functions. *Hum Reprod*. 1993;8(12):2061-7.
10. Smith SL, Kennedy PR, Stacey KB, Worboys JD, Yarwood A, Seo S, et al. Diversity of peripheral blood human NK cells identified by single-cell RNA sequencing. *Blood Adv*. 2020;4(7):1388-406.
11. Björkström NK, Ljunggren HG, Michaëlsson J. Emerging insights into natural killer cells in human peripheral tissues. *Nat Rev Immunol*. 2016;16(5):310-20.
12. Horowitz A, Strauss-Albee DM, Leipold M, Kubo J, Nemat-Gorgani N, Dogan OC, et al. Genetic and Environmental Determinants of Human NK Cell Diversity Revealed by Mass Cytometry. *Sci Transl Med*. 2013;5(208).
13. Male V, Moffett A. Natural Killer Cells in the Human Uterine Mucosa. *Annu Rev Immunol*. 2023;41:127-51.
14. Hanna J, Goldman-Wohl D, Hamani Y, Avraham I, Greenfield C, Natanson-Yaron S, et al. Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. *Nat Med*. 2006;12(9):1065-74.
15. Vacca P, Cantoni C, Prato C, Fulcheri E, Moretta A, Moretta L, et al. Regulatory role of NKp44, NKp46, DNAM-1 and NKG2D receptors in the interaction between NK cells and trophoblast cells. Evidence for divergent functional profiles of decidual versus peripheral NK cells. *International Immunology*. 2008;20(11):1395-405.
16. El Costa H, Tabiasco J, Berrebi A, Parant O, Aguerre-Girr M, Piccinni MP, et al. Effector functions of human decidual NK cells in healthy early pregnancy are dependent on the specific engagement of natural cytotoxicity receptors. *J Reprod Immunol*. 2009;82(2):142-7.
17. Zhou J, Yan P, Ma W, Li J. Cytokine modulation and immunoregulation of uterine NK cells in pregnancy disorders. *Cytokine Growth Factor Rev*. 2025;81:40-53.
18. King A, Allan DSJ, Bowen M, Powis SJ, Joseph S, Verma S, et al. HLA-E is expressed on trophoblast and interacts with CD94/NKG2 receptors on decidual NK cells. *European Journal of Immunology*. 2000;30(6):1623-31.
19. Kwak-Kim J, Gilman-Sachs A. Clinical implication of natural killer cells and reproduction. *Am J Reprod Immunol*. 2008;59(5):388-400.

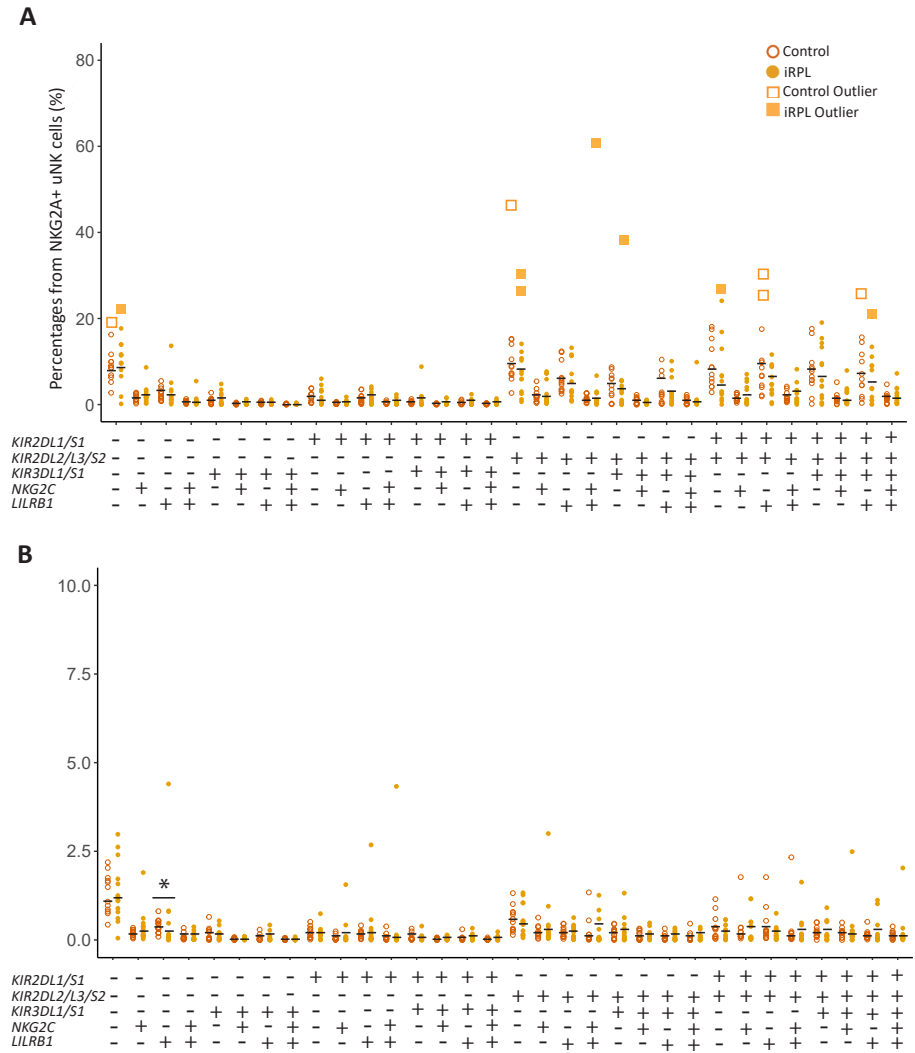
20. Quenby S, Bates M, Doig T, Brewster J, Lewis-Jones DI, Johnson PM, et al. Pre-implantation endometrial leukocytes in women with recurrent miscarriage. *Hum Reprod.* 1999;14(9):2386-91.
21. Sfakianoudis K, Rapani A, Grigoriadis S, Pantou A, Maziotis E, Kokkini G, et al. The Role of Uterine Natural Killer Cells on Recurrent Miscarriage and Recurrent Implantation Failure: From Pathophysiology to Treatment. *Biomedicines.* 2021;9(10).
22. Seshadri S, Sunkara SK. Natural killer cells in female infertility and recurrent miscarriage: a systematic review and meta-analysis. *Hum Reprod Update.* 2014;20(3):429-38.
23. Tang AW, Alfirevic Z, Quenby S. Natural killer cells and pregnancy outcomes in women with recurrent miscarriage and infertility: a systematic review. *Hum Reprod.* 2011;26(8):1971-80.
24. Von Woon E, Greer O, Shah N, Nikolaou D, Johnson M, Male V. Number and function of uterine natural killer cells in recurrent miscarriage and implantation failure: a systematic review and meta-analysis. *Hum Reprod Update.* 2022;28(4):548-82.
25. van der Molen RG, Schutten JH, van Cranenbroek B, ter Meer M, Donckers J, Scholten RR, et al. Menstrual blood closely resembles the uterine immune micro-environment and is clearly distinct from peripheral blood. *Hum Reprod.* 2014;29(2):303-14.
26. Ivarsson MA, Stiglund N, Marquardt N, Westgren M, Gidlof S, Bjorkstrom NK. Composition and dynamics of the uterine NK cell KIR repertoire in menstrual blood. *Mucosal Immunol.* 2017;10(2):322-31.
27. Hosseini S, Zarnani AH, Asgarian-Omran H, Vahedian-Dargahi Z, Eshraghian MR, Akbarzadeh-Pasha Z, et al. Comparative analysis of NK cell subsets in menstrual and peripheral blood of patients with unexplained recurrent spontaneous abortion and fertile subjects. *J Reprod Immunol.* 2014;103:9-17.
28. Benner M, Feyaerts D, Lopez-Rincon A, van der Heijden OWH, van der Hoorn ML, Joosten I, et al. A combination of immune cell types identified through ensemble machine learning strategy detects altered profile in recurrent pregnancy loss: a pilot study. *F S Sci.* 2022;3(2):166-73.
29. Tong XH, Gao M, Du XH, Lu FT, Wu LM, Wei HM, et al. Analysis of uterine CD49a NK cell subsets in menstrual blood reflects endometrial status and association with recurrent spontaneous abortion. *Cell Mol Immunol.* 2021;18(7):1838-40.
30. Marron K, Harrity C. Potential utility of a non-invasive menstrual blood immunophenotype analysis in reproductive medicine. *Reprod Fertil.* 2022;3(4):255-61.
31. Vomstein K, Egerup P, Kolte AM, Behrendt-Moller I, Boje AD, Bertelsen ML, et al. Biopsy-free profiling of the uterine immune system in patients with recurrent pregnancy loss and unexplained infertility. *Reprod Biomed Online.* 2023;47(2).
32. Mansour L, Alkhuriji A, Babay ZA, Alqadheeb S, Al-Khulaifi F, Al-Talhi R, et al. Association of Killer Immunoglobulin-Like Receptor and Human Leukocyte Antigen Class I Ligand with Recurrent Abortion in Saudi Women. *Genet Test Mol Biomarkers.* 2020;24(2):78-84.
33. Fukui A, Funamizu A, Fukuhara R, Shibahara H. Expression of natural cytotoxicity receptors and cytokine production on endometrial natural killer cells in women with recurrent pregnancy loss or implantation failure, and the expression of natural cytotoxicity receptors on peripheral blood natural killer cells in pregnant women with a history of recurrent pregnancy loss. *J Obstet Gynaecol Res.* 2017;43(11):1678-86.
34. Beziat V, Liu LL, Malmberg JA, Ivarsson MA, Sohlberg E, Bjorklund AT, et al. NK cell responses to cytomegalovirus infection lead to stable imprints in the human KIR repertoire and involve activating KIRs. *Blood.* 2013;121(14):2678-88.
35. Sivori S, Parolini S, Falco M, Marcenaro E, Biassoni R, Bottino C, et al. 2B4 functions as a co-receptor in human NK cell activation. *Eur J Immunol.* 2000;30(3):787-93.

36. Hanna J, Goldman-Wohl D, Hamani Y, Avraham I, Greenfield C, Natanson-Yaron S, et al. Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. *Nat Med.* 2006;12(9):1065-74.
37. McNerney ME, Lee KM, Kumar V. 2B4 (CD244) is a non-MHC binding receptor with multiple functions on natural killer cells and CD8+T cells. *Mol Immunol.* 2005;42(4):489-94.
38. Benoit L, Wang XX, Pabst HF, Dutz J, Tan R. Cutting edge: Defective NK cell activation in X-linked lymphoproliferative disease. *J Immunol.* 2000;165(7):3549-53.
39. Vacca P, Pietra G, Falco M, Romeo E, Bottino C, Bellora F, et al. Analysis of natural killer cells isolated from human decidua: evidence that 2B4 (CD244) functions as an inhibitory receptor and blocks NK-cell function. *Blood.* 2006;108(13):4078-85.
40. Feyaerts D, Benner M, Comitini G, Shadmanfar W, van der Heijden OWH, Joosten I, et al. NK cell receptor profiling of endometrial and decidual NK cells reveals pregnancy-induced adaptations. *Frontiers in Immunology.* 2024;15.
41. Gumá M, Angulo A, Vilches C, Gómez-Lozano N, Malats N, López-Botet M. Imprint of human cytomegalovirus infection on the NK cell receptor repertoire. *Blood.* 2004;104(12):3664-71.
42. Lopez-Vergès S, Milush JM, Schwartz BS, Pando MJ, Jarjoura J, York VA, et al. Expansion of a unique CD57+NKG2Chigh natural killer cell subset during acute human cytomegalovirus infection. *P Natl Acad Sci USA.* 2011;108(36):14725-32.
43. Kurioka A, Cosgrove C, Simoni Y, van Wilgenburg B, Geremia A, Björkander S, et al. CD161 Defines a Functionally Distinct Subset of Pro-Inflammatory Natural Killer Cells. *Frontiers in Immunology.* 2018;9.
44. Bister J, Filipovic I, Sun D, Crona-Guterstam Y, Cornillet M, Ponzetta A, et al. Tissue-specific nonheritable influences drive endometrial immune system variation. *Sci Immunol.* 2024;9(94):eadj7168.
45. Feyaerts D, van der Meer A, Joosten I, van der Molen RG. Selective expansion and CMV-dependency in pregnancy trained human endometrial NK cells. *Cell Mol Immunol.* 2019;16(4):410-1.
46. Gamliel M, Goldman-Wohl D, Isaacson B, Gur C, Stein N, Yamin R, et al. Trained Memory of Human Uterine NK Cells Enhances Their Function in Subsequent Pregnancies. *Immunity.* 2018;48(5):951-+.
47. Feyaerts D, Benner M, Comitini G, Shadmanfar W, van der Heijden OWH, Joosten I, et al. NK cell receptor profiling of endometrial and decidual NK cells reveals pregnancy-induced adaptations. *Front Immunol.* 2024;15:1353556.
48. Toth B, Vomstein K, Togawa R, Bottcher B, Hudalla H, Strowitzki T, et al. The impact of previous live births on peripheral and uterine natural killer cells in patients with recurrent miscarriage. *Reproductive Biology and Endocrinology.* 2019;17(1).

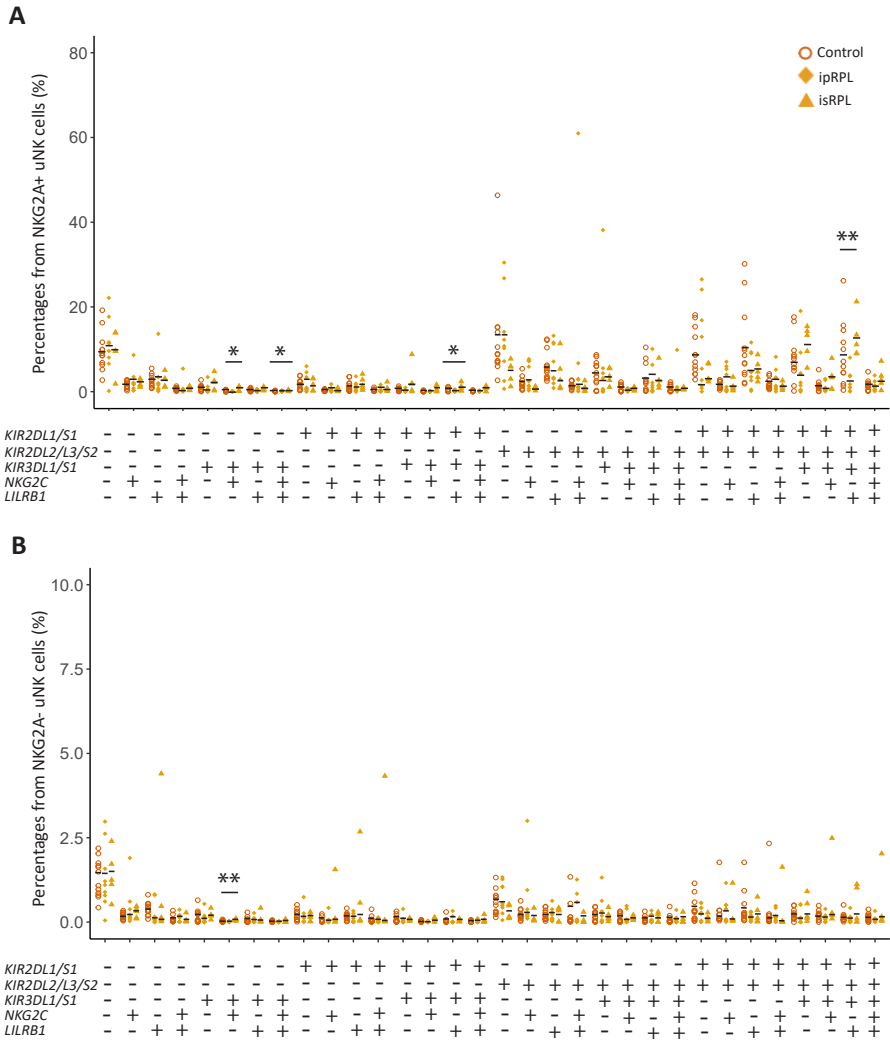
Supplementary Material



Supplementary Figure 1: Expression intensities of NKRs in control and iRPL women. A) Median fluorescence intensity (MFI) of Nkp46, **B)** Nkp30, **C)** CD161, and **D)** NKG2D.



Supplementary Figure 2: Co-expression of KIRs, LILRB1, and NK2C in NK2A[±] uNK cells.
A) The tree plot indicating frequencies of NK2A⁺ uNK cell clusters between control (red-round) and iRPL women (orange round). **B)** The tree plot indicating frequencies of NK2A⁻ uNK cell clusters between control and iRPL women. To determine outliers Tukey's range test was performed for NK2A[±] uNK cells in both control (orange empty square) and iRPL women (orange full square). For statistical analysis Mann-Whitney U test was performed (*<0.05; **< 0.01).



Supplementary Figure 3: Co-expression of KIRs, LILRB1, and NKG2C in uNK cells of ipRPL, isRPL and healthy controls. A) The tree plot indicating frequencies of NKG2A⁺ uNK cell clusters between control, ipRPL and isRPL (Control-red round, ipRPL-orange diamond, isRPL- orange triangle). **B)** The tree plot indicating frequencies of NKG2A⁺ uNK cell clusters between control, ipRPL and isRPL. For statistical analysis Kruskal Wallis test was performed (*<0.05; **< 0.01).

Supplementary Table 1. List of monoclonal antibodies

Fluorochrome	Antibody	Clone	Company
FITC	CD16	3G8	Beckman Coulter
PE	HLA-DR	immu-357	Beckman Coulter
ECD	CD14	UCHT1	Beckman Coulter
PE-Cy5.5	CD4	13B8.2	Beckman Coulter
Pe-Cy7	CD25	M-A251	BD Biosciences
APC	CD56	N901	Beckman Coulter
APC-AF700	CD8	B9.11	Beckman Coulter
APC-AF750	CD19	J3-119	Beckman Coulter
PB	CD3	UCHT1	Beckman Coulter
KO	CD45	J33	Beckman Coulter
ECD	CD3	UCHT1	Beckman Coulter
PE-Cy5.5	CD4	13B8.2	Beckman Coulter
APC	CD161	191B8	Miltenyi
APC-AF700	CD8	B9.11	Beckman Coulter
PE	CD336 (NKp44)	Z231	Beckman Coulter
ECD	CD56	N901	Beckman Coulter
PE-Cy5.5	CD337 (NKp30)	Z25	Beckman Coulter
PE-Cy7	CD335 (NKp46)	BAB281	Beckman Coulter
APC	CD314 (NKG2D)	ON72	Beckman Coulter
APC-AF700	CD244 (2B4)	C1.7.1	Beckman Coulter
APC-AF750	CD3	UCHT1	Beckman Coulter
PB	CD161	191B8	Beckman Coulter
PE	CD159c (NKG2C)	134522	R&D Systems
PE-Cy5.5	CD85j (ILT2)	HPF1.4	Beckman Coulter
PE-Cy7	KIR2DL2/S2/L3 (CD158b1/b2)	GL183	Beckman Coulter
APC	KIR3DL1/S1 (CD158e1/e2)	Z27.3.7	Beckman Coulter
APC-AF700	KIR2DL1/S1 (CD158a,h)	EB6.B.3.1.1	Beckman Coulter
PB	CD159a (NKG2A)	Z199.1.10	Beckman Coulter

Supplementary Table 2. Genotyping Table

SAMPLE-ID	Haplotype	2D11	2D12	2D13	2D14	2D15	2D51	2D52	2D53	2D54	2D55	3D11	3D12	3D13	3D51	2DP1	3DP1	Maternal HLA-C	Partner's HLA-C	CMV
<i>Control women (n=16)</i>																				
HC16	AB																	C1C1	C1C1	Positive
HC18	AB																	C2C1	C2C1	Negative
HC19	AB																	C2C2	C1C1	Positive
HC21	AA																	C1C1	C1C2	NA
HC22	AA																	C1C1	C1C1	Negative
HC23	AA																	C1C1	NA	Negative
HC24	AB																	C1C1	NA	Negative
HC29*	NA																	C1C2	C1C1	Negative
HC31	BB																	NA	NA	Positive
HC32	BB																	C1C2	NA	Negative
HC34	AB																	C2C2	C2C2	Positive
HC35	AB																	C1C2	NA	Positive
HC32	AB																	C2C2	NA	Positive
HC34	AB																	C2C1	C1C1	Negative
HC56*	NA																	NA	NA	Negative
HC57*	NA																	NA	NA	Negative
<i>IRPL women (n=20)</i>																				
IRPL1	AA																	C2C2	C2C2	NA
IRPL2	AB																	C2C2	NA	Positive
IRPL3	AB																	C2C1	C2C1	Negative
IRPL4	AA																	C1C2	C1C2	Positive
IRPL5	AA																	C1C1	C2C2	NA
IRPL6	AB																	C1C1	C1C1	Negative
IRPL7	AB																	C1C1	C1C1	Negative
IRPL8	AB																	C1C2	C1C2	NA
IRPL9*	NA																	NA	NA	Positive
IRPL10*	NA																	NA	NA	NA
IRPL11	AB																	C2C1	C1C1	NA
IRPL12	AA																	C1C1	C1C1	Negative
IRPL13	AB																	C1C2	NA	NA
IRPL14*	NA																	NA	NA	Negative
IRPL20	AB																	C2C1	C2C1	Negative
IRPL21	AB																	C1C2	C2	Negative
IRPL22*	NA																	NA	NA	Positive
IRPL25*	AB																	NA	NA	Negative
IRPL26	AB																	C1C2	C2C2	Negative
IRPL27*	AB																	C2C1	NA	Positive

Participants written in red are isRPL patients
*Genotyping and HLA-C typing are not available



An aerobe exercise intervention for optimizing metabolic, cardiovascular and immune status: protocol of an intervention study with a multi-systemic approach for women with unexplained recurrent pregnancy loss

Denise Habets^{1,2}, Aysel Gurbanova^{3*}, Amber Lombardi^{1,2*}, Salwan Al-Nasiry⁴, Marc Spaanderman^{2,4,5}, Renate van der Molen^{3**}, Lotte Wieten^{1,2**}, Tess Meuleman⁵

¹ Department of Transplantation Immunology, Maastricht University Medical Center+, Maastricht, the Netherlands

² GROW – Research Institute for Oncology and Reproduction, Maastricht University, Maastricht, the Netherlands

³ Department of Laboratory Medicine, Laboratory of Medical Immunology, Radboud University Medical Center, Nijmegen, the Netherlands

⁴ Department of Obstetrics and Gynecology, Maastricht University Medical Center+, Maastricht, the Netherlands

⁵ Department of Obstetrics and Gynecology, Radboud University Medical Center, Nijmegen, the Netherlands

*Shared second author, **Shared penultimate author

Frontiers in Medicine (Lausanne). 2025 Feb 13;12:1397039. doi: 10.3389/fmed.2025.1397039.

PMID: 40018355; PMCID: PMC11866123.

Abstract

Introduction - Women confronted with recurrent pregnancy loss (RPL) are often desperately searching for a possible explanation and hoping they will someday fulfill a healthy pregnancy. Unfortunately, in more than 50% of these women no cause for their losses can be identified after clinical investigations and therefore clinicians have no treatment options to help these women. Although adaptations in several systems such as the metabolic, the cardiovascular, and the immune system are highly important to support early pregnancy, especially the contribution of a specific subset of immune cells in the uterus known as CD56^{bright} Natural Killer (NK) cells has gained a lot of interest, investigating separate RPL associated factors might not be the way forward. Moreover, a readily available and non-invasive exercise intervention might optimize all systems simultaneously, reducing metabolic, cardiovascular and immunological risk factors contributing to RPL. Therefore, we propose an aerobe exercise intervention and study the influence on the cardiovascular, the metabolic, and the immune system, with a particular focus on endometrial CD56^{bright} NK cells, in women with unexplained RPL.

Methods and analysis - In this exercise intervention study, women with unexplained RPL will receive two short questionnaires to assess baseline characteristics. Moreover, they will receive 1. an immunological assessment (by sampling menstrual blood, peripheral blood and a vaginal swab) 2. an assessment of the cardiovascular system (by transvaginal ultrasound to assess uterine artery perfusion, by measuring hemodynamic and autonomic nerve system responses during a tilt test and by maximum stress test on a cycle ergometer) and 3. a metabolic assessment (by sampling peripheral blood, urine and by measuring body characteristics) before and after intervention. The intervention consists of 12-weeks moderate exercise training based on 50-65% of heart rate reserve. One year after the end of the intervention women will receive a final short questionnaire regarding possible subsequent pregnancy outcome.

Ethics and dissemination - This study is approved by the Medical Ethics Committee of the RUMC (registration number: NL77307.091.21, date of approval 19/10/2021) and the MUMC+ (registration number: NL77307.091.21, date of approval 12/05/2022). All women will give written informed consent before participation. Results of the study will be shared with the scientific community and with patient platforms to provide more insight in RPL related mechanisms and to support shared decision-making between clinicians and patients in order to select the most suitable treatment option based on both best available evidence and informed patient preferences.

Trial registration number - NCT06007560, registered 22nd of August 2023.

Introduction

Recurrent Pregnancy Loss (RPL), a distressing pregnancy complication defined as two or more losses before 24 weeks of gestation, nowadays affects 1-2% of all couples trying to conceive (1). Sadly, in at least 50% of these couples, no underlying deficit is found which leaves clinicians without means to improve their pregnancy outcome (2). It has been suggested that non-receptive adjustments of different maternal systems, such as the metabolic system (3,4), the cardiovascular system (5,6) and the immune system (7,8,9), underlie a proportion of these unexplained pregnancy losses, as all these systems need to adapt properly for early pregnancy to be successful.

During early pregnancy, the maternal metabolic system needs to undergo changes towards an anabolic state for the increasing energy demand of the developing embryo (10). In addition, the maternal cardiovascular system needs to undergo hemodynamic changes to ensure an increasing systemic blood supply to support both the developing embryo and placenta (11). As for the immune system, the composition of the uterine immune cell population is truly unique since it has to tolerate the presence of a semi-allogenic fetus, while at the same time providing sufficient immunity to infectious uterine threats (12,13). Hereby playing a fundamental role in early pregnancy.

In the uterus, the abundance of a specific immune cell population rapidly increases in the endometrium during the mid-secretory phase of the menstrual cycle and even up to 70% in the decidua during early pregnancy (14). This population of uterine Natural Killer (NK) cells is characterized by high levels of CD56 thereby also called CD56^{bright} NK cells, in contrast to the NK cell population in peripheral blood that mainly consists of CD56^{dim} NK cells (15). It is commonly thought that these CD56^{bright} uterine NK cells have an immunomodulatory function with the ability to secrete anti-inflammatory and growth factors, hereby playing a crucial role in embryo implantation by regulating adequate trophoblast invasion and remodeling of spiral arteries in order to establish proper early placentation (16). Early pregnancy safe harvesting of human CD56^{bright} uterine NK cells without endangering ongoing pregnancy is hardly feasible, however in non-pregnant endometrium it is possible to obtain samples through biopsies. Nevertheless, biopsies from endometrial tissue are invasive and painful, limiting their use for research purposes. Instead of biopsies, endometrial NK (eNK) cells can be easily isolated from menstrual blood and have shown to have the same phenotype as cells isolated from biopsies even allowing for collection of ~10 times more lymphocytes than biopsies (17,18).

As the metabolic, the cardiovascular and the immune system seemingly play a major role in sustaining successful pregnancy, research in these various systems has gained interest for pregnancy complications such as RPL. Women with RPL have been shown to have an increased incidence of metabolic disorders such as hyperhomocysteinemia, altered amino acid metabolism and an increased risk for developing the metabolic syndrome (3,19,20). Moreover, in women with RPL, several studies have found cardiovascular aberrations (21), amongst reduced circulatory plasma volume, reduced uteroplacental perfusion, increased total peripheral resistance, and hypertension (5,22,23,24), also increasing their risk for cardiovascular diseases later in life (25,26). In addition, alterations in uterine NK cell function and phenotype in patients with RPL have also been observed. Lower numbers of the CD56^{bright} NK cell subset and increased numbers of the CD56^{dim} NK cell subset with high expression of the activating CD16 receptor were found in the endometrium and decidua of women with RPL compared to controls without a history of RPL (9,27,28,29). Furthermore, enhanced secretion of the pro-inflammatory cytokines IFN- γ and TNF- α by uterine NK cells was found in women with RPL compared to women with a previous normal pregnancy (30).

Although these prior studies emphasize the contribution of the cardiovascular, metabolic and immune system in relation to RPL, no study to date has looked at the added value of interplay between these different systems. Even taking into consideration the importance of a common regulator of these various systems; the sympathetic nervous system (31). Sympathetic hyperactivity has been linked to both cardiovascular and metabolic disorders and sympathetic dysfunction has been a significant contributor to the pathophysiology of immune mediated inflammatory diseases (32,33,34). Valuable work to develop effective therapeutic strategies for reversal of pathological sympathetic hyperactivity (35) is ongoing, nevertheless emerging evidence from recent animal and human studies seems to highlight the importance of exercise training as a widely prescribed non-pharmacological therapeutic strategy (36,37,38).

Nowadays, it is becoming generally accepted that exercise is associated with beneficial effects on the cardiovascular and metabolic system and even short workouts modulate immune responses that may have clinical impact³⁹. More notably, exercise is known to promote healthy reproduction by reducing the risk on pregnancy complications such as preeclampsia and gestational diabetes (40). For women with RPL specifically, inconclusive effects have been found and to date no studies investigate the impact of exercise on the chance of a live birth for RPL women (1,40). Moreover, the effect of exercise on CD56^{bright} eNK cells has

never been studied although it has been found that NK cells in the periphery are the most responsive immune cells to exercise, displaying an activation and acute mobilization to the circulation during physical exertion most pronounced during moderate training (41,42,43).

As a low number or altered function of CD56^{bright} eNK cells, in addition to the presence of metabolic and cardiovascular risk factors might contribute to RPL, we aim to gain new insights in the link between metabolic, cardiovascular and immune status of women with unexplained RPL with a unique multi-systemic approach and try to improve their overall health status with a moderate aerobic exercise intervention.

Methods and analysis

Study objectives and design

The aim of this multi-center interventional cohort study is to explore differences in immune parameters, in particular in CD56^{bright} eNK cells, and in cardiovascular, and metabolic parameters in women with RPL before and after an exercise intervention. Women referred to the department of Obstetrics and Gynecology of the Radboud University Medical Centre (RUMC) and the Maastricht University Medical Centre+ (MUMC+) for RPL will be included when no identifiable cause for their losses can be determined by clinical investigations.

According to current (inter)national guidelines of the European Society of Human Reproduction and Embryology (ESHRE), the clinical work-up includes a standardized history from the couple, screening for uterine abnormalities, anti-phospholipid syndrome, thyroid abnormalities, and parental karyotyping if indicated (1).

After obtaining written informed consent, a questionnaire to assess general baseline characteristics and a short questionnaire to assess short health-enhancing physical activity (SQUASH) (44) will be conducted. Subsequently, peripheral blood and menstrual blood will be collected for assessment of the immune system and a vaginal swab will be collected to additionally evaluate the vaginal microbiome as the microbiome is known to play a fundamental role on the induction, training, and function of the immune system (45). Assessment of the cardiovascular system will be conducted by transvaginal ultrasound of both uterine arteries and by measuring hemodynamic responses during tilt test to determine hemodynamic parameters and sympathetic nervous system activity and responsiveness. Additionally, a

maximum stress test on a cycle ergometer will be performed to determine the maximum rate of oxygen (VO₂max) the subject's body is able to use during exercise. Metabolic status will be assessed by measuring metabolic components in peripheral blood, urine and by measurements of body characteristics.

The intervention consists of 12-week heartrate-controlled cycle training program. Heart rate will be monitored by a Polar H10 heartrate monitor chest strap. If women don't want to participate in the training intervention, they will be asked to participate in a time-control group without intervention. In this group, women will receive a similar immune, cardiovascular and metabolic assessment, thereby acting as a timeline control group for the training intervention. Comparability between groups will be on baseline characteristics prior to the intervention. For both the intervention and control group, women will be asked to additionally donate menstrual blood at 4 and 8 weeks during the 12-week interval in order to assess intra menstrual cycle variation of eNK cells. Subsequently, all women will receive an identical immune, cardiovascular and metabolic assessment after 12 weeks and one year after intervention, a short questionnaire for monitoring subsequent pregnancy outcomes. See Figure 1 for a schematic overview of study design.

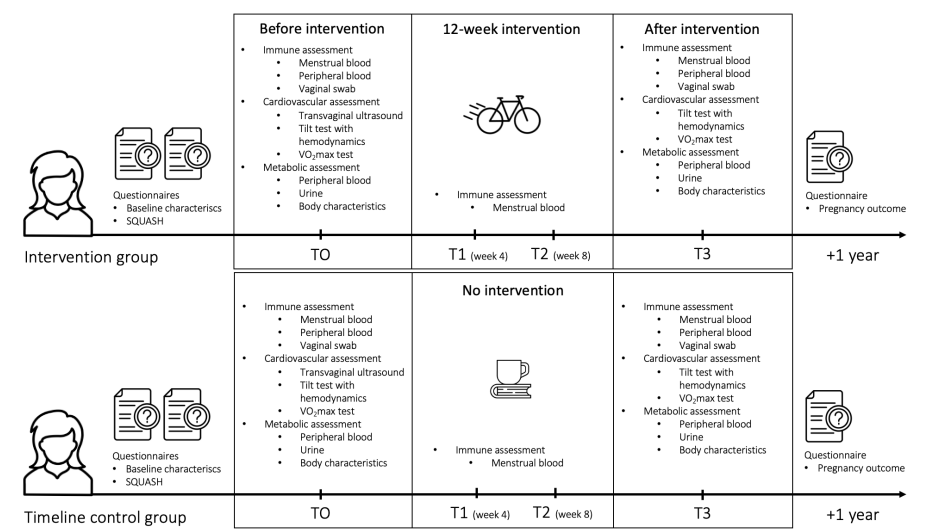


Figure 1. Schematic overview of study design.

Primary objective

To determine the changes in CD56^{bright} eNK cell number, phenotype and function before and after a moderate aerobic exercise intervention in women with unexplained RPL.

Secondary objectives

- To study baseline characteristics and baseline levels of physical activity in women with unexplained RPL.
- To study immunological effects of an aerobic exercise intervention in women with unexplained RPL and compare these between endometrial and peripheral samples.
- To compare immunological effects in women who undergo an aerobic exercise intervention and a timeline control group who do not follow an aerobic exercise intervention.
- To evaluate additional effects of an aerobic exercise intervention on the vaginal microbiota (in relationship to eNK cell population) as it can influence the maternal immune system.
- To evaluate additional effects of an aerobic exercise intervention on markers of cardiovascular and metabolic health that are known to be associated with adverse pregnancy outcomes in women with unexplained RPL.
- To study additional effects of an aerobic exercise intervention on sympathetic nervous system activity, as the sympathetic autonomic system appears to be an important modulator of the immune system, the cardiovascular system, and the metabolic system.
- To study additional effects of an aerobic exercise intervention on physical fitness levels measured by maximal oxygen consumption.
- To monitor pregnancy outcome up to 1 year after an aerobic exercise intervention.
- To investigate correlations between the immunological, cardiovascular and metabolic effects of an aerobic exercise intervention in women with unexplained RPL.

Sample size

The effect of this interventional cohort study on CD56^{bright} eNK cells is hard to predict due to the novelty of this study using uterine NK cells as our primary objective. As for peripheral NK cells, a large meta-analysis showed an overall striking increase in cell count during moderate exercise training (186% to 344% of initial resting counts) (43). Since it is believed that training evokes redistribution of cells between blood and tissues due to increased hemodynamics (46), we hypothesize that moderate exercise possibly enables mobilization of NK cells to the endometrium and therefore affects CD56^{bright} eNK cell numbers. Moreover, after moderate exercise training, numbers of peripheral NK cells overall decreased to their initial NK cell count along with an analogous depression of cytolytic activity before complete

recovery (43). Although at first these changes per training seem unlikely to have a major influence on general population health, it could be that a cumulative period of immune depression becomes of greater importance, especially to reproductive health. As these studies from this meta-analysis included around 20 subjects to show an effect on peripheral NK cells, we will aim to end up with 20 women for data analysis.

Additionally, we performed a pilot study to evaluate the feasibility of training women with RPL, in order to determine the modifiability of risk factors for RPL (evaluations of CD56^{bright} eNK cells were not part of this pilot study). We found that already after 1 month of supervised training, 9 out of 12 women significantly showed an improved VO₂max (26.7 versus 27.4, $p=0.024$) and one woman was unable to complete her trainings (unpublished data). Therefore, we expect that after a longer period of physical training at least 75% of the included women will improve their physical fitness (47).

In conclusion, we expect a clinically significant training-induced improvement with the inclusion of 30 women, assuming a similar effect on eNK cells as in peripheral blood. This allows for a drop-out rate of approximately 10%, leaving around 20 subjects for analysis hereby taking into account a compliance of 75% to the training intervention.

Study population and recruitment

Eligible women with two or more unexplained pregnancy losses, will be included during a visit at the RPL clinic at MUMC+ or first trimester pregnancy clinic at the RUMC. These women are mostly referred by their general practitioner or other regional clinics in the southern or eastern part of the Netherlands. A pregnancy prior to a pregnancy loss, must be confirmed at least by either serum or urine beta-human chorionic gonadotropin, as ectopic and molar pregnancies are excluded in this definition. According to current (inter)national guidelines, the clinical work-up of women with RPL includes a standardized history from the couple, screening for uterine abnormalities, screening for anti-phospholipid syndrome, thyroid abnormalities, and parental karyotyping if indicated. Only women with unexplained RPL, not aiming to conceive within the study time frame of 3 to 4 months are eligible to participate. Furthermore, to ensure that there is no influence of a recent pregnancy loss or birth, women should be at least 2-3 months after their last pregnancy loss or birth. Further exclusion criteria are age above 40 years, body mass index above 40, current use of immune suppressive medication or hormonal contraceptives, current infection with the human immunodeficiency

virus or symptomatic genital infections such as chlamydia or gonorrhea, pre-existent diabetes mellitus, autoimmune disease or overt cardiovascular disease, and vaccination 1 month prior to or during sampling and intervention.

On average, 30 new couples who experienced RPL visit our clinic every month, of which approximately 15 women have unexplained RPL. We estimate that monthly approximately 4 out of these 15 eligible women would like to participate in the intervention study. Additionally, we anticipate extra participants via RiNet (Reproductive Immunology network in the Netherlands, www.RiNet.nl) and via Freya (Dutch association for women with fertility problems, www.freya.nl). In our clinical experience we observed profound willingness in this specific patient group to participate in scientific studies which potentially help them and future patients. Our inclusion rate is however anticipated on the more modest side, because we do respect practical concerns, mainly related to time investment issues in couples who would like to conceive on short notice.

A total of 30 women with unexplained RPL will be included in this study to participate in the intervention group, additionally a maximum of 30 women will be included in the timeline control group.

Study procedures and data collection

In short, a questionnaire on baseline characteristics and on baseline exercise levels will be conducted before intervention. For immunological assessment, menstrual blood will be sampled before, during, and after intervention. Peripheral blood and a vaginal swab will be sampled before and after intervention. For assessment of the cardiovascular system, a transvaginal ultrasound examination of both uterine arteries will be performed before intervention. Hemodynamic responses during a tilt test and maximal oxygen consumption during a VO_2max test will be measured both before and after intervention. For metabolic assessment, measurements of metabolic components in peripheral blood and urine plus body characteristics will be determined before and after intervention. The intervention consists of 12-weeks moderate training. One year after intervention women will receive a final short questionnaire regarding pregnancy outcome.

Questionnaires

Before the intervention women will receive two questionnaires; one for assessing baseline characteristics such as age, medication use, information on gynecological history and obstetric history and one for standardized assessing baseline exercise

levels. A year after completion of the intervention, women will receive a short digital questionnaire with questions about subsequent pregnancy outcome.

Immune assessment

- Menstrual blood and peripheral blood

Peripheral blood will be collected during a visit to the RUMC or MUMC+. Menstrual blood will be self-collected by the subject during 4 cycles before (T0), during (T1: week 4 in intervention, T2: week 8 in intervention) and after the intervention (T3). Women will receive verbal and written instructions on how to collect menstrual blood. Each cycle menstrual blood will be collected in 3 blocks of 12 hours (total of 36 hours) by use of a menstrual cup. A menstrual cup is a small silicon cup that can be used similarly like a tampon. All materials necessary to collect menstrual blood, including one menstrual cup since this cup can be re-used, will be send to the participant by postal service. When collection of the samples is completed, the material will be delivered in the hospital or sent to the lab directly by postal service. Our study group has gained experience with this protocol in previous studies (9,17).

Peripheral blood mononuclear cells (PBMC) and menstrual mononuclear cells (MMC) will be isolated from the peripheral and menstrual blood by density gradient centrifugation. Number, phenotype, and function of these cells will be studied using flow cytometry. In short, NK cell receptors related to activation, inhibition, and memory function, will be analysed. Functionality of NK cells will be determined by measuring the intracellular production of cytokines. Furthermore, cytotoxic potential of NK cells will be investigated by culturing NK cells with K562 target cells and degranulation potential will be assessed by CD107 expression and cytokine analysis such as IFN- γ production of different NK cell subsets. Additionally, menstrual mononuclear cells will be used for metabolomic analyses, targeting several lipids including glycerophospholipid and sphingolipid metabolism and microbiome related metabolites.

- Vaginal swab

Vaginal swabs for microbiome analyses will be collected with Copan flocced swabs and placed in eNAT buffer and stored at -20°C. DNA extraction of the vaginal swabs will be performed using a buccal swab extraction kit. Briefly, collected swabs samples will be thawed, vortexed and incubated with lysis buffer, proteinase K and elution buffer. For interspace profiling, amplification of the intergenic spaces (IS) regions will be performed with the IS-pro assay. IS-pro is a eubacterial technique based on the detection and categorisation of the length of the 16S-23S rRNA gene IS region, as the length of this IS region is specific for each bacterial species. Phylum-specific fluorescently labelled PCR primers will be used for taxonomic classification.

Cardiovascular assessment

- Transvaginal ultrasound

To evaluate blood flow to the uterus, the resistive index (RI) and pulsatility index (PI) of both uterine arteries will be examined by transvaginal route. After obtaining a sagittal image of the uterus, cervical canal and detection of the internal ostium, the uterine artery will be identified via color flow mapping and will be measured by pulsed-wave Doppler flow velocity waveforms of the ascending branch of both uterine arteries.

- Hemodynamic responses during tilt test

A tilt test will be performed under standardized environmental conditions by an experienced researcher. During the tilt test a woman first remains supine for 20 minutes, after which her body posture will passively be changed from 20° head-down tilt (−20°) to 60° head-up tilt (+60°), in steps of 20° at 8-minute intervals. Heart rate and arterial blood pressure will be measured continuously using a monitoring device, called a Finometer, attached to a finger. The activity of the sympathetic nervous system will be derived by means of an arithmetic processing of the beat-to-beat changes in heart rate and blood pressure over time (47).

- VO₂max test

Physical fitness will be determined as the peak oxygen uptake (VO₂max: milliliter minute^{−1} kilogram^{−1}) during a maximal cycling test on a cycle ergometer. The VO₂max test will be performed under standardized environmental conditions and by an experienced researcher. During the test several parameters will be measured continuously. These non-invasive measurements include breath oxygen uptake by means of analysis of inhalation (O₂ input) of and exhalation (CO₂ output) and heart rate plus rhythm by electrocardiogram. The VO₂max test will endure cycling in total for 10 to 30 minutes, with every minute an increase of 10 watts until total exhaustion.

Metabolic assessment

- Peripheral blood and urine

Collection of peripheral blood will be done during a visit to the RUMC or MUMC+ at a similar timepoint as for the immune assessment so that women need to donate blood only once. A first-morning urine sample will be self-collected. Blood and urine samples will be sent to the central diagnostics laboratory of the RUMC or MUMC+ for standardized assessment of metabolic components such as triglycerides and cholesterol levels, glucose, insulin, and creatinine.

- Body characteristics

Measurements of body characteristics such as weight, height and waist-hip circumference will be performed.

Intervention

Our moderate exercise intervention will consist of a 12-week heartrate-controlled training program for a cycle trainer. Trainings will take place at the Radboud Sportcentrum in Nijmegen, at the UM Sports in Maastricht or in exceptional cases at a local sports facility of choice. During the intervention, the subjects will be asked to follow the same diet as prior to study inclusion and are instructed not to participate in novel exercise regimes in addition to the training program that they were given.

The training program is personalized to heartrate reserve (HHR) and can be calculated in the following manner: $HHR = HR_{\max} - HR_{\text{rest}}$, in which $HR_{\max}$ is the maximal heartrate measured during the $VO_{2\max}$ test before intervention and HR_{rest} is the heartrate determined at rest. The program consists of 60 minutes of cycling for 2 times (week: 1-6) to 3 times (week: 7-12) per week. Each training will start with a warming-up of 5 minutes on 40% of the individual HHR, followed by a training intensity of 50 minutes on 50-65% of HRR and a cooling down of 5 minutes on 40% HRR. For convenience, heartrate zones according the HRR will be predetermined. During each training heartrate can be monitored continuously and recorded by a Polar chest band and results can be viewed on the subject's personal Polar account.

Each week, the subject will be asked to send results of the trainings to the researcher so read out of heartrates can be evaluated to ensure adequate adherence to the training protocol. During weekly consultations by phone, the researcher will monitor compliance and motivate subjects to complete the intervention.

Statistical analysis

Normality of data will be evaluated with Kolmogorov-Smirnov tests or visual inspection of histograms. Descriptive data will be expressed as mean $\pm$ standard deviation for parametric continuous variables or as median and interquartile range for non-parametric continuous parameters or percentage for categorical variables. Data of immune, metabolic, and cardiovascular assessments will be compared before and after intervention by parametric paired T-tests or non-parametric Wilcoxon signed rank tests. Differences between intervention and time control group will be compared by parametric T-tests or non-parametric Mann Whitney U tests. Correlations will be assessed by univariate and multivariate logistic regression analysis. Statistical significance will be defined as $P < 0.05$ using SPSS version 28.0.

Data management

Data will be collected in a single electronic cloud-based clinical data management platform; Castor EDC by the coordinating investigators of the RUMC and MUMC+. Furthermore, coordinating investigators will maintain adequate study records, including laboratory forms, signed informed consents, and will ensure that the subject's anonymity will be guaranteed by confidentially coding patient data.

All data will be stored and archived through the data management infrastructure of the institute, which will be kept for at least 15 years (following guidelines for minimal risk research Kwaliteitsborging Mensgebonden Onderzoek 2.0 + institutional DR policy). Materials will be stored for further investigation concerning this protocol with their corresponding code at -20°C, -80°C or within a liquid nitrogen storage for a maximum of 10 years. Following publication, raw and processed data will be archived for scientific integrity, which is only accessible upon request through the coordinating investigators and the researchers responsible for the data.

Adverse events

All adverse events (AE) and serious adverse events (SAE) reported spontaneously by the subject or observed by the coordinating investigator (or his staff) will be recorded without undue delay after obtaining knowledge of the events. However, we expect the number of (S)AE's during the study to be minimal. As the training will be performed on a stationary cycling trainer and training will only be on 50-66% of HRR, the risk on a heavy sport injury is expected to be negligible. We anticipate some mild muscle ache in the first weeks of the training protocol, especially in previously sedentary women. We try to minimize any sport related risks and injuries by starting with cycling training two times a week in the first 6 weeks and increase after 6 weeks to 3 times a week to allow a more gradual increase in physical fitness. Additionally, each training will start with a warming-up of 5 minutes and end with a cooling down of 5 minutes. Training will take place in a center with routine surveillance of sport instructors, which could be consulted in case of injury. Participants will be actively asked to report any soreness, injuries or adverse effects during the weekly support session with the researcher. The principal investigator will report all SAEs to the sponsor without undue delay after obtaining knowledge of the events.

Monitoring

A certified independent party of the RUMC will monitor the study in both the RUMC as the MUMC+ according to the monitor plan. A first visit was planned before start of the study, a second visit was planned after inclusion of the first 5 subjects.

Interim assessment is not planned yet. Safety surveillance by a safety monitoring board is not implemented given the low risk of adverse events.

Patient and public involvement

RPL is one of the most complex and challenging scenarios in reproductive medicine. Understanding the mechanisms behind RPL and identifying mediators or effectors and validating targets for prevention or therapy will have a profound impact on the couples' decision making on future family planning. However, identifying one single causal factor is challenging to say the least. A multidisciplinary approach seems to be the way forward and can be of great value for identifying a subset of women with RPL with an underlying high-risk etiology. To date, there are no multi-systemic diagnostic or intervention strategies available for women with RPL. However, since there is a limited availability of predictors and possibilities for diagnosis and treatment of RPL, it is important to investigate novel factors that can offer more perspective for these women. Therefore, this intervention study with a multi-systemic approach could provide new insights. Information and results of the study will therefore be shared on a national patient platform for women with reproductive infertility (www.freya.nl) and on a personalized website that will temporarily be active (www.hmovestudie.nl).

Ethics and dissemination

This study is approved by the Medical Ethics Committee of the RUMC (registration number: NL77307.091.21, date of approval 19/10/2021) and the MUMC+ (registration number: NL77307.091.21, date of approval 12/05/2022). Furthermore, this study is registered at clinicaltrials.gov with unique protocol ID: HMOVE (registration date 22nd of August 2023). Written informed consent will be obtained of all subjects before participation. This study will be conducted according to the Declaration of Helsinki and in accordance with the Medical Research Involving Human Subjects Act (WMO) and the guidelines for Good Clinical Practice (GCP). Results will be shared with the scientific community through peer-reviewed publications and shared with patients at patient meetings in the RUMC and MUMC+ in order to share new insights on optimizing care for couples with RPL.

References

1. ESHRE Guideline Group on RPL, Bender Atik R, Christiansen OB, Elson J, Kolte AM, Lewis S, Middeldorp S, Nelen W, Peramo B, Quenby S, Vermeulen N, Goddijn M. ESHRE guideline: recurrent pregnancy loss. *Hum Reprod Open*. 2018;2018(2).
2. El Hachem H, Crepau V, May-Panloup P, Descamps P, Legendre G, Bouet PE. Recurrent pregnancy loss: current perspectives. *Int J Womens Health*. 2017 May 17;9:331-345.
3. Hilali NG, Sak S, Incebiyik A, Uyanikoglu H, Sak ME, Incebiyik H, Sabuncu T. Recurrent pregnancy loss and metabolic syndrome. *Ginekolo Pol*. 2020;91(6):320-323.
4. Wang LL, Liu H, Zhao SJ, Shen L, Xie T, Luo J, Mor G, Liao AH. The metabolic landscape of decidua in recurrent pregnancy loss using a global metabolomics approach. *Placenta*. 2021 Sep 1;112:45-53.
5. Habets DHJ, Schiffer VMMM, Kraneburg LPA, de Krom FJW, Gürtekin I, van Bree BE, van Golde RJT, Wieten L, Spaanderman MEA, Al-Nasiry S. Preconceptional evaluation of women with recurrent pregnancy loss: the additional value of assessing vascular and metabolic status. *BMC Pregnancy Childbirth*. 2022 Jan 27;22(1):75.
6. Wagner MM, Jukema JW, Hermes W, le Cessie S, de Groot CJM, Bakker JA, et al. Assessment of novel cardiovascular biomarkers in women with a history of recurrent miscarriage. *Pregnancy hypertension*. 2018;11:129-35.
7. Ticconi C, Pietropolli A, Di Simone N, Piccione E, Fazleabas A. Endometrial Immune Dysfunction in Recurrent Pregnancy Loss. *Int J Mol Sci*. 2019;20(21): 5332.
8. Habets DHJ, Schlütter A, van Kuijk SMJ, Spaanderman MEA, Al-Nasiry S, Wieten L. Natural killer cell profiles in recurrent pregnancy loss: Increased expression and positive associations with TACTILE and LILRB1. *Am J Reprod Immunol*. 2022 Nov;88(5):e13612.
9. Benner M, Feyaerts D, Lopez-Rincon A, van der Heijden OWH, van der Hoorn ML, Joosten I, Ferwerda G, van der Molen RG. A combination of immune cell types identified through ensemble machine learning strategy detects altered profile in recurrent pregnancy loss: a pilot study. *F S Sci*. 2022 May;3(2):166-173.
10. Lain KY, Catalano PM. Metabolic Changes in Pregnancy. *Clin Obstet Gynaecol*. 2007;50(4):938-48.
11. Troiano NH. Physiologic and Hemodynamic Changes During Pregnancy. *AACN Adv Crit Care*. 2018 Fall;29(3):273-283.
12. Al-Nasiry S, Ambrosino E, Schlaepfer M, Morré SA, Wieten L, Voncken JW, Spinelli M, Mueller M, Kramer BW. The Interplay Between Reproductive Tract Microbiota and Immunological System in Human Reproduction. *Front Immunol*. 2020 Mar 16;11:378.
13. Benner M, Ferwerda G, Joosten I, van der Molen RG. How uterine microbiota might be responsible for a receptive, fertile endometrium. *Hum Reprod Update*. 2018 Jul 1;24(4):393-415.
14. Lee JY, Lee M, Lee SK. Role of endometrial immune cells in implantation. *Clin Exp Reprod Med*. 2011 Sep;38(3):119-25.
15. Mselle TF, Meadows SK, Eriksson M, Smith JM, Shen L, Wira CR, et al.. Unique Characteristics of Nk Cells Throughout the Human Female Reproductive Tract. *Clin Immunol* (2007) 124(1):69–76.
16. Xie M, Li Y, Meng YZ, Xu P, Yang YG, Dong S, He J, Hu Z. Uterine Natural Killer Cells: A Rising Star in Human Pregnancy Regulation. *Front Immunol*. 2022 Jun 1;13:918550.
17. van der Molen RG, Schutten JH, van Cranenbroek B, ter Meer M, Donckers J, Scholten RR, van der Heijden OW, Spaanderman ME, Joosten I. Menstrual blood closely resembles the uterine immune micro-environment and is clearly distinct from peripheral blood. *Hum Reprod*. 2014 Feb;29(2):303-14.
18. Feyaerts D, Kuret T, van Cranenbroek B, van der Zeeuw-Hingrez S, van der Heijden OWH, van der Meer A, Joosten I, van der Molen RG. Endometrial natural killer (NK) cells reveal a tissue-specific receptor repertoire. *Hum Reprod*. 2018 Mar 1;33(3):441-451.

19. Coumans ABC, Huijgens PC, Jakobs C, Schats R, de Vries JIP, van Pampus MG, et al. Haemostatic and metabolic abnormalities in women with unexplained recurrent abortion. *Human Reproduction*. 1999;14(1):211-4.
20. Banerjee P, Dutta M, Srivastava S, Joshi M, Chakravarty B, Chaudhury K. 1H NMR Serum Metabonomics for Understanding Metabolic Dysregulation in Women with Idiopathic Recurrent Spontaneous Miscarriage during Implantation Window. *Journal of Proteome Research*. 2014;13(6):3100-6.
21. Donckers J, van Drongelen J, Spaanderman MEA, Scholten RR. Unexplained recurrent pregnancy loss and markers of arterial vascular health: An experimental case-control study. *BJOG*. 2023 Oct 18.
22. Donckers J, Scholten RR, Oyen WJ, Hopman MT, Lotgering FK, Spaanderman ME. Unexplained first trimester recurrent pregnancy loss and low venous reserves. *Hum Reprod*. 2012;27(9):2613-8.
23. Habara T, Nakatsuka M, Konishi H, Asagiri K, Noguchi S, Kudo T. Elevated blood flow resistance in uterine arteries of women with unexplained recurrent pregnancy loss. *Hum Reprod*. 2002;17(1):190-4.
24. Dendrinis S, Creatsas G, Makrakis E, Hassiakos D, Baka S, Botsis D. Increased impedance in the uterine arteries of women with unexplained recurrent pregnancy loss. *Eur J Obstet Gynecol Reprod Biol*. 2006;127(2):266-8.
25. Wright CE, Enquobahrie DA, Prager S, Painter I, Kooperberg C, Wild RA, Park K, Sealy-Jefferson S, Kernic MA. Pregnancy loss and risk of incident CVD within 5 years: Findings from the Women's Health Initiative. *Front Cardiovasc Med*. 2023 Feb 21;10:1108286.
26. Wagner MM, Beshay MM, Rooijakkers S, Hermes W, Jukema JW, Le Cessie S, De Groot CJM, Ballieux BEPB, Van Lith JMM, Bloemenkamp KWM. Increased cardiovascular disease risk in women with a history of recurrent miscarriage. *Acta Obstet Gynecol Scand*. 2018 Oct;97(10):1192-1199.
27. Lachapelle MH, Miron P, Hemmings R, Roy DC. Endometrial T, B, and NK cells in patients with recurrent spontaneous abortion. Altered profile and pregnancy outcome. *J Immunol*. 1996;156(10):4027-34.
28. Giuliani E, Parkin KL, Lessey BA, Young SL, Fazleabas AT. Characterization of uterine NK cells in women with infertility or recurrent pregnancy loss and associated endometriosis. *Am J Reprod Immunol*. 2014;72(3):262-9.
29. Wang F, Jia W, Fan M, Shao X, Li Z, Liu Y, et al. Single-cell Immune Landscape of Human Recurrent Miscarriage. *Genomics Proteomics Bioinformatics*. 2021;19(2):208-22.
30. Liu J, Dong P, Jia N, Wen X, Luo L, Wang S, et al. The expression of intracellular cytokines of decidual natural killer cells in unexplained recurrent pregnancy loss. *J Matern Fetal Neonatal Med*. 2022;35(16):3209-15.
31. Courtar DA, Spaanderman ME, Aardenburg R, Janssen BJ, Peeters LL. Low plasma volume coincides with sympathetic hyperactivity and reduced baroreflex sensitivity in formerly preeclamptic patients. *J Soc Gynecol Investig*. 2006 Jan;13(1):48-52.
32. Grassi G, Seravalle G, Mancia G. Sympathetic activation in cardiovascular disease: evidence, clinical impact and therapeutic implications. *Eur J Clin Invest*. 2015 Dec;45(12):1367-75.
33. Thorp AA, Schlaich MP. Relevance of Sympathetic Nervous System Activation in Obesity and Metabolic Syndrome. *J Diabetes Res*. 2015;2015:341583.
34. Bucsek MJ, Giridharan T, MacDonald CR, Hylander BL, Repasky EA. An overview of the role of sympathetic regulation of immune responses in infectious disease and autoimmunity. *Int J Hyperthermia*. 2018 Mar;34(2):135-143.
35. Scholten RR, Hopman MT, Lotgering FK, Spaanderman ME. Aerobic Exercise Training in Formerly Preeclamptic Women: Effects on Venous Reserve. *Hypertension*. 2015 Nov;66(5):1058-65.
36. Pedersen BK, Saltin B. Evidence for prescribing exercise as therapy in chronic disease. *Scand J Med Sci Sports*. 2006;16(Suppl 1):3-63.
37. Fisher JP, Young CN, Fadel PJ. Central sympathetic overactivity: maladies and mechanisms. *Auton Neurosci*. 2009 Jun 15;148(1-2):5-15. D

38. Scholten RR, Thijssen DJ, Lotgering FK, Hopman MT, Spaanderman ME. Cardiovascular effects of aerobic exercise training in formerly preeclamptic women and healthy parous control subjects. *Am J Obstet Gynecol*. 2014 Nov;211(5):516.e1-516.e11.
39. Ballard-Barbash R, Friedenreich CM, Courneya KS, Siddiqi SM, McTiernan A, Alfano CM. Physical activity, biomarkers, and disease outcomes in cancer survivors: a systematic review. *J Natl Cancer Inst*. 2012 Jun 6;104(11):815-40.
40. Schlüssel MM, Souza EB, Reichenheim ME, Kac G. Physical activity during pregnancy and maternal-child health outcomes: a systematic literature review. *Cad Saude Publica*. 2008;24 Suppl 4:s531-44.
41. Idorn M, Hojman P. Exercise-Dependent Regulation of NK Cells in Cancer Protection. *Trends Mol Med*. 2016 Jul;22(7):565-577.
42. Idorn M, Thor Straten P. Exercise and cancer: from "healthy" to "therapeutic"? *Cancer Immunol Immunother*. 2017 May;66(5):667-671.
43. Shephard RJ, Shek PN. Effects of exercise and training on natural killer cell counts and cytolytic activity: a meta-analysis. *Sports Med*. 1999 Sep;28(3):177-95.
44. Campbell N, Gaston A, Gray C, Rush E, Maddison R, Prapavessis H. The Short Questionnaire to Assess Health-Enhancing (SQUASH) Physical Activity in Adolescents: A Validation Using Doubly Labeled Water. *J Phys Act Health*. 2016 Feb;13(2):154-8.
45. Belkaid Y, Hand TW. Role of the microbiota in immunity and inflammation. *Cell*. 2014 Mar 27;157(1):121-41.
46. Simpson RJ, Kunz H, Agha N, Graff R. Exercise and the Regulation of Immune Functions. *Prog Mol Biol Transl Sci*. 2015;135:355-80.
47. Krabbendam I, Maas ML, Thijssen DH, Oyen WJ, Lotgering FK, Hopman MT, Spaanderman ME. Exercise-induced changes in venous vascular function in nonpregnant formerly preeclamptic women. *Reprod Sci*. 2009 Apr;16(4):414-20.



Impact of a Moderate-Intensity Aerobic Exercise Intervention on Systemic and Uterine Natural Killer Cells in Women with Unexplained Recurrent Pregnancy Loss

Aysel Gurbanova^{†1}, Amber E. M. Lombardi^{†2,3}, Denise H. J. Habets^{2,3},
Salwan Al-Nasiry^{3,4}, Marc E. A. Spaanderman^{3,4,5}, Marien I. de Jonge¹,
Tess Meuleman⁵, Lotte Wieten^{††2,3} and Renate G. van der Molen ^{††1}

¹ Department of Laboratory Medicine, Laboratory of Medical Immunology, Radboud University Medical Center, Nijmegen, the Netherlands

² Department of Transplantation Immunology, Maastricht University Medical Center+, Maastricht, the Netherlands

³ GROW – Research Institute for Oncology and Reproduction, Maastricht University, Maastricht, the Netherlands

⁴ Department of Obstetrics and Gynecology, Maastricht University Medical Center+, Maastricht, the Netherlands

⁵ Department of Obstetrics and Gynecology, Radboud University Medical Center, Nijmegen, the Netherlands

[†]These authors contributed equally to this work and share first authorship

^{††}These authors contributed equally to this work and share last authorship

Frontier in Immunology 2025 Jun 4:16:1602939.
doi: 10.3389/fimmu.2025.1602939. eCollection 2025.

Abstract

Recurrent pregnancy loss (RPL) is associated with altered immune phenotypes and functions. It has been proposed that physical exercise might impact the immune system. Therefore, we evaluated the effect of a personalized 3-month moderate-intensity aerobic exercise intervention on the immune system of women with unexplained RPL (uRPL). Given the suggested supportive role of Natural Killer (NK) cells during early pregnancy, we focused on numerical, phenotypic, and functional changes in peripheral NK (pNK) and uterine NK (uNK) cells. Mononuclear cells were isolated from peripheral blood (PB) (n=23) and menstrual blood (MB) (n=22) of women with uRPL. NK cell phenotypes were assessed with comprehensive flow cytometry panels. NK cell function was assessed with degranulation assays and intracellular staining of interferon- γ (IFN- γ), perforin and granzyme-B in a subgroup of women due to lower availability of samples (n=12). Attendance to the exercise intervention was overall 95%, which resulted in effects on the phenotype and function of pNK cells. We found a significant reduction in the median fluorescent intensity of CD161 (464 vs 410, $p=0.011$), NKp30 (432 vs 376, $p=0.018$), and NKG2A (886 vs 732, $p=0.039$) in pNK cells after exercise, while no differences were observed in uNK cells. We also observed decreased percentages of IFN- γ^+ pNK cells (49% vs 25.2%, $p=0.027$) after exercise. Our study shows promising results, suggesting that exercise can impact pNK cell phenotype and function in women with uRPL. Following the changes in pNK phenotype and function suggest a lower pro-inflammatory state post-exercise. Whether these exercise-induced phenotypic and functional changes of pNK cells impact subsequent pregnancies remains to be studied.

The study details are available through [Home | ClinicalTrials.gov](#), trial ID: HMOVE

Introduction

Recurrent pregnancy loss (RPL) is a pregnancy disorder affecting 1-2% of women attempting to conceive and is defined by the failure of two or more clinically recognized pregnancies before 24 weeks of gestation (1). The etiologies for RPL include advanced maternal age, abnormal parental karyotyping (if indicated), endocrine disorders, uterine abnormalities, antiphospholipid syndrome, lifestyle factors, and embryonic factors (1). In addition, women with RPL have a higher incidence of metabolic disorders (2-4), and an elevated risk of developing cardiovascular disease (5-7). However, in approximately 50% of cases, the underlying cause remains unknown, referred to as “unexplained” RPL (uRPL) (8).

It is well-recognized that the maternal immune system plays a critical role in the establishment and maintenance of pregnancy (9, 10). During early pregnancy, 70% of all infiltrating leukocytes are uterine Natural Killer (uNK) cells (11). They are primarily CD56^{bright}CD16⁻ and exhibit functional and phenotypic differences compared to peripheral NK (pNK) cells, which are predominantly CD56^{dim}CD16⁺ (12). It is known that uNK cells are involved in regulating trophoblast invasion and spiral artery remodelling (13-16). Numerous studies have investigated the role of NK cells in the etiology of RPL by examining uterine tissue and peripheral blood (PB) (15, 17-19). Some of these studies reported altered numbers and percentages of NK cells in the endometrium and decidua (20, 21) of women with RPL compared to controls. Additionally, several studies have demonstrated altered NK cell receptor expression (20, 22-25) and increased NK cell cytotoxicity in women with RPL (26-28). However, there are also studies indicating no changes in NK cells of women with RPL (29, 30).

Multiple studies have shown that physical exercise can modulate the immune system and influence the distribution, phenotype, and activity of different immune cell subsets, as summarized in various reviews (31-33). NK cells seem to be particularly responsive to exercise, acutely mobilizing into the circulation immediately after exercise (34-36). Notably, this mobilization primarily involves more mature and differentiated NKG2A⁻/Killer Immunoglobulin-like (KIR)⁺ NK cells compared to the less mature NKG2A⁺/KIR⁻ NK cells in PB (37). It is hypothesized that after rapid mobilization to the bloodstream NK cells redistribute to peripheral tissues, as a drop in circulating NK cells to baseline levels was observed 2 hours after acute exercise in men (38). Moreover, a recent meta-analysis showed that the cytolytic activity of NK cells increases after acute exercise (39). However, the

effects of exercise on tissue-resident immune cells, such as those in the uterine environment, remain understudied.

Considering altered numbers, phenotypes and function of NK cells have been reported in uRPL, we aimed to investigate the potential effects of a moderate-intensity aerobic exercise intervention on NK cell percentages, phenotype and function. Moreover, we have also investigated the impact of exercise on different T- and B-cell population frequencies. In this feasibility study, we focused on the impact of a three-month moderate-intensity aerobic exercise intervention on both peripheral immune cells and uterine immune cells by sampling menstrual blood (MB) as source of uterine-derived immune cells (40).

Materials and methods

Study population

In this multi-center intervention study, 49 women with uRPL were included (Supplementary Figure 1). uRPL was defined as having two or more pregnancy losses before 24 weeks of gestation without an identifiable cause. Women were referred to the department of Obstetrics and Gynecology at either the Radboud University Medical Centre (RUMC) or the Maastricht University Medical Centre+ (MUMC+) and were included when no cause for their miscarriages could be identified according to the guidelines of the European Society of Human Reproduction and Embryology (ESHRE). The clinical work-up included a standardized medical history of the couple, screening for uterine abnormalities, thyroid abnormalities, anti-phospholipid syndrome, paternal screening for sperm DNA fragmentation and parental karyotyping (if indicated) (41). Participants were excluded from the study if they met one or more of the exclusion criteria presented in Supplementary Table 1.

This study was conducted in accordance with the Declaration of Helsinki, the Medical Research Involving Human Subjects Act, and the guidelines for Good Clinical Practice (GCP). After obtaining informed consent, according to the Medical Ethical Committee of the Radboud University Medical Centre and Maastricht University Medical Centre+ NL77307.091.21, women received a questionnaire for assessing baseline characteristics. PB and MB were collected for immunophenotyping.

The intervention consisted of a 12-week, personalized, moderate-intensity, aerobic exercise program for a stationary bike. The exercise program was personalized to individual heart rate reserves (HRR). The first six weeks the participants were

instructed to train two times a week for 50 minutes at 50-60% of their HRR, the subsequent six weeks for three times a week for one 50 minutes at 50-60% HRR. Their heart rate was monitored during exercise using a Polar H10 heartrate monitor chest strap (Polar Electro, Inc, Kempele Finland). Women needed to attend a minimum of 80% of 30 prescribed trainings after completion of the intervention in order to be eligible for data analysis. All women were asked not to change their pre-existing training schedules (if applicable) and not to change their diet during the intervention. Before and after the 12-week exercise intervention, identical immune analyses were conducted.

Of the 49 women who were included in the study, 24 women completed the intervention (data is shown only of these women) (Table 1). Both PB and MB samples were collected. However, due to collection issues, PB was available from 23 participants, and MB was available from 22 participants. Different figures might show variable numbers of women included in analyses if unreliable individual markers were excluded due to technical staining issues. Sufficient remaining cells (n=12) were frozen for later functional analyses.

Isolation of menstrual blood mononuclear cells and peripheral blood mononuclear cells

To study uterine lymphocytes, MB was collected using a menstrual cup (40) at 3 time points of 12 hours right after the start of menses, for a total of 36 hours. After every 12 hours, the cup was emptied in a new collection tube, containing RPMI1640 medium (ThermoFisher Scientific, Waltham, MA, USA) supplemented with 10% human pooled serum (HPS) (Zen-Bio, Durham, NC, USA), 1 mM pyruvate (ThermoFisher Scientific), 2 mM GlutaMAX™ (ThermoFisher Scientific), 100 U/mL penicilin/100 µg/mL streptomycin (ThermoFisher Scientific), and 0.3% sodium citrate (Merck, Darmstadt, Germany). PB (10 mL) was collected in ethylene diamine tetra acetic acid (EDTA) tubes.

PB was diluted 1:1 with phosphate buffered saline (PBS) (Fresenius Kabi, GmbH, Graz, Austria). The collected MB from 3 different timepoints was first pooled, after which it was washed with PBS. After centrifugation, the pellet of the MB was filtered through a 70 µm cell strainer (PluriSelect Life Science, Leipzig, Germany) by creating a vacuum using a connector ring (PluriSelect) and a 30 mL syringe (Terumo Corporation, Tokyo, Japan). The filtered MB was then incubated for 20 minutes at room temperature with RosetteSep™ Human Granulocyte Depletion Cocktail (STEMCELL Technologies, Vancouver, Canada) according to manufacturer's instructions, after which the sample was diluted with an equal volume of PBS+2%HPS.

Subsequently, MB mononuclear cells (MBMCs) and PB mononuclear cells (PBMCs) were isolated using Lymphoprep (STEMCELL Technologies) according to manufacturer's instructions. The samples were centrifuged for 15 min at 800g, no brake at 20°C. Isolated MBMCs and PBMCs were washed twice with PBS+2%HPS, and resuspended in RPMI1640+10% HPS.

Antibodies and flow cytometry analyses

Immunophenotyping of MBMCs and PBMCs was done using flow cytometry. For the phenotyping, 500.000 MBMCs and 250.000 PBMCs were used and stained in 50 µL and 25 µL respectively. First, the cells were stained for 30 minutes at 4°C with eBioscience™ Fixable Viability Dye eFluor 780 (ThermoFisher Scientific) to stain for dead cells. Subsequently, the cells were stained for 30 minutes at 4°C for specific surface markers stated in Supplementary Table 2. Because of the use of multiple BD Horizon™ Brilliant Violet Dyes (BD, Franklin Lakes, NJ, USA), BD Horizon™ Brilliant Staining Buffer (BD) was used in combination with regular FACS buffer (PBS+1%BSA+0.02% Sodium Azide (Merck, Rahway, NJ, USA)) according to manufacturer's recommendations. After surface staining, all cells were fixated and permeabilized using the eBioscience™ Foxp3/Transcription Factor Staining Buffer Set according to the manufacturer's instructions (ThermoFisher Scientific). After fixation/permeabilization, part of the cells were stained for the transcription factor FoxP3 (ThermoFisher Scientific). All samples were measured with standardized settings on the BD FACSLytic™ (BD). Data was analyzed using the FlowJo™ v10.8 software (Flowjo™, Ashland, OR, USA) as either a percentage or as Median Fluorescent Intensity (MFI). MFIs of immune markers were normalized against MFIs of Fluorescence Minus One (FMO) controls, critical to ensure that the signals observed are truly due to the marker of interest and not artefacts from overlapping fluorescent markers. Certain markers for specific signals, timepoints or participants could not be analyzed due to various reasons such as sample or reagent availability. PBMCs and MBMCs that were not directly used in the flow cytometry staining were frozen in Recovery™ Cell Culture Freezing Medium (ThermoFisher Scientific).

Degranulation assay and staining of intracellular molecules

To assess the functional capacity of NK cells in MB and PB, we performed a CD107a degranulation assay and an intracellular staining on frozen PBMCs and MBMCs. Following thawing of a subgroup of available samples (n=12), 200,000 cells were plated in a round-bottom 96-wells plate. Cells that were used to investigate CD107a and interferon-γ (IFN-γ) expression were stimulated with 100 U/mL IL-2 and 10 ng/mL IL-15. An unstimulated condition was taken as a control. The cells that were used to investigate granzyme B and perforin expression were left unstimulated. The

cells were incubated at 37°C and 5% CO₂. After 20 hours of incubation, 25 µL of a 1:50 dilution of CD107a-PE (BD Biosciences) was added to the respective wells. One hour later, 20,000 K562 target cells and 5 µg/mL of Brefeldin A and 5 µg/mL of Monensin were added to inhibit the internalization of CD107a molecules and enhance the detection of intracellular proteins. The cells in the wells for investigation of granzyme B and perforin expression were not stimulated with K562. The cells were then incubated for an additional 3 hours at 37°C in 5% CO₂. Following the incubation period, cell viability was assessed, and surface markers were stained with the following antibodies: anti-CD16 Alexa Fluor 700 (BioLegend), anti-CD45 Krome Orange (Beckman Coulter), anti-CD3 BV605 (BD Biosciences), and anti-CD56 BV711 (BD Biosciences). Subsequently, intracellular staining was performed using anti-granzyme B PerCP-Cy5.5 (BioLegend), anti-IFNγ PE-Cy7 (ThermoFisher Scientific), and anti-perforin Pacific Blue (BioLegend) (Supplementary Table 2). All samples were analyzed on a BD FACSLytic™ flow cytometer (BD Biosciences).

Statistics

Percentages of immune cells or percentages/MFIs of immune markers before and after exercise were analyzed with a Wilcoxon signed-rank test. All analyses were performed using IBM SPSS statistics, version 29.0.0.0 (SPSS Inc, Chicago, IL, USA) and P-values <0.05 were considered statistically significant. The boxplots were prepared in RStudio using ggplot2 package (42, 43).

Results

Moderate-intensity aerobic exercise does not alter immune cell frequencies in MB and PB of women with uRPL

To determine the effect of exercise on specific immune cell subsets, we analyzed frequencies of T, B, and NK cells using high-dimensional flow cytometry in both PB and MB from women with uRPL (Figure 1A). Baseline characteristics, of the included 24 women who completed the intervention with an overall attendance of 95%, are shown in Table 1.

The role of NK cells in PB and MB are different. While the majority of pNK cells consist of a subset of CD56^{dim}CD16⁺ NK cells with high cytotoxic capabilities, uNK cells in MB mainly have a CD56^{bright}CD16⁻ phenotype and a immunoregulatory functions (12). Results of these two subsets (Figure 1B) revealed no significant differences in the frequency of total CD56⁺CD3⁻, CD56^{bright}CD16⁻ and CD56^{dim}CD16⁺ NK cells after intervention in either PB or MB (Figure 1C-E). Interestingly, we observed significant

heterogeneity in the baseline frequencies of uNK cells among women with uRPL, as well as in their response to the intervention. While some women exhibited increased frequencies of uNK cells, others showed a decrease post-intervention.

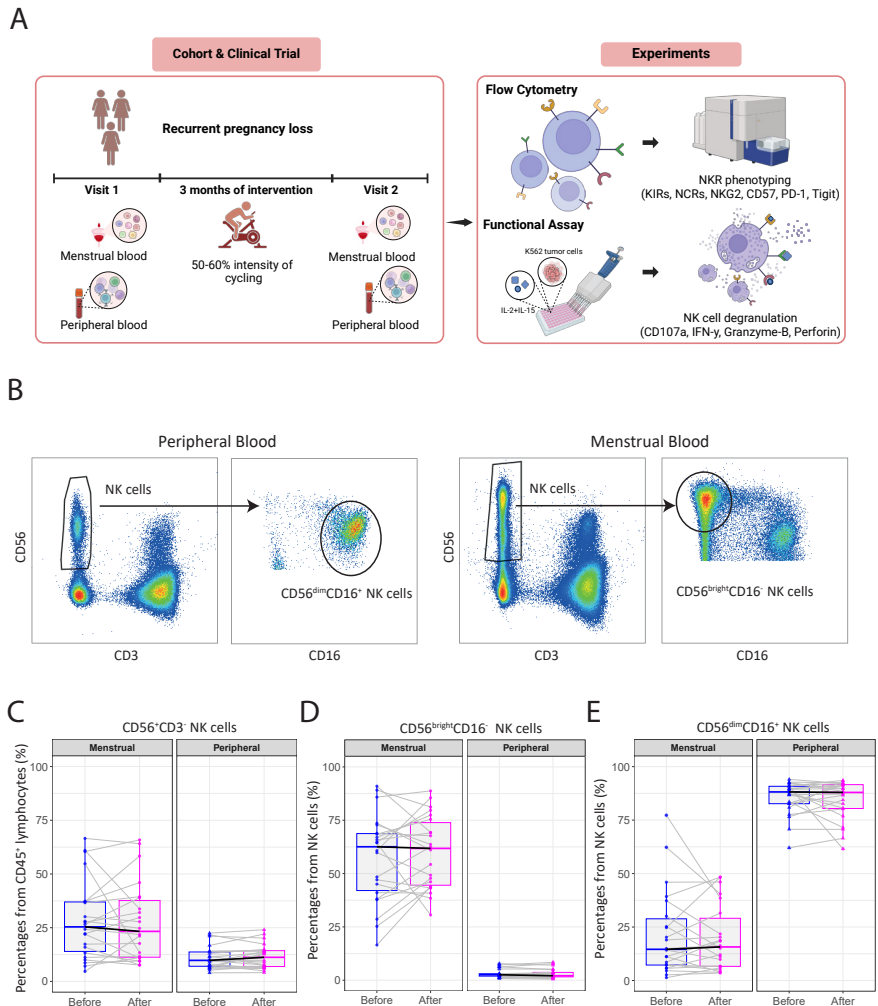


Figure 1. The moderate-intensity aerobic exercise intervention does not alter immune cell frequencies in MB or PB of women with uRPL. A) Overview of the study and the experiments performed on the isolated immune cells. B) Representative dot plots showing the gating strategies for NK cell subsets in PB and MB. C) The percentage of total CD56⁺CD3⁺ NK cells from CD45⁺ lymphocytes in MB and PB before and after the moderate-intensity aerobic exercise intervention. D) The percentage of CD56^{bright}CD16⁺ NK cells of total NK cells. E) The percentage of CD56^{dim}CD16⁺ NK cells of total NK cells. PB n=23, MB n=22. The dots represent the uterine NK cells, the triangles the peripheral NK cells (blue=before intervention, magenta=after intervention). Boxplots visualize the median (bold black line) and the interquartile ranges. A Paired Wilcoxon signed-rank test was used to determine statistical significance.

Table 1. Baseline characteristics of the study population

	Intervention (n=24)
Age (years)	35.0 (30.5-38.0)
BMI (kg/m ²)	25.0 (21.7-28.2)
Chronic disease	16.7% IgA nephropathy (1/24) Congenital hypothyroidism (1/24) Polycystic Ovary Syndrome (1/24) Celiac disease (1/24)
Medication use	20.8% Thyromimetics (3/24) Anti-depressants (1/24) Acetylsalicylic acid (1/24)
Smoking	
- Current	8.3%
- Former	20.8%
- Non-smoker	70.8%
Alcohol use	50%
Regular menstrual cycle	79.2%
Gravida	5.5 (4.3-7.8)
Para	0.0 (0.0-1.0)
Pregnancy losses	4.0 (3.3-7.0)
- Primary RPL	54.2%
- Secondary RPL	45.8%
- Early miscarriage (<10 weeks)	4.0 (2.0-7.0)
- Late miscarriage (≥10 weeks)	0.0 (0.0-1.0)

Medication use means the percentage of people that are currently using medication. Alcohol use means the percentage of people that consume at least one glass of alcohol/week. Gravida is number of pregnancies, para is the number of live births. Data are presented as either a percentage (chronic disease, medication use, smoking, alcohol use, regular menstrual cycle, and primary vs secondary RPL), or as a median with interquartile range (age, BMI, gravida, para, pregnancy losses, early miscarriage, and late miscarriage).

Likewise, the frequencies of total CD3⁺ T cells and their subsets (including conventional effector CD4⁺, cytotoxic CD8⁺ T, and CD127^{low}CD25⁺FoxP3⁺ regulatory T cells) remained similar in both PB and MB when comparing pre- and post-intervention measurements (Supplementary Figure 2A-C). Although there was a trend towards an increased frequency of total CD19⁺CD20⁺ B cells in PB as well as MB, the frequencies of B cell subsets did not change significantly after intervention (Supplementary Figure 2D-F).

CD161 (KLRB1) and NKp30 expression by pNK cells is decreased after a moderate-intensity aerobic exercise intervention in women with uRPL

To study the effect of the intervention on the phenotype of pNK and uNK cells, we performed comprehensive immunophenotyping by flow cytometry to evaluate changes in activating and inhibitory NK cell receptors (NKR), frequently described to be involved in the regulation of NK cell activity.

We found a significant decrease in median fluorescent intensity (MFI) of CD161 (KLRB1) in total pNK cells (464 [364-623] to 410 [277-483] ; $p=0.011$), in the CD56^{bright}CD16⁻ pNK cell subset (353 [247-478] to 296 [193-367]; $p=0.033$), and in the CD56^{dim}CD16⁺ pNK cell subset (493 [393-648] to 424 [296-559]; $p=0.035$), while no significant differences were observed in uNK cells post-intervention (Figure 2A-D).

Among the natural cytotoxicity receptors (NCRs) analyzed, the MFI of NKp30 significantly decreased in total pNK cells (432 [317-494] to 376 [252-481]; $p=0.018$) and in the CD56^{dim}CD16⁺ pNK subset (468 [290-550] to 369 [228-520]; $p=0.004$) post-intervention, whereas this was not observed in uNK cells (Figure 2E-H). No significant changes were found in the expression intensities of other NCRs (NKp44 and NKp46) after intervention (Supplementary Figure 3A-B).

Moderate-intensity aerobic exercise impacts NKG2A and NKG2C expression by pNK cells in women with uRPL

To study the effect of exercise on NK cell markers involved in maturation and memory, we analyzed the frequency and expression intensity of NKG2A and NKG2C before and after intervention. While the frequencies of NKG2A⁺ NK cells did not significantly change after intervention in either MB or PB (Supplementary Figure 4A-C), a marked decrease in the MFI of NKG2A was observed in total pNK cells (886 [781-1218] to 732 [555-962]; $p=0.039$) and CD56^{bright}CD16⁺ pNK cells (2472 [1583-3116] to 1497 [1113-1790]; $p=0.034$) (Figure 3A-C). In contrast, uNK cells did not show any significant changes in NKG2A expression (Figure 3A-D). Furthermore, no significant changes were observed in both frequency and expression intensity of NKG2C in both MB and PB, except for a slight increase the percentage of in NKG2C⁺ CD56^{dim}CD16⁺ pNK cells (5.4 [3.3-13.7] to 5.9 [2-8.9]; $p=0.021$) (Supplementary Figure 4F).

Next we investigated co-expression of NKR by analyzing the expression of NKG2A, NKG2C, and KIRs, on CD56^{dim}CD16⁺ pNK cells and CD56^{bright}CD16⁻ uNK cells. No significant changes were found in the CD56^{dim}CD16⁺ pNK cells or CD56^{bright}CD16⁻

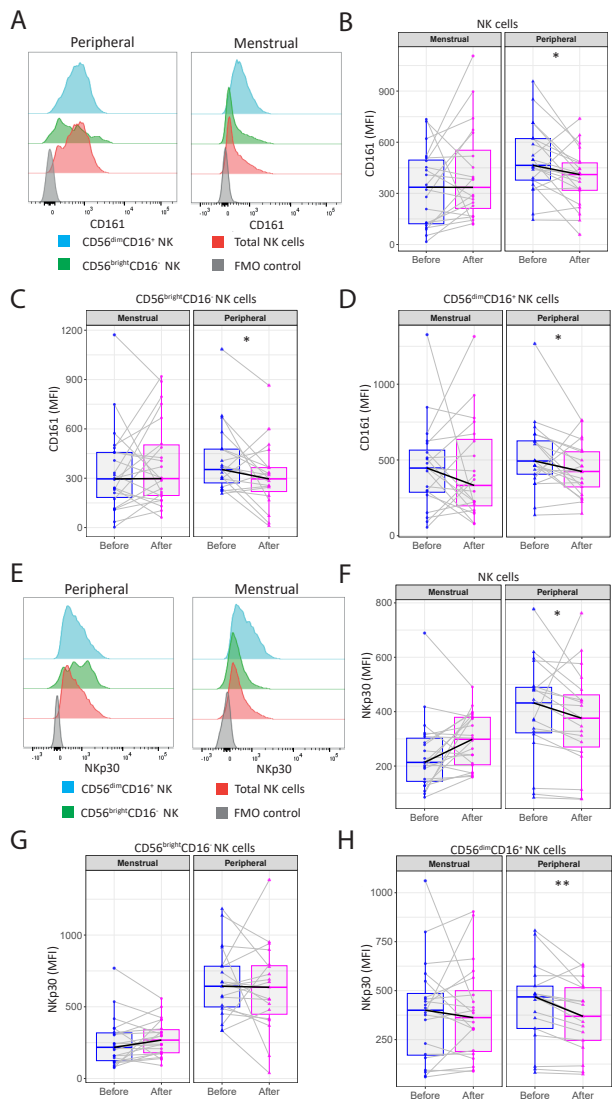


Figure 2

Figure 2. CD161 (KLRB1) and NKp30 expression by peripheral NK cells is decreased after the moderate-intensity aerobic exercise intervention in women with uRPL. A) Representative histograms of CD161 expression by total NK cells (red), CD56^{dim}CD16⁺ NK cells (blue), CD56^{bright}CD16⁻ NK cells (green), and the fluorescence minus one (FMO) control (gray) of MB and PB. B) Median fluorescent intensity (MFI) of CD161 by total uterine NK (uNK) and peripheral NK (pNK) cells before and after the moderate-intensity aerobic exercise intervention. C) MFI of CD161 by CD56^{bright}CD16⁻ NK cells. D) MFI of CD161 by CD56^{dim}CD16⁺ NK cells. E) Representative histograms of NKp30 expression by total NK cells (red), CD56^{dim}CD16⁺ NK cells (blue), CD56^{bright}CD16⁻ NK cells (green), and the FMO control (gray) of MB and PB. F) MFI of NKp30 by NK cells. G) MFI of NKp30 by CD56^{bright}CD16⁻ NK cells. H) MFI of NKp30 by CD56^{dim}CD16⁺ NK cells. PB n=21, MB n=22. All MFIs were normalized to the MFI of the FMO control. The dots represent the uNK cells, the triangles the pNK cells (blue=before intervention, magenta=after intervention). Boxplots visualize the median (bold black line) and the interquartile ranges. A Paired Wilcoxon signed-rank test was performed to determine statistical significance (*p<0.05, **p<0.005).

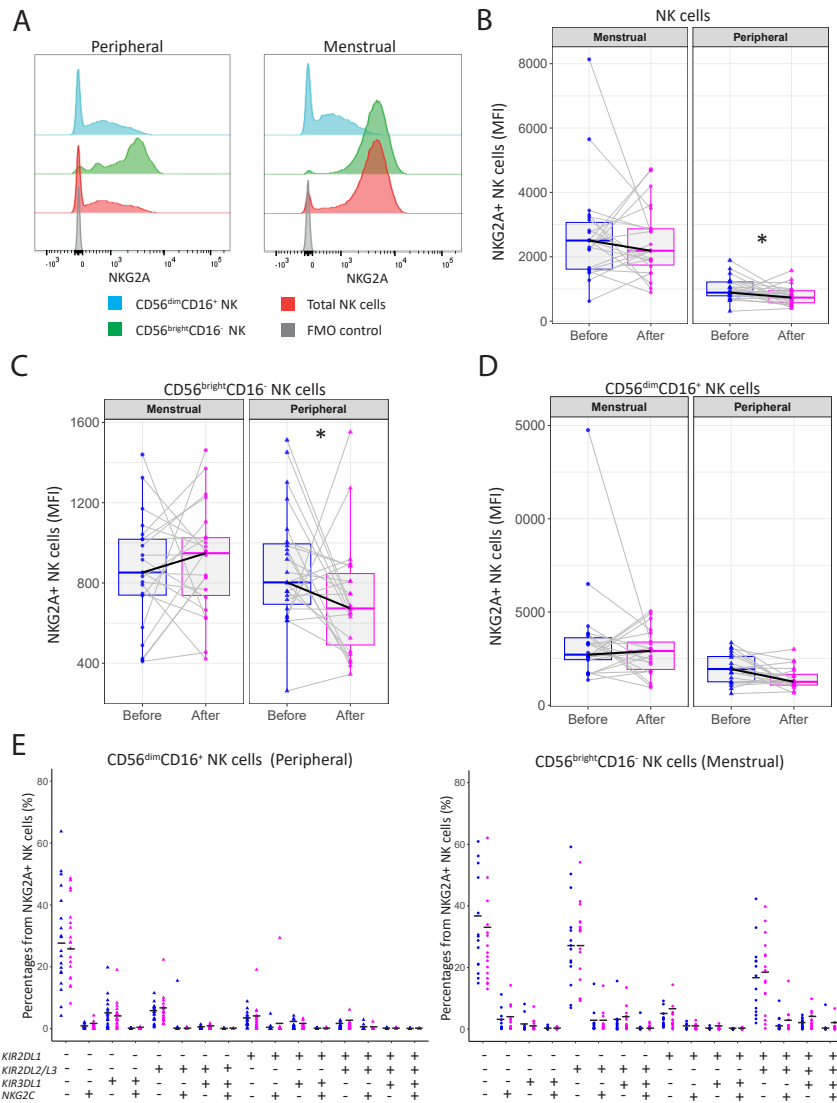


Figure 3. NKG2A expression by peripheral NK is decreased after the moderate-intensity aerobic exercise intervention in women with uRPL. A) Representative histograms of NKG2A expression by total NK cells (red), CD56^{dim}CD16⁺ NK cells (blue), CD56^{bright}CD16⁻ NK cells (green), and the fluorescence minus one (FMO) control (gray) of MB and PB. B) Median fluorescent intensity (MFI) of NKG2A by total NK cells in MB and PB before and after the moderate-intensity aerobic exercise intervention. C) MFI of NKG2A by CD56^{bright}CD16⁻ NK cells. D) MFI of NKG2A by CD56^{dim}CD16⁺ NK cells. PB n=23, MB n=22. E) Tree plots showing percentages of NK cell clusters positive for NKR (KIR2DL1, KIR2DL2/L3, KIR3DL1, NKG2C) in NKG2A⁺ CD56^{dim}CD16⁺ peripheral NK (pNK) cells and CD56^{bright}CD16⁻ uterine NK (uNK) cells (PB n=18, MB n=16). MFIs were normalized to the MFI of the FMO control. The dots represent the uNK cells, the triangles the pNK cells (blue=before intervention, magenta=after intervention). Boxplots visualize the median (bold black line) and the interquartile ranges. A Paired Wilcoxon signed-rank test was performed to determine statistical significance (*p<0.05).

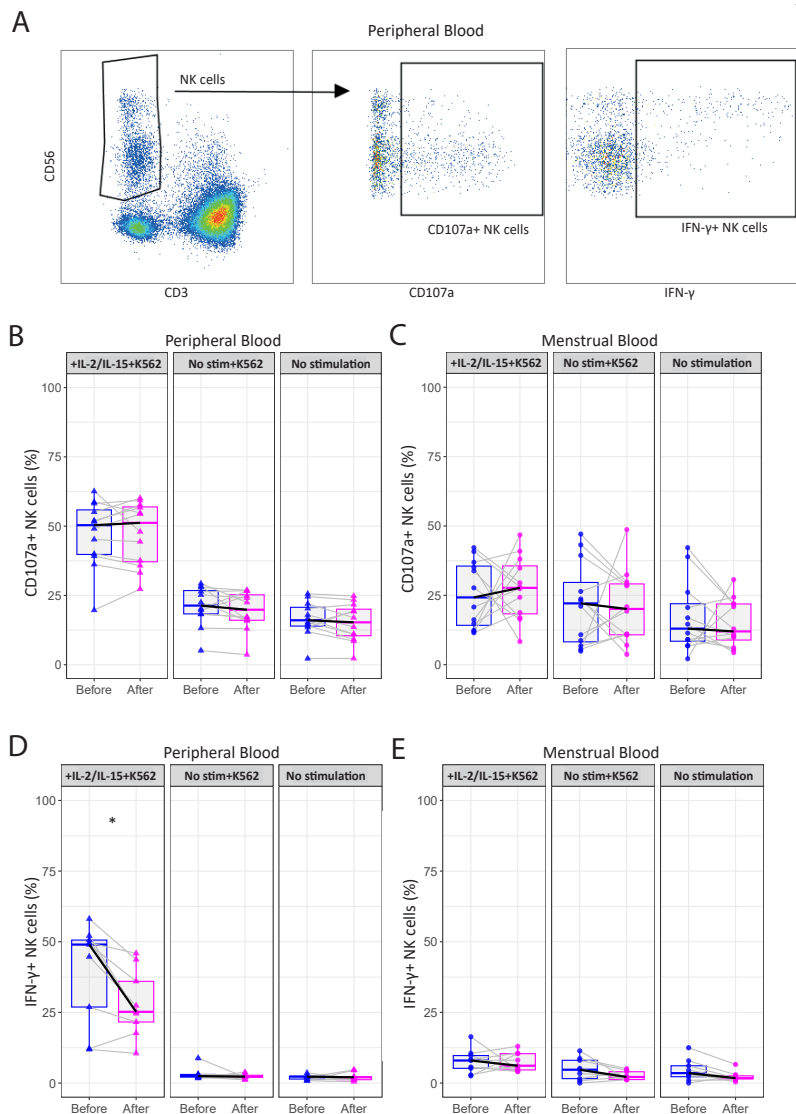


Figure 4. The moderate-intensity aerobic exercise intervention reduces the IFN- γ production by peripheral NK cells in women with uRPL. A) Representative dot plots showing the gating strategy for determining IFN- γ ⁺ and CD107a⁺ peripheral NK (pNK) cells after 24h of stimulation with IL-2 and IL-15 and a 4h co-culture with K562 tumor cells. B) Percentages of CD107a⁺ pNK cells stimulated with IL-2 and IL-15, and co-cultured with K562 (IL-2/IL-15+K562, left panel), only co-cultured with K562 (No stim+K562, central panel), and unstimulated (No stimulation, right panel) conditions. C) Percentages of CD107a⁺ uterine NK (uNK) cells. D) Percentages of IFN- γ ⁺ pNK cells. E) Percentages of IFN- γ ⁺ uNK cells. PB n=12, MB n=12. The dots represent the uNK cells, the triangles the pNK cells (blue=before intervention, magenta=after intervention). Boxplots visualize the median (bold black line) and the interquartile ranges. A Paired Wilcoxon signed-rank test was used for statistical significance (*p<0.05).

uNK cells populations (Figure 3E). Additionally, the frequencies of CD57 and KIRs remained unchanged (Supplementary Figure 3C-F).

Exercise reduces the IFN- γ production by pNK cells in uRPL women

Lastly, we investigated the effect of the intervention on NK effector function by determining the levels of intracellular granzyme B and perforin. In addition, we analyzed NK cell degranulation (CD107a) and IFN- γ production upon stimulation with K562 target cells or cytokines (IL-2 and IL-15) before and after intervention. Our analysis revealed no significant changes in CD107a expression intensity or frequency in either pNK or uNK cells after intervention (Figure 4B-C, Supplementary Figure 5A). Notably, stimulation with K562 cells did not enhance CD107a expression in uNK cells compared to the unstimulated conditions (Figure 4C). IFN- γ production was significantly reduced by pNK cells (35.8 [3.5-50.4] to 23.1 [3.2-33.9]; $p=0.028$) after intervention, with no substantial change observed in uNK cells (Figure 4D and E). Overall, uNK cells demonstrated lower IFN- γ production compared to their peripheral counterparts upon stimulation and co-culture. Granzyme B and perforin production remained unchanged post-intervention in unstimulated pNK cells (Supplementary Figure 4B and C).

Discussion

This study investigated the effects of a moderate-intensity aerobic exercise intervention on the immune profiles of women with uRPL, focusing on immune cell frequencies, NK cell receptor repertoire, and NK cell functional responses. To our knowledge, this is the first study investigating the impact of an exercise intervention strategy on systemic (PB) and uterine (MB) immune cells, particularly in women with uRPL. The use of MB, a less commonly studied but highly relevant derivate of uterine tissue in reproductive research, can aid in providing better understanding whether exercise could impact the uterine immune environment. By conducting comprehensive analyses of NK cell receptor repertoires and functional degranulation assays in both PB and MB, we aim to provide new insights into characterization of potential immunomodulatory effects of exercise in women with uRPL.

The moderate-intensity aerobic exercise intervention was shown to induce phenotypic and functional changes in pNK cells. Firstly, we found a significant decrease in the expression of NKp30 by pNK cells after intervention, while no significant changes were detected in uNK cells. NKp30 is a critical activating receptor expressed on both mature resting and activated NK cells (44). Depending

on the isoform, NKp30 can either enhance NK cell activation or promote the secretion of immunosuppressive cytokines, such as IL-10 (45). In the context of uRPL, some studies have reported a higher expression of NKp30 compared to healthy controls (46, 47), however, others have reported no significant differences (25, 48). In addition to a decreased expression of NKp30, we also found a reduced expression of CD161 (KLRB1) by pNK cells after exercise. Previously, Ntrivalas *et al* investigated CD161 expression in uRPL patients and healthy controls but did not find any differences (49). It has been shown that high CD161 expression on NK cells correlates with pro-inflammatory NK cells indicating high cytokine responsiveness (50, 51). Crosslinking of CD161 with its ligand lectin-like transcript 1 (LLT1) can increase IFN- γ expression (50, 52). Indeed, our functional assay revealed decreased IFN- γ production by pNK cells following exercise suggesting potential association with decreased expression of CD161. In RPL patients, higher levels of IFN- γ have been found compared to controls, and this increase has been positively correlated with NK cell levels (53). However, it is important to note that only a small proportion of samples could be used in the functional assays. This reduces the ability to detect more subtle effects. In addition, we found significant decrease in expression intensity of NKG2A in pNK cells following intervention. Exercise has been shown to preferentially mobilize more mature and differentiated NKG2A⁺/KIR⁺ NK cells in PB (37). While we did not observe changes in the frequencies of KIRs after exercise, the decrease in NKG2A expression suggests a shift towards a more systemic mature NK cell phenotype, consistent with previous findings (34). Interestingly, NKG2A expression and frequency was not altered in uNK cells, however, a trend was visible towards an increase of NKG2A expression by CD56^{bright}CD16⁺ NK cells.

In this study, we collected paired PB and MB samples from the same menstrual cycle for each participant to compare the impact of exercise in systemic and uterine immune environment. Interestingly, the changes in the function and receptor expression of NK cells were confined to pNK cells and were not detected in uNK cells. It is well-established that pNK cells and uNK cells are distinct cell subsets (12), albeit the origin of uNK cells remains uncertain— some studies suggest that uNK cells originate from recruitment of CD34⁺CD117⁺CD94⁺ NK precursor cells from the PB to the uterus (54-56), while others propose that these cells directly derive from CD34⁺ precursor cells present in the uterus (57, 58). This distinction could raise relevant questions about the diverse responsiveness of systemic versus tissue resident cells to exercise. Some studies have shown that pNK cells are functionally responsive to exercise (59-61). To the best of our knowledge, this is the first study to report effects of exercise on uterine NK cells. Therefore, in the future, it would be interesting to further investigate the differential responses of pNK and uNK cells to exercise.

The effect of exercise on immune cell numbers and function can vary significantly depending on the type, intensity, and duration of the exercise (62-65). Previous studies have reported increased leukocyte mobilization, particularly of NK cells, immediately after acute or high-intensity exercise (62, 63, 66, 67). However, we did not observe an increase in NK cells frequencies which might be attributed to a different duration and the choice for a moderate intensity exercise regimen. Additionally, it was shown that immune cell numbers typically peak shortly after an exercise session and later return to baseline levels (38, 67). Since we did not collect blood immediately after the final training session, the timing of our sample collection may explain the differences in our findings. Interestingly, we observed that the frequencies of immune cells varied between women with uRPL. Some exhibited increased NK cell percentages, while others showed reduced percentages, suggesting that the effects of exercise may differ between individuals. This variability could possibly be attributed to the lifestyle of women prior to the intervention. Previously, it has been shown that people with a sedentary lifestyle exhibited higher C-reactive protein and IL-6 levels, which could indicate low-grade inflammation (68, 69). Moreover, it has been shown that age can also contribute to differential immune responses to exercise. A study from de Almeida-Neto *et al* showed that men of older chronological age (34.6 ± 8.3) versus males of a younger age (21.8 ± 1.8) responded differently to exercise in their T cell compartment (70). This highlights, that even within a narrow age-range, immune responses to exercise can differ. In our study, we included women with different lifestyles and ages (between 28 and 40 years old). Therefore, it would be relevant to study possible associations between, among others, prior lifestyle and chronological age in relation to the response of immune cells to an exercise intervention in more detail in the future.

It is important to highlight several limitations that may have influenced the study's conclusions. The first limitation is that we do not know if the changes induced by exercise are specific for uRPL patients. It may be that these changes also occur in women with uncomplicated pregnancies or, for pNK cells, also in age-matched men. Inclusion of a control group with non-uRPL women or males would be needed to further elucidate this. Second, there are no reference values for the markers that were affected by exercise. Furthermore, we were not able to evaluate multiple consecutive samples to determine the sample variation over time in individual patients by taking samples over multiple menstrual cycles or by sampling an intervention control group with uRPL that did not undergo the exercise intervention. For those markers, it would be interesting to include a larger cohort that could help confirm whether the changes we observe are purely exercise-mediated or whether

additional factors may be involved as well. Some studies have already investigated the effects of the menstrual cycle on the expression of different uterine and peripheral NK cell receptors, including KIRs and NKG2A. Both studies showed that the expression was stable over different menstrual cycles (71, 72). Another point of attention is that there could have been a selection bias. It has been suggested that the endometrium of women with uRPL may be less selective for embryo quality, allowing low-quality embryos to implant (73, 74). Since these embryos may fail to develop properly, miscarriage is more likely to occur. In this study, participants were allowed to conceive during the intervention, leading to a high drop-out rate primarily due to pregnancy. As a result, we not only ended up with a relatively small sample size, but we also lost a subgroup of women who may have a higher propensity to conceive easily. As a follow up it would be interesting to perform more in-depth analysis of NK cell phenotypes or function in subgroups of women for example based on number of miscarriages, age, lifestyle factors. In the current study, our groups were underpowered to do this type of analysis. Finally, we were not able yet to correlate immune changes observed in response to the intervention with pregnancy outcomes due to the short follow-up of our study, currently limiting our ability to directly link these findings to clinical benefit. Despite these limitations, our intervention strategy may offer broader health benefits for women with uRPL, as regular physical activity is known to reduce risk factors commonly associated with RPL, such as metabolic syndrome and cardiovascular disease.

In conclusion, our study showed systemic effects of moderate-intensity aerobic exercise on immune phenotype and function in women with uRPL. Although it is difficult to translate these results into clinical significance, this research has offered new insights into the differential impact of exercise on both systemic pNK and tissue-resident uNK cells. Future research should focus on increasing the sample size, including a control cohort, and correlating the pregnancy outcomes to the exercise-dependent changes to identify the clinical relevance of these immune changes in pregnancy success or failure in women with uRPL.

Data availability statement

Data is available upon request by contacting the corresponding author.

Ethics statement

This study was approved by Ethics Committee of the RUMC and the MUMC+ under registration number: NL77307.091.21. The study was conducted in accordance with the local legislation and institutional requirements. The participants provided written informed consent to participate in this study.

Author contributions

AG: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. AL: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. DH: Conceptualization, Resources, Validation, Writing – review and editing. SA-N: Conceptualization, Writing – review and editing. MS: Conceptualization, Resources, Writing – review and editing. MJ: Writing – review and editing. TM: Conceptualization, Funding acquisition, Resources, Writing – review and editing. LW: Conceptualization, Resources, Writing – review and editing. RM: Conceptualization, Resources, Writing – review and editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work was supported by the Academic Incentive Radboud UMC/Maastricht UMC+ 2022.

Conflict of interest

The 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.

Acknowledgements

We would like to thank all the women who participated in the study. We would also like to express our gratitude to the Radboud University Sports Department for providing free training sessions to participants enrolled at the Radboud UMC. Additionally, we would like to thank all the people who helped with inclusion of the participants and processing of the samples: Anne van Uden, Imke van Wandeloo, Battice Westendorp, Lotte Jongen, Sanne Mol, Anouck van der Vlist, Claire Wenzler, Dieke Merks, Femke van Dalssen, and Vera Valckx.

References

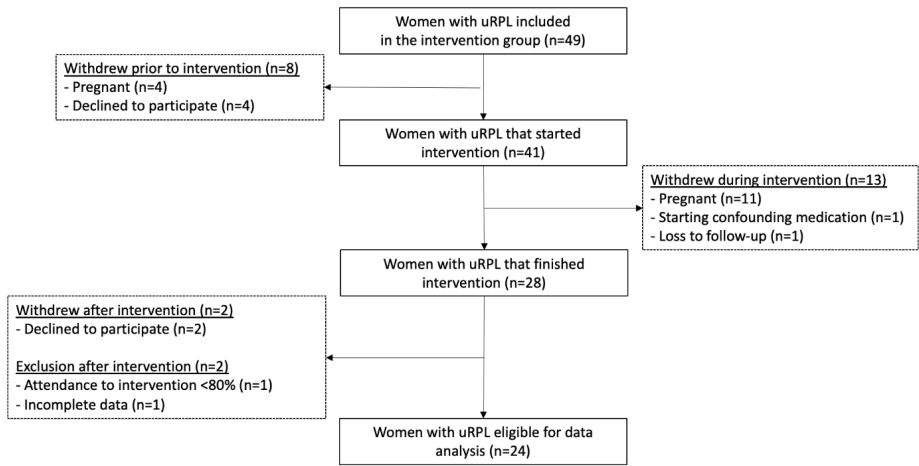
1. RPL EGGo, Bender Atik R, Christiansen OB, Elson J, Kolte AM, Lewis S, et al. ESHRE guideline: recurrent pregnancy loss: an update in 2022. *Hum Reprod Open*. 2023;2023(1):hoad002.
2. Coumans ABC, Huijgens PC, Jakobs C, Schats R, de Vries JIP, van Pampus MG, et al. Haemostatic and metabolic abnormalities in women with unexplained recurrent abortion. *Hum Reprod*. 1999;14(1):211-4.
3. Hantoushzadeh S, Kohandel Gargari O, Shafiee A, Seighali N, Ghaemi M. Glucose metabolism tests and recurrent pregnancy loss: evidence from a systematic review and meta-analysis. *Diabetol Metab Syndr*. 2023;15(1):3.
4. Liu YJ, Du MY, Gan YX, Bao SH, Feng LP, Zhang J. Triglyceride Induced Metabolic Inflammation: Potential Connection of Insulin Resistance and Recurrent Pregnancy Loss. *Front Endocrinol*. 2021;12.
5. Westergaard D, Nielsen AP, Mortensen LH, Nielsen HS, Brunak S. Phenome-Wide Analysis of Short- and Long-Run Disease Incidence Following Recurrent Pregnancy Loss Using Data From a 39-Year Period. *J Am Heart Assoc*. 2020;9(8):e015069.
6. Parikh NI, Jeppson RP, Berger JS, Eaton CB, Kroenke CH, LeBlanc ES, et al. Reproductive Risk Factors and Coronary Heart Disease in the Women's Health Initiative Observational Study. *Circulation*. 2016;133(22):2149-58.
7. Wang YX, Minguez-Alarcon L, Gaskins AJ, Wang L, Ding M, Missmer SA, et al. Pregnancy loss and risk of cardiovascular disease: the Nurses' Health Study II. *Eur Heart J*. 2022;43(3):190-9.
8. Christiansen OB. Special Issue Recurrent Pregnancy Loss: Etiology, Diagnosis, and Therapy. *J Clin Med*. 2021;10(21).
9. Mor G, Cardenas I. The Immune System in Pregnancy: A Unique Complexity. *Am J Reprod Immunol*. 2010;63(6):425-33.
10. Mor G, Cardenas I, Abrahams V, Guller S. Inflammation and pregnancy: the role of the immune system at the implantation site. *Ann Ny Acad Sci*. 2011;1221:80-7.
11. Moffett-King A. Natural killer cells and pregnancy. *Nat Rev Immunol*. 2002;2(9):656-63.
12. Gaynor LM, Colucci F. Uterine Natural Killer Cells: Functional Distinctions and influence on Pregnancy in Humans and Mice. *Frontiers in Immunology*. 2017;8.
13. Fraser R, Whitley GS, Johnstone AP, Host AJ, Sebire NJ, Thilaganathan B, et al. Impaired decidual natural killer cell regulation of vascular remodelling in early human pregnancies with high uterine artery resistance. *J Pathol*. 2012;228(3):322-32.
14. Faas MM, de Vos P. Uterine NK cells and macrophages in pregnancy. *Placenta*. 2017;56:44-52.
15. Hanna J, Goldman-Wohl D, Hamani Y, Avraham I, Greenfield C, Natanson-Yaron S, et al. Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. *Nat Med*. 2006;12(9):1065-74.
16. Robson A, Harris LK, Innes BA, Lash GE, Aljunaidy MM, Aplin JD, et al. Uterine natural killer cells initiate spiral artery remodeling in human pregnancy. *Faseb J*. 2012;26(12):4876-85.
17. Seshadri S, Sunkara SK. Natural killer cells in female infertility and recurrent miscarriage: a systematic review and meta-analysis. *Hum Reprod Update*. 2014;20(3):429-38.
18. Tang AW, Alfirevic Z, Quenby S. Natural killer cells and pregnancy outcomes in women with recurrent miscarriage and infertility: a systematic review. *Hum Reprod*. 2011;26(8):1971-80.
19. Tuckerman E, Mariee N, Prakash A, Li TC, Laird S. Uterine natural killer cells in peri-implantation endometrium from women with repeated implantation failure after IVF. *J Reprod Immunol*. 2010;87(1-2):60-6.
20. Wang S, Li YP, Ding B, Zhao YR, Chen ZJ, Xu CY, et al. Recurrent miscarriage is associated with a decline of decidual natural killer cells expressing killer cell immunoglobulin-like receptors specific for human leukocyte antigen C. *J Obstet Gynaecol Re*. 2014;40(5):1288-95.
21. Eskicioglu F, Özdemir AT, Özdemir RB, Turan GA, Akan Z, Hasdemir SP. The association of HLA-G and immune markers in recurrent miscarriages. *J Matern-Fetal Neo M*. 2016;29(18):3056-60.

22. Giuliani E, Parkin KL, Lessey BA, Young SL, Fazleabas AT. Characterization of Uterine NK Cells in Women with Infertility or Recurrent Pregnancy Loss and Associated Endometriosis. *Am J Reprod Immunol.* 2014;72(3):262-9.
23. Ghafourian M, Karami N, Khodadadi A, Nikbakht R. Increase of CD69, CD161 and CD94 on NK Cells in Women with Recurrent Spontaneous Abortion and in Vitro Fertilization Failure. *Iran J Immunol.* 2014;11(2):84-96.
24. Habets DHJ, Schlütter A, van Kuijk SMJ, Spaanderman MEA, Al-Nasiry S, Wieten L. Natural killer cell profiles in recurrent pregnancy loss: Increased expression and positive associations with TACTILE and LILRB1. *Am J Reprod Immunol.* 2022;88(5).
25. Fukui A, Funamizu A, Fukuhara R, Shibahara H. Expression of natural cytotoxicity receptors and cytokine production on endometrial natural killer cells in women with recurrent pregnancy loss or implantation failure, and the expression of natural cytotoxicity receptors on peripheral blood natural killer cells in pregnant women with a history of recurrent pregnancy loss. *J Obstet Gynaecol Res.* 2017;43(11):1678-86.
26. Shakhar K, Ben-Eliyahu S, Loewenthal R, Rosenne E, Carp H. Differences in number and activity of peripheral natural killer cells in primary versus secondary recurrent miscarriage. *Fertil Steril.* 2003;80(2):368-75.
27. Karami N, Boroujerdnia MG, Nikbakht R, Khodadadi A. Enhancement of peripheral blood CD56(dim) cell and NK cell cytotoxicity in women with recurrent spontaneous abortion or in vitro fertilization failure. *J Reprod Immunol.* 2012;95(1-2):87-92.
28. Yoo JH, Kwak-Kim J, Han AR, Ahn H, Cha SH, Koong MK, et al. Peripheral blood NK cell cytotoxicities are negatively correlated with CD8(+) T cells in fertile women but not in women with a history of recurrent pregnancy loss. *Am J Reprod Immunol.* 2012;68(1):38-46.
29. Hosseini S, Zarnani AH, Asgarian-Omran H, Vahedian-Dargahi Z, Eshraghian MR, Akbarzadeh-Pasha Z, et al. Comparative analysis of NK cell subsets in menstrual and peripheral blood of patients with unexplained recurrent spontaneous abortion and fertile subjects. *J Reprod Immunol.* 2014;103:9-17.
30. Benner M, Feyaerts D, Lopez-Rincon A, van der Heijden OWH, van der Hoorn ML, Joosten I, et al. A combination of immune cell types identified through ensemble machine learning strategy detects altered profile in recurrent pregnancy loss: a pilot study. *F S Sci.* 2022;3(2):166-73.
31. Chastin SFM, Abaraogu U, Bourgois JG, Dall PM, Darnborough J, Duncan E, et al. Effects of Regular Physical Activity on the Immune System, Vaccination and Risk of Community-Acquired Infectious Disease in the General Population: Systematic Review and Meta-Analysis. *Sports Med.* 2021;51(8):1673-86.
32. Gustafson MP, Wheatley-Guy CM, Rosenthal AC, Gastineau DA, Katsanis E, Johnson BD, et al. Exercise and the immune system: taking steps to improve responses to cancer immunotherapy. *J Immunother Cancer.* 2021;9(7).
33. Scheffer DDL, Latini A. Exercise-induced immune system response: Anti-inflammatory status on peripheral and central organs. *Biochim Biophys Acta Mol Basis Dis.* 2020;1866(10):165823.
34. Idorn M, Hojman P. Exercise-Dependent Regulation of NK Cells in Cancer Protection. *Trends in Molecular Medicine.* 2016;22(7):565-77.
35. Quintana-Mendias E, Rodriguez-Villalobos JM, Gastelum-Arellanez A, Cervantes N, Carrasco-Legleu CE, Espino-Solis GP. The Effect of Acute Physical Exercise on Natural Killer Cells Populations and Cytokine Levels in Healthy Women. *Sports (Basel).* 2023;11(10).
36. Millard AL, Valli PV, Stussi G, Mueller NJ, Yung GP, Seebach JD. Brief Exercise Increases Peripheral Blood NK Cell Counts without Immediate Functional Changes, but Impairs their Responses to ex vivo Stimulation. *Front Immunol.* 2013;4:125.
37. Bigley AB, Rezvani K, Chew C, Sekine T, Pistillo M, Crucian B, et al. Acute exercise preferentially redeploys NK-cells with a highly-differentiated phenotype and augments cytotoxicity against lymphoma and multiple myeloma target cells. *Brain Behav Immun.* 2014;39:160-71.

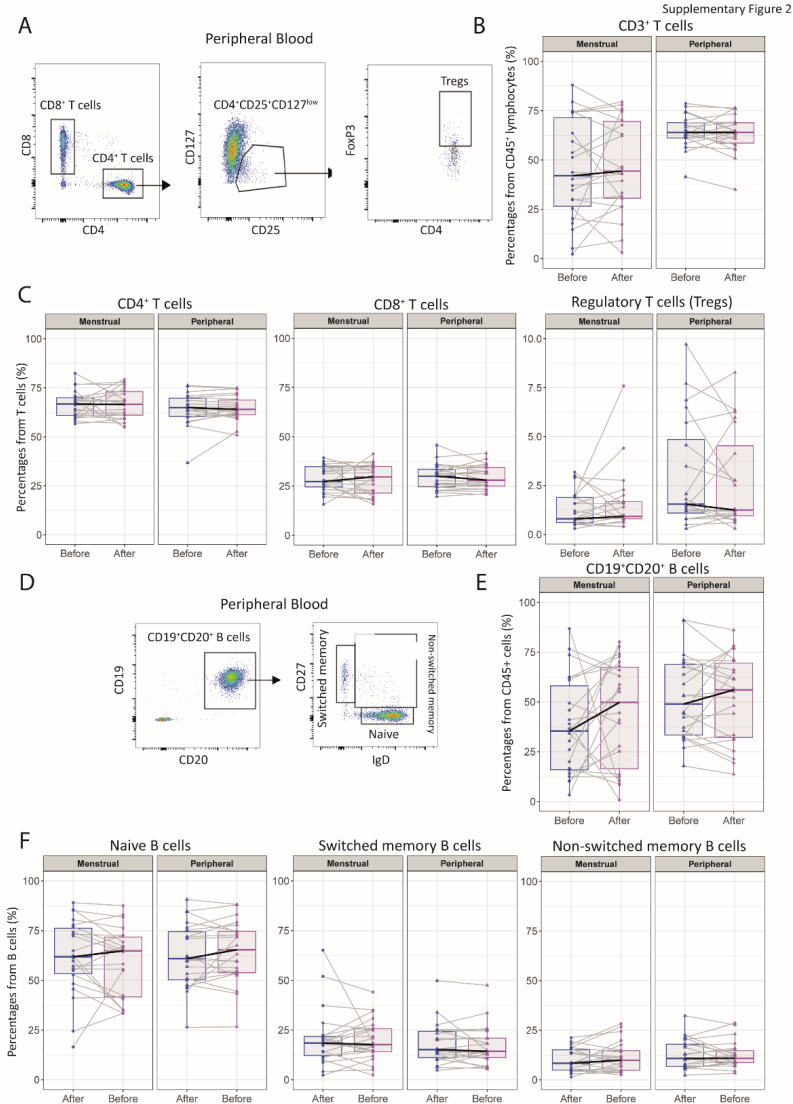
38. Hanson ED, Sakkal S, Que S, Cho E, Spielmann G, Kadife E, et al. Natural killer cell mobilization and egress following acute exercise in men with prostate cancer. *Exp Physiol.* 2020;105(9):1524-39.
39. Rumpf C, Proschinger S, Schenk A, Bloch W, Lampit A, Javelle F, et al. The Effect of Acute Physical Exercise on NK-Cell Cytolytic Activity: A Systematic Review and Meta-Analysis. *Sports Med.* 2021;51(3):519-30.
40. van der Molen RG, Schutten JH, van Cranenbroek B, ter Meer M, Donckers J, Scholten RR, et al. Menstrual blood closely resembles the uterine immune micro-environment and is clearly distinct from peripheral blood. *Hum Reprod.* 2014;29(2):303-14.
41. RPL EGG0, Bender Atik R, Christiansen OB, Elson J, Kolte AM, Lewis S, et al. ESHRE guideline: recurrent pregnancy loss. *Hum Reprod Open.* 2018;2018(2):hoy004.
42. Villanueva RAM, Chen ZJ. ggplot2: Elegant Graphics for Data Analysis, 2nd edition. *Meas-Interdiscip Res.* 2019;17(3):160-7.
43. Wickham H. ggplot2: Elegant Graphics for Data Analysis. *Use R.* 2009:1-212.
44. Barrow AD, Martin CJ, Colonna M. The Natural Cytotoxicity Receptors in Health and Disease. *Front Immunol.* 2019;10:909.
45. Delahaye NF, Rusakiewicz S, Martins I, Ménard C, Roux S, Lyonnet L, et al. Alternatively spliced NKp30 isoforms affect the prognosis of gastrointestinal stromal tumors. *Nat Med.* 2011;17(6):700-+.
46. Comins-Boo A, Cristobal I, Fernandez-Arquero M, Rodriguez de Frias E, Calvo Urrutia M, Pilar Suarez L, et al. Functional NK surrogate biomarkers for inflammatory recurrent pregnancy loss and recurrent implantation failure. *Am J Reprod Immunol.* 2021;86(2):e13426.
47. Zhang Y, Huang C, Lian R, Xu J, Fu Y, Zeng Y, et al. The low cytotoxic activity of peripheral blood NK cells may relate to unexplained recurrent miscarriage. *Am J Reprod Immunol.* 2021;85(6):e13388.
48. Habets DHJ, Schlutter A, van Kuijk SMJ, Spaanderman MEA, Al-Nasiry S, Wieten L. Natural killer cell profiles in recurrent pregnancy loss: Increased expression and positive associations with TACTILE and LILRB1. *Am J Reprod Immunol.* 2022;88(5):e13612.
49. Ntrivalas EI, Bowser CR, Kwak-Kim J, Beaman KD, Gilman-Sachs A. Expression of killer immunoglobulin-like receptors on peripheral blood NK cell subsets of women with recurrent spontaneous abortions or implantation failures. *Am J Reprod Immunol.* 2005;53(5):215-21.
50. Kurioka A, Cosgrove C, Simoni Y, van Wilgenburg B, Geremia A, Björkander S, et al. CD161 Defines a Functionally Distinct Subset of Pro-Inflammatory Natural Killer Cells. *Frontiers in Immunology.* 2018;9.
51. Konjevic G, Mirjagic Martinovic K, Vuletic A, Jurisic V, Spuzic I. Distribution of several activating and inhibitory receptors on CD3-CD16+ NK cells and their correlation with NK cell function in healthy individuals. *J Membr Biol.* 2009;230(3):113-23.
52. Mathew PA, Chuang SS, Vaidya SV, Kumaresan PR, Boles KS, Pham HTK. The LLT1 receptor induces IFN- γ production by human natural killer cells. *Molecular Immunology.* 2004;40(16):1157-63.
53. Zargar M, Ghafourian M, Behrahi F, Nikbakht R, Salehi AM. Association of recurrent implantation failure and recurrent pregnancy loss with peripheral blood natural killer cells and interferon-gamma level. *Obstet Gynecol Sci.* 2024;67(1):112-9.
54. Male V, Hughes T, McClory S, Colucci F, Caligiuri MA, Moffett A. Immature NK cells, capable of producing IL-22, are present in human uterine mucosa. *J Immunol.* 2010;185(7):3913-8.
55. Cerdeira AS, Rajakumar A, Royle CM, Lo A, Husain Z, Thadhani RI, et al. Conversion of peripheral blood NK cells to a decidual NK-like phenotype by a cocktail of defined factors. *J Immunol.* 2013;190(8):3939-48.
56. Keskin DB, Allan DSJ, Rybalov B, Andzelmm MM, Stern JNH, Kopcow HD, et al. TGF β promotes conversion of CD16+ peripheral blood NK cells into CD16- NK cells with similarities to decidual NK cells. *PNAS.* 2007;104(9):3378-83.
57. McMenamin M, Lysakova-Devine T, Wingfield M, O'Herlihy C, O'Farrelly C. Endometrial aspiration biopsy: a non-invasive method of obtaining functional lymphoid progenitor cells and mature natural killer cells. *Reprod Biomed Online.* 2012;25(3):322-8.

58. Vacca P, Vitale C, Montaldo E, Conte R, Cantoni C, Fulcheri E, et al. CD34+ hematopoietic precursors are present in human decidua and differentiate into natural killer cells upon interaction with stromal cells. *Proc Natl Acad Sci U S A*. 2011;108(6):2402-7.
59. Jan P, Asp S, Daugaard JR, Richter EA, Klokke M, Pedersen BK. Effect of eccentric exercise on natural killer cell activity. *J Appl Physiol*. 1995;78(4):1442-6.
60. Nieman DC, Henson DA, Sampson CS, Herring JL, Suttles J, Conley M, et al. The Acute Immune Response to Exhaustive Resistance Exercise. *Int J Sports Med*. 1995;16(5):322-8.
61. McFarlin BK, Flynn MG, Phillips MD, Stewart LK, Timmerman KL. Chronic Resistance Exercise Training Improves Natural Killer Cell Activity in Older Women. *J Gerontol*. 2005;60A(10):1315-8.
62. Lancaster GI, Halson SL, Khan Q, Drysdale P, Wallace F, Jeukendrup AE, et al. Effects of acute exhaustive exercise and chronic exercise training on type 1 and type 2 T lymphocytes. *Exerc Immunol Rev*. 2004;10:91-106.
63. Graff RM, Kunz HE, Agha NH, Baker FL, Laughlin M, Bigley AB, et al. beta(2)-Adrenergic receptor signaling mediates the preferential mobilization of differentiated subsets of CD8+ T-cells, NK-cells and non-classical monocytes in response to acute exercise in humans. *Brain Behav Immun*. 2018;74:143-53.
64. McFarlin BK, Flynn MG, Phillips MD, Stewart LK, Timmerman KL. Chronic resistance exercise training improves natural killer cell activity in older women. *J Gerontol a-Biol*. 2005;60(10):1315-8.
65. Rowbottom DG, Green KJ. Acute exercise effects on the immune system. *Med Sci Sport Exerc*. 2000;32(7):S396-S405.
66. Neves PRD, Tenorio TRD, Lins TA, Muniz MTC, Pithon-Curi TC, Botero JP, et al. Acute effects of high- and low-intensity exercise bouts on leukocyte counts. *J Exerc Sci Fit*. 2015;13(1):24-8.
67. Natale VM, Brenner IK, Moldoveanu AI, Vasiliou P, Shek P, Shephard RJ. Effects of three different types of exercise on blood leukocyte count during and following exercise. *Sao Paulo Med J*. 2003;121(1):9-14.
68. Colbert LH, Visser M, Simonsick EM, Tracy RP, Newman AB, Kritchevsky SB, et al. Physical Activity, Exercise, and Inflammatory Markers in Older Adults: Findings from The Health, Aging and Body Composition Study. *J Am Geriatr Soc*. 1995;43(7):1098-104.
69. Henson J, Yates T, Edwardson CL, Khunti K, Talbot D, Gray LJ, et al. Sedentary time and markers of chronic low-grade inflammation in a high risk population. *PLoS One*. 2013;8(10):e78350.
70. de Almeida-Neto PF, Goncalves CAM, Wilde P, Jaggars JR, Junior GBC, de Farias Sales VS, et al. Influence of age and fitness level on immune responses of T and NK cells in healthy physically active subjects after strenuous aerobic exercise: a cross-sectional study. *Front Immunol*. 2023;14:1252506.
71. Ivarsson MA, Stiglund N, Marquardt N, Westgren M, Gidlof S, Bjorkstrom NK. Composition and dynamics of the uterine NK cell KIR repertoire in menstrual blood. *Mucosal Immunol*. 2017;10(2):322-31.
72. Feyaerts D, Kuret T, van Cranenbroek B, van der Zeeuw-Hingrez S, van der Heijden OWH, van der Meer A, et al. Endometrial natural killer (NK) cells reveal a tissue-specific receptor repertoire. *Hum Reprod*. 2018;33(3):441-51.
73. Salker M, Teklenburg G, Molokhia M, Lavery S, Trew G, Aojanepong T, et al. Natural selection of human embryos: impaired decidualization of endometrium disables embryo-maternal interactions and causes recurrent pregnancy loss. *PLoS One*. 2010;5(4):e10287.
74. Quenby S, Vince G, Farquharson R, Aplin J. Recurrent miscarriage: a defect in nature's quality control? *Hum Reprod*. 2002;17(8):1959-63.

Supplementary Material

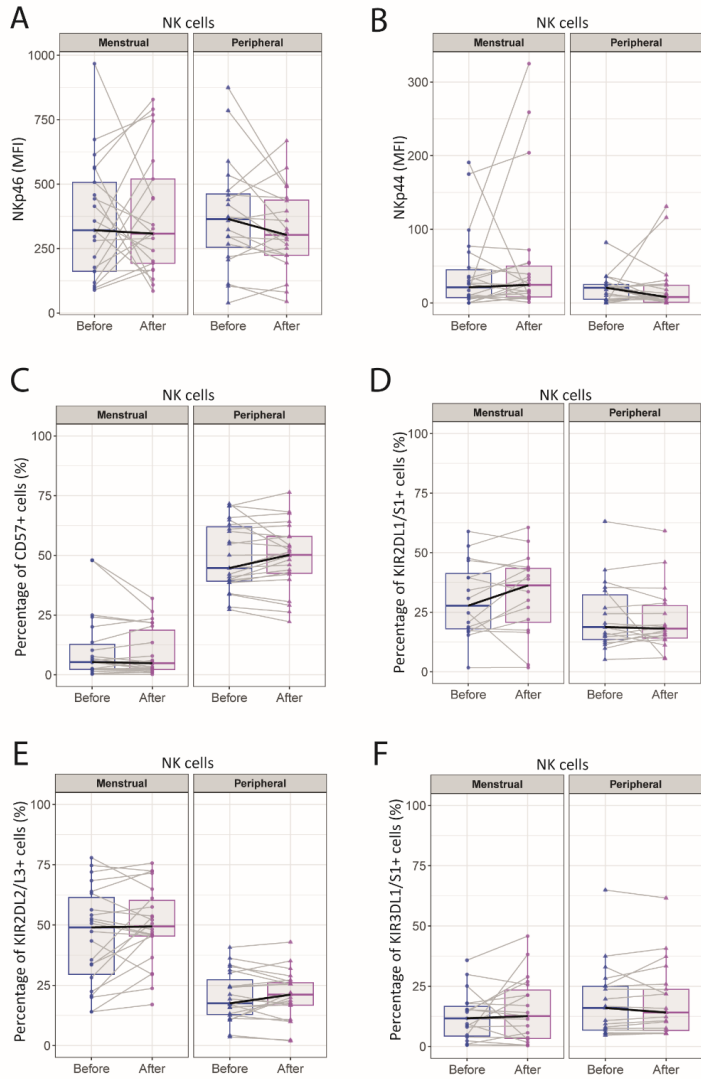


Supplementary Figure 1. Flow chart of study population

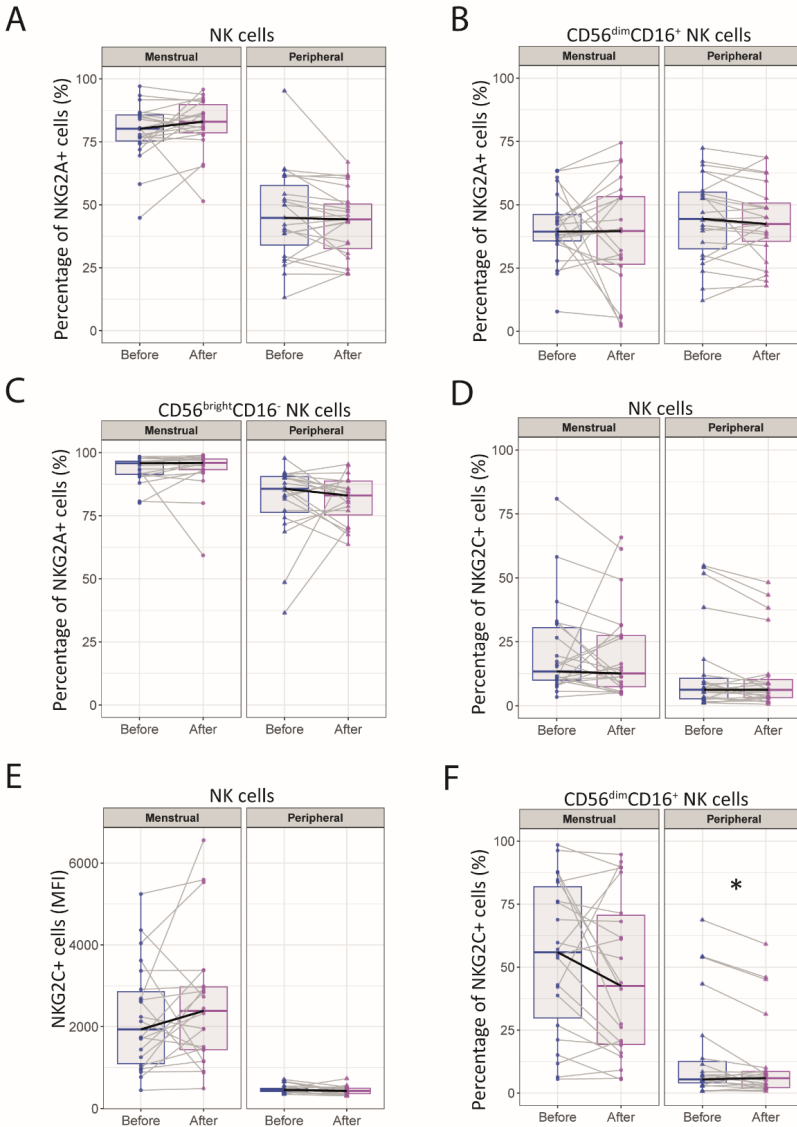


Supplementary Figure 2. Frequencies of different T cell and B cell subsets in MB and PB before and after the moderate-intensity aerobic exercise intervention in women with uRPL.

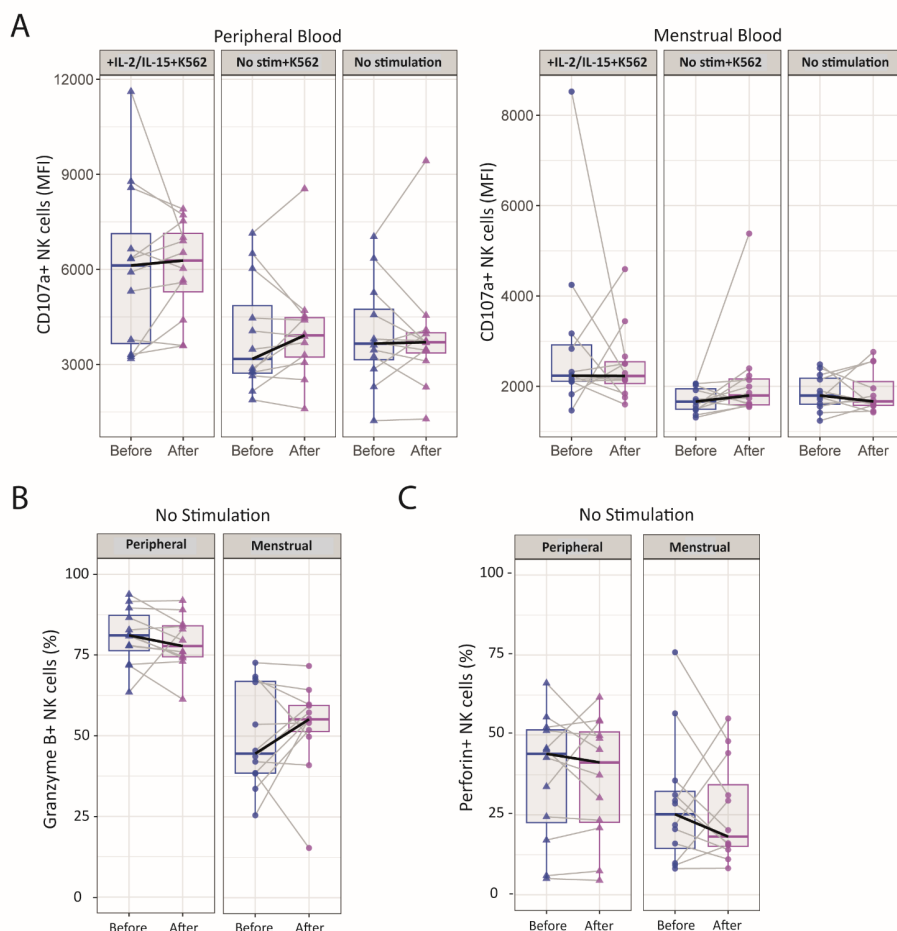
A) Representative dot plots showing the gating strategy for determining T cell subsets. B) Percentages of CD56⁺CD3⁺ T cells of CD45⁺ lymphocytes in MB and PB before and after the moderate-intensity aerobic exercise intervention. C) Percentages of CD4⁺ T cells, CD8⁺ T cells, and CD125^{low}CD25⁺ regulatory T cells. D) Representative dot plots showing the gating strategy for determining B cell subsets. E) Percentages of CD19⁺CD20⁺ B cells of CD45⁺ lymphocytes in MB and PB. F) Percentages of naive B cells, switched memory B cells, and non-switched memory B cells of CD19⁺CD20⁺ B cells. PB n=22, MB n=22. The dots represent the uterine NK cells, the triangles the peripheral NK cells (blue=before intervention, magenta=after intervention). Boxplots visualize the median (bold black line) and the interquartile ranges. A Paired Wilcoxon signed-rank test was performed to determine statistical significance.



Supplementary Figure 3. Expression of NKp46, NKp30, CD57, and killer immunoglobulin-like receptors by uterine NK and peripheral NK cells before and after the moderate-intensity aerobic exercise intervention in women with uRPL. Median fluorescent intensity (MFI) of A) NKp46 and B) NKp44 by NK cells in MB and PB before and after the moderate-intensity aerobic exercise intervention. Percentages of C) CD57⁺, D) KIR2DL1⁺, E) KIR2DL2/L3⁺, and F) KIR3DL1⁺ NK cells in MB and PB. PB n=21, MB n=22. MFIs were normalized to the MFI of the fluorescence minus one control (FMO). The dots represent the uterine NK cells, the triangles the peripheral NK cells (blue=before intervention, magenta=after intervention). Boxplots visualize the median (bold black line) and the interquartile ranges. A Paired Wilcoxon signed-rank test was performed to determine statistical significance.



Supplementary Figure 4. Expression of NKG2A and NKG2C by uterine NK and peripheral NK cells before and after the moderate-intensity aerobic exercise intervention in women with uRPL. Percentages of NKG2A⁺ A) CD56⁺CD3⁺ total NK cells, B) CD56^{dim}CD16⁺ NK cells, and C) CD56^{bright}CD16⁺ NK cells in MB and PB before and after the moderate-intensity aerobic exercise intervention. D) Percentages of NKG2C⁺CD56⁺CD3⁺ total NK cells. E) Median fluorescent intensity (MFI) of NKG2C in total NK cells. F) Percentage of NKG2C⁺CD56^{dim}CD16⁺ NK cells in MB and PB. PB n=23, MB n=22. The dots represent the uterine NK cells, the triangles the peripheral NK cells (blue=before intervention, magenta=after intervention). Boxplots visualize the median (bold black line) and the interquartile ranges. MFIs were normalized to the MFI of the fluorescent minus one (FMO) control. A Paired Wilcoxon signed-rank test was performed to determine statistical significance (*p<0.05).



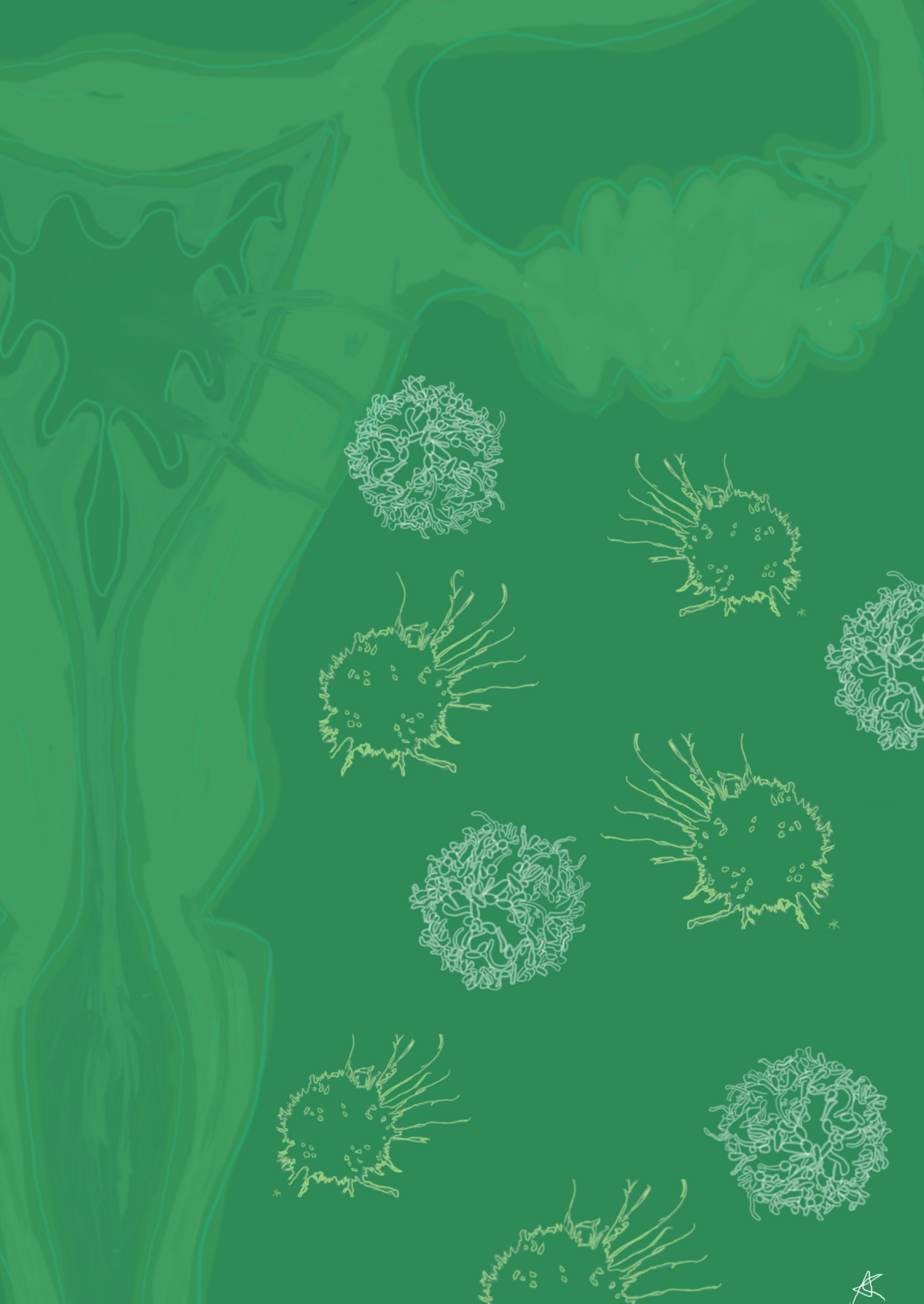
Supplementary Figure 5. Expression of functional makers CD107a, granzyme-B, and perforin by uterine NK and peripheral NK cells before and after the moderate-intensity aerobic exercise intervention in women with uRPL. A) Median Fluorescent Intensity (MFI) of CD107a⁺ NK cells from MB and PB in the IL-2 and IL-15 stimulated and co-cultured with K562 (IL-2/IL-15+K562, left panel), unstimulated and co-cultured with K562 (No stim+K562, central panel), and unstimulated (No stimulation, right panel) conditions. B) Percentages of granzyme B⁺ unstimulated NK cells in PB and MB. C) PB n=12, MB, n=12. Percentages of perforin⁺ unstimulated NK cells in PB and MB. MFIs were normalized to the MFI of the fluorescence minus one (FMO) control. The dots represent the uterine NK cells, the triangles the peripheral NK cells (blue=before intervention, magenta=after intervention). Boxplots visualize the median (bold black line) and the interquartile ranges. A Paired Wilcoxon signed-rank test was performed to determine statistical significance.

Supplementary Table 1. Exclusion criteria

Exclusion criteria
Age > 40 years
BMI > 40
Current use of immunosuppressive or biological drugs
Current use of hormonal contraceptives
HIV positivity
Current or recent (< 2 weeks) symptomatic genital infection
Pre-existent diabetes mellitus, autoimmune disease, or overt cardiovascular disease
Vaccinations within 1 month prior to or during sampling and intervention
Breastfeeding
Current or recent (< 3 months ago) pregnancy
(Physical) inabilities to follow the moderate-intensity aerobic cycling training
Participants who are not capable of signing the informed consent

Supplementary Table 2. List of antibodies used in flow cytometry experiments

Antibody	Fluorochrome	Clone
CD45	Krome Orange	J33
CD3	BV605	HIT3a
CD3	PE-Cy7	UCHT1
CD56	BV711	NCAM16.2
CD16 (FcγRII)	APC-H7	3G8
CD4	PE-Cy7	SFC112T4D1
CD8	APC-AF700	B9.11
CD19	APC-R700	H1B19
CD20	BV786	2H7
HLA-DR	FITC	G46-6
CD38	PE	HB.7
IgD	APC	IA6-2
CD27	BV421	M-T271
CD127	AF647	eBioRDR5
CD197 (CCR7)	BV421	G043H7
FoxP3	PE	PCH101
CD45RA	FITC	ALB11
CD25	PerCP-Cy5.5	M-A251
CD158a (KIR2DL1)	FITC	REA284
CD159c (NKG2C)	AF700	134522
CD336 (NKP44)	PE	Z231
CD337 (NKP30)	PerCP-Cy5.5	P30-15
CD335 (NKP46)	PE-Cy7	BAB281
CD161 (KLRB1)	eFluor 450	HP-3G10
Perforin	Pacific Blue	dG9
Granzyme B	PerCP-Cy5.5	QA16A02
IFN-γ	PE-Cy7	4S.B3
CD107a	PE	H4A3
CD16	AF700	3G8



Cellular fingerprinting reveals a dominant role for the IL-6-JAK-STAT3 axis in relation to T-cell lineage commitment in endometriosis

Aysel Gurbanova¹, Emma Huijs², Johanna Franz^{3,4,5}, Esther van Rijssen², Tal Pecht^{3,4,5}, Janneke S. Hoogstad-van Evert^{2,6}, Annemiek W. Nap², Ruben L. Smeets¹, Hans Koenen¹, Marien I. de Jonge¹ and Renate G. van der Molen¹

¹ Department of Laboratory Medicine, Laboratory for Medical Immunology, Radboud University Medical Center, Nijmegen, The Netherlands

² Department of Obstetrics & Gynecology, Radboud University Medical Center, Nijmegen, The Netherlands

³ Systems Medicine, German Center for Neurodegenerative Diseases (DZNE), Bonn, Germany

⁴ Department for Genomics and Immunoregulation, Life & Medical Sciences (LIMES) Institute, University of Bonn, Bonn, Germany

⁵ Institute of Clinical Chemistry and Clinical Pharmacology, University Hospital Bonn, Bonn, Germany, Bonn, Germany

⁶ Department of Obstetrics & Gynecology, Amphia, Breda, The Netherlands

Manuscript in preparation

Abstract

Endometriosis is a chronic, estrogen-dependent disease characterized by inflammation and immune dysregulation. Cytokine-mediated activation of the JAK–STAT signaling pathway plays a significant role in driving inflammation and modulating immune responses. In this study, we examined STAT3 phosphorylation in immune cells from individuals with endometriosis after stimulation with inflammatory cytokines. First, we showed that plasma samples from individuals with endometriosis contained significantly higher IL-6 and MMP-10 concentrations ($p=0.021$ and $p=0.034$, respectively), than controls. Cytokine stimulation of peripheral blood mononuclear cells (PBMC) obtained from individuals with endometriosis induced significantly higher STAT3 phosphorylation (p-STAT3) compared to controls ($p=0.035$), with IL-6 alone sufficient to elicit this response. Notably, the selective JAK1 inhibitor, Upadacitinib, reduced pSTAT3 to baseline levels. Furthermore, pSTAT3 levels positively correlated with the level of FoxP3 and ROR- γ t expression, suggesting a potential role for STAT3 signaling in shaping T-cell lineage commitment toward T helper 17 (Th17) or regulatory T (Treg) cell fates. Overall, these findings identify the JAK–STAT3 axis as a key immunoregulatory pathway and a promising therapeutic target in the treatment of endometriosis.

Keywords: endometriosis, inflammation, JAK-STAT, IL-6, Upadacitinib

Introduction

Endometriosis is a chronic inflammatory disease affecting approximately 2–10% of women of reproductive age (1). It is classically characterized by the presence of endometrial-like tissue outside the uterus, most commonly in the peritoneal cavity, and is associated with pain symptoms and infertility (2, 3). However, growing evidence suggests that endometriosis is not merely a localized pelvic disorder but a systemic condition marked by immune dysfunction and chronic inflammation (4, 5). Although the exact origin of endometriosis remains debated, hypotheses such as retrograde menstruation, coelomic metaplasia, and stem cell migration have been proposed (6). One of the hallmark features of endometriosis is persistent inflammation, observed both locally in the peritoneal cavity and systemically (7). Yet, it remains unclear whether inflammation initiates the disease or perpetuates its progression.

Levels of inflammatory mediators including interleukin (IL)-1 β , IL-6, IL-18, TNF- α , c-reactive protein (CRP), Vascular Endothelial Growth Factor (VEGF), and various chemokines have been consistently investigated in the plasma, peritoneal fluid (PF), and endometrial tissue of individuals with endometriosis (8–12). However, the results of these studies are often conflicting, primarily due to variations in the study design, control group, and disease stage. Inflammatory mediators are often involved in crucial intracellular signaling pathways, which regulate immune cell responses. Janus kinase/signal transducer and activator of transcription (JAK–STAT) signaling pathway is regarded as one of the main communication nodes in the cell function. JAK-STAT is activated by hormones, cytokines and growth factors (13) and is implicated in malignancies and autoimmune disorders (14–16). Upon binding to their respective ligand JAKs recruit STATs that are further phosphorylated and transported to the nucleus to regulate genes involved in immune cell functions. The signal transducer and activator of transcription (STAT) family comprises of STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6, each playing lineage-specific roles in immune cell differentiation and function (17).

In endometriosis, aberrant STAT3 signaling has been implicated in both immune dysfunction and lesion persistence (18, 19). Notably, increased levels of phosphorylated STAT3 (p-STAT3) have been detected in the eutopic endometrium of individuals with adenomyosis across both the proliferative and secretory phases compared to healthy controls (20). While p-STAT3 plays a physiological role in endometrial receptivity and decidualization during normal pregnancy, its persistent activation in endometriosis may promote cell survival and disease progression (21). STAT3 is known to influence T cell lineage commitment by promoting differentiation

toward the T helper 17 (Th17) phenotype by inducing RAR-related orphan receptor gamma (ROR- γ t) expression (22). Moreover, sustained inflammatory signaling through the STAT3 pathway has been shown to inhibit FoxP3 expression and promote effector T cell expansion, thereby contributing to immune dysregulation in chronic inflammatory conditions (22). Despite evidence of increased p-STAT3 in endometrial tissues, it remains unclear whether STAT3 is activated in peripheral immune cells, especially T cells, in individuals with endometriosis.

It was shown that T cell profiles are altered in endometriosis, with an increased peritoneal CD4/CD8 T-cell ratio and accumulation of Th17 cells correlating with advanced disease stages (23, 24). Findings regarding regulatory T (Treg) cells in endometriosis vary depend on the sample type investigated. Some studies report increased frequencies of FoxP3⁺ Tregs in the endometrial tissue located within the uterus (i.e. eutopic endometrium) or peritoneal fluid (PF) (25), whereas others describe reduced Treg frequencies in blood accompanied by higher percentages in PF (24). In addition to conventional T cells, mucosal-associated invariant T (MAIT) cells have been implicated in the inflammatory processes underlying endometriosis. A recent study has reported increased frequencies of MAIT cells in the PF of women with endometriosis compared to healthy controls (26). Given the critical role of T cells in endometriosis, understanding how STAT3 influences T-cell lineage commitment could provide valuable insights into disease mechanisms and inform potential therapeutic strategies.

In this study, we investigated STAT3 activation and its role in T cell differentiation of individuals suffering from endometriosis, with a focus on the Th17 and Treg cell lineages.

Materials & Methods

Study Population

This study was conducted at the Radboud University Medical Center in Nijmegen, the Netherlands, between February 2023 and June 2024. Ethical approval was obtained from the regional ethics committee METC Oost-Nederland (reference number: 2022-13650). Two groups were recruited: women with endometriosis and healthy controls. Participants were enrolled through the outpatient clinic at Radboudumc and via social media platforms of the Dutch patient advocacy group, the Endometriosis Society. Interested women were screened based on predefined inclusion and exclusion criteria. Eligible participants in the endometriosis group

were women with a confirmed diagnosis of endometriosis or adenomyosis, established by transvaginal ultrasound, Magnetic Resonance Imaging (MRI), or surgical findings, and who reported pain symptoms with a Numeric Rating Scale (NRS) score greater than four. Healthy controls were recruited from the same cohort and were matched for age and reproductive status but had no history of endometriosis.

All participants had to be at least 16 years old, nulligravida, premenopausal, and have a Body Mass Index (BMI) between 18 and 30 kg/m². Exclusion criteria for both groups included current pregnancy, a diagnosis of Crohn's disease, ulcerative colitis, short bowel syndrome, other chronic inflammatory diseases, celiac disease, or active smoking.

OLINK proximity assay for measurement of inflammatory proteins

PB was drawn during different stages of menstrual cycle and collected in tubes containing Sodium Heparin (BD Vacutainer). To collect plasma, PB was centrifuged for 10 min at 20 °C, 2204 rpm. The collected plasma was stored at –80°C. To measure pro-inflammatory markers in the plasma, 92-plex Olink inflammation panel (Olink Proteomics AB) was used.

Isolation and freezing of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated using Lymphoprep™ (STEMCELL Technologies) following the manufacturer's instructions. Samples were centrifuged for 15 minutes at 800 × g, without brake, at 20°C. The isolated PBMCs were washed twice with PBS supplemented with 2% heat-inactivated pooled human serum (HPS) and subsequently resuspended in RPMI 1640 medium containing 10% HPS. Cells were cryopreserved at a concentration of 10 million cells per milliliter in Recovery™- cell freezing medium (Gibco, Life Technologies). The samples were initially stored at –80°C for 24 hours before being transferred to liquid nitrogen for long-term storage.

Immunophenotyping with 42-color flow cytometry

For immunophenotyping immune cell subsets in PB, we used a previously published flow cytometry panel and modified the panel to suit the specific aims of this study (27). Frozen PBMCs were thawed following a standardized protocol. Culture medium was pre-warmed in a 37°C water bath while samples were retrieved from liquid nitrogen. Ampoules were thawed until nearly fully liquid, after which 1 mL of warm culture medium was added dropwise and gently resuspended. The cell suspension was transferred to 15 mL Falcon tubes containing 5 mL of medium

and centrifuged at $452 \times g$ for 5 minutes at room temperature. The wash step was repeated twice using fresh culture medium, followed by a final wash in PBS.

Cells were counted using a DxH 500 Hematology Analyzer (Beckman Coulter). For staining, 500,000 cells were incubated with 125 μL of a surface antibody cocktail for 30 minutes at room temperature in the dark. Unstained controls received 125 μL of PBS. After washing, a second surface antibody cocktail (125 μL) was added and incubated under the same conditions. Cells were washed again with 3 mL of Fluorescence-Activated Cell Sorting (FACS) buffer (0.2% Bovine Serum Albumin (BSA) in PBS), resuspended in 300 μL of FACS buffer, and analyzed on the Sony ID7000 spectral flow cytometer. Antibody details for the flow cytometry can be found in Supplementary Table 1. Samples were processed in multiple batches, each including a PBMC control to monitor inter-batch variability.

Measuring phosphorylation levels of STAT3 with flow cytometry

To assess p-STAT3 (Y705), 250,000 PBMCs were stimulated for 30 minutes at 37°C with either 50 ng/mL IL-6 alone or a cytokine cocktail containing 50 ng/mL IL-6, 10 ng/mL IFN- γ , 10 ng/mL IFN- α , and 10 ng/mL TNF- α . Following stimulation, cells were centrifuged and washed with PBS, then stained with surface marker antibodies for 30 minutes at 4°C in the dark. After surface staining, cells were washed twice with PBS containing 0.2% BSA. Cells were subsequently fixed using pre-warmed BD Cytofix™ (BD Biosciences) for 10 minutes at 37°C , then permeabilized with BD Phosflow Perm buffer (10 $\times$ diluted in PBS, BD Biosciences) for 15 minutes on ice. After two additional washes with PBS + 0.2% BSA, cells were stained with antibodies targeting intracellular phosphorylated STAT proteins for 30 minutes at 4°C in the dark. Finally, cells were washed twice and resuspended in 150 μL of PBS + 0.2% BSA prior to acquisition. Samples were measured using the Sony ID7000 spectral flow cytometer. Antibody details for the staining can be found in Supplementary Table 2.

Inhibition of STAT3 phosphorylation

To inhibit p-STAT3 (Y705), cells were treated with Upadacitinib at concentrations of 1 μM or 10 μM for 30 minutes at 37°C . Following this treatment, p-STAT3 (Y705) was assessed using the stimulation and staining protocol as described above.

Assessment of correlation between p-STAT3 and FoxP3 and ROR- γt

To assess correlation between p-STAT3 (Y705) and T cell markers (FoxP3 and ROR- γt), 250,000 PBMCs were cultured for 30 minutes, 24h, and 48h at 37°C without any stimulation. Following incubation, cells were stained for T cell specific markers

using similar flow cytometry protocol mentioned above. Antibody details for the staining can be found in Supplementary Table 3.

Data analysis and statistics

The 42-color flow cytometry data were analyzed using the *cyCONDOR* package (v0.1.6) (28). Immune cell subset frequency and median fluorescence intensity (MFI) of surface and intracellular markers were extracted. OLINK NPX (log2) data were analyzed for individuals with endometriosis and healthy controls. After standard Olink normalization/QC, proteins with low detectability were removed, and values below LOD were handled per Olink guidance. Global patterns were summarized with z-scored (by protein) heatmaps and PCA, and differential proteins were visualized in volcano plots of Δ NPX versus $-\log_{10}(p)$. For p-STAT3 analyses, MFI of p-STAT3 was compared between individuals with endometriosis and healthy controls were performed using the Wilcoxon rank-sum for unpaired data and p-values < 0.05 were considered statistically significant. Correction for multiple testing has been performed for OLINK and immunophenotyping data using Benjamini–Hochberg FDR. Since our goal was to explore the role of p-STAT3 (Y705), multiple testing correction was not applied to phosphorylation, inhibition, and timepoint experiments. All statistical analyses and plots were generated in R version 4.4.1.

Results

Levels of IL-6 and MMP-10 are significantly higher in individuals with endometriosis compared to healthy controls

To assess systemic inflammation, we compared plasma inflammatory proteins between individuals with endometriosis (n=17) and healthy controls (n=9). A heatmap of NPX values (z-scored by protein) showed more inflammatory profile in individuals with endometriosis compared to healthy controls (Supplementary Figure 1). Principle component analysis (PCA) similarly revealed substantial overlap between groups, except for two outliers within the endometriosis cohort (Figure 1a). Despite this overlap, several proteins including OPG, IL-18, IFN- γ , CCL4, CDCP1, MMP-10, and IL-6 were nominally different between groups (Figure 1b). After Benjamini–Hochberg FDR correction, only IL-6 and MMP-10 remained significantly increased in individuals with endometriosis compared to controls (unadjusted $p=0.021$ and $p=0.034$, respectively; $q<0.05$, Figure 1c-d).

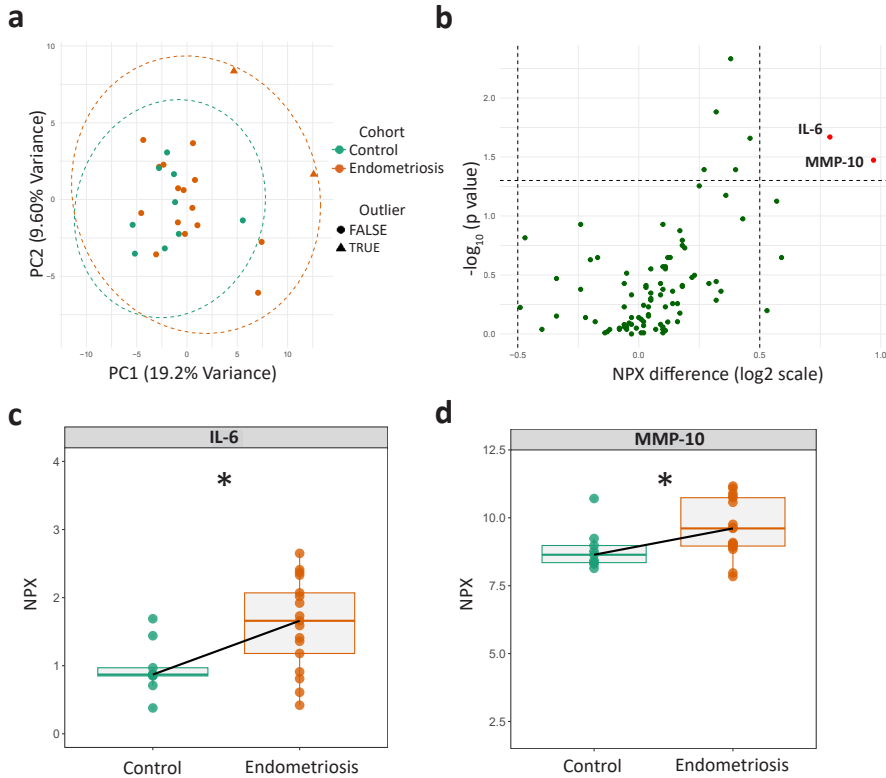


Figure 1. Overview of inflammatory markers in individuals with endometriosis and healthy controls. a) Heatmap showing row-scaled NPX values of inflammatory proteins measured for individuals with endometriosis (orange; n=17) and healthy controls (green; n = 9). b) Principal component analysis (PCA) plot based on inflammatory protein profiles. Outliers for each cohort are indicated with triangles. c) Volcano plot displaying $\log_2$ fold changes versus $-\log_{10}$ p-values of inflammatory proteins. Red dots represent proteins that changed significantly ($p \leq 0.05$) between healthy controls and endometriosis. d) Boxplot showing IL-6 levels measured in plasma from individuals with endometriosis and controls. e) Boxplot showing MMP-10 levels measured in plasma from individuals with endometriosis and controls. Statistical analyses were performed using the Wilcoxon signed-rank test and correction for multiple testing was applied using FDR. * $p \leq 0.05$; ** $p \leq 0.01$.

The percentage of MAIT cells is significantly lower in individuals with endometriosis

We next explored systemic immune cell composition using a 42 parameter (40 color) high-dimensional flow cytometry panel in participants not using hormonal contraception. Initial clustering based on marker expression intensities identified 12 major immune cell populations (Figure 2b). Overall, the composition of systemic immune cell subsets were similar between individuals with endometriosis and healthy controls (n=13 and n=5, respectively; Figure 2a-c). The frequencies of immune cells

between endometriosis and healthy controls were comparable (Figure 2d). Although the percentage of MAIT cells did not remain significant after correction for multiple testing, they were reduced in endometriosis (unadjusted $p=0.035$, Figure 2e).

Within the T-cell compartment, higher-resolution clustering identified 17 T cell subsets (Figure 2f, Supplementary Figure 1b). No significant differences were observed in the frequencies of these cell subsets (Supplementary Figure 2a). The Th1:Th2 ratio were comparable between endometriosis and healthy controls (Supplementary Figure 2c). In addition, we observed a slight increase in Th17:Treg ratio in individuals with endometriosis, however this difference was not statistically significant (Figure 2g). Although the percentages of Th17 was similar between cohorts, the percentages of Tregs were higher in endometriosis (Supplementary Figure 2 d-e).

Individuals with endometriosis have significantly higher p-STAT3 upon stimulation

As STAT3 is implicated in immune dysregulation and the survival of ectopic lesions in endometriosis, we examined its phosphorylation status (18, 19). PBMCs were stimulated for 30 min with a cytokine cocktail (IL-6, IFN- γ , IFN- α , TNF- α , endometriosis $n=10$, control $n=10$). Relative to the unstimulated condition, pSTAT3 increased upon stimulation, and addition of Upadacitinib reduced p-STAT3 to baseline (Figure 3a). The level of p-STAT3 in monocytes did not differ between endometriosis and controls (Figure 3b). B cells and NK cells in endometriosis showed higher p-STAT3 trends that did not reach significance (Figure 3c–d). Notably, the level of p-STAT3 was significantly higher in T cells from endometriosis ($p = 0.035$; Figure 3e). Finally, IL-6 alone was sufficient to induce STAT3 phosphorylation in T cells (endometriosis $n=3$, control $n=3$) (Figure 3f).

p-STAT3 is positively correlated with protein expression of ROR- γ t and FoxP3

Given the role of p-STAT3 in T-cell lineage commitment, we quantified ROR- γ t and FoxP3 protein expression at 30 min, 24 h, and 48 h (endometriosis $n=5$; control $n=5$) (22). The expression intensities of ROR- γ t increased at 24h and 48h timepoint compared to 30 min (Figure 4a-c). We observed that without any stimulation STAT3 is phosphorylated at 24h and 48h (Supplementary Figure 3a-b). Therefore, we did not stimulate the PBMCs any further. While at 30 min the expression intensity of ROR- γ t was similar between cohorts, at 24h timepoint individuals with endometriosis had lower ROR- γ t which went back to similar levels at 48h (Figure 4c). Individuals with endometriosis had lower frequencies of ROR- γ t⁺CCR6⁺ cells at all timepoints

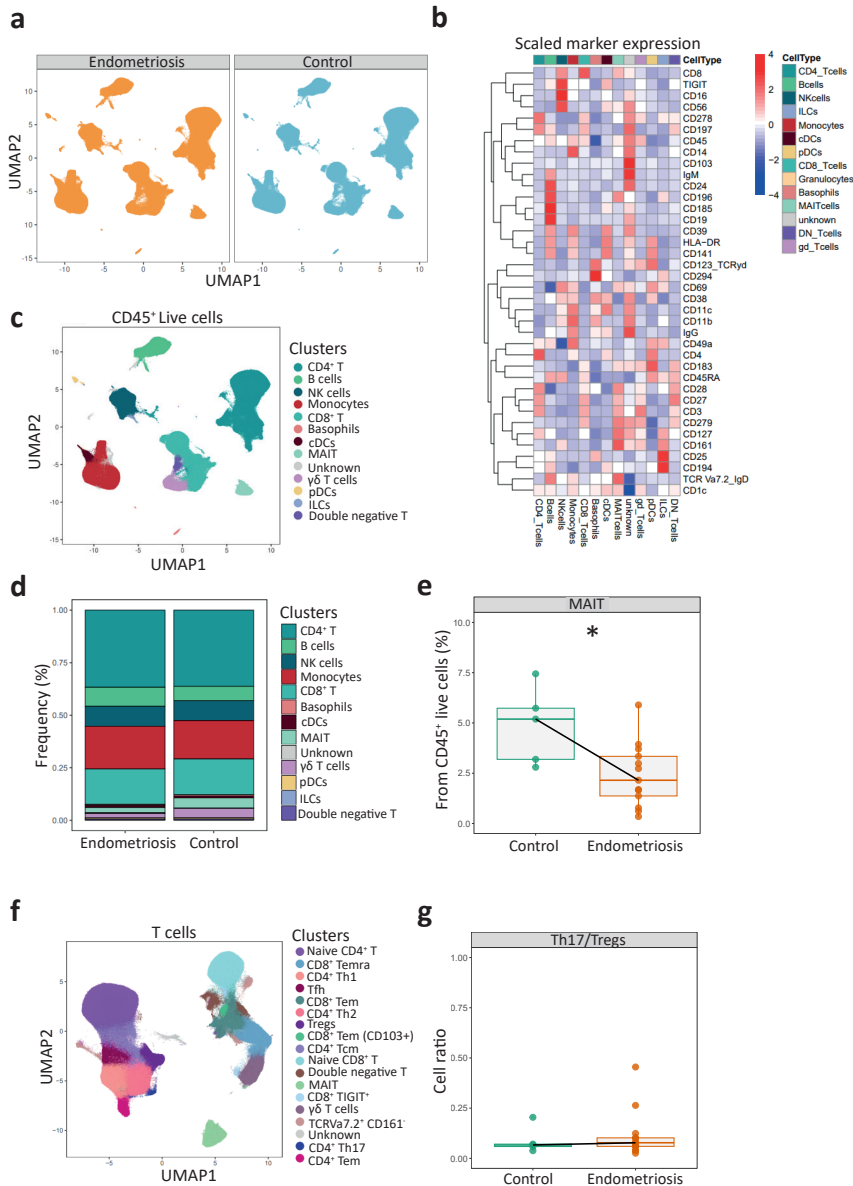


Figure 2. Immune landscape of blood in individuals with endometriosis and controls. a) UMAP plot showing the peripheral immune cell landscape for individuals with endometriosis (orange) and controls (blue). b) Heatmap showing scaled marker expression for each subset. c) Twelve immune cell clusters identified and embedded onto the UMAP plot. d) Stacked bar plot representing the relative proportions of the 12 immune cell clusters for endometriosis and healthy controls. e) Boxplot showing the frequency of MAIT cells for individuals with endometriosis (orange, n=13) and controls (green; n=5). f) UMAP plot of T cell subclusters, with 17 distinct clusters identified. g) Boxplot showing the Th17:Treg cell ratio. Statistical analysis was performed using the Wilcoxon signed-rank test and correction for multiple testing was applied using FDR. * $p \leq 0.05$; ** $p \leq 0.01$.

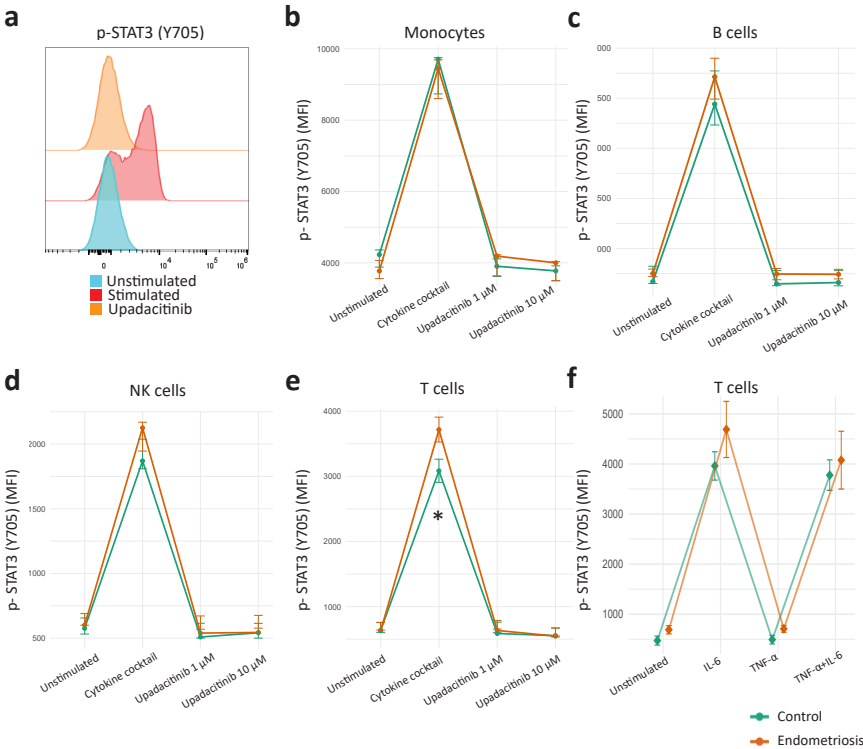


Figure 3. STAT3 phosphorylation is significantly higher on T cells of individuals with endometriosis. a) Histogram showing median expression intensity (MFI) of p-STAT3 for unstimulated (no-stimulation; blue), stimulated (stimulation with cytokine cocktail; red), and Upadacitinib (addition of inhibitor; orange) conditions. b) Line plot showing p-STAT3 in monocytes of individuals with endometriosis (orange; n=10) and controls (green; n=10). c) Line plot showing p-STAT3 in B cells. d) Line plot showing p-STAT3 in NK cells. e) Line plot showing p-STAT3 in T cells. f) Line plot showing p-STAT3 in T cells upon stimulation with either IL-6, TNF- α , or IL-6+TNF- α . Statistical analysis was performed using the Wilcoxon signed-rank test. * $p \leq 0.05$; ** $p \leq 0.01$.

(Figure 4d). However, these results were not statistically significant. Finally, p-STAT3 and ROR- γ t were positively correlated for both endometriosis and controls (Figure 4e).

The expression intensities of FoxP3 increased over time (Figure 4 f-h). When comparing individuals with endometriosis with controls, a trend towards lower FoxP3 expression in CD127^{low}CD25⁺ Tregs was observed at all timepoints in individuals with endometriosis (Figure 4h). Similarly, a trend towards decreased Treg frequencies was observed across all timepoints in the endometriosis cohort (Figure 4i). Finally, a positive correlation between p-STAT3 and FoxP3 expression was observed in both endometriosis and healthy controls (Figure 4j).

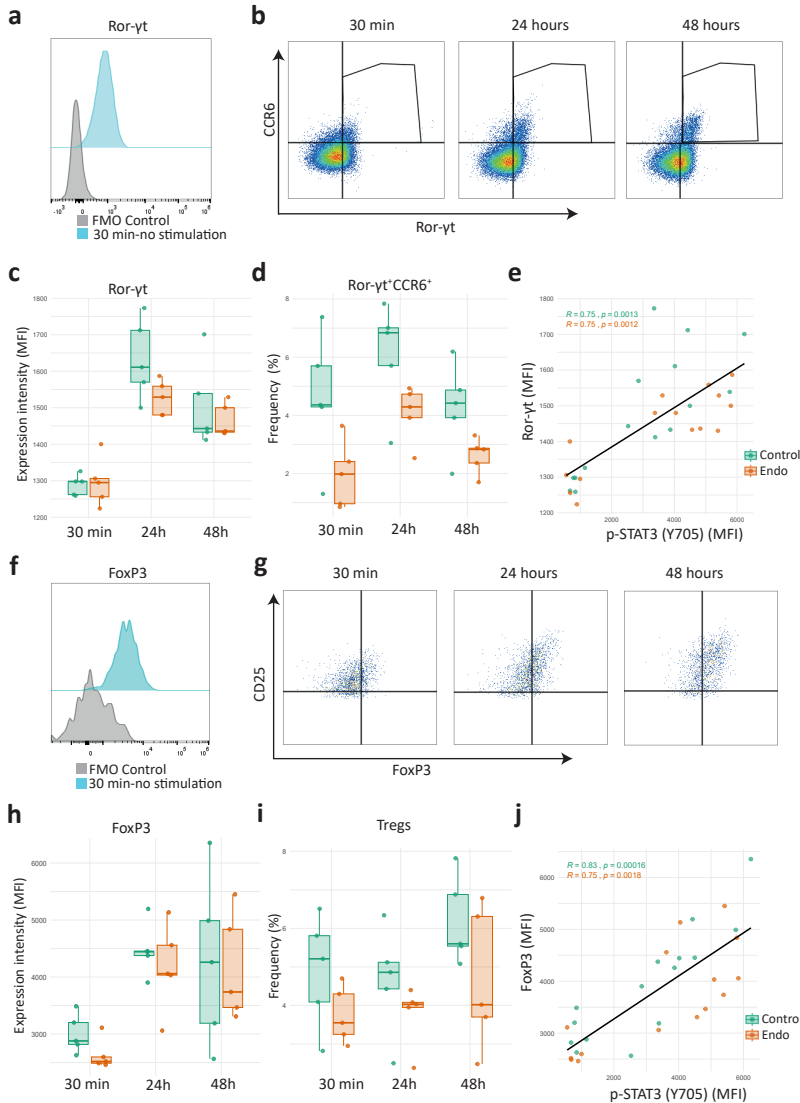


Figure 4. STAT3 phosphorylation is significantly higher in T cells of individuals with endometriosis.

a) Histogram showing median expression intensity (MFI) of ROR- γ t in fluorescent minus one (FMO; grey) control at 30 min without stimulation (blue). b) Dot plots showing expression of ROR- γ t in 30 min, 24h, and 48h. c) Boxplot showing expression intensity of ROR- γ t in CCR6⁺ROR- γ t⁺CD4⁺ T cells at 30 min, 24h, and 48h for endometriosis (orange; n=5) and controls (green; n=5). d) Boxplot showing frequency of CCR6⁺ROR- γ t⁺CD4⁺ T cells at 30 min, 24h, and 48h. e) Scatter plot showing correlation between p-STAT3 and ROR- γ t. f) Histogram showing MFI of FoxP3 in FMO control (grey) and 30 min no stimulation condition (blue). g) Dot plots showing expression of FoxP3 in 30 min, 24h, and 48h. h) Boxplot showing expression intensity of FoxP3 in CD127^{low}CD25⁺CD4⁺ Tregs at 30 min, 24h, and 48h for endometriosis (orange; n=5) and controls (green; n=5). i) Boxplot showing frequency of Tregs at 30 min, 24h, and 48h. j) Scatter plot showing correlation between p-STAT3 and FoxP3 expression. Statistical analysis was performed using the Wilcoxon signed-rank test. *p ≤ 0.05; **p ≤ 0.01.

Discussion

In this study, we examined how inflammatory cues influence immune cell differentiation in individuals suffering from endometriosis. Upon cytokine stimulation, p-STAT3 in T cells of blood increased significantly in individuals with endometriosis compared to healthy controls, and IL-6 alone was sufficient to induce STAT3 phosphorylation. Pharmacological inhibition of the JAK-STAT pathway with Upadacitinib reduced p-STAT3 levels to baseline, might suggest a potential therapeutic avenue. Unexpectedly, p-STAT3 correlated positively with both ROR- γ t and FoxP3, which contrasts with the canonical model in which IL-6/STAT3 signaling promotes Th17 development while antagonizing Treg differentiation. Together, these findings point to heightened STAT3 responsiveness in T cells from individuals with endometriosis and highlight the need for further studies to clarify how STAT3 activity modulates Th17/Treg differentiation and lineage balance in this disease.

Investigation of inflammatory proteins in the plasma revealed significantly higher levels of IL-6 in individuals with endometriosis compared to healthy controls. Indeed, IL-6 was reported previously to be elevated in plasma, serum, and PF of individuals with endometriosis (29-31). It is a key inflammatory cytokine involved in JAK-STAT cascade regulating inflammation and immune responses (32). In line with higher circulating IL-6 and its established function as a major driver of STAT3 activation, we found that IL-6 stimulation alone induced p-STAT3 in T cells from individuals with endometriosis. Unfortunately, our study was underpowered to confirm whether this increase was statistically significant. It was previously shown that p-STAT3 plays roles in endometrial receptivity and decidualization process during normal pregnancy. Paradoxically, hyperactivation of STAT3 in endometriosis can promote cell survival (21). This hyperactivation of STAT3 is thought to be driven by hypoxia-induced IL-6 production from ectopic lesions, which perpetuates a positive feedback loop of inflammation and immune imbalance (18, 33).

In addition to elevated IL-6, MMP-10 protein levels were significantly higher in individuals with endometriosis. Increased MMP-10 expression was previously reported in the eutopic endometrium of individuals with endometriosis, linking it to enhanced tissue remodeling and invasion (34). Interestingly, MMP-10 can be transcriptionally induced by TGF- β signaling (35). Moreover, TGF- β itself has been shown to interact with the IL-6/STAT3 signaling pathway either by promoting IL-6 production or by potentiating STAT3 phosphorylation (36, 37). Together, these observations suggest a potential TGF- β -IL-6/STAT3-MMP-10 signaling crosstalk contributing to the inflammatory and fibrotic milieu characteristic of endometriosis.

The cytokine milieu and STAT activation status are key determinants of lineage commitment of T cells into Th1, Th2, Th17, or regulatory T cells (Tregs). STAT1 and STAT4 promote Th1 differentiation (38), STAT6 supports Th2 polarization (39), and STAT3, particularly in the presence of IL-6 and TGF- β , skews cells toward a Th17 phenotype by inducing ROR- γ t expression (22). In contrast, activated STAT3 has been shown to inhibit FoxP3 expression on Tregs and promote effector T cell expansion exacerbating immune dysregulation in chronic inflammatory conditions (22). Consistent with these findings, we observed positive correlation between p-STAT3 and ROR- γ t expression. However, p-STAT3 levels also correlated positively with FoxP3 expression intensity. Given that correlations were measured at 24–48 h in PBMC cultures, it is plausible that cytokines released by non-T cells activated additional STAT pathways (e.g., STAT1/STAT5), complicating the interpretation of STAT3's specific contribution to T-cell lineage commitment.

Despite the known importance of T cells in the pathogenesis of endometriosis, studies on Treg frequencies and function have yielded inconsistent results (40). Some report increased proportions of CD25^{high}FoxP3⁺ Tregs in PF and reduced proportions in PB (24, 41), while others observe no significant differences in PB compared to controls (42). These discrepancies may stem from differences in study design, timing within the menstrual cycle, and disease stage heterogeneity. In our study, we did not find significant differences in the percentages of Tregs or Th17:Treg ratio in blood between individuals with endometriosis and controls. However, we saw trends towards lower FoxP3 expression in individuals with endometriosis. Further studies focusing on functions of these cells might give more information about their role in endometriosis.

Current pharmacological treatment options for the management of the endometriosis symptoms are limited to hormonal treatments, analgesics and non-steroidal anti-inflammatory drugs (NSAID) (43). These treatment options do not eradicate the disease underscoring the need for new strategies. Given the central role of JAK-STAT signaling pathway in driving inflammation and influencing immune cell responses, pharmacological inhibition of this pathway might be a promising therapeutic strategy. Few studies have investigated targeted inhibition of JAK-STAT pathway in endometriosis using either human tissues or mouse models. In experimental mouse models, treatment with the JAK inhibitor tofacitinib led to lesion regression and reduced adhesions while lowering STAT3 activity (44). Similarly, the direct STAT3 inhibitor Stattic, reduced disease burden and attenuated IL-6/TGF- β signaling in a mouse model, supporting in vivo proof-of-concept for this therapeutic approach (45). Complementary in vitro studies using

human endometriosis-derived stromal cells demonstrated that pharmacological targeting of this axis with WP1066 (JAK2/STAT3 inhibitor) reduces p-JAK2/p-STAT3 and downregulated pro-inflammatory responses (46). Related work has also implicated Niclosamide (47) and Cryptotanshinone (48) as modulators of STAT3-driven signaling in preclinical models. Consistent with these findings, in our cohort short-term inhibition with the selective JAK1 inhibitor, Upadacitinib, reduced STAT3 phosphorylation to baseline, indicating on-target activity. Taken together, these mouse and human data support testing JAK/STAT3-targeted therapies to see how they affect inflammation, immune function, and clinical outcomes in endometriosis.

In conclusion, we show that individuals suffering from endometriosis display a T cell fingerprint which is dominated by the IL-6-JAK-STAT3 axis and is underlined by the correlation with ROR- γ t and the presence of Th17 cells. By modulation of this pathway via Upadacitinib, STAT3 activation can be normalized, which could be a promising but further studied strategy to modulate pathological T cell development in endometriosis in the future.

Data availability statement

Data is available upon request by contacting the corresponding author.

Author contributions

AG: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. EH: Conceptualization, Investigation, Methodology, Writing – review & editing. JF: Formal Analysis, Writing – review and editing. ER: Formal Analysis, Writing – review and editing. TP: Resources, Writing – review and editing. JH: Conceptualization, Writing – review and editing. AN: Resources, Writing – review and editing. RS: Conceptualization, Methodology, Writing – review and editing. HK: Conceptualization, Methodology, Resources, Writing – review and editing. MJ: Conceptualization, Investigation, Methodology, Resources, Writing – review & editing. RM: Conceptualization, Investigation, Methodology, Resources, Writing – review & editing.

Funding

The author(s) declare that no financial support was received for this research.

Conflict of interest

The author(s) 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.

Acknowledgements

The author(s) would like to thank all participants for their contribution to this study. In addition, the authors would also like to thank Laurien Waaijer and Guilherme Gouveia for providing antibodies and inhibitors and help with the data interpretation.

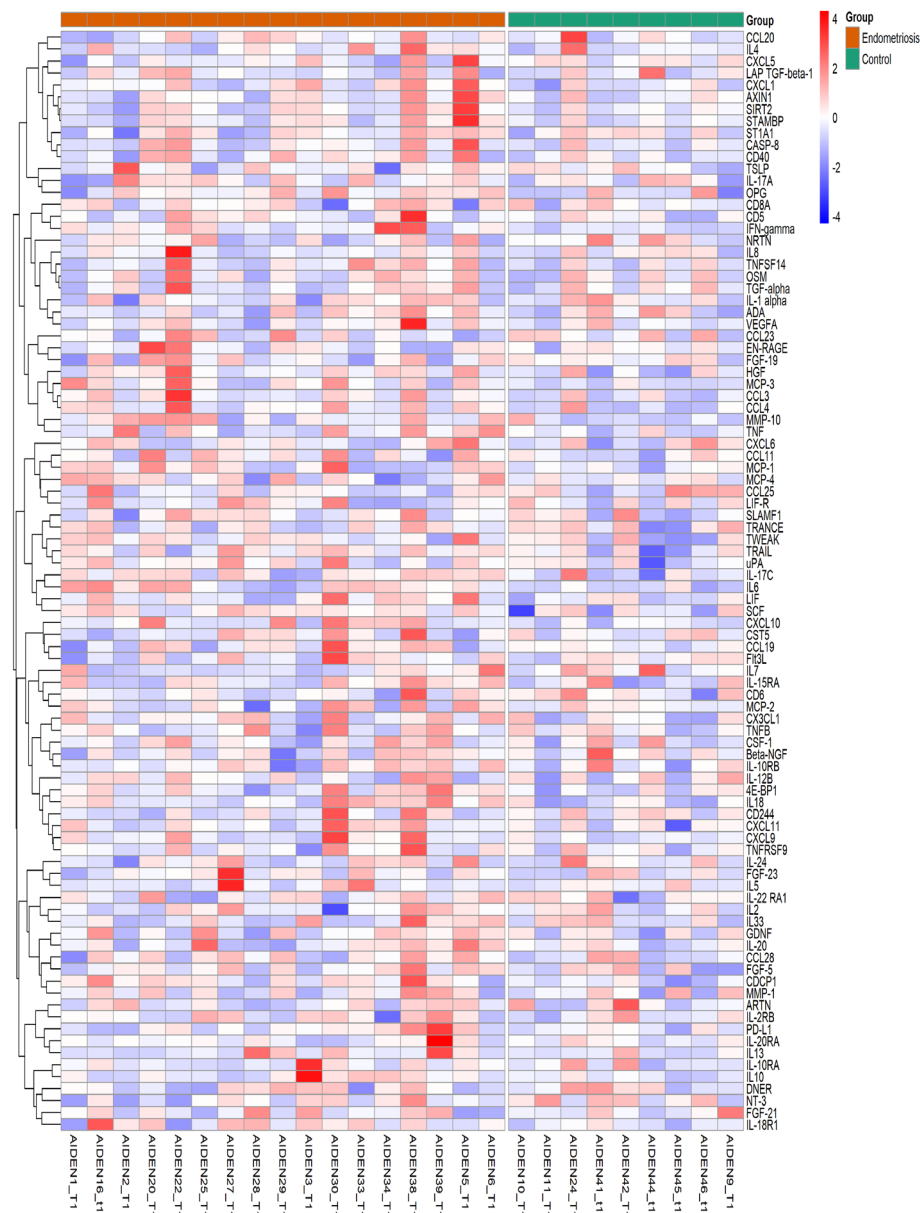
References

1. Shafrir AL, Farland LV, Shah DK, Harris HR, Kvaskoff M, Zondervan K, et al. Risk for and consequences of endometriosis: A critical epidemiologic review. *Best Pract Res Clin Obstet Gynaecol*. 2018;51:1-15.
2. Vercellini P, Vigano P, Somigliana E, Fedele L. Endometriosis: pathogenesis and treatment. *Nat Rev Endocrinol*. 2014;10(5):261-75.
3. Ashrafi M, Sadatmahalleh SJ, Akhoond MR, Talebi M. Evaluation of Risk Factors Associated with Endometriosis in Infertile Women. *Int J Fertil Steril*. 2016;10(1):11-21.
4. Taylor HS, Kotlyar AM, Flores VA. Endometriosis is a chronic systemic disease: clinical challenges and novel innovations. *Lancet*. 2021;397(10276):839-52.
5. Symons LK, Miller JE, Kay VR, Marks RM, Liblik K, Koti M, et al. The Immunopathophysiology of Endometriosis. *Trends Mol Med*. 2018;24(9):748-62.
6. Saunders PTK, Horne AW. Endometriosis: Etiology, pathobiology, and therapeutic prospects. *Cell*. 2021;184(11):2807-24.
7. Lebovic DI, Mueller MD, Taylor RN. Immunobiology of endometriosis. *Fertil Steril*. 2001;75(1):1-10.
8. Wu MY, Ho HN. The role of cytokines in endometriosis. *Am J Reprod Immunol*. 2003;49(5):285-96.
9. Pizzo A, Salmeri FM, Ardità FV, Sofo V, Tripepi M, Marsico S. Behaviour of cytokine levels in serum and peritoneal fluid of women with endometriosis. *Gynecol Obstet Invest*. 2002;54(2):82-7.
10. Kalu E, Sumar N, Giannopoulos T, Patel P, Croucher C, Sherriff E, et al. Cytokine profiles in serum and peritoneal fluid from infertile women with and without endometriosis. *J Obstet Gynaecol Res*. 2007;33(4):490-5.
11. Tarokh M, Ghaffari Novin M, Poordast T, Tavana Z, Nazarian H, Norouzian M, et al. Serum and Peritoneal Fluid Cytokine Profiles in Infertile Women with Endometriosis. *Iran J Immunol*. 2019;16(2):151-62.
12. Mu F, Harris HR, Rich-Edwards JW, Hankinson SE, Rimm EB, Spiegelman D, et al. A Prospective Study of Inflammatory Markers and Risk of Endometriosis. *Am J Epidemiol*. 2018;187(3):515-22.
13. Darnell JE, Jr. STATs and gene regulation. *Science*. 1997;277(5332):1630-5.
14. Villarino AV, Kanno Y, O'Shea JJ. Mechanisms and consequences of Jak-STAT signaling in the immune system. *Nat Immunol*. 2017;18(4):374-84.
15. Banerjee S, Biehl A, Gadina M, Hasni S, Schwartz DM. Erratum to: JAK-STAT Signaling as a Target for Inflammatory and Autoimmune Diseases: Current and Future Prospects. *Drugs*. 2017;77(11):1261.
16. O'Shea JJ, Schwartz DM, Villarino AV, Gadina M, McInnes IB, Laurence A. The JAK-STAT pathway: impact on human disease and therapeutic intervention. *Annu Rev Med*. 2015;66:311-28.
17. Hu X, Li J, Fu M, Zhao X, Wang W. The JAK/STAT signaling pathway: from bench to clinic. *Signal Transduct Target Ther*. 2021;6(1):402.
18. Kim BG, Yoo JY, Kim TH, Shin JH, Langenheim JF, Ferguson SD, et al. Aberrant activation of signal transducer and activator of transcription-3 (STAT3) signaling in endometriosis. *Hum Reprod*. 2015;30(5):1069-78.
19. Yoo JY, Jeong JW, Fazleabas AT, Tayade C, Young SL, Lessey BA. Protein Inhibitor of Activated STAT3 (PIAS3) Is Down-Regulated in Eutopic Endometrium of Women with Endometriosis. *Biol Reprod*. 2016;95(1):11.
20. Hiraoka T, Hirota Y, Aikawa S, Iida R, Ishizawa C, Kaku T, et al. Constant Activation of STAT3 Contributes to the Development of Adenomyosis in Females. *Endocrinology*. 2022;163(5).
21. Xu Y, Wu F, Qin C, Lin Y. Paradoxical role of phosphorylated STAT3 in normal fertility and the pathogenesis of adenomyosis and endometriosis. *Biol Reprod*. 2024;110(1):5-13.
22. Egwuagu CE. STAT3 in CD4+ T helper cell differentiation and inflammatory diseases. *Cytokine*. 2009;47(3):149-56.

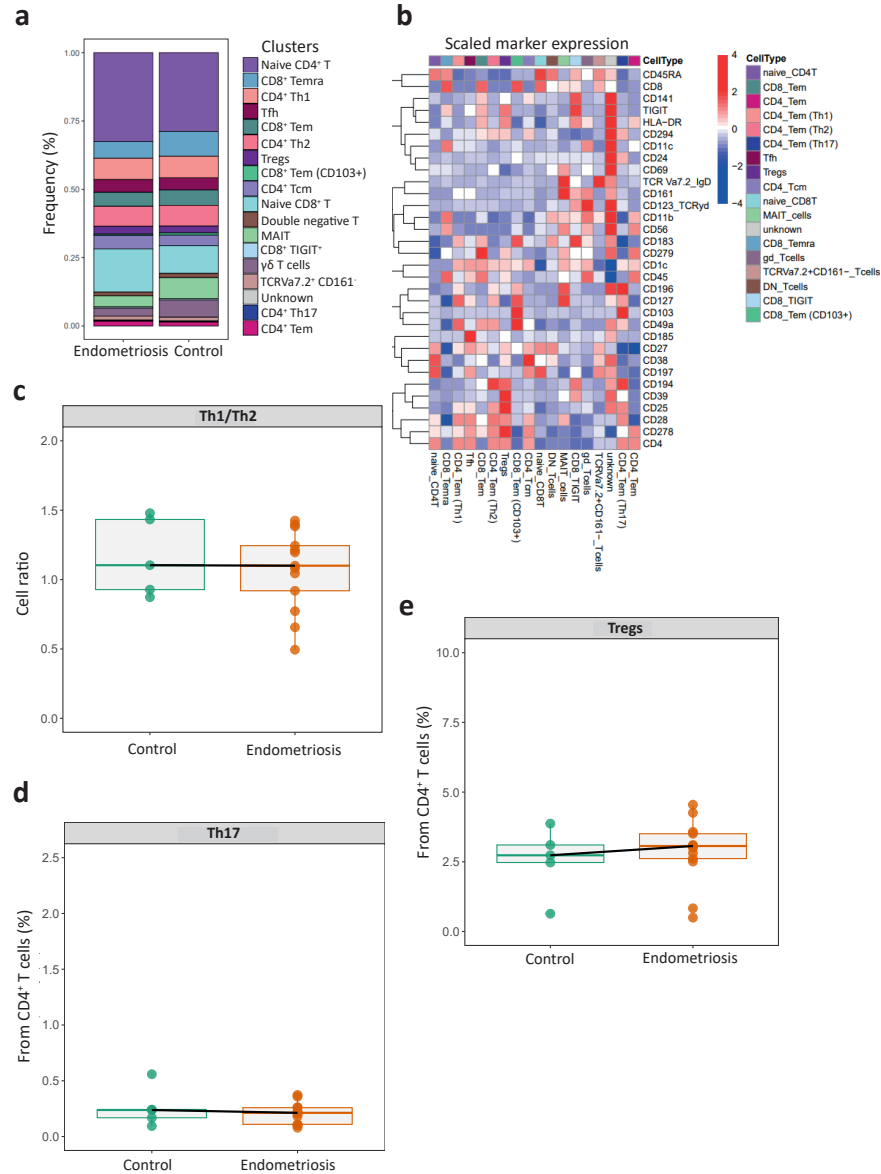
23. Szylo K, Tchorzewski H, Banasik M, Glowacka E, Lewkowicz P, Kamer-Bartosinska A. The involvement of T lymphocytes in the pathogenesis of endometriotic tissues overgrowth in women with endometriosis. *Mediators Inflamm.* 2003;12(3):131-8.
24. Olkowska-Truchanowicz J, Bocian K, Maksym RB, Bialoszewska A, Wlodarczyk D, Baranowski W, 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.
25. Berbic M, Hey-Cunningham AJ, Ng C, Tokushige N, Ganewatta S, Markham R, et al. The role of Foxp3+ regulatory T-cells in endometriosis: a potential controlling mechanism for a complex, chronic immunological condition. *Hum Reprod.* 2010;25(4):900-7.
26. Li C, Lu Z, Bi K, Wang K, Xu Y, Guo P, et al. CD4(+)/CD8(+) mucosa-associated invariant T cells foster the development of endometriosis: a pilot study. *Reprod Biol Endocrinol.* 2019;17(1):78.
27. Waaijer LA, van Cranenbroek B, Koenen H. OMIP-112: 42-Parameter (40-Color) Spectral Flow Cytometry Panel for Comprehensive Immunophenotyping of Human Peripheral Blood Leukocytes. *Cytometry A.* 2025;107(4):226-32.
28. Kroger C, Muller S, Leidner J, Krober T, Wernat-Herresthal S, Spintge JB, et al. Unveiling the power of high-dimensional cytometry data with cyCONDOR. *Nat Commun.* 2024;15(1):10702.
29. Martinez S, Garrido N, Coperias JL, Pardo F, Desco J, Garcia-Velasco JA, et al. Serum interleukin-6 levels are elevated in women with minimal-mild endometriosis. *Hum Reprod.* 2007;22(3):836-42.
30. Li S, Fu X, Wu T, Yang L, Hu C, Wu R. Role of Interleukin-6 and Its Receptor in Endometriosis. *Med Sci Monit.* 2017;23:3801-7.
31. Jiang J, Jiang Z, Xue M. Serum and peritoneal fluid levels of interleukin-6 and interleukin-37 as biomarkers for endometriosis. *Gynecol Endocrinol.* 2019;35(7):571-5.
32. Basu A, Das AS, Sharma M, Pathak MP, Chattopadhyay P, Biswas K, et al. STAT3 and NF-kappaB are common targets for kaempferol-mediated attenuation of COX-2 expression in IL-6-induced macrophages and carrageenan-induced mouse paw edema. *Biochem Biophys Res.* 2017;12:54-61.
33. Hsiao KY, Chang N, Tsai JL, Lin SC, Tsai SJ, Wu MH. Hypoxia-inhibited DUSP2 expression promotes IL-6/STAT3 signaling in endometriosis. *Am J Reprod Immunol.* 2017;78(4).
34. Luddi A, Marrocco C, Governini L, Semplici B, Pavone V, Luisi S, et al. Expression of Matrix Metalloproteinases and Their Inhibitors in Endometrium: High Levels in Endometriotic Lesions. *Int J Mol Sci.* 2020;21(8).
35. Ishikawa F, Miyoshi H, Nose K, Shibamura M. Transcriptional induction of MMP-10 by TGF- β , mediated by activation of MEF2A and downregulation of class IIa HDACs. *Oncogene.* 2010;29(6):909-19.
36. Liu RY, Zeng Y, Lei Z, Wang L, Yang H, Liu Z, et al. JAK/STAT3 signaling is required for TGF-beta-induced epithelial-mesenchymal transition in lung cancer cells. *Int J Oncol.* 2014;44(5):1643-51.
37. Yamada D, Kobayashi S, Wada H, Kawamoto K, Marubashi S, Eguchi H, et al. Role of crosstalk between interleukin-6 and transforming growth factor-beta 1 in epithelial-mesenchymal transition and chemoresistance in biliary tract cancer. *Eur J Cancer.* 2013;49(7):1725-40.
38. Glimcher LH, Townsend MJ, Sullivan BM, Lord GM. Recent developments in the transcriptional regulation of cytolytic effector cells. *Nat Rev Immunol.* 2004;4(11):900-11.
39. Flavell RA, Li B, Dong C, Lu HT, Yang DD, Enslen H, et al. Molecular basis of T-cell differentiation. *Cold Spring Harb Symp Quant Biol.* 1999;64:563-71.
40. Knez J, Kovacic B, Goropevsek A. The role of regulatory T-cells in the development of endometriosis. *Hum Reprod.* 2024.
41. Podgaec S, Rizzo LV, Fernandes LFC, Baracat EC, Abrao MS. CD4+CD25highFoxp3+ Cells Increased in the Peritoneal Fluid of Patients with Endometriosis. *American Journal of Reproductive Immunology.* 2012;68(4):301-8.
42. Hanada T, Tsuji S, Nakayama M, Wakinoue S, Kasahara K, Kimura F, et al. Suppressive regulatory T cells and latent transforming growth factor-beta-expressing macrophages are altered in the peritoneal fluid of patients with endometriosis. *Reprod Biol Endocrinol.* 2018;16(1):9.

43. Garcia-Izquierdo L, Marin-Sanchez P, Garcia-Penarrubia P, Martinez-Esparza M. New Potential Pharmacological Options for Endometriosis Associated Pain. *Int J Mol Sci.* 2024;25(13).
44. Kotlyar AM, Mamillapalli R, Flores VA, Taylor HS. Tofacitinib alters STAT3 signaling and leads to endometriosis lesion regression. *Mol Hum Reprod.* 2021;27(4).
45. Inui H, Kawakita T, Murayama M, Nakagawa T, Sasada H, Shinohara A, et al. Effects of STAT Inhibitors in Mouse Models of Endometriosis. *Reprod Sci.* 2023;30(8):2449-56.
46. Hu Y, Chen H, Jin L, Chi X, Zhao J, Cao Q. Hypomethylation of IL6ST promotes development of endometriosis by activating JAK2/STAT3 signaling pathway. *PLoS One.* 2025;20(1):e0317569.
47. Prather GR, MacLean JA, 2nd, Shi M, Boadu DK, Paquet M, Hayashi K. Niclosamide As a Potential Nonsteroidal Therapy for Endometriosis That Preserves Reproductive Function in an Experimental Mouse Model. *Biol Reprod.* 2016;95(4):76.
48. Xie L, Zhong Y, Chen Y, Wang Y, Xian P, Liu S, et al. Cryptotanshinone alleviates immunosuppression in endometriosis by targeting MDSCs through JAK2/STAT3 pathway. *Phytomedicine.* 2025;136:156227.

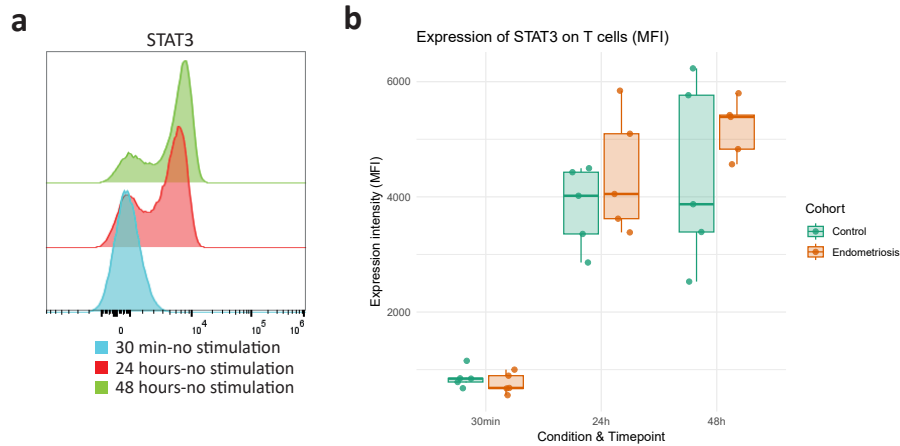
Supplementary Material



Supplementary Figure 1. Heatmap showing row-scaled NPX values of inflammatory proteins measured for endometriosis (orange, n=17) and healthy controls (green, n=9).



Supplementary Figure 2. Immune landscape of blood in individuals with endometriosis and controls. a) Stacked bar plot indicating frequencies of 17 T cell subsets. b) Heatmap showing scaled marker expression for each cell subset. c) Boxplot showing Th1:Th2 ratio in endometriosis (orange; n=13) and controls (green; n=5). d) Boxplot showing frequency of Th17 cells. e) Boxplot showing frequency of Tregs. Statistical analysis was performed using the Wilcoxon signed-rank test. *p ≤ 0.05; **p ≤ 0.01.



Supplementary Figure 3. STAT3 phosphorylation is significantly higher in T cells of individuals with endometriosis. a) Histogram showing median expression intensity (MFI) of p-STAT3 at 30 min (blue), 24h (red), and 48h (green) b) Boxplot showing MFI of p-STAT3 in T cells at 30 min, 24h, and 48h for endometriosis (orange; n=5) and controls (green; n=5). Statistical analysis was performed using the Wilcoxon signed-rank test. * $p \leq 0.05$; ** $p \leq 0.01$.

Supplementary Table 1. Antibody details for the 42-color flow cytometry panel.

Fluorochrome	Marker	Company
BD OptiBuild™ BUV615	TCR-γδ	BD
BD OptiBuild™ BUV496	CD28	BD
BD OptiBuild™ BUV563	CD196	BD
BD OptiBuild™ BUV615	CD123	BD
BD OptiBuild™ BUV661	IgG	BD
BD OptiBuild™ BUV737	CD103	BD
BD OptiBuild™ RY586	CD56	BD
BD Horizon™ BV480	CD185	BD
BD OptiBuild™ BV750	CD294	BD
BD Horizon™ RB780	CD69	BD
BD Horizon™ BUV395	CD183	BD
BD OptiBuild™ BUV805	CD24	BD
PerCP/Cyanine5,5	CD16	Biolegend
PE/Fire™ 640	CD19	Biolegend
PE/Fire™ 700	CD45RA	Biolegend
PE/Cyanine5	CD278	Biolegend
Brilliant Violet 785™	CD279	Biolegend
PE/Cyanine7	CD11c	Biolegend
PE/Dazzle™ 594	CD141	Biolegend
PerCP	CD8	Biolegend
Spark Blue™ 550	CD3	Biolegend
Brilliant Violet 570™	CD127	Biolegend
APC	TCR Vα7,2	Biolegend
Spark NIR™ 685	CD197	Biolegend
Brilliant Violet 605™	CD25	Biolegend
Brilliant Violet 650™	CD38	Biolegend
PE/Fire™ 810	CD194	Biolegend
Spark Blue™ 574	CD45	Biolegend
Brilliant Violet 711™	Tigit	Biolegend
Spark Violet™ 538	CD14	Biolegend
Zombie NIR™ Fixable Viability Kit	Fix viability dye	Biolegend
Alexa Fluor 700	IgM	Biolegend
PerCP/Fire™ 806	CD4	Biolegend
APC	IgD	Biolegend
APC/Fire™ 810	CD11b	Biolegend
FITC	CD27	Biolegend
PE	CD49a	Biolegend
Brilliant Violet 421™	CD39	Biolegend
Brilliant Violet 510™	CD326 (EpCAM)	Biolegend
NovaFluor™ Yellow 730	CD1c	Invitrogen
NovaFluor™ Blue 660-40S	HLA- DR	Thermofisher
eFluor™ 450	CD161	Thermofisher

Supplementary Table 2. Antibody details for the assessment of p-STAT3.

Fluorochrome	Marker	Company
BUV496	CD19	BD
APC-Cy7	CD16	BD
AF488	STAT3 (pY705)	BD
Alexa Fluor 700	CD8	Biolegend
Spark NIR 685	CD3	Biolegend
Spark Violet 538	CD14	Biolegend
Brilliant Violet 750	CD56	Biolegend
Alexa Fluor 647	CD4	Biolegend
Zombie-NIR	Fix Viability Dye	Biolegend

Supplementary Table 3. Antibody details for the assessment of correlation between p-STAT3, FoxP3, and ROR- γ t.

Fluorochrome	Marker	Company
Alexa Fluor 700	CD8	Biolegend
SparkBlue™ 550	CD3	Biolegend
Briljant Violet 750™	CD56	Biolegend
Alexa Fluor 647	CD4	Biolegend
Zombie-NIR	Fix Viability Dye	Biolegend
Spark NIR™ 685	CD197	Biolegend
Brilliant Violet 570™	CD127	Biolegend
Brilliant Violet 605™	CD25	Biolegend
PE/Fire 810™	CD194	Biolegend
BD Horizon™ BUV395	CD183	BD
BD OptiBuild™ BUV563	CD196	BD
AF488	STAT3	BD
BD Horizon™ PE-CF594	ROR- γ t	BD
PE	FoxP3	Ebioscience
	Rat Serum	Ebioscience



Impact of an Anti-Inflammatory Diet on quality of life, pain, and inflammation in ENdometriosis: AIDEN-study

Emma Huijs^{#1}, Aysel Gurbanova^{#2}, Janneke S. Hoogstad-van Evert³, Johanna Franz^{4,5,6}, Loutje van der Sman^{1,2}, Laurien Waaijer², Quirijn de Mast⁷, Clara Belzer⁹, Nicole M. de Roos⁸, Tal Pecht^{4,5,6}, Renate G. van der Molen^{†2}, Annemiek W. Nap^{†1}

¹ Department of Obstetrics & Gynecology, Radboud University Medical Center, Nijmegen, The Netherlands

² Department of Laboratory Medicine, Laboratory for Medical Immunology, Radboud University Medical Center, Nijmegen, The Netherlands

³ Department of Obstetrics & Gynecology, Amphia, Breda, The Netherlands

⁴ Systems Medicine, German Center for Neurodegenerative Diseases (DZNE), Bonn, Germany

⁵ Department for Genomics and Immunoregulation, Life & Medical Sciences (LIMES) Institute, University of Bonn, Bonn, Germany

⁶ Institute of Clinical Chemistry and Clinical Pharmacology, University Hospital Bonn, Bonn, Germany

⁷ Department of Internal Medicine, Radboud University Medical Center, Nijmegen, The Netherlands

⁸ Department of Human Nutrition and Health, Wageningen University and Research, Wageningen, The Netherlands

⁹ Laboratory of Microbiology, Wageningen University and Research, Wageningen, The Netherlands

[#]Shared first co-authorship

[†]Shared last co-authorship

Abstract

Endometriosis is a chronic, estrogen-dependent inflammatory disease and the most common benign gynecologic condition. Due to limited treatment options, many individuals with endometriosis pursue self-management to control symptoms and improve health-related quality of life (HRQoL). Although various diets are linked to symptom relief, solid evidence is scarce. It remains unclear whether benefits reflect diet-driven physiological changes or the psychological impact of self-management. In this prospective explorative feasibility study, 23 individuals with endometriosis completed a 12-week anti-inflammatory diet intervention (AIDEN-intervention). Primary outcomes were changes in average and maximum pain scores measured with a numerical rating scale (NRS); secondary outcomes assessed HRQoL, chronic inflammation, immune system, vaginal and gut microbiomes. The AIDEN-intervention reduced NRS pain scores and improved HRQoL. Inflammatory proteins showed no significant change. However, CD8+ Mucosal-Associated Invariant T (MAIT) cell percentages increased in blood while decreasing in the uterus after the AIDEN-intervention. Gut and vaginal microbiomes showed no significant shifts. These findings suggest that an AIDEN-intervention is a viable symptom-management strategy, while physiological mechanisms underlying benefit require further study.

Clinical Trial Register number [EUdraCT 2022-13492](#).

Keywords: dietary intervention, anti-inflammatory, endometriosis, pain, quality of life, self-management, inflammation, MAIT cells, microbiome

Introduction

Endometriosis is a chronic, estrogen-dependent inflammatory disease characterized by the presence of endometrial-like tissue outside the uterine cavity (1). Common symptoms are severe (pelvic) pain, dysmenorrhea, dyspareunia, and dysuria, though it may also include gastrointestinal symptoms, infertility, migraine, and fatigue (2, 3). Beyond these physical symptoms, endometriosis substantially impairs health-related quality of life (HRQoL), affecting physical, psychological, and social well-being (4, 5). This debilitating disease impacts approximately 10% of reproductive age individuals that were assigned female at birth (from here referred to as women), affecting around 190 million people worldwide (1). Unfortunately, there is no cure for endometriosis and current hormonal and surgical therapies often fail to provide lasting relief. Up to 50% of women experience recurrent or persistent symptoms (1). Consequently, many women diagnosed with endometriosis turn to self-management strategies, and dietary adjustments are among the most common (6, 7).

Diets rich in omega-3 fatty acids, vitamin D, dietary fiber, and antioxidants (e.g., vitamins C and E, beta-carotene, selenium, zinc, polyphenols) have been linked to reduce endometriosis-related pain (8, 9). Additionally, probiotic-rich foods (e.g., yogurt, kefir) and flavonoid-rich foods can enhance Natural Killer (NK) cell activity (10, 11), while nutrients such as vitamins A and D, polyunsaturated fatty acids, and probiotics promote regulatory T (Treg) cell development (12). By contrast, diets high in pro-inflammatory components, such as saturated- and trans fatty acids and processed meats, may exacerbate systemic inflammation (13-16). These pro-inflammatory components can furthermore promote a pro-inflammatory gut microbiome and increase intestinal permeability, of which both are closely linked to dysbiosis (17). In contrast, fiber-rich plant-based and Mediterranean diets, rich in anti-inflammatory components can positively influence microbial diversity and influence bacterial metabolites linked to inflammation (18, 19). Because of their immunomodulatory effects and their influence on inflammatory processes, both macro- and micronutrients can profoundly affect the regulation of pro- and anti-inflammatory pathways (20-23). This is particularly relevant to endometriosis where immune dysregulation and chronic inflammation are key features in the pathophysiology of the disease.

Peritoneal lesions as well as the eutopic endometrium of individuals with endometriosis harbor increased numbers of macrophages compared to eutopic endometrium from controls (24, 25). NK cells in the peritoneal fluid (PF) of individuals with endometriosis are reduced in number and function, and their cytokine release

facilitates endometrial cell adhesion, invasion, and neovascularization (26, 27). T cell profiles are also skewed, where an elevated peritoneal CD4/CD8 T-cell ratio and accumulation of T helper 17 (Th17) cells has been shown to correlate with advanced stages of endometriosis (28, 29). Concurrent increases in Treg cells foster local immune tolerance, and B cells may contribute to inflammation through cytokine release and autoantibody production (26, 30). In addition to conventional T cells, mucosal-associated invariant T (MAIT) cells have been implicated in the inflammatory processes underlying endometriosis. A recent study has reported increased frequencies of MAIT cells in the PF of individuals with endometriosis compared to healthy controls(31). Interestingly, MAIT cells have been associated with microbial imbalance in the gut microbiome, also known as dysbiosis (32). MAIT cells are likely to be one of the first immune cells exposed to translocated gut microbes and inflammatory signals. Dysbiosis at mucosal surfaces can influence MAIT cell frequency and function both locally and systemically (33). Notably, women with endometriosis exhibit distinct microbial compositions in the vaginal, cervical, and gut microbiota compared to healthy controls(34). It is suggested that dysbiosis may contribute to the pathogenesis of endometriosis by promoting local and systemic immune dysfunction (35).

While various dietary adjustments have been reported by women with endometriosis to be beneficial in alleviating their symptoms, robust scientific evidence remains limited (8, 36, 37). Furthermore, it is unclear whether any reported symptom relief arises from direct biological effects of dietary adjustments or from the psychosocial benefits of active self-management. However, given the inflammatory nature of endometriosis and the emerging evidence suggesting that dietary factors can modulate systemic inflammation, diet presents as a promising target for pain management in endometriosis. We hypothesize that an anti-inflammatory diet may reduce chronic inflammation and influence immune cell and microbiome composition, thereby alleviating pain and enhancing HRQoL in endometriosis.

To explore this, we conducted an explorative feasibility study examining the effects of an anti-inflammatory diet intervention (AIDEN-intervention) on pain symptoms and HRQoL in individuals with endometriosis. Additionally, we evaluated the impact on chronic inflammation, immune system and the composition of the vaginal and gut microbiome.

Materials and methods

Study population

This prospective explorative feasibility study, with a pre-post design, was performed at the Radboud University Medical Center Nijmegen, the Netherlands. It was conducted between February 2023 and June 2024. Ethical approval was granted by the regional ethics committee 'METC Oost-Nederland' in the Netherlands (reference number: 2022-13650) and registered at the EU Clinical Trials Register (reference number EUdraCT 2022-13492). Women with endometriosis were recruited at the Radboud University Medical Center and the social media channels of the Dutch patient representative group for endometriosis, the Endometriosis Society. Eligible participants were screened based on specific inclusion and exclusion criteria. Women with a confirmed diagnosis of endometriosis or adenomyosis through ultrasound, Magnetic Resonance Imaging (MRI), or surgery and experiencing pain symptoms with a Numerical Rating Scale (NRS) score greater than four could participate. Furthermore, participants were at least 16 years old, nulligravida, premenopausal, had a Body Mass Index (BMI) between 18 and 30 kg/m², committed to not changing their use (or non-use) of dietary supplements, and willing to obey the study protocol. Additionally, eligible participants needed to be proficient in Dutch or English to comprehend the AIDEN-intervention and provide written informed consent. Women were excluded if they were pregnant, had been diagnosed with an eating disorder, inflammatory bowel disease, short bowel syndrome, another chronic inflammatory disease, celiac disease, followed a vegan diet, smoked, had a Food Frequency Questionnaire (FFQ) Eetscore-FFQ greater than 140, participated in another intervention study, or had surgery during the study period.

Study design

This exploratory feasibility study was conducted to generate preliminary insights and aimed to include twenty individuals with endometriosis, with the sample size informed by a similar feasibility study in this population(38). Participants attended a study visit upon inclusion (Baseline) and a follow-up visit one to four weeks later (T0), with the timing of the follow-up visit determined by their menstrual cycle. After this run-in period participants started with the AIDEN-intervention for 12 weeks, which was finished by a final visit (T1). Participants were provided with instructions for the collection of feces, vaginal swab, and menstrual effluent (ME). These samples were collected by the participant before each visit. In addition, peripheral blood (PB) was drawn from participants during every visit. At the time of these study visits participants completed a series of online questionnaires. These questionnaires covered various aspects, including demographic characteristics, diet quality, pain symptoms, HRQoL, sleep quality, and physical activity levels.

AIDEN-intervention

During the run-in period (Baseline to T0), participants completed a phone consultation with the dietitian, during which their eating habits and a 24-hour recall were assessed. This information was used to create a personalized nutrition plan, which was further discussed during an in-person consultation at the start of the AIDEN-intervention (T0). Participants received tailored dietary advice, along with an information folder containing recipes, food substitutions, and two-week meal plans.

A certified dietitian specialized in endometriosis provided ongoing support throughout the intervention. In addition to the T0 visit, participants had three individual consultations (via videocall or in person) at the beginning, middle, and end of the AIDEN-intervention. Adherence was monitored using unannounced 24-hour recalls. Additional phone consultations were available upon request. Participants were asked to avoid changes in physical activity, maintain their current supplement use and record the use of medications, including analgesics.

The anti-inflammatory diet used in the AIDEN-intervention is based on the Dutch Healthy Diet Index 2015(39) (DHD2015) and shares features with the EAT-Lancet, Mediterranean, and Healthy Eating Index dietary patterns. It promotes high intake of vegetables, fruits, legumes, nuts, fish, whole grains, and dairy, while limiting red and processed meat, sugary drinks, salt, added sugars, and saturated fats(40). Making it a predominantly plant-based diet. Dietary guidelines and study design are summarized in Figure 1.

Data collection

Questionnaires

The primary outcome of this study was the NRS pain score, which was assessed to evaluate average and maximum pain levels experienced in the last month. Clinically relevant differences were based on the cut-off values mentioned in the study of Farrar et al., indicating a reduction of 30% or 2 points (41). For HRQoL the Endometriosis Health Profile-30 (EHP-30), and the Short Form-36 (SF-36) were administered. The EHP-30 is an endometriosis specific HRQoL questionnaire, which consists of five domains. Lower scores indicate better health and a lower impact of endometriosis symptoms, while higher scores indicate a lower HRQoL. The SF-36 assesses eight domains of physical and mental health, from which the Physical Component Summary (PCS) and Mental Component Summary (MCS) scores are calculated. A score of fifty represents the population average, with higher scores indicating better health and lower scores reflecting poorer health. Sleep quality was evaluated using the

Pittsburgh Sleep Quality Index (PSQI), in which lower scores suggest better sleep quality, and a total score greater than five indicates a poor sleep quality. Physical activity level was measured with the International Physical Activity Questionnaire Short Form (IPAQ-SF). Diet quality was assessed three times throughout the study period using the Eetscore-FFQ, a validated and reproducible questionnaire designed to assess adherence to the DHD2015. This index emphasized dietary patterns aligned with anti-inflammatory principles and consists of 16 dietary components. For each component a total score of ten points can be given, with a higher score indicating a better adherence to the DHD2015. A total score is calculated by summing all components, resulting in an Eetscore-FFQ range from 0 – 160⁴². Based on the data from the four 24h-recalls specific macro- and micronutrient intake was measured using Compl-eat™. In addition, a dietary evaluation questionnaire was administered at T1 to assess the AIDEN-interventions taste, affordability, and feasibility.

Biological data

Olink proximity assay for measurement of inflammatory proteins

For the collection of PB, tubes containing Sodium Heparin (BD Vacutainer) were used. To collect plasma, PB was centrifuged for 10 min at 20 °C, 2204 rpm. The collected plasma was stored at –80°C. To measure pro-inflammatory markers in the plasma, 92-plex Olink inflammation panel (Olink Proteomics AB) was used.

Isolation of immune cells from peripheral blood and menstrual effluent

To study the composition of uterine immune cells, participants collected ME at T0 and T1 using a menstrual cup (AllMatters OrganiCup), right after the start of menses, for a total of 36 hours (42). Every 12 hours, the cup was emptied in a new collection tube, containing RPMI1640 medium (ThermoFisher Scientific, Waltham, MA, USA) supplemented with 10% human pooled serum (HPS) (Zen-Bio, Durham, NC, USA), 1 mM pyruvate (ThermoFisher Scientific), 2 mM GlutaMAX™ (ThermoFisher Scientific), 100 U/mL penicilin/100 µg/mL streptomycin (ThermoFisher Scientific), and 0.3% sodium citrate (Merck, Darmstadt, Germany). PB (10 mL) was collected in ethylene diamine tetra acetic acid (EDTA) tubes and stored at room temperature.

The collected ME from was first pooled together and centrifuged. After centrifugation, the pellet of the ME was filtered through a 70 µm cell strainer (PluriSelect Life Science, Leipzig, Germany) by creating a vacuum using a connector ring (PluriSelect) and a 30 mL syringe (Terumo Corporation, Tokyo, Japan). The filtered ME was then incubated for 20 minutes at room temperature with RosetteSep™ Human Granulocyte Depletion Cocktail (STEMCELL Technologies, Vancouver, Canada) according to manufacturer's

instructions, after which the sample was diluted with an equal volume of Phosphate-Buffered Saline (PBS) 2% Horse Serum.

Subsequently, ME mononuclear cells (MMCs) and PB mononuclear cells (PBMCs) were isolated using Lymphoprep (STEMCELL Technologies) according to manufacturer's instructions. The samples were centrifuged for 15 min at 800g, no brake at 20°C. Isolated MMCs and PBMCs were frozen in Recovery™ cell freezing medium (Gibco, Life Technologies) and stored at –80°C for 24 hours before being transferred to liquid nitrogen for long-term storage.

Immunophenotyping with high dimensional flow cytometry

For the immunophenotyping of immune cell subsets in PB and ME, a modified version of a previously published 40-color flow cytometry panel was used (43). For staining, 500,000 cells were incubated with 125 µL of a surface antibody cocktail for 30 minutes at room temperature in the dark. Unstained controls were incubated with 125 µL of PBS. After washing, a second surface antibody cocktail (125 µL) was added and incubated under the same conditions. Cells were washed again with 3 mL of Fluorescence-Activated Cell Sorting (FACS) buffer (0.2% Bovine Serum Albumin (BSA) in PBS), resuspended in 300 µL of FACS buffer, and measured on the Sony ID7000 spectral flow cytometer (Sony Biotechnology). Samples were acquired in multiple batches, each including a control sample to monitor inter-batch variability.

Gut and vaginal microbiome

Participants were asked to collect vaginal swabs during the luteal phase of their menstruation, and feces was collected maximum 24 hours prior to the study visit. Samples were stored in the fridge until the time of the study visit. The microbiome composition of fecal samples and vaginal swabs was determined through 16S rRNA gene profiling. DNA was isolated from vaginal swabs (eNAT® swabs, Copan) using Qiagen DNA isolation kit. A total of 250 µg of the fecal sample was homogenized using bead beating, and DNA was isolated with a Maxwell 16 system (Promega Corporation) according to the manufacturer's protocol. Amplification of the V4 hypervariable regions of the 16S rRNA gene was performed using barcoded primers 515F (5'-GTGCCAGCMGCCGCGGTAA-) and 806R (5'-GTGCCAGCMGCCGCGGTAA-). Libraries consisted of uniquely barcoded samples, negative controls and two mock communities to assess quality control. This amplicon mixture was sequenced using a commercially available platform (HiSeq 2000; Illumina, Inc).

Data analysis and statistics

Questionnaire data was collected using Castor EDC (Castor Electronic Data Capture, Ciwit BV, Amsterdam, the Netherlands) and analyzed using IBM SPSS for Windows, version 29.0 (IBM Corp., Armonk, NY, USA). Baseline characteristics were presented as continuous variables using descriptive statistics with mean and standard deviation (SD) for normally distributed data and median with interquartile ranges (IQR) for non-normally distributed data. Categorical variables were presented as absolute numbers and percentages. Data were calculated using the paired samples Student's *t*-test for normally distributed variables, and the Wilcoxon Signed-rank test for non-normally distributed variables/parameters. Two-sided *p*-values of ≤ 0.05 were considered statistically significant.

Olink Normalized Protein eXpression (NPX) (log2) data were analyzed in a paired pre-post design. After standard Olink normalization and quality control, proteins with low detectability were removed, and values below Limit of Detection (LOD) were handled per Olink guidance. Global patterns were summarized with z-scored (by protein) heatmaps and Principle component analysis (PCA), and differential proteins were visualized in volcano plots of Δ NPX (post-pre) versus $-\log_{10}(p)$. Statistical analysis of cell type abundances and protein levels were done using paired Wilcoxon Signed-Rank test and resulting *p*-values were adjusted using Benjamin-Hochberg procedure and a FDR-cutoff of 5%. Two-sided *p*-values of ≤ 0.05 were considered statistically significant.

Prior to the flow cytometry analysis, debris was removed according to *FSC-Area* and *SSC-Area*. Singlets were gated (*SSC-Area* vs. *SSC-Height*) and dead cells were removed. After unmixing of the data FCS files were exported for unsupervised analysis using the *cyCONDOR* package (v0.1.6) (44), which was done separately for the PBMC and MMC data. Briefly, data was imported into the *cyCONDOR* ecosystem (max. of 90 000 cells/sample for PBMCs and 30,000 cells/sample for MMCs) followed by auto-logical transformation using the *prep_fcd* function. PCA was performed excluding live/dead, FSC and SSC parameters (and CD326 for the PBMC data) using the *run_PCA* function. Non-linear dimensionality reduction was performed by Uniform Manifold Approximation and Projection (UMAP) using the *runUMAP* function with default settings and all principle components as input. The implemented *harmonize_PCA* function was used to correct for experimental batches. Clustering by the *runPhenograph* function ($k = 90$) was followed by manual cluster annotation into broad cell types based on known cell type markers. Subsequently, the dataset was subsetted for the main identified cell types (B cells, T cells, NK cells, myeloid cells) and each subset was further analyzed separately as

described above to identify cell type subsets. For visualization of all identified cell type subsets, the fine level cell type annotations for each subset were combined and mapped onto the total data set UMAP space and visualized using the *plot_dim_red* function. Two-sided p-values of ≤ 0.05 were considered statistically significant.

For the analysis of the gut- and vaginal microbiome data processing was performed using the NG-Tax semantic framework (open-source) on demultiplexed fastq files with default settings, except for the read length, which was adjusted to 125 base pairs (45). Taxonomy of amplicon sequence variants (ASVs) was assigned using the SILVA 138.1 reference database (46). Relative abundance of top 15 species at genus level was calculated and plots were generated in R (v5.5.1) using packages *phyloseq* (v1.52.0)(47) and *microbiome* (v1.30.0). Beta-diversity was calculated using the Bray–Curtis dissimilarity, generating a principal coordinates analysis (PCoA) ordination plot with the R package *vegan* (v2-7.1). Differential abundance analysis was done using the R package *DESeq2* (v1.48.2) (48).

Results

In total 56 women with endometriosis were assessed for eligibility. Twenty-three women fulfilled the inclusion criteria and started the AIDEN-intervention. Eventually, 18 of them completed the intervention (78.3%), and were available for analysis. A flow chart of the in- and exclusion process is shown in Supplementary Figure 1 and cohort characteristics are presented in Table 1.

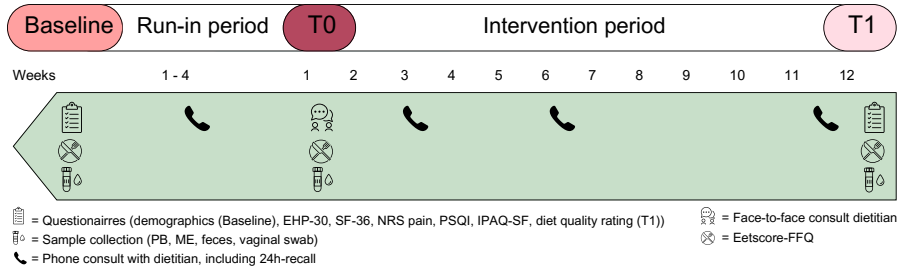
AIDEN-intervention reduced pain levels of individuals with endometriosis

After the AIDEN-intervention a clinically relevant improvement was observed in NRS pain scores. Average pain scores significantly decreased with 30%, from 5.7 ± 1.9 to 4.0 ± 1.9 ($p = 0.018$; Figure 2a). For maximum pain scores, there was a decrease of almost two points, 8.6 ± 1.1 to 6.7 ± 2.9 ($p = 0.012$; Figure 2b).

AIDEN-intervention increased health-related quality of life of individuals with endometriosis

A significant improvement in HRQoL was observed at the end of the AIDEN-intervention, using the SF-36 and EHP-30 core questionnaire. After the AIDEN-intervention there was no significant improvement on the eight domains of the SF-36. However, the MCS improved significantly ($p = 0.019$) (Supplementary Table 2). The total mean score of the EHP-30 improved significantly from 45.0 ± 14.1 to

29.6 ± 18.1 (p = 0.003), which was reflected in all five subdomains showing a significant improvement (p = ≤ 0.001 – p = 0.036; Table 2).



The AIDEN-intervention anti-inflammatory diet

A predominantly plant-based diet with 3 to 6 regular meals a day. Includes vegetables, fruits, legumes, nuts, fish, whole grains, and adequate dairy intake. Limits red and processed meat, alcohol, sugary drinks, salt, added sugars, and saturated fats.

Fruits, 200 g/day: of which 1 portion red fruit	Vegetables, 300 g/day	Legumes, 150 g/week (uncooked)
Nuts and seeds, 25 g/day	Whole grains, 90 g/day	Fats and oils, 10 - 20 g/day
Red unprocessed meat, max. 100 - 125 g/week	Dairy, 450 ml/day	Fish, 100 - 200 g/week: of which 1 portion fat fish
Poultry unprocessed, max. 200 g/week	Eggs, max. 6 - 10 p/week	Liquids: water, tea and coffee at least 1.5 p/day

Figure 1. Overview of the study design. EHP-30 = Endometriosis Health Profile-30; SF-36 = Short Form-36; NRS = Numerical Rating Scale, PSQI = Pittsburgh Sleep Quality Index, IPAQ-SF = International Physical Activity Questionnaire Short Form, FFQ = Food Frequency Questionnaire.

Diet quality and adherence

After the AIDEN-intervention a significant improvement ($p \leq 0.001$) in the mean Eatscore-FFQ was observed, increasing by 34.7 points from 106.3 ± 15.5 to 141 ± 10.2 on the 160-point scale (Figure 2c). Following the AIDEN-intervention, the Eatscore-FFQ showed a significant increase in 11 out of the 16 individual components of the Eatscore-FFQ. There was a significant increase in the intake of vegetables, fruits, whole grains, legumes, nuts, dairy, fish, and oils and fats, along with a significant decrease in the intake of processed meats, sugary drinks, and unhealthy choices (Table 3).

These results were confirmed by the analysis of the 24-hour recalls in Compl-eat™, data are presented in Supplementary Table 3 and 4. Adhering to the AIDEN-intervention resulted in a significant increase in the intake of vegetables, dairy, fruits, and legumes ($p = \leq 0.001 - p = 0.021$), and a decrease in the intake of meat and poultry ($p \leq 0.001$). This resulted in a significant increase in the intake of several macro- and micronutrients, specifically in the intake of plant protein, dietary fibers,

calcium, total iron, non-heme iron, and vitamin C ($p \leq 0.001 - p = 0.010$). Meanwhile, the intake of animal protein, total fat, saturated fat, and heme iron decreased ($p = \leq 0.001 - p = 0.034$).

Table 1. Participant characteristics (n=18)

Characteristics	Participants
Age (years), median (IQR)	27.5 (12.0)
BMI (kg/m ²), median (IQR)	23.0 (3.2)
Endometriosis confirmed, n (%)	
Ultrasound	6 (33.3)
MRI	6 (33.3)
Laparoscopic	6 (33.3)
Location of endometriosis*, n (%)	
Peritoneal endometriosis	8 (44.4)
Ovarian endometriosis	8 (44.4)
Deep infiltrating endometriosis	8 (44.4)
Adenomyosis	5 (27.8)
Other	2 (11.1)
Type of management, n (%)	
Medication (IUD, oral contraceptives, pain killers)	8 (44.4)
History of surgery	6 (33.3)
Pelvic floor physical therapy	2 (11.1)
Previous dietary intervention	3 (16.7)
Food allergy and/or intolerance	
Crustaceans and shellfish	2 (11.1)
Hazelnuts	1 (5.6)
Lactose intolerance	1 (5.6)
Pork	1 (5.6)
Use of supplements n (%)	
Magnesium	7 (38.9)
Vitamins B (B11, B12, complex)	7 (38.9)
Multivitamin	5 (27.8)
Omega-3	4 (22.2)
Vitamin D	3 (16.7)
Iron	3 (16.7)
Vitamin C	2 (11.1)
Probiotics	2 (11.1)
Zinc	2 (11.1)
Tryptophan	2 (11.1)
Other**	4 (22.2)

IQR = inter quartile range, MRI = magnetic resonance imaging

*Some patients had endometriosis at multiple locations

**Mentioned by one participant, for example serrapeptase, lysine, ubiquinol, creatin, chloride

The results of the evaluation of the diet were positive and are shown in Table 4. On a scale of 1-10, the average rating of the diet was 8.3 ± 1.05 and 8.1 ± 0.87 for taste. Out of the 18 patients, 88% (strongly) agreed to advise the diet to other women diagnosed with endometriosis.

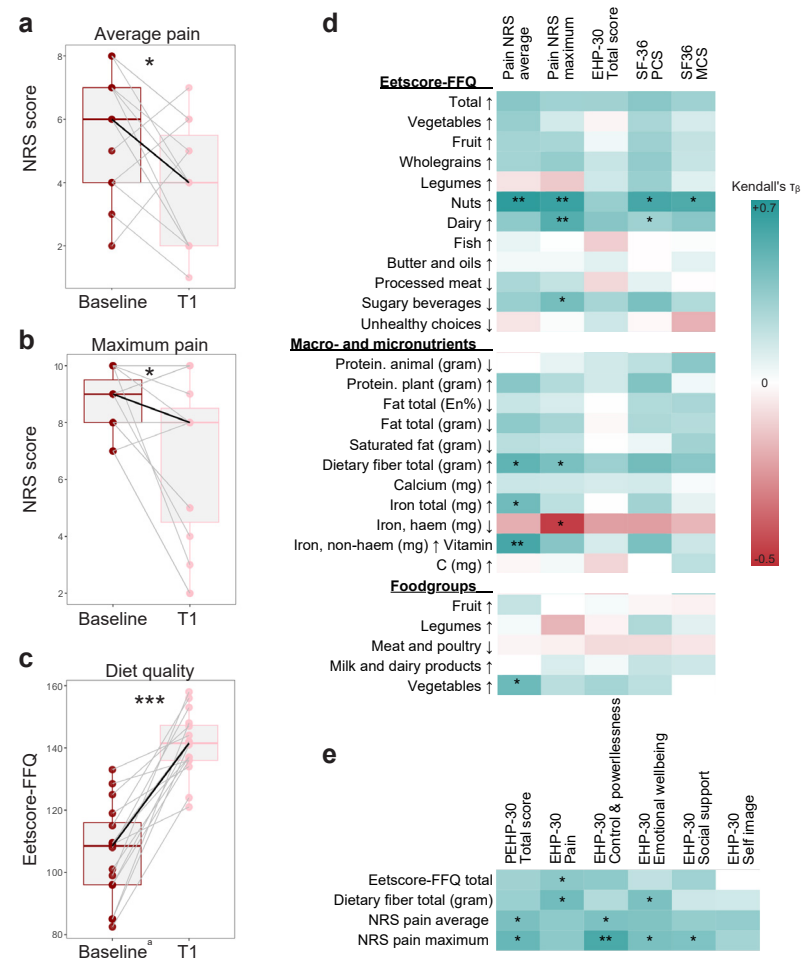


Figure 2. Outcomes of pain and diet quality before and after AIDEN-intervention. a) Average and b) maximum NRS pain score during the last month, at Baseline and after (T1) the AIDEN-intervention. c) Eetscore-FFQ indicating diet quality at Baseline and after (T1) AIDEN-intervention. d) Heatmap defined by Kendall's τ_B correlations between significantly changed food groups and nutrients measured with the Eetscore-FFQ or Compl-eat™ and average and maximum NRS pain scores and outcomes of the HRQoL assessment measured with the EHP-30 and SF-36. Red and cyan colors represent negative and positive association, respectively. The color intensity is proportional to the degree of association. To improve the interpretability the color scales for the SF-36 have been reversed. This is because a better score for the SF-36 is positive, which is the opposite of the EHP-30 and pain scores where a lower score is positive. Asterisks indicate correlation significance: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, ↑ indicates a higher intake after the AIDEN-intervention, and ↓ indicates a lower intake after the AIDEN-intervention.

Table 2. Health-related quality of life, EHP-30

	Baseline, mean (SD)	T1, mean (SD)	Mean difference (95% CI)	p-value (two-sided) ^a
Pain	46.0 (14.5)	28.4 (16.5)	17.6 (8.43 to 26.80)	≤ 0.001
Control & powerlessness	50.5 (20.5)	30.2 (23.6)	20.3 (9.50 to 31.13)	0.001
Emotional wellbeing	37.8 (13.5)	26.3 (16.1)	11.5 (0.84 to 22.08)	0.036
Social support	46.1 (23.7)	30.5 (24.2)	15.6 (1.92 to 29.32)	0.028
Self image	44.8 (16.9)	32.8 (23.5)	12.0 (1.99 to 21.97)	0.022
Total	45.0 (14.1)	29.6 (18.1)	15.4 (6.04 to 24.76)	0.003

^a Measured with the paired samples *t*-test.

EHP-30: Endometriosis Health Profile questionnaire (range 0-100).

Table 3. Eetscore-FFQ, total and subdomains scores

	Baseline	T0	Average of Baseline and T0	T1 n=16	Mean difference (95% CI)	p-value (two-sided)
Eetscore-FFQ (0 - 160), mean (SD)						
Total score	107.9 (16.8)	104.8 (15.2)	106.3 (15.5)	141.0 (10.2)	-34.7 (-44.03 to -25.34)	≤ 0.001^a
Eetscore-FFQ 16 domains (0 - 10), median (IQR)						
Vegetables	7.2 (2.9)	6.8 (2.5)	6.8 (4.6)	10.0 (2.5)		0.016^b
Fruit	6.2 (3.5)	6.5 (2.9)	6.9 (5.8)	10.0 (1.2)		0.001^b
Wholegrains	7.1 (2.0)	7.2 (2.9)	6.9 (3.7)	10.0 (0.0)		0.001^b
Legumes	7.0 (4.1)	5.4 (3.8)	6.6 (5.5) ^c	10.0 (0.0)		0.002^b
Nuts	5.6 (4.4)	6.8 (3.8)	7.2 (8.3)	10.0 (0.0)		0.003^b
Dairy	3.9 (3.1)	3.3 (2.7)	2.8 (4.4)	7.7 (5.5)		0.003^b
Fish	5.2 (3.4)	5.2 (3.3)	5.0 (4.0)	10.0 (2.5)		0.003^b
Tea	7.3 (3.0)	6.3 (3.4)	7.9 (6.5)	6.7 (6.7)		0.505 ^b
Butter and oils	5.3 (4.9)	4.2 (4.7)	5.1 (9.7)	10.0 (4.8)		0.050^b
Coffee	7.2 (2.6)	7.5 (2.6)	6.3 (5.0)	10.0 (5.0)		0.180 ^b
Red meat	9.9 (0.3)	9.4 (2.5)	10.0 (0.0)	10.0 (0.0)		0.102 ^b
Processed meat	5.4 (3.6)	5.4 (3.6)	6.5 (5.6)	9.8 (1.6)		≤ 0.001^b
Sugary beverages	8.0 (2.9)	8.6 (2.4)	9.6 (3.1)	10.0 (0.0)		0.003^b
Alcohol	8.8 (2.9)	8.1 (4.1)	10.0 (0.0)	10.0 (0.0)		0.273 ^b
Salt	9.1 (0.7)	8.9 (1.6)	9.3 (0.9)	9.5 (0.0)		0.175 ^b
Unhealthy choices	5.0 (4.6)	5.3 (4.5)	7.1 (9.0)	10.0 (0.0)		≤ 0.001^b

^a Paired Samples *t*-test.

^b Wilcoxon Signed-ranks test, asymp. Sig (2-tailed).

^c Wilcoxon Signed-ranks test, asymp. Sig (2-tailed), *p* = 0.042.

FFQ = Food Frequency Questionnaire

AIDEN-intervention improves sleep quality, while physical activity level and BMI kg/m² remain unchanged

To evaluate additional factors affecting health, sleep quality, physical activity levels, and BMI were measured before and after the AIDEN-intervention. For physical activity levels measured with the IPAQ-SF and BMI no significant differences were observed before and after the AIDEN-intervention. Sleep quality assessed with the PSQI, showed a significant improvement after the AIDEN-intervention ($p = 0.033$) (Supplementary Table 5).

A more plant-based food choices plays a role in reducing NRS pain scores

Kendall's τ_b was performed to measure the correlation between the intake of specific nutrients, food groups, Eetscore-FFQ, NRS pain scores and HRQoL (Figure 2d and 2e; Supplementary Table 6). This indicated a correlation between the Eetscore-FFQ total and the EHP-30 subdomain pain, as well as for dietary fiber intake. The EHP-30 subdomains control & powerlessness and emotion appeared to be most strongly correlated with the changed NRS pain scores (Figure 2e). Furthermore, an increased intake of nuts, vegetables and dairy products were correlated with lower NRS pain scores. A higher intake of non-heme iron (predominantly found in plant-based foods; vegetables, legumes, grains, nuts and seeds) and dietary fiber, and a lower intake of heme iron (primarily present in animal products) showed a correlation with NRS pain scores.

Inflammatory protein profile before and after AIDEN-intervention

To assess the impact of the AIDEN-intervention on systemic inflammation high-sensitivity C-reactive protein (hs-CRP) was measured (Figure 3a), this showed no significant difference. To obtain a more comprehensive evaluation of the inflammatory protein levels in plasma samples. NPX values were measured, which revealed individual variation in protein expression, yet no consistent clustering by timepoint emerged (Supplementary Figure 2). Similarly, PCA showed a high degree of overlap between samples collected before and after the AIDEN-intervention, indicating limited global shifts in the inflammatory proteome (Figure 3b).

After correction for multiple testing, no significant results were observed. Nonetheless, four proteins demonstrated statistically significant changes in expression levels after the AIDEN-intervention prior to FDR correction (Figure 3b). Among these, Interleukin-17C (IL-17C) showed a decrease ($p = 0.016$), while Leukemia Inhibitory Factor Receptor (LIF-R), Tumor Necrosis Factor (TNF), and TNF-related apoptosis-inducing ligand (TRAIL) exhibited increased levels ($p = 0.025$, 0.024 , and 0.036 , respectively) (Figure 3c).

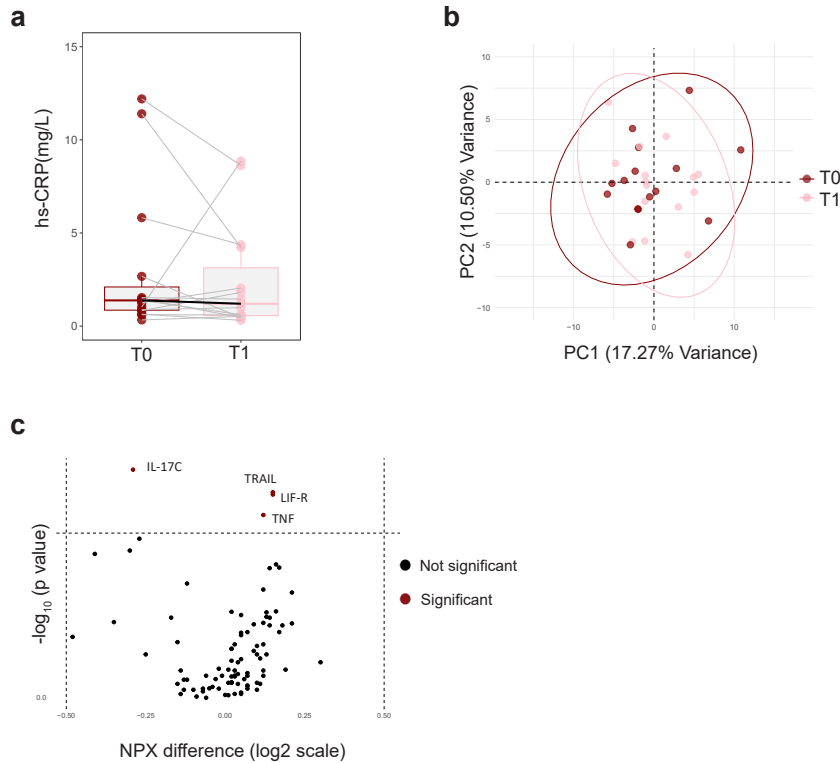


Figure 3. Changes in inflammatory markers before and after AIDEN-intervention. a) Boxplot showing hs-CRP levels measured before (T0) and after (T1) the AIDEN-intervention (n = 16). b) Principal component analysis (PCA) plot based on inflammatory protein profiles at T0 (dark red) and T1 (pink). c) Volcano plot displaying log2 fold changes versus $-\log_{10}$ p-values of inflammatory proteins. Red dots represent proteins that changed significantly after the intervention. Statistical analyses were performed using the Wilcoxon signed-rank test. * $p \leq 0.05$; ** $p \leq 0.01$.

Selective modulation of MAIT cells in PB after AIDEN-intervention

Subsequent analysis focused on the influence on systemic immune cell composition using a 42 parameter (40 color) high-dimensional flow cytometry panel which was performed only on the samples of participants who were non-users of hormonal contraceptives. Overall, the composition of PB immune cell subsets remained largely unchanged following the AIDEN-intervention (Figure 4a). Initial clustering was based on marker expression intensities identified 14 major immune cell populations (Figure 4b, Supplementary Figure 3a), with comparable frequencies observed before and after the AIDEN-intervention (Figure 4c). Interestingly, a trend toward an increased monocyte-to-lymphocyte ratio post-intervention was observed (Figure 4d).

Table 4. Evaluation of the AIDEN-intervention

Overall rating (0 – 10), mean (SD)		8.3 (1.05)	
Taste rating (0 – 10), mean (SD)		8.1 (0.87)	
Taste compared to former diet		Much less good	0%
		Somewhat less good	6%
		Equal	47%
		Better	41%
		Much better	6%
Positive aspects regarding taste*		Negative aspects regarding taste*	
New recipes	41%	Less use of flavorings: salt, sugar etc.	29%
Fruit	29%	Legumes	24%
Dairy	29%		
Affordability			
Overall		Not at all	0%
		Hardly	6%
		Reasonably	35%
		Highly	59%
		To a very large extent	0%
Affordability compared to former diet		Much lower	0%
		Narrowly lower	12%
		Equal	35%
		Narrowly higher	47%
		Much higher	6%
Feasibility			
Overall		Not at all	0%
		Hardly	0%
		Reasonably	12%
		Highly	59%
		To a very large extent	29%
Advisability		Strongly disagree	0%
		Disagree	0%
		Neutral	12%
		Agree	41%
		Strongly agree	47%
<u>Dietary recommendations that were easy to apply*</u>		<u>Dietary recommendations that were less applicable*</u>	
Combining different products, variation	47%	Eating less red meat, 100 grams/week	29%
Eating regularly	29%	Eating red fruit	23%
Eating fruit, 2-3 portions/day	29%		
Eating fish, one a week	23%		
Eating nuts, 25 grams/day	23%		
Eating wholegrain products	23%		

*All other aspects were mentioned less than 18% (n=3)

Furthermore, the main immune compartments were further stratified into 34 finer subsets through unsupervised clustering (Supplementary Figure 3b). Within the T cell compartment, 16 distinct clusters were identified (Figure 4e). While the Th1:Th2 ratio appeared lower after the AIDEN-intervention, this reduction was not statistically significant (Figure 4f). In addition, a reduction in naïve CD4⁺ T cell percentages was observed, accompanied by increased proportions of CD4⁺ Th1 and Th2 cells ($p = 0.037$, 0.014 , and 0.037 , respectively), although these differences did not remain statistically significant after FDR correction (Supplementary Figure 3c).

Notably, MAIT cells exhibited diet-associated changes. Five MAIT cell clusters were identified, among which the frequency of CD8⁺ MAIT cells significantly increased following the intervention (from 68.6% to 71.1%; $p = 0.049$, FDR-adjusted, Figure 4g–h).

MAIT-cell composition shifts in ME after AIDEN-intervention

To investigate the impact of the AIDEN-intervention on the local immune environment, uterine immune cell subsets collected from ME were analyzed. Overall, the composition of these immune cells remained largely unchanged comparing before and after the AIDEN-intervention (Figure 5a). Initial unsupervised clustering identified eight immune cell populations and one cluster of epithelial-stromal like cells in ME (Figure 5b), with comparable frequencies across timepoints (Figure 5c). With fine clustering 28 immune cell clusters were identified (Supplementary Figure 4 and 5).

Given the alterations observed in peripheral T cell subsets. Subsequently, the analysis focused on ME T cells, clustering analysis revealed 11 distinct T cell subsets (Figure 5d). Among these, MAIT cells showed a notable reduction in frequency following the AIDEN-intervention (from 3.3% to 2.3%; $p = 0.008$). However, this change did not remain significant following FDR correction (Figure 5e).

Further analysis of MAIT cell subsets revealed a shift in composition: CD8⁺ MAIT cells were reduced, while double-negative (DN) MAIT cells increased after the AIDEN-intervention (Figure 5e).

The composition of the gut and vaginal microbiota showed minor changes after the AIDEN intervention

Gut and vaginal microbiota were profiled at the genus level from paired fecal samples and vaginal swabs collected before and after the 12-week AIDEN-intervention. Stacked bar plots display the relative abundance of the top 15 genera (remaining taxa pooled as “Others”) per individual and as cohort mean (Figure 6); mean relative abundances are provided in Supplementary Table 7 and 8.

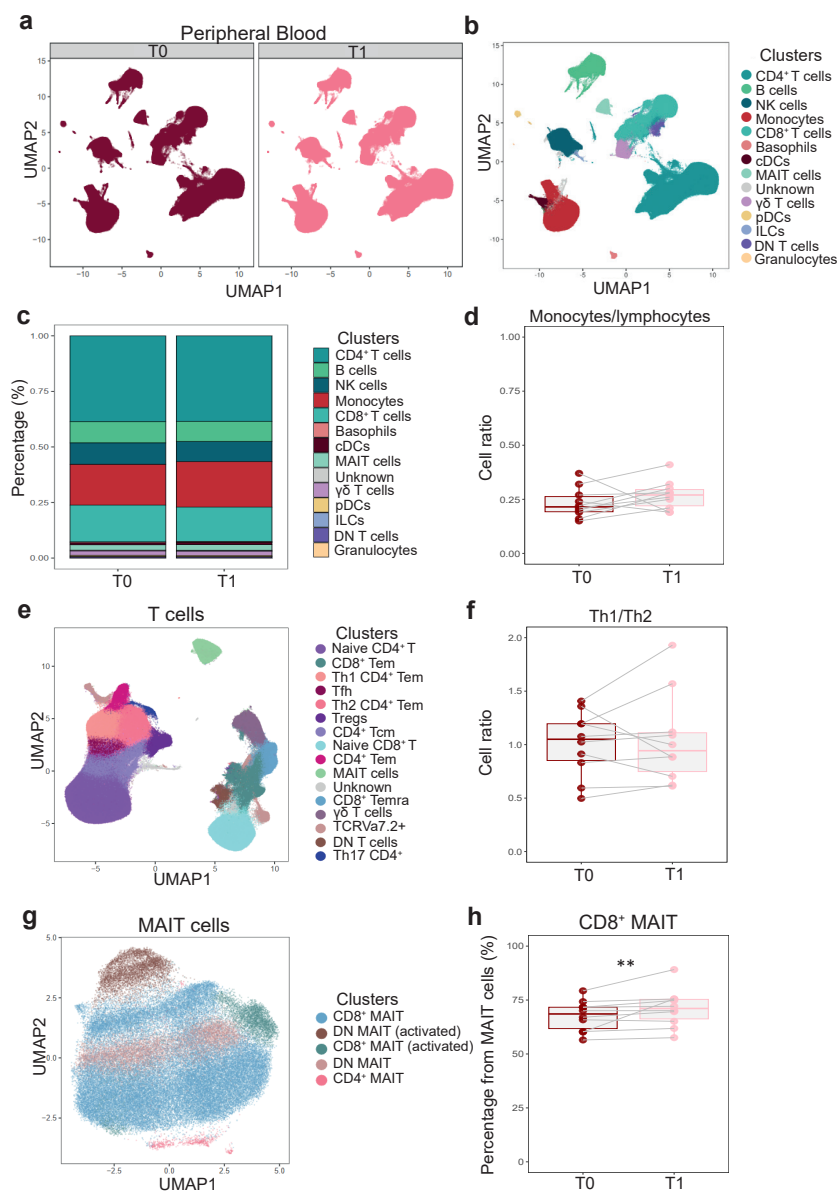


Figure 4. Systemic immune cell subsets before and after the AIDEN-intervention. a) UMAP plot showing the peripheral immune cell landscape before (T0) and after (T1) the intervention (n = 10). b) Fourteen immune cell clusters identified and embedded onto the UMAP plot. c) Stacked bar plot representing the relative proportions of the 14 immune cell clusters at T0 and T1. d) Boxplot showing the monocyte-to-lymphocyte ratio before (dark red) and after (pink) the intervention. e) UMAP plot of T cell subclusters, with 16 distinct clusters identified. f) Boxplot showing the Th1-to-Th2 cell ratio before and after the intervention. g) UMAP plot indicating MAIT cell subclusters (5 clusters identified). h) Boxplot depicting the percentage of CD8⁺ MAIT cells relative to total MAIT cells at T0 and T1. Statistical analysis was performed using the Wilcoxon signed-rank test. * p ≤ 0.05; ** p ≤ 0.01.

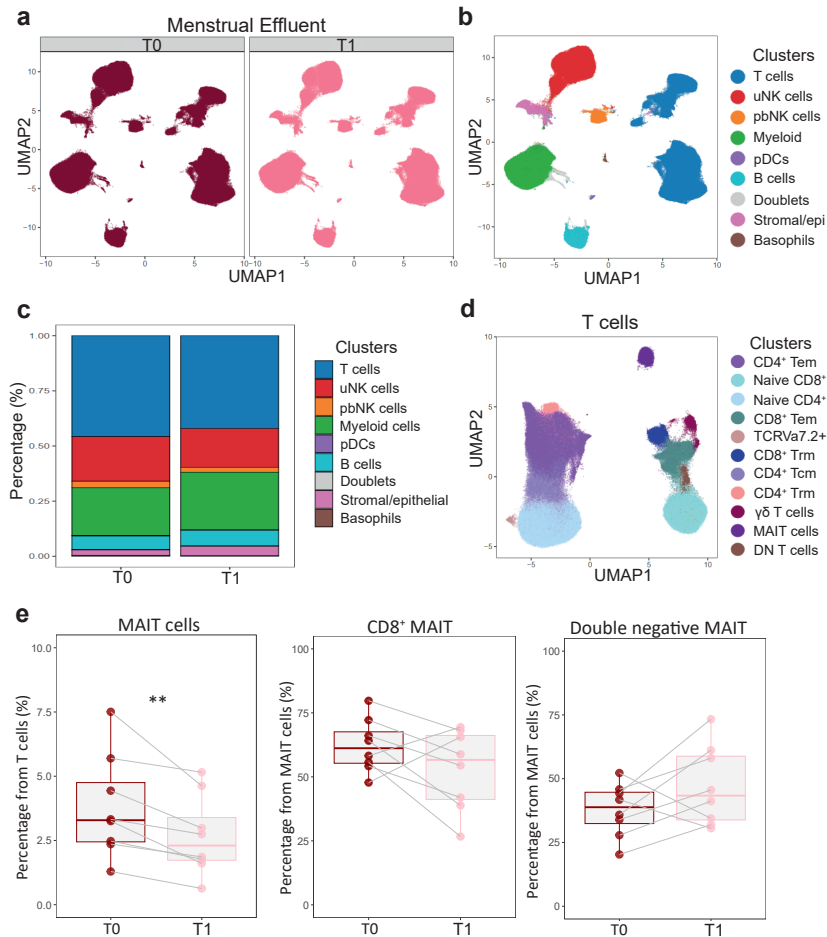


Figure 5. Uterine immune cell subsets before and after the AIDEN-intervention. a) UMAP plot showing the menstrual effluent derived immune cell landscape before (T0) and after (T1) the intervention (n = 8). b) Nine immune cell clusters identified and embedded onto the UMAP plot. c) Stacked bar plot representing the relative proportions of the 9 immune cell clusters at T0 and T1. d) UMAP plot of T cell subclusters, with 11 distinct clusters identified. e) Boxplot depicting the percentage of MAIT, CD8⁺ MAIT, and double negative (DN) MAIT cells at T0 (dark red) and T1 (pink). Statistical analysis was performed using the Wilcoxon signed-rank test. * $p \leq 0.05$; ** $p \leq 0.01$.

The gut microbiome composition showed substantial inter-individual variability both before and after the AIDEN-intervention (Figure 6a), limiting detection of consistency shifts; accordingly, no genus met significance by DESeq2, and beta-diversity analyses (PCoA) showed no separation by time point. In contrast, the vaginal microbiome was consistently *Lactobacillus*-dominated, with similar profiles before and after the AIDEN-intervention (Figure 6b).

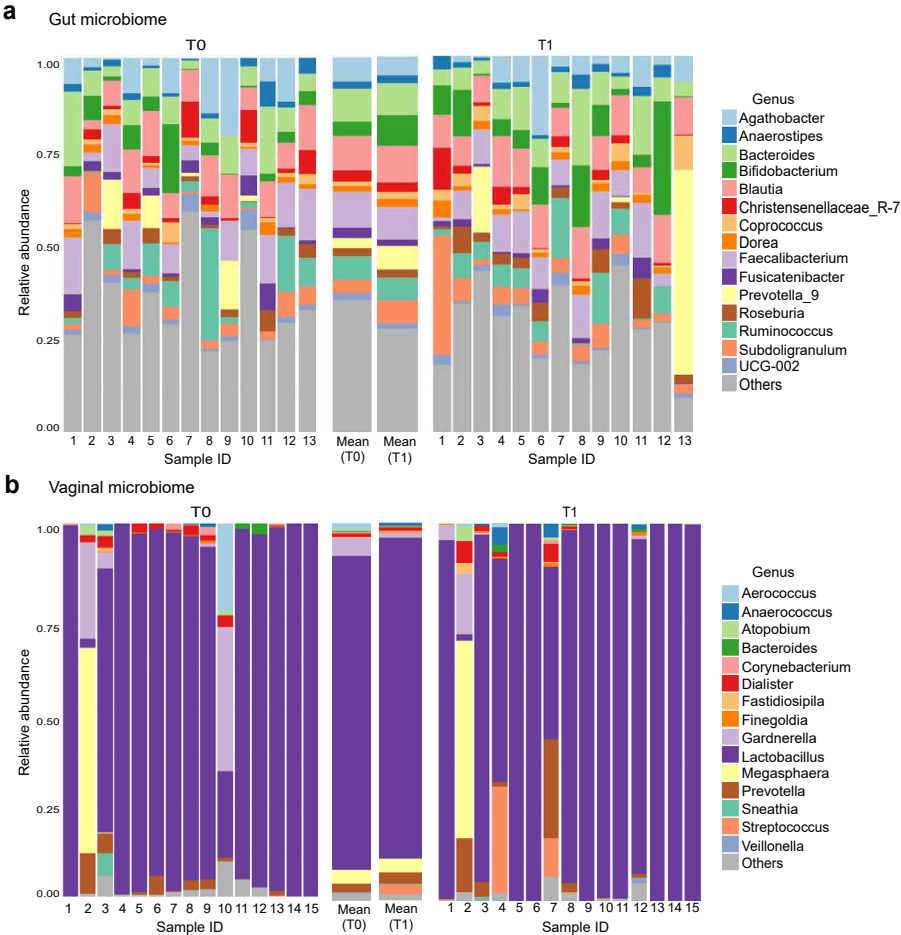


Figure 6. Gut and vaginal microbiome before and after AIDEN-intervention. Relative abundance analysis of the level of bacterial genus (top-15) of the a) gut (n=13) and b) vaginal microbiome (n=15) before (T0) and after (T1) AIDEN-intervention.

Although not statistically significant, *Bifidobacterium* showed a reproducible upward trend: mean relative abundance increased from 0.0397 to 0.0755 after the AIDEN-intervention, with concordant within-participant increases across individuals (Supplementary Table 7). No comparable pattern was observed for other genera.

Discussion

This explorative feasibility study investigated the effect of an AIDEN- intervention for 12 weeks on endometriosis-related pain symptoms, HRQoL, gut- and vaginal microbiome and inflammatory markers in women with endometriosis. The findings of this study show that women with endometriosis who improve their diet quality by a higher intake of anti-inflammatory nutrients and a lower intake of pro-inflammatory nutrients, based on the DHD2015 experienced a decrease in NRS pain scores, and an improvement in HRQoL. Furthermore, participants who followed the AIDEN-intervention were highly satisfied: they evaluated the diet as tasty, affordable, and feasible.

Although the AIDEN-intervention had a notable impact on endometriosis-related pain symptoms and improved HRQoL, its small sample size may have contributed to modest measurable effects on systemic inflammatory markers and immune parameters. A systematic review of 20 randomized controlled trials demonstrated that anti-inflammatory diets can significantly modulate levels of inflammatory proteins such as interleukins, IFN- γ , CRP, and TNF- α (49). In line with these findings, we observed a trend toward decreased levels of IL-17C following the AIDEN-intervention. The level of IL-17 is known to be elevated either in endometrial lesions, PF, or blood of women with endometriosis (50-52), suggesting that an anti-inflammatory diet may help reduce inflammation. However, no significant changes were observed in IL-6 or hs-CRP levels, key inflammatory markers consistently reported to be elevated in endometriosis (53, 54). Furthermore, we observed increased levels of LIF-R, TRAIL, and TNF- α . LIF-R, the receptor for LIF, is involved in decidualization, endometrial immune regulation, and tissue remodeling (55). Notably, prior studies report reduced LIF in ectopic tissues of women with endometriosis while LIF-R is up-regulated (56). Consistent with this biology, higher LIF-R here may indicate greater tissue sensitivity to LIF signaling. TNF- α , while a canonical pro-inflammatory cytokine implicated in lesion dynamics and immune-cell recruitment, can also reflect enhanced immune surveillance when moderately elevated (57). Meanwhile, TRAIL, a TNF-family ligand, induces apoptosis in stromal cells (58) and has been reported as suppressed in some endometriosis cohorts (59), suggesting impaired apoptotic clearance. Together, the increases in TNF- α and TRAIL may therefore reflect more effective immune surveillance (TNF- α) and improved apoptotic control of ectopic tissue (TRAIL), rather than a simple escalation of inflammation.

The most notable immunological impact of the AIDEN-intervention was observed in MAIT cells, both systemically and within the uterine environment. MAIT cells are a non-conventional T cell subset found in blood and mucosal tissues, where they contribute to antimicrobial defense and inflammatory responses through rapid cytokine production and cytotoxic activity (60, 61). They are constantly present in the endometrium, and their frequency remains stable across menstrual cycle phases and independent of sex hormones. It has been suggested that endometrial MAIT cells represent a transient tissue-resident population that can be replenished from circulation (62). Although the role of MAIT cells in endometriosis pathophysiology remains unclear, previous studies have reported elevated frequencies of MAIT cells in both blood and PF of women with endometriosis, with an increased proportion of CD8⁺ MAIT cells and reduced double-negative (DN) MAIT cells in PF (31). In our study a reduction in total uterine MAIT cells, along with decreased CD8⁺ and increased DN MAIT cell proportions in ME was observed. Interestingly, the percentage of CD8⁺ MAIT cells in PB were increased after the AIDEN-intervention. Taken together, these data suggests that the AIDEN-intervention likely reduces recruitment of CD8⁺ MAIT cells to the uterus, leading to fewer MAITs in the uterus and more in blood. However, this mechanism needs to be further elucidated.

MAIT cells are known to expand in response to commensal microbial colonization, particularly in the presence of riboflavin-producing bacteria (63). However, in our study no significant changes in genus-level composition of both gut and vaginal microbiome were observed. Even though the composition of microbiome did not change significantly, microbial metabolites such as riboflavin-pathway derived MR1 ligands and short-chain fatty acids could shift and influence immune cells. Future studies should incorporate targeted metabolomics and pathway-level profiling (e.g., riboflavin biosynthesis genes) to resolve microbiome–MAIT cell interaction.

After the AIDEN-intervention, a trend toward increased relative abundance of the SCFA-producing genus *Bifidobacterium* was observed. SCFAs are well-established immunomodulators acting on both innate and adaptive immune compartments (64). Their intestinal production depends on substrate availability for SCFA-producing bacteria, primarily derived from dietary fiber (65). Consistent with this, participants increased their intake of fiber-rich foods such as fruits, vegetables, whole grains, and legumes, and total dietary fiber during the AIDEN-intervention. Although we did not directly quantify SCFA levels, future studies should assess whether enhanced fiber intake and shifts in *Bifidobacterium* contribute to the clinical effects observed.

A key strength of our study is its integrative design, combining questionnaires and biological samples to explore both symptom relief and potential underlying biological mechanisms. By including both systemic and uterine immune parameters, using ME as a non-invasive method to assess the uterine immune system (42). This provides a comprehensive picture of the diet's potential immunological effects. The study also benefits from a standardized yet personalized nutritional approach. All participants were supervised by the same dietitian, using a shared dietary protocol tailored to individual needs. This personalized approach mirrors real-world dietary counseling and may enhance the generalizability of our findings to endometriosis patients seeking lifestyle-based treatment options. Moreover, this individualized approach may stimulate individuals with endometriosis to adhere to the diet for a prolonged period of time. However, this individualization also poses limitations. The consultation with the dietitian may have influenced self-reported outcomes such as pain and HRQoL through increased feelings of support or social desirability. In addition, lower scores on the EHP-30 subdomain control & powerlessness were observed which reflects a stronger sense of control and empowerment.

Fostering a greater sense of control is an important psychological factor in pain management and, in addition to any biological mechanisms, may have contributed to the reduction in NRS pain scores and the improvement in HRQoL. This is consistent with the findings of Ahmed et al (66). Actively involving individuals with endometriosis in their own healthcare, through self-management interventions can empower them to improve control their pain symptoms which leads to better HRQoL.

As a feasibility study, the sample size was small, which may have limited detectable effects on systemic inflammatory markers and immune parameters. Moreover, our strict inclusion and exclusion criteria yielded a relatively homogeneous cohort. Given the limited sample size and the inherent heterogeneity of endometriosis, we were unable to perform subgroup analyses (e.g., by disease subtype). It is also plausible that a particularly motivated subset, individuals willing to change their diet, self-selected into the study. Although this may limit generalizability to the broader endometriosis population, it reflects the group most likely to consider an anti-inflammatory diet as a treatment option. Finally, the absence of a healthy control group and a non-intervention control group constrains our ability to attribute observed changes specifically to the dietary intervention.

The impact of dietary interventions on endometriosis-related pain symptoms and HRQoL is shown in a few studies. However, none of them investigated a specific anti-inflammatory diet. In an exploratory observational study by Krabbenborg et al.,

which assessed diet quality, pain symptoms and HRQoL, no specific dietary adjustment was found to increase HRQoL while various dietary changes were reported to improve endometriosis-related pain (7). Nevertheless, evidence from related dietary approaches supports our findings. Cirillo et al. found a trend toward pain reduction with adherence to the Mediterranean diet (MD)(67), a dietary pattern with similarities to the anti-inflammatory diet, though HRQoL was not assessed. In contrast, Haaps et al. investigated the impact of low-FODMAP and endometriosis-specific diets on HRQoL and, similar to our results, reported significant improvements in pain, emotional well-being, powerlessness, and self-image domains of the EHP-30 questionnaire(68). Notably, their study did not find improvements in social support, whereas our participants reported significant gains in this domain possibly due to the continuous involvement of a supportive dietitian.

Although the AIDEN-intervention produced only modest changes in systemic inflammatory markers and immune parameters, larger controlled trials are warranted to confirm these trends and clarify underlying mechanisms. Nevertheless, an anti-inflammatory diet remains a promising non-pharmacological approach to alleviating endometriosis-related pain and improving HRQoL.

Acknowledgements

The authors would like to thank all participants for their contribution to this study. In addition, the authors would also like to thank Jet van Willigen at Wageningen University and Research for processing all the 24h-recall data in Compl-eat™; Maryse D. Berkhout at Wageningen University and Research for help with the microbiome analysis, and Kleopatra Tzai at Radboudumc for help with sample processing.

Author contributions

EH: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. AG: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. JH: Conceptualization, Investigation, Methodology, Validation, Writing – review & editing. JF: Formal Analysis, Writing – review and editing. LS: Formal Analysis, Writing – review and editing. LW: Resources, Writing – review and editing. QM: Conceptualization, Writing – review and editing. CB: Resources, Writing – review and editing. NR: Conceptualization, Resources, Writing – review and editing. TP: Resources, Writing – review and editing. RM: Conceptualization, Investigation, Methodology, Resources, Writing – review & editing. AN: Conceptualization, Investigation, Methodology, Resources, Writing – review & editing.

Competing interests

The authors declare no competing interests.

Data availability statement

Data is available upon request by contacting the corresponding author.

Funding

No funding was acquired for this research.

References

1. Zondervan KT, Becker CM, Missmer SA. Endometriosis. *N Engl J Med*. 2020;382(13):1244-56.
2. Rolla E. Endometriosis: advances and controversies in classification, pathogenesis, diagnosis, and treatment. *F1000Res*. 2019;8.
3. Benagiano G, Bianchi P, Guo SW. Endometriosis in adolescent and young women. *Minerva Obstet Gynecol*. 2021;73(5):523-35.
4. Della Corte L, Di Filippo C, Gabrielli O, Reppuccia S, La Rosa VL, Ragusa R, et al. The Burden of Endometriosis on Women's Lifespan: A Narrative Overview on Quality of Life and Psychosocial Wellbeing. *Int J Environ Res Public Health*. 2020;17(13).
5. Missmer SA, Tu FF, Agarwal SK, Chapron C, Soliman AM, Chiuve S, et al. Impact of Endometriosis on Life-Course Potential: A Narrative Review. *Int J Gen Med*. 2021;14:9-25.
6. Armour M, Sinclair J, Chalmers KJ, Smith CA. Self-management strategies amongst Australian women with endometriosis: a national online survey. *BMC Complement Altern Med*. 2019;19(1):17.
7. Krabbenborg I, de Roos N, van der Grinten P, Nap A. Diet quality and perceived effects of dietary changes in Dutch endometriosis patients: an observational study. *Reprod Biomed Online*. 2021;43(5):952-61.
8. Huijs E, Nap A. The effects of nutrients on symptoms in women with endometriosis: a systematic review. *Reprod Biomed Online*. 2020;41(2):317-28.
9. Piecuch M, Garbicz J, Waliczek M, Malinowska-Borowska J, Rozentryt P. I Am the 1 in 10-What Should I Eat? A Research Review of Nutrition in Endometriosis. *Nutrients*. 2022;14(24).
10. Burkard M, Leischner C, Lauer UM, Busch C, Venturelli S, Frank J. Dietary flavonoids and modulation of natural killer cells: implications in malignant and viral diseases. *J Nutr Biochem*. 2017;46:1-12.
11. Lee A, Lee YJ, Yoo HJ, Kim M, Chang Y, Lee DS, et al. Consumption of Dairy Yogurt Containing *Lactobacillus paracasei* ssp. *paracasei*, *Bifidobacterium animalis* ssp. *lactis* and Heat-Treated *Lactobacillus plantarum* Improves Immune Function Including Natural Killer Cell Activity. *Nutrients*. 2017;9(6).
12. Issazadeh-Navikas S, Teimer R, Bockermann R. Influence of dietary components on regulatory T cells. *Mol Med*. 2012;18(1):95-110.
13. Calder PC, Ahluwalia N, Brouns F, Buetler T, Clement K, Cunningham K, et al. Dietary factors and low-grade inflammation in relation to overweight and obesity. *Brit J Nutr*. 2011;106:S1-S78.
14. Minihaan AM, Vinoy S, Russell WR, Baka A, Roche HM, Tuohy KM, et al. Low-grade inflammation, diet composition and health: current research evidence and its translation. *Br J Nutr*. 2015;114(7):999-1012.
15. Casas R, Sacanella E, Urpi-Sarda M, Corella D, Castaner O, Lamuela-Raventos RM, et al. Long-Term Immunomodulatory Effects of a Mediterranean Diet in Adults at High Risk of Cardiovascular Disease in the PREvencion con Dieta MEDiterranea (PREDIMED) Randomized Controlled Trial. *J Nutr*. 2016;146(9):1684-93.
16. Bahr LS, Franz K, Mahler A. Assessing the (anti)-inflammatory potential of diets. *Curr Opin Clin Nutr Metab Care*. 2021;24(5):402-10.
17. Bibbo S, Ianiro G, Giorgio V, Scaldaferri F, Masucci L, Gasbarrini A, et al. The role of diet on gut microbiota composition. *Eur Rev Med Pharmacol Sci*. 2016;20(22):4742-9.
18. Brown K, DeCoffe D, Molcan E, Gibson DL. Diet-Induced Dysbiosis of the Intestinal Microbiota and the Effects on Immunity and Disease (vol 4, pg 1095, 2012). *Nutrients*. 2012;4(11):1552-3.
19. Beam A, Clinger E, Hao L. Effect of Diet and Dietary Components on the Composition of the Gut Microbiota. *Nutrients*. 2021;13(8).

20. Barrea L, Muscogiuri G, Frias-Toral E, Laudisio D, Pugliese G, Castellucci B, et al. Nutrition and immune system: from the Mediterranean diet to dietary supplementary through the microbiota. *Crit Rev Food Sci Nutr*. 2021;61(18):3066-90.
21. Shao T, Verma HK, Pande B, Costanzo V, Ye W, Cai Y, et al. Physical Activity and Nutritional Influence on Immune Function: An Important Strategy to Improve Immunity and Health Status. *Front Physiol*. 2021;12:751374.
22. Temba GS, Kullaya V, Pecht T, Mmbaga BT, Aschenbrenner AC, Ulas T, et al. Urban living in healthy Tanzanians is associated with an inflammatory status driven by dietary and metabolic changes. *Nat Immunol*. 2021;22(3):287-300.
23. Pahwa H, Sharan K. Food and nutrition as modifiers of the immune system: A mechanistic overview. *Trends Food Sci Tech*. 2022;123:393-403.
24. Berbic M, Schulke L, Markham R, Tokushige N, Russell P, Fraser IS. Macrophage expression in endometrium of women with and without endometriosis. *Hum Reprod*. 2009;24(2):325-32.
25. Tran LV, Tokushige N, Berbic M, Markham R, Fraser IS. Macrophages and nerve fibres in peritoneal endometriosis. *Hum Reprod*. 2009;24(4):835-41.
26. Abramiuk M, Grywalska E, Malkowska P, Sierawska O, Hryniewicz R, Niedzwiedzka-Rystwej P. The Role of the Immune System in the Development of Endometriosis. *Cells*. 2022;11(13).
27. Azeze GG, Wu L, Alemu BK, Wang CC, Zhang T. Changes in the number and activity of natural killer cells and its clinical association with endometriosis : systematic review and meta-analysis. *F&S Rev*. 2024;5(2).
28. Hirata T, Osuga Y, Takamura M, Saito A, Hasegawa A, Koga K, et al. Interleukin-17F increases the secretion of interleukin-8 and the expression of cyclooxygenase 2 in endometriosis. *Fertil Steril*. 2011;96(1):113-7.
29. Ahn SH, Monsanto SP, Miller C, Singh SS, Thomas R, Tayade C. Pathophysiology and Immune Dysfunction in Endometriosis. *Biomed Res Int*. 2015;2015:795976.
30. Riccio L, Santulli P, Marcellin L, Abrao MS, Batteux F, Chapron C. Immunology of endometriosis. *Best Pract Res Clin Obstet Gynaecol*. 2018;50:39-49.
31. Li C, Lu Z, Bi K, Wang K, Xu Y, Guo P, et al. CD4(+)/CD8(+) mucosa-associated invariant T cells foster the development of endometriosis: a pilot study. *Reprod Biol Endocrinol*. 2019;17(1):78.
32. El Morr Y, Furstenheim M, Mestdagh M, Franciszkievicz K, Salou M, Morvan C, et al. MAIT cells monitor intestinal dysbiosis and contribute to host protection during colitis. *Sci Immunol*. 2024;9(96):eadi8954.
33. Jabeen MF, Hinks TSC. MAIT cells and the microbiome. *Front Immunol*. 2023;14:1127588.
34. Ata B, Yildiz S, Turkgeldi E, Brocal VP, Dinleyici EC, Moya A, et al. The Endobiota Study: Comparison of Vaginal, Cervical and Gut Microbiota Between Women with Stage 3/4 Endometriosis and Healthy Controls. *Sci Rep*. 2019;9(1):2204.
35. Jiang I, Yong PJ, Allaire C, Bedaiwy MA. Intricate Connections between the Microbiota and Endometriosis. *Int J Mol Sci*. 2021;22(11).
36. Vennberg Karlsson J, Patel H, Premberg A. Experiences of health after dietary changes in endometriosis: a qualitative interview study. *BMJ Open*. 2020;10(2):e032321.
37. Nirgianakis K, Egger K, Kalaitzopoulos DR, Lanz S, Bally L, Mueller MD. Effectiveness of Dietary Interventions in the Treatment of Endometriosis: a Systematic Review. *Reprod Sci*. 2022;29(1):26-42.
38. Armour M, Cave AE, Schabrun SM, Steiner GZ, Zhu X, Song J, et al. Manual Acupuncture Plus Usual Care Versus Usual Care Alone in the Treatment of Endometriosis-Related Chronic Pelvic Pain: A Randomized Controlled Feasibility Study. *J Altern Complement Med*. 2021;27(10):841-9.
39. Kromhout D, Spaaij CJK, de Goede J, Weggemans RM, Brug J, Geleijnse JM, et al. The 2015 Dutch food-based dietary guidelines. *Eur J Clin Nutr*. 2016;70(8):869-78.
40. de Rijk MG, Slotegraaf AI, Brouwer-Brolsma EM, Perenboom CWM, Feskens EJM, de Vries JHM. Development and evaluation of a diet quality screener to assess adherence to the Dutch food-based dietary guidelines. *Br J Nutr*. 2021;128(8):1-11.

41. Farrar JT, Young JP, Jr., LaMoreaux L, Werth JL, Poole MR. Clinical importance of changes in chronic pain intensity measured on an 11-point numerical pain rating scale. *Pain*. 2001;94(2):149-58.
42. van der Molen RG, Schutten JH, van Cranenbroek B, ter Meer M, Donckers J, Scholten RR, et al. Menstrual blood closely resembles the uterine immune micro-environment and is clearly distinct from peripheral blood. *Hum Reprod*. 2014;29(2):303-14.
43. Waaijer LA, van Cranenbroek B, Koenen H. OMIP-112: 42-Parameter (40-Color) Spectral Flow Cytometry Panel for Comprehensive Immunophenotyping of Human Peripheral Blood Leukocytes. *Cytometry A*. 2025;107(4):226-32.
44. Kroger C, Muller S, Leidner J, Krober T, Warnat-Herresthal S, Spintge JB, et al. Unveiling the power of high-dimensional cytometry data with cyCONDOR. *Nat Commun*. 2024;15(1):10702.
45. Poncheewin W, Hermes GDA, van Dam JCJ, Koehehorst JJ, Smidt H, Schaap PJ. NG-Tax 2.0: A Semantic Framework for High-Throughput Amplicon Analysis. *Front Genet*. 2019;10:1366.
46. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res*. 2013;41(Database issue):D590-6.
47. McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One*. 2013;8(4):e61217.
48. Love MI, Anders S, Kim V, Huber W. RNA-Seq workflow: gene-level exploratory analysis and differential expression. *F1000Res*. 2015;4:1070.
49. Mukherjee MS, Han CY, Sukumaran S, Delaney CL, Miller MD. Effect of anti-inflammatory diets on inflammation markers in adult human populations: a systematic review of randomized controlled trials. *Nutr Rev*. 2022;81(1):55-74.
50. Zhang X, Xu H, Lin J, Qian Y, Deng L. Peritoneal fluid concentrations of interleukin-17 correlate with the severity of endometriosis and infertility of this disorder. *BJOG*. 2005;112(8):1153-5.
51. Luddi A, Marrocco C, Governini L, Semplici B, Pavone V, Luisi S, et al. Expression of Matrix Metalloproteinases and Their Inhibitors in Endometrium: High Levels in Endometriotic Lesions. *Int J Mol Sci*. 2020;21(8).
52. Rafi U, Ahmad S, Bokhari SS, Iqbal MA, Zia A, Khan MA, et al. Association of Inflammatory Markers/ Cytokines with Cardiovascular Risk Manifestation in Patients with Endometriosis. *Mediators Inflamm*. 2021;2021:3425560.
53. Mihalyi A, Gevaert O, Kyama CM, Simsa P, Pochet N, De Smet F, et al. Non-invasive diagnosis of endometriosis based on a combined analysis of six plasma biomarkers. *Hum Reprod*. 2010;25(3):654-64.
54. Mosbah A, Nabel Y, Khashaba E. Interleukin-6, intracellular adhesion molecule-1, and glycodelin A levels in serum and peritoneal fluid as biomarkers for endometriosis. *Int J Gynaecol Obstet*. 2016;134(3):247-51.
55. Shuya LL, Menkhorst EM, Yap J, Li P, Lane N, Dimitriadis E. Leukemia inhibitory factor enhances endometrial stromal cell decidualization in humans and mice. *PLoS One*. 2011;6(9):e25288.
56. Zutautas KB, Sisnett DJ, Miller JE, Lingegowda H, Childs T, Bougie O, et al. The dysregulation of leukemia inhibitory factor and its implications for endometriosis pathophysiology. *Front Immunol*. 2023;14:1089098.
57. Miller JE, Ahn SH, Monsanto SP, Khalaj K, Koti M, Tayade C. Implications of immune dysfunction on endometriosis associated infertility. *Oncotarget*. 2017;8(4):7138-47.
58. Pimentel JM, Zhou JY, Wu GS. The Role of TRAIL in Apoptosis and Immunosurveillance in Cancer. *Cancers (Basel)*. 2023;15(10).
59. Kim H, Ku SY, Suh CS, Kim SH, Kim JH, Kim JG. Association between endometriosis and polymorphisms in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), TRAIL receptor and osteoprotegerin genes and their serum levels. *Arch Gynecol Obstet*. 2012;286(1):147-53.

60. Kjer-Nielsen L, Patel O, Corbett AJ, Le Nours J, Meehan B, Liu L, et al. MR1 presents microbial vitamin B metabolites to MAIT cells. *Nature*. 2012;491(7426):717-23.
61. Godfrey DI, Koay HF, McCluskey J, Gherardin NA. The biology and functional importance of MAIT cells. *Nat Immunol*. 2019;20(9):1110-28.
62. Bister J, Crona Guterstam Y, Strunz B, Dumitrescu B, Haij Bhattarai K, Ozenci V, et al. Human endometrial MAIT cells are transiently tissue resident and respond to *Neisseria gonorrhoeae*. *Mucosal Immunol*. 2021;14(2):357-65.
63. Treiner E, Duban L, Bahram S, Radosavljevic M, Wanner V, Tilloy F, et al. Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1. *Nature*. 2003;422(6928):164-9.
64. Fusco W, Lorenzo MB, Cintoni M, Porcari S, Rinninella E, Kaitsas F, et al. Short-Chain Fatty-Acid-Producing Bacteria: Key Components of the Human Gut Microbiota. *Nutrients*. 2023;15(9).
65. O'Riordan KJ, Collins MK, Moloney GM, Knox EG, Aburto MR, Fulling C, et al. Short chain fatty acids: Microbial metabolites for gut-brain axis signalling. *Mol Cell Endocrinol*. 2022;546:111572.
66. Ahmad M, Sechi C, Guerriero S, Guicciardi M, Vismara L. Patient engagement, quality of life and chronic pain: A cross-sectional study on endometriosis. *J Health Psychol*. 2025;30(8):1990-2000.
67. Cirillo M, Argento FR, Becatti M, Fiorillo C, Coccia ME, Fatini C. Mediterranean Diet and Oxidative Stress: A Relationship with Pain Perception in Endometriosis. *Int J Mol Sci*. 2023;24(19).
68. van Haaps AP, Wijbers JV, Schreurs AMF, Vlek S, Tuynman J, De Bie B, et al. The effect of dietary interventions on pain and quality of life in women diagnosed with endometriosis: a prospective study with control group. *Hum Reprod*. 2023;38(12):2433-46.

Supplementary material

Supplementary Table 1. Antibody details

Fluorochrome	Marker	Company
PerCP/Cyanine5,5	CD16	Biolegend
PE/Fire™ 640	CD19	Biolegend
PE/Fire™ 700	CD45RA	Biolegend
PE/Cyanine5	CD278	Biolegend
Brilliant Violet 785™	CD279	Biolegend
PE/Cyanine7	CD11c	Biolegend
PE/Dazzle™ 594	CD141	Biolegend
PerCP	CD8	Biolegend
Spark Blue™ 550	CD3	Biolegend
Brilliant Violet 570™	CD127	Biolegend
APC	TCR Va7,2	Biolegend
Spark NIR™ 685	CD197	Biolegend
Brilliant Violet 605™	CD25	Biolegend
Brilliant Violet 650™	CD38	Biolegend
PE/Fire™ 810	CD194	Biolegend
Spark Blue™ 574	CD45	Biolegend
Brilliant Violet 711™	Tigit	Biolegend
Spark Violet™ 538	CD14	Biolegend
Zombie NIR™ Fixable Viability Kit	Fix viability dye	Biolegend
BD OptiBuild™ BUV615	TCR-γδ	BD
NovaFluor™ Blue 660-40S	HLA-DR	Thermofisher
eFluor™ 450	CD161	Thermofisher
BD OptiBuild™ BUV496	CD28	BD
BD OptiBuild™ BUV563	CD196	BD
BD OptiBuild™ BUV615	CD123	BD
BD OptiBuild™ BUV661	IgG	BD
BD OptiBuild™ BUV737	CD103	BD
BD OptiBuild™ RY586	CD56	BD
BD Horizon™ BV480	CD185	BD
BD OptiBuild™ BV750	CD294	BD
BD Horizon™ RB780	CD69	BD
BD Horizon™ BUV395	CD183	BD
Alexa fluor 700	IgM	Biolegend
BD OptiBuild™ BUV805	CD24	BD
PerCP/Fire™ 806	CD4	Biolegend
NovaFluor™ Yellow 730	CD1c	Invitrogen
APC	IgD	Biolegend

Supplementary Table 1. Continued

Fluorochrome	Marker	Company
APC/Fire™ 810	CD11b	Biolegend
FITC	CD27	Biolegend
PE	CD49a	Biolegend
Brilliant Violet 421™	CD39	Biolegend
Brilliant Violet 510™	CD326 (EpCAM)	Biolegend

Supplementary Table 2. Health-related quality of life, SF-36

	Baseline, mean (SD)	T1, mean (SD)	Mean difference (95% CI)	P-value (two-sided)
Physical Component Summary (PCS)	47.8 (6.7)	47.3 (8.7)	0.42 (-3.43 to 4.27)	0.820 ^a
Mental Component Summary (MCS)	38.9 (10.1)	44.5 (10.4)	-5.63 (-10.20 to -1.05)	0.019^a
<i>Subdomains of the SF-36</i>	Baseline, median (IQR)	T1, median (IQR)		
Physical functioning	95.0 (10.0)	95.0 (13.8)		0.952 ^b
Role limitations due to physical health	75.0 (93.8)	87.5 (100.0)		0.887 ^b
Role limitations due to emotional problems	83.3 (91.7)	100.0 (25.0)		0.054 ^b
Energy/fatigue	45.0 (18.8)	52.5 (32.5)		0.068 ^b
Emotional wellbeing	60.0 (29.0)	66.0 (30.0)		0.529 ^b
Social functioning	62.5 (25.0)	75.0 (37.5)		0.066 ^b
Pain	50.0 (12.5)	41.25 (47.5)		0.972 ^b
General health	60.0 (27.5)	57.5 (22.8)		0.390 ^b

^a Paired Samples t-test.

^b Wilcoxon Signed-ranks test, asymp. Sig (2-tailed).

SF-36: Short Form-36.

Supplementary Table 3. Food group consumption Compl-eat™

Foodgroups	Baseline, mean (SD)	Intervention period ^a, mean (SD)	Mean difference (95% CI)	P-value (two-sided) ^b
Alcoholic beverages	11.1 (32.3)	5.6 (23.6)	5.6 (-6.16 to 17.27)	0.331
Bread	78.3 (46.5)	76.1 (26.9)	2.3 (-22.66 to 27.18)	0.851
Cereal products and binding agents	84.4 (45.1)	103.1 (37.1)	-18.7 (-41.77 to 4.38)	0.106
Cheese	32.5 (31.0)	33.7 (25.9)	-1.2 (-10.96 to 8.55)	0.798
Eggs	29.5 (32.9)	37.9 (30.1)	-8.4 (-20.91 to 4.13)	0.176
Fats and oils	14.6 (7.2)	18.2 (6.3)	-3.5 (-7.74 to 0.65)	0.093
Fish	19.9 (46.9)	23.6 (38.0)	-3.7 (-22.56 to 15.08)	0.680
Fruit	179.4 (119.4)	280.3 (55.5)	-100.8 (-161.15 to -40.49)	0.003
Legumes	64.8 (70.2)	98.4 (54.3)	-33.6 (-60.08 to -7.04)	0.016
Meat and poultry	124.4 (65.1)	58.1 (54.6)	66.4 (36.19 to 96.55)	≤0.001
Meat and dairy substitutes	81.9 (102.7)	98.3 (54.6)	-16.3 (-51.45 to 18.82)	0.341
Milk and dairy products	163.9 (159.1)	269.7 (110.1)	-105.8 (-182.87 to -28.69)	0.010
Non-alcoholic drinks	1203.4 (836.8)	1448.7 (685.8)	-245.3 (-670.83 to 180.22)	0.241
Nuts and seeds	26.8 (18.6)	27.6 (12.6)	-0.9 (-10.60 to 8.89)	0.856
Pastry, cake and biscuits	20.2 (31.1)	16.2 (33.0)	4.0 (-3.74 to 11.70)	0.292
Potatoes	74.2 (80.2)	60.6 (76.5)	13.6 (-9.80 to 37.02)	0.237
Processed meats	9.4 (12.4)	6.7 (12.2)	2.8 (-0.44 to 5.99)	0.086
Savory sandwich fillings	14.2 (18.3)	13.9 (14.6)	0.3 (-11.67 to 12.23)	0.961
Savory sauces	5.3 (10.4)	2.8 (6.3)	2.5 (-1.14 to 6.14)	0.166
Savory snacks and salty bites	25.3 (47.0)	15.6 (40.4)	9.7 (-5.16 to 24.60)	0.186
Soups	32.9 (77.9)	24.6 (66.1)	8.3 (-16.16 to 32.83)	0.483

Supplementary Table 4. Continued

Foodgroups	Baseline, mean (SD)	Intervention period ^a, mean (SD)	Mean difference (95% CI)	P-value (two-sided) ^b
Sugar, candy, sweet spreads, and sweet sauces	0.0 (0.0)	0.0 (0.0)	0	-
Vegetables	235.5 (174.4)	401.3 (72.1)	-165.8 (-251.50 to -80.04)	≤ 0.001

^a Mean score of the three 24h-recalls during the AIDEN-intervention period. The intervention period is a combination of three 24h-recalls.

^b Paired Samples t-test.

Supplementary Table 4. Macro- and micronutrient intake Compl-eat™

Macro- and micronutrients	Baseline, mean (SD)	Intervention period ^a, mean (SD)	Mean difference (95% CI)	P-value (two-sided) ^b
Energy (kcal)	1765 (504)	1600 (221)	157 (-90.00 to 403.59)	0.198
Protein total (gram)	79.9 (24.1)	78.3 (13.6)	1.0 (-11.01 to 13.07)	0.859
Protein total (En%)	18.6 (4.1)	19.9 (2.8)	-1.5 (-3.79 to 0.78)	0.183
Protein, plant (gram)	29.5 (9.4)	43.4 (9.3)	-13.8 (-20.14 to -7.54)	≤ 0.001
Protein, animal (gram)	48.8 (19.5)	34.2 (8.0)	14.6 (4.18 to 25.09)	0.009
Fat total (gram)	79.5 (33.4)	61.3 (15.0)	17.9 (1.52 to 34.21)	0.034
Fat total (En%)	38.7 (6.8)	33.4 (5.1)	5.2 (1.72 to 8.58)	0.006
Saturated fat (gram)	24.9 (11.7)	13.7 (3.8)	11.0 (5.43 to 16.59)	≤ 0.001
MUFA's	32.0 (14.8)	28.1 (7.1)	4.1 (-2.94 to 11.13)	0.236
PUFA's	15.8 (8.0)	14.4 (4.4)	1.1 (-3.26 to 5.41)	0.609
Carbohydrates total (gram)	169.0 (44.5)	164.4 (20.6)	4.3 (-18.52 to 27.13)	0.696
Carbohydrates total (En%)	39.8 (7.4)	42.0 (3.5)	-1.9 (-4.75 to 0.86)	0.162
Dietary fiber total (gram)	21.4 (6.8)	34.9 (6.1)	-13.2 (-17.02 to -9.46)	≤ 0.001
Calcium (mg)	601.7 (257.5)	899.5 (223.8)	-322.1 (-473.21 to -171.03)	≤ 0.001
Iron total (mg)	10.1 (3.1)	12.6 (3.1)	-2.4 (-4.18 to -0.67)	0.010
Iron, haem (mg)	1.2 (1.2)	0.1 (0.2)	1.1 (0.51 to -1.98)	≤ 0.001
Iron, non-haem (mg)	8.9 (2.6)	12.5 (2.7)	-3.5 (-5.09 to -1.98)	≤ 0.001
Vitamin E (mg)	13.6 (6.2)	14.3 (3.4)	-1.0 (-4.14 to 2.07)	0.492
Vitamin C (mg)	68.9 (34.8)	130.3 (44.7)	-62.5 (-88.96 to -35.97)	≤ 0.001

^a Mean score of the three 24h-recalls during the AIDEN-intervention period. The intervention period is a combination of three 24h-recalls.

^b Paired Samples t-test.

MUFA = monounsaturated fatty acid

PUFA = polyunsaturated fatty acid

Supplementary Table 5. Sleep quality (PSQI), physical activity (IPAQ-SF) and BMI (kg/m²)

	Baseline, median (IQR)	Baseline, median (IQR)	P-value (two-sided) ^a
PSQI (0 - 21)			
Total score	6.5 (4.25)	4.0 (2.75)	0.033
IPAQ-SF (0 - 10)			
MET-min/week	3298.5 (3133.3)	3093.0 (2426.3)	0.134
BMI			
kg/m ²	23.0 (2.8)	21.9 (3.7)	0.053

^aWilcoxon Signed-ranks test, asymp. Sig (2-tailed)

PSQI = Pittsburgh Sleep Quality Index

IPAQ-SF = International Physical Activity Questionnaire Short Form

BMI = Body Mass Index

Supplementary Table 6. Kendall's tau-b significant correlations

	Kendall's tau-b	Sig. 2-tailed	CI-95%
Significant correlations nutrients, foodgroups, pain and HRQoL			
FFQ nuts and Pain NRS average	0.664	0.002	0.388 to 0.831
FFQ nuts and Pain NRS maximum	0.624	0.002	0.329 to 0.809
FFQ nuts and SF-36 PCS	-0.418	0.028	-0.675 to -0.071
FFQ nuts and SF-36 MCS	-0.400	0.035	-0.663 to -0.050
FFQ dairy and Pain NRS maximum	0.544	0.007	0.215 to 0.762
FFQ dairy and EHP-30 Total score	0.383	0.038	0.030 to 0.652
FFQ sugary beverages and Pain NRS maximum	0.439	0.035	0.080 to 0.697
Dietary fiber total (gram) and Pain NRS average	0.505	0.013	0.164 to 0.738
Dietary fiber total (gram) and Pain NRS maximum	0.423	0.035	0.060 to 0.687
Iron total (mg) and Pain NRS average	0.455	0.026	0.100 to 0.707
Iron. haem (mg) and Pain NRS maximum	-0.460	0.023	-0.710 to -0.106
Iron. non-haem (mg) and Pain NRS average	0.589	0.004	0.278 to 0.788
Vegetables and Pain NRS average	0.463	0.023	0.110 to 0.712
Significant correlations EHP-30 Total and subdomain			
Eetscore-FFQ Total and EHP-30 pain	0.367	0.048	0.011 to 0.640
Dietary fiber total (gram) and EHP-30 pain	0.450	0.015	0.110 to 0.696
Dietary fiber total (gram) and EHP-30 emotional wellbeing	0.417	0.024	0.070 to 0.674
Pain NRS average and EHP-30 Total score	0.421	0.039	0.058 to 0.686

Supplementary Table 6. Continued

	Kendall's tau-b	Sig. 2-tailed	CI-95%
Pain NRS average and EHP-30 Control & powerlessness	0.412	0.044	0.048 to 0.680
Pain NRS maximum and EHP-30 Total score	0.483	0.016	0.136 to 0.725
Pain NRS maximum and EHP-30 Control & powerlessness	0.576	0.004	0.260 to 0.781
Pain NRS maximum and EHP-30 Emotional wellbeing	0.423	0.035	0.060 to 0.687
Pain NRS maximum and EHP-30 Social support	0.406	0.048	0.041 to 0.676

Supplementary Table 7. Relative abundance (mean) of gut microbiome

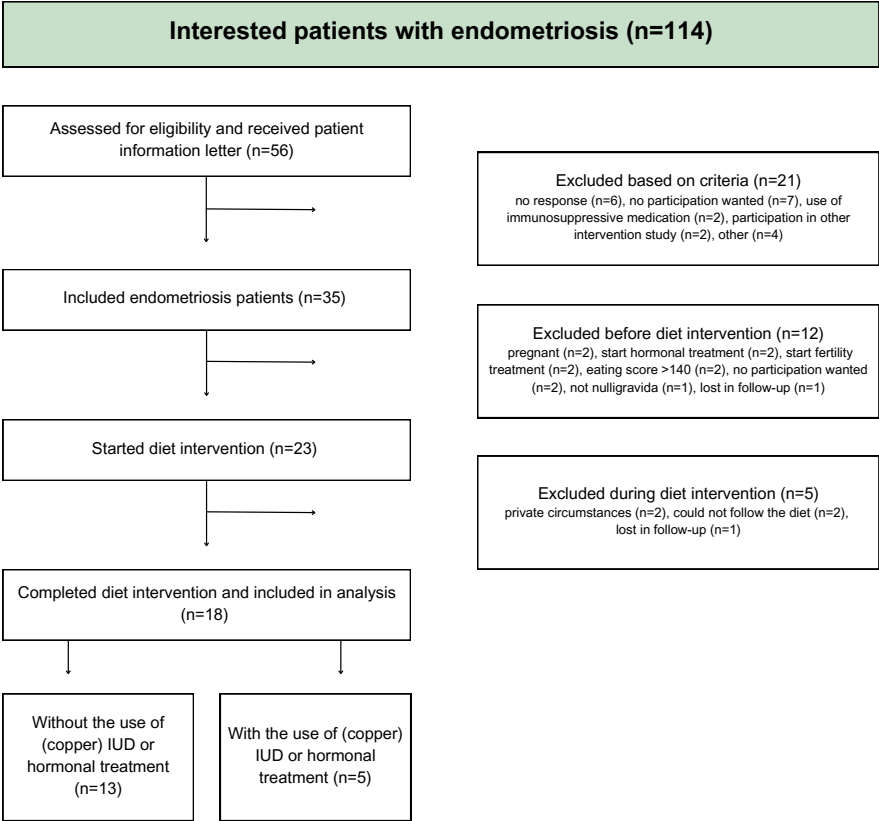
	T0, mean	T1, mean
g__Agathobacter	0,0651	0,0519
g__Anaerostipes	0,0193	0,0179
g__Bacteroides	0,0785	0,0832
g__Bifidobacterium	0,0397	0,0755
g__Blautia	0,0897	0,0976
g__Christensenellaceae_R-7_group	0,0301	0,0232
g__Coprococcus	0,0141	0,0190
g__Dorea	0,0151	0,0183
g__Faecalibacterium	0,0895	0,0877
g__Fusicatenibacter	0,0245	0,0159
g__Prevotella_9	0,0284	0,0527
g__Roseburia	0,0217	0,0339
g__Ruminococcus	0,0657	0,0576
g__Subdoligranulum	0,0373	0,0586
g__UCG-002	0,0207	0,0141
Others	0,3607	0,2930

Supplementary Table 8. Relative abundance (mean) of vaginal microbiome

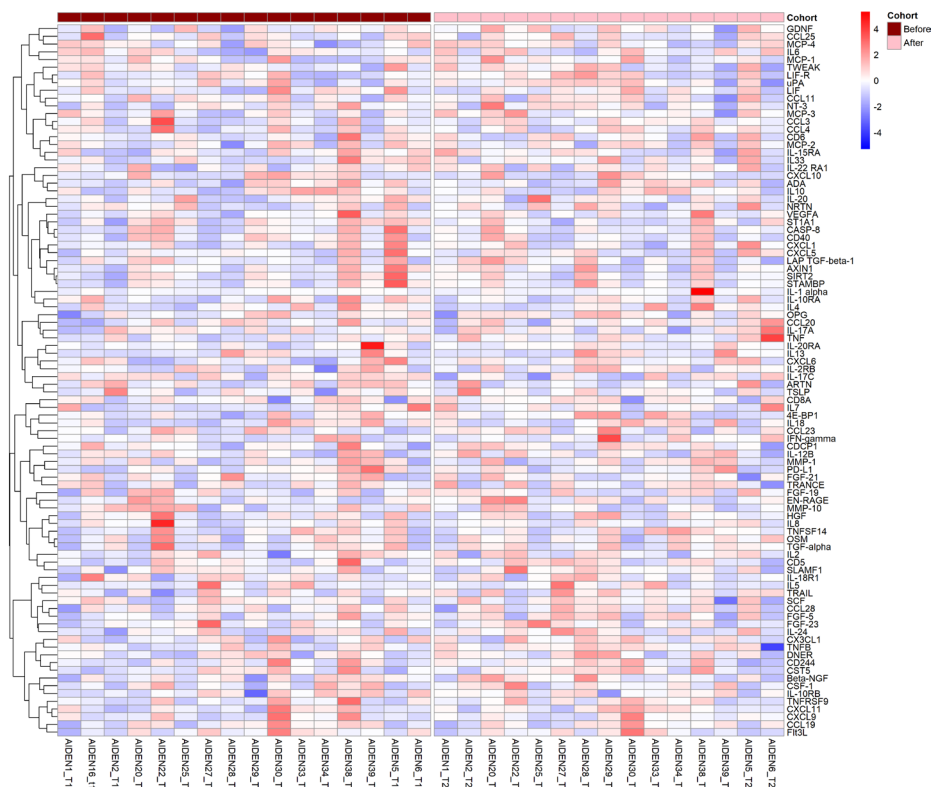
	T0, mean	T1, mean
g__Aerococcus	0,0160	0,0014
g__Anaerococcus	0,0017	0,0062
g__Atopobium	0,0034	0,0031
g__Bacteroides	0,0026	0,0022
g__Corynebacterium	0,0032	0,0009

Supplementary Table 8. Continued

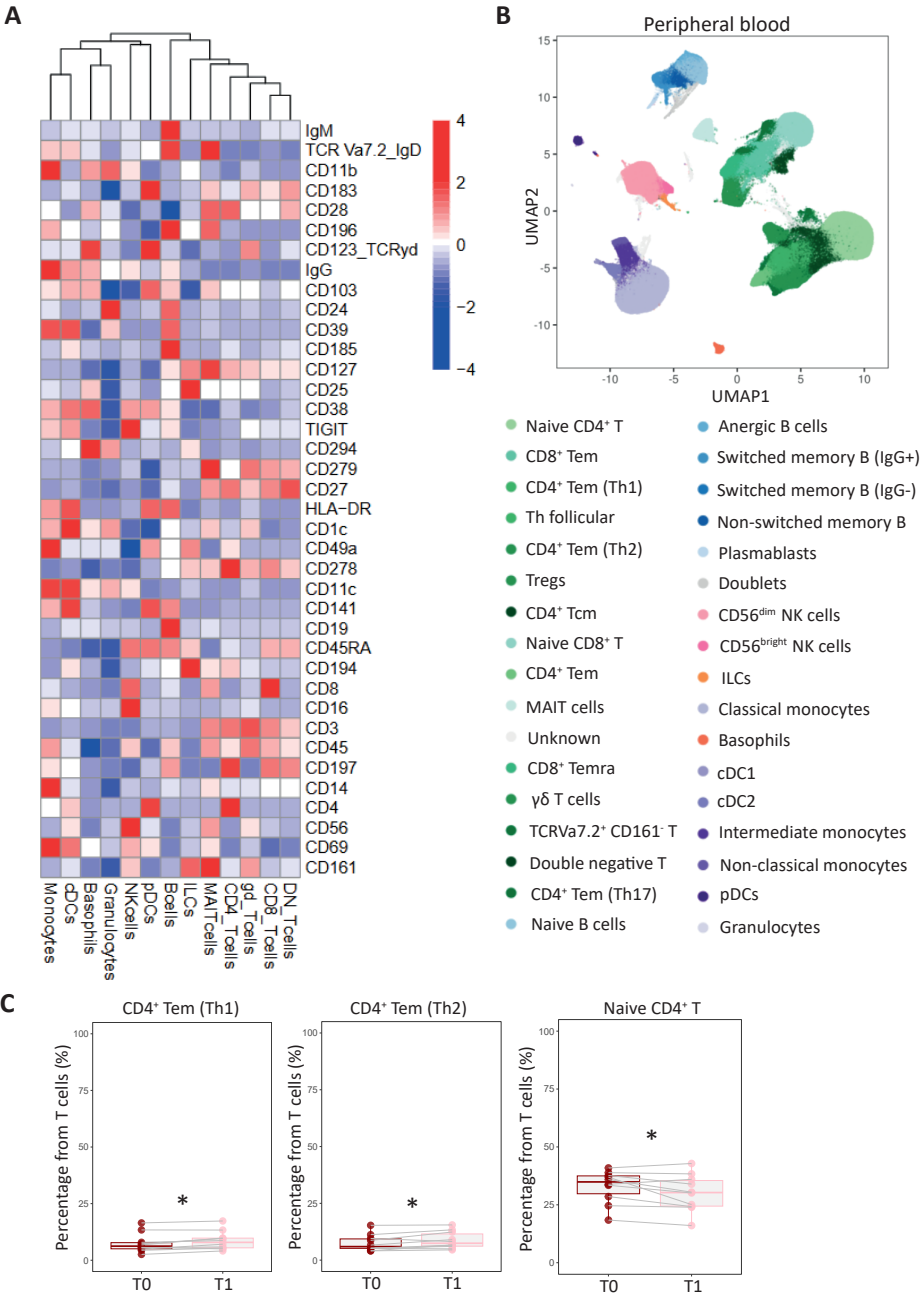
	T0, mean	T1, mean
g__Dialister	0,0112	0,0094
g__Fastidiosipila	0,0001	0,0021
g__Finegoldia	0,0012	0,0019
g__Gardnerella	0,0466	0,0142
g__Lactobacillus	0,8319	0,8477
g__Megasphaera	0,0369	0,0347
g__Prevotella	0,0204	0,0331
g__Sneathia	0,0040	0,0003
g__Streptococcus	0,0001	0,0256
g__Veillonella	0,0001	0,0013
Others	0,0200	0,0160



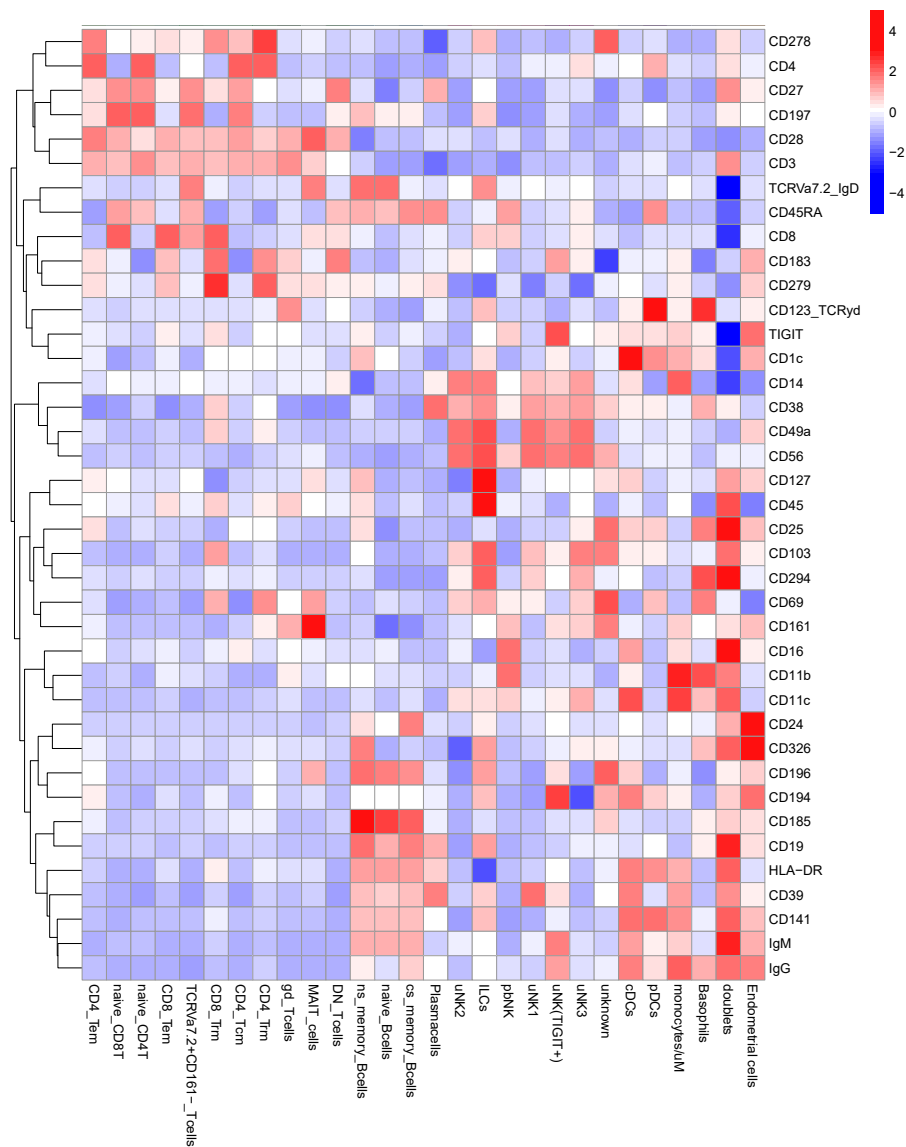
Supplementary Figure 1. Flow chart of the inclusion and exclusion criteria. IUD: Intrauterine Device.



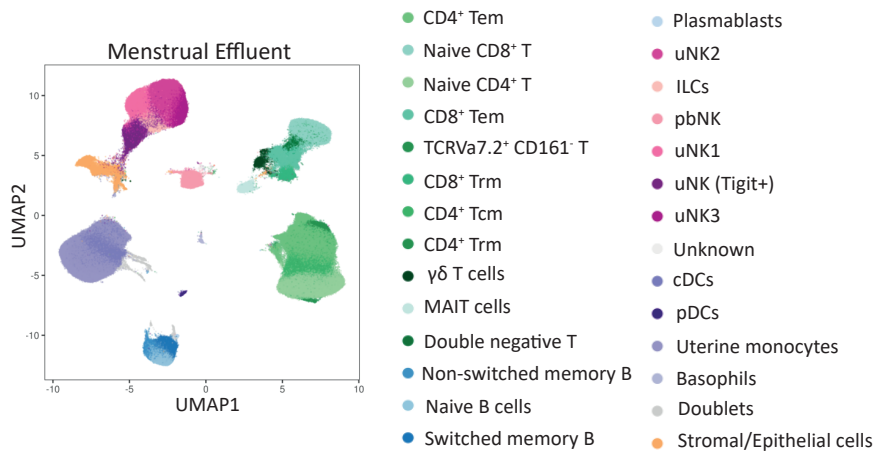
Supplementary Figure 2. Heatmap showing row-scaled NPX values of inflammatory proteins measured before (T0) and after (T1) the AIDEN-intervention (n=16).



Supplementary Figure 3. Systemic immune cell subsets before and after AIDEN-intervention. A) Heatmap representing scaled mean expression of markers in peripheral blood. B) UMAP representing 32 identified immune cell clusters. C) Boxplots representing percentages of Th1, Th2, and Naive CD4⁺ T cells at T0 (dark red) and T1 (pink). Wilcoxon signed-rank test. * $p \leq 0.05$; ** $p \leq 0.01$



Supplementary Figure 4. Heatmap representing scaled mean expression of immune cell markers in menstrual effluent.



Supplementary Figure 5. UMAP representing 28 identified clusters in menstrual effluent.



CHAPTER 8

Discussion

Throughout history, women's reproductive health has often been overlooked in both scientific research and medical practice. Endometriosis and recurrent pregnancy loss (RPL) affect millions of women worldwide, yet they remain poorly understood and insufficiently addressed. Dysregulation of the immune system is thought to play a key role in the pathophysiology of these reproductive health disorders. Understanding the mechanisms by which immune cells contribute to disease development is therefore essential for identifying new therapeutic opportunities. However, the limited knowledge available has hindered the development of effective treatment strategies. This gap highlights the urgent need for innovative approaches that not only target disease mechanisms but also empower women to manage their own symptoms. In this thesis, we describe the immunological mechanisms that may contribute to endometriosis and RPL and outline lifestyle interventions that could modulate the immune system. These interventions represent a novel, cost-effective, and low-risk strategy that supports women to take a more active role in managing their health.

Distinct phenotypes and functional profiles of uterine versus systemic immune cells

It is well established that the function and phenotype of immune cells within mucosal tissues, like the uterus, including the endometrium and decidua, differ significantly from those of the systemic compartment, peripheral blood (PB) immune cells (1). As observed in **Chapters 5** and **7**, the immune response to aerobic exercise and diet interventions also varies between these compartments, underscoring the importance of distinguishing systemic from local uterine immune system. In this thesis, menstrual blood (MB) was employed as a non-invasive source to access uterine immune cells; thus our discussion is mainly restricted to the non-pregnant uterus (endometrium). During menstruation, endometrial immune cells are shed into MB, making it a valuable resource for studying uterine immune cells, and several studies have shown that the immune cell profile of MB closely resembles that of endometrial biopsies (2, 3).

In **Chapter 3**, the most striking differences between MB and PB immune cells were observed in the NK cell population of both healthy controls and RPL patients. Uterine NK (uNK) cells, the predominant immune cell population in the uterus, display a more immunoregulatory phenotype characterized by reduced cytotoxicity and secretion of cytokines that promote trophoblast invasion and vascular remodeling (4-6). Compared to PB, the proportion of NK cells in the uterus is markedly higher (1), and uNK cells express tissue-residency markers such as CD69 and CD103, which are largely absent in peripheral NK (pNK) cells (7). They also exhibit increased expression

of inhibitory and activation markers including KIRs, LILRB1, Ki-67, Nkp30, and NKG2A, alongside reduced expression of activating receptors such as NKG2D, CD161, and the transcription factor T-bet (8, 9). By contrast, pNK cells are typically characterized by lower NKG2A expression and higher levels of CD57, CD16, and DNAM-1 (10, 11). These phenotypic and functional differences between pNK and uNK cells raise the question of whether they reflect distinct ontogenies or whether the uterine microenvironment reprograms circulating NK cells into a specialized uNK phenotype upon entrance into the uterine tissue.

The developmental origin of uNK cells remains a subject of debate, with several non-mutually exclusive hypotheses proposed. One possibility is that they arise from circulating CD34⁺ hematopoietic progenitor cells (HPCs) or NK-lineage committed precursors that migrate to the uterine mucosa and differentiate locally. Supporting this view, *in vivo* bone marrow transfer studies have demonstrated that HPC-derived NK cells can populate the endometrium and decidua (12, 13). In parallel, NK cell precursors have been identified in PB and have shown to differentiate into mature NK cells in the presence of IL-15, indicating that local cytokine signals can drive their maturation within the uterine microenvironment (14). Another hypothesis is that pNK cells can differentiate into uNK cells when exposed to the uterine microenvironment. Indeed, experimental exposure of pNK cells to TGF- β 1, possibly produced by decidual stromal cells, induces a uNK-like phenotype, suggesting that circulating NK cells can adapt functionally and phenotypically to the uterine milieu (15). There is evidence that uNK populations can be replenished from the circulation, pointing to a dynamic interplay between systemic recruitment and local differentiation (16). Together, these findings indicate that uNK cells may not be derived from a single origin but instead reflect a composite pool shaped by both progenitor recruitment and local reprogramming signals.

Beyond NK cells, T cells are also an important constituent of the uterine immune system. Relative to PB, the overall frequency of T cells is lower in the endometrium (1). Endometrial T cells predominantly display a tissue-resident phenotype (CD69⁺, CD103⁺, CD62L⁻), whereas circulating T cells are typically CD69⁻, CD103⁻, and CD62L⁺ (17). Unlike PB, where CD4⁺ T cells predominate, the endometrium is enriched in CD8⁺ T cells, resulting in a lower CD4⁺:CD8⁺ ratio and reflecting a distinct tissue-resident immune landscape (18). However, the CD4⁺:CD8⁺ T-cell ratio in MB more closely resembles blood, reflecting its mixed composition of circulating and endometrial immune cells (2). In addition, there are more Th1 and less Th2 type T cells in the endometrium compared to blood (1). The frequency of regulatory T (Treg) cells is generally comparable between blood and the endometrium (1).

Together, these observations highlight that uterine immune system is not merely a reflection of the systemic immune system but rather represents a uniquely adapted compartment.

Factors shaping uterine immune cell phenotypes and functions

Hormonal signals play a central role in shaping uterine immune system, regulating both the abundance and function of local immune cells throughout the menstrual cycle and pregnancy (19, 20). Rather than being static, uterine immune populations are highly dynamic, during pregnancy they adopt a more tolerogenic phenotype that facilitates implantation and fetal tolerance (1). This plasticity reflects cyclical fluctuations in estradiol and progesterone, which create distinct immune environments. Estradiol is generally associated with a pro-inflammatory environment, whereas progesterone promotes an anti-inflammatory state through the regulation of cytokines and chemokines (21). In particular, progesterone upregulates CX3CL1, CCL7, CCL14, and CCL4 in the mid-secretory endometrium, thereby attracting and retaining uNK cells (22). It also induces stromal cell secretion of IL-15 (23, 24), a cytokine essential for uNK proliferation and differentiation, providing a mechanistic explanation for the marked expansion of uNK cells in progesterone-rich states such as the mid-secretory phase and early pregnancy.

In line with this, our data in **Chapter 2** show that women using combined oral contraceptives (COCs) had lower frequencies of uNK cells than women not using COCs. This difference may be attributable to suppression of the menstrual cycle and the continuous presence of synthetic progesterone and estrogen. Similar findings were reported by Bister et al., who observed reduction in the percentage of uNK cells (25). This observation may not be the direct impact of the hormones because classical progesterone receptor isoforms (PR_A and PR_B) have been detected only in mature KIR⁺ pNK cells and not in uNK cells (26). However, this effect may occur indirectly mediated through reduced IL-15 production by endometrial stromal cells in response to altered progesterone levels (25). Unfortunately, in our study blood samples were not available to explore systemic effects of COC use. Nevertheless, given the presence of progesterone receptors on circulating immune cells, hormonal treatments are likely to affect systemic immune responses as well.

Beyond hormones, CMV infection induces phenotypic changes in circulating NK cells, giving rise to a subset characterized by high NKG2C expression, co-expression of CD57 and KIRs, and reduced expression of NKG2A, NCRs, and CD161 (27-29). CMV can also infect both the endometrium and decidua leading to the expansion of NKG2C⁺LILRB1⁺ uNK cells (30, 31). However, CMV induced uNK cell expansion

is not often observed in non-pregnant endometrium (25). Given the monthly regeneration of the endometrium, endometrial NK cells may not experience sufficient or sustained CMV antigen exposure, therefore, prolonged exposure such as pregnancy may be necessary to acquire a CMV-specific NKG2C⁺/LILRB1⁺ signature. Indeed, Feyaerts et al. observed an increased presence of NKG2C⁺LILRB1⁺ uNK cells in previously pregnant CMV seropositive (CMV⁺) women compared with CMV⁺ women without prior pregnancies (32). Gamliel et al. similarly observed an expansion of these cells in both the decidua and endometrium of multigravida CMV⁺ women (33). In **Chapter 3**, we did not find significant differences in the frequencies of NKG2C⁺ or NKG2C⁺LILRB1⁺ uNK cells between CMV⁺ and CMV seronegative (CMV⁻) women, consistent with previous findings (25). A key limitation of the study is that we could not stratify by prior live birth due to the small sample size; this likely limited our ability to detect effects related to previous live birth. Together with previous studies, these data support a model in which CMV infection may be insufficient on its own and prior live birth may be required to license or expand the NKG2C⁺LILRB1⁺ uNK subset.

Pregnancy has been shown to expand NKG2C⁺LILRB1⁺ uNK cells as well as uNK cells carrying KIRs specific for self-HLA-C (32-34). These changes can persist after pregnancy, potentially enhancing NK cell function in subsequent gestations (33). In **Chapter 3**, we describe a higher abundance of NKG2A⁺KIR⁺LILRB1⁺NKG2C⁻ uNK cells in both CMV⁺ women and women with secondary idiopathic RPL (isRPL), suggesting that CMV seropositivity, previous live birth or both may contribute to this phenotype. In addition, we found differences in the expression intensities of NKp44 and CD161 in uNK cells of women with primary idiopathic RPL (ipRPL) and isRPL suggesting these biologically represent two distinct sub-groups. However, due to limited sample size, we could not definitively assess the relative impact of pregnancy history and CMV infection. To address this question, future studies should investigate uNK cell phenotypes in large cohorts of women of reproductive age before and after their first pregnancy, with careful assessment of maternal CMV status. Moreover, CMV status should be systematically considered in future research on uterine immunity, given its significant influence on uNK cell phenotypes as demonstrated by our findings.

The impact of exercise intervention on systemic immune cells of women with iRPL

Since NK cells play a pivotal role in implantation, in **Chapter 3** we describe the investigation of their phenotype and abundance in women with iRPL. Both pNK and uNK cells have been implicated in iRPL, with deviations in number and function

reported across studies (35-37). A meta-analysis indicated higher pNK cell numbers in women with RPL compared to controls, although the data were heterogeneous (38), and pNK levels did not predict subsequent miscarriage (39). Findings for uNK cells are similarly inconsistent: studies using MB reported either no differences (40, 41), decreased (42), or increased uNK levels in iRPL compared to controls (3). Further analyses revealed altered uNK composition, with higher percentages of CD56^{dim}CD16⁺ and reduced CD56^{bright}CD16^{+/-} cells in iRPL. In our analyses, overall pNK and uNK cell abundance did not differ between women with iRPL and parous controls. However, phenotypic profiling revealed significantly reduced expression of 2B4 and NKp44 on uNK cells in iRPL. While 2B4 is best known as a co-stimulatory receptor on mature pNK cells (43, 44), in uNK cells it functions as an inhibitory receptor, limiting excessive activation and contributing to maternal-fetal tolerance (45). Endometrial NK cells show lower 2B4 expression than decidual NK cells (34), but its precise functional role in the uterine compartment remains unclear. Nevertheless, reduced 2B4 expression in iRPL may impair trophoblast tolerance and thereby compromise implantation success. Given these observations, there is an urgent need for minimally invasive, cost-effective approaches to monitor, modulate NK cell function and improve pregnancy outcome.

Successful pregnancy requires coordinated adaptations across maternal metabolic, cardiovascular, and the immune systems (46-49). Aberrancies in these systems, particularly during early pregnancy, can lead to pregnancy loss (41, 50-52). In **Chapter 4**, we evaluate a moderate-intensity aerobic exercise intervention (HMove) as a multi-systemic approach for targeting all three systems in women with iRPL. Exercise is well known to improve metabolic and cardiovascular health, and may also modulate the immune system. The primary aim of HMove was to determine whether moderate-intensity aerobic exercise could influence pNK and uNK phenotypes in women with iRPL. A key strength of HMove is the comprehensive, paired dataset collected before and after the intervention, encompassing gynecological/clinical history, fitness measures, metabolic and cardiovascular indices, and immune profiling. Integrating these data may enable identification of patient profiles to predict responsiveness to an exercise-based intervention. The main limitation was recruitment, which specifically affected the timeline control (iRPL without exercise). The small size of this group limited our ability to disentangle exercise effects from variation between sampling points. Future studies should design randomized controlled trial (RCT) with time-matched iRPL control and healthy control group to be able to find exercise driven changes.

Multiple studies have shown that physical exercise can modulate the systemic immune system, influencing the distribution, phenotype, and activity of different

immune cell subsets (53-55). NK cells appear particularly responsive, they mobilize into the circulation immediately after exercise (56, 57), with mobilization favoring more mature NKG2A⁻KIR⁺ NK cells over the less mature NKG2A⁺KIR⁻ subset in peripheral blood (58). Once in the bloodstream, NK cells are thought to redistribute to peripheral tissues. However, the effects of exercise on tissue-resident immune cells, such as those in the uterine environment, remain largely unexplored (59). The intensity and duration of exercise may also influence mobilization and function of NK cells. Acute higher-intensity exercise (≥ 70 –80 % VO_2max) led to a more pronounced mobilization of NK cells into the circulation and greater increases in NK-cell cytotoxic activity compared with moderate exercise (50–65 % VO_2max) (60, 61). By contrast, resting NK cell function did not differ after 12 weeks of either high- or moderate-intensity training (62). In **Chapter 5**, we show that a three-month moderate-intensity aerobic exercise intervention did not alter the abundance of NK cells but did reduce the expression intensity of NKP30, NKG2A, and CD161 in pNK cells. We also observed decreased IFN- γ production in pNK cells, while uNK cells remained unaffected. These results suggest that chronic moderate exercise dampens the cytotoxic and inflammatory potential of pNK cells, whereas uNK cells show limited responsiveness over the timeframe studied, an important consideration given their unique tissue-resident characteristics. pNK and uNK cells already display distinct sensitivity to environmental cues, and uterine immune cells are replenished each menstrual cycle and shed during menstruation. Consequently, longer interventions may be required before measurable changes in uNK cells become apparent.

Our cohort consisted of a heterogeneous population, including women with very low VO_2max suggesting a sedentary lifestyle and those with medium to high VO_2max . The HMove intervention significantly improved VO_2max levels, particularly in participants with low baseline VO_2max , while showing little effect in others. Furthermore, at the 1-year follow-up, 12 of the 26 women, who completed the intervention, had miscarriages, while the remaining participants had either successful or ongoing pregnancies. This suggests that our exercise program may be beneficial for a subset of women. Future studies should stratify participants by active versus sedentary lifestyle and correlate their immune profiles with these lifestyle categories. This approach could help identify lifestyle patterns associated with a more favorable immune profile that may support reproductive success.

Immune modulation of endometriosis through an anti-inflammatory diet

Pain is the primary symptom of endometriosis, severely limiting quality of life and social well-being. Conventional hormonal and surgical therapies often fail to

provide lasting relief, prompting many patients to adopt self-management strategies, with dietary modifications being a common approach. While such interventions are believed to alleviate symptoms, robust scientific evidence remains limited. To address this, we investigated the impact of a three-month anti-inflammatory diet (AIDEN) intervention on symptoms, immune responses, and the microbiota of women with endometriosis, as described in **Chapter 7**. We observed that AIDEN intervention reduced pain symptoms, although its impact on immune parameters and microbiota were relatively modest.

Interestingly, systemic and uterine immune cells responded differently to the intervention. MAIT cells increased in peripheral blood but decreased in the uterus. MAIT cells, which recognize microbial-derived intermediates, play a key role in the defense against bacterial, fungal, and viral pathogens. Their relevance in the immunopathogenesis of endometriosis has been highlighted by others, reporting elevated MAIT frequencies in the peritoneal fluid of affected women compared to healthy controls (63). Furthermore, microbial imbalance, or dysbiosis, particularly in the gut (64) and at other mucosal surfaces, can alter MAIT cell frequency and function both locally and systemically (65). MAIT cells are consistently present in the endometrium, with their frequency remaining stable across menstrual cycle phases and independent of sex hormones. Interestingly, it has been suggested that endometrial MAIT cells constitute a transient tissue-resident population that can be replenished from circulation (66). To further explore this biology in the context of dietary modulation, we analyzed both gut and vaginal microbiota before and after the AIDEN intervention. Unfortunately, we did not find any significant changes in genus-level composition of both gut and vaginal microbiome. Even though the composition of microbiome did not change, microbial metabolites such as riboflavin-pathway derived MR1 ligands and short-chain fatty acids could shift and influence immune cells (65). Future studies with larger cohorts should incorporate targeted metabolomics and pathway-level profiling (e.g., riboflavin biosynthesis genes) to resolve microbiome-immune cell interaction to determine whether microbial-immune modulation can be harnessed to develop targeted, evidence-based dietary therapies for endometriosis.

While dietary interventions such as AIDEN are low-risk adjunct strategies for symptom relief, they do not eradicate endometriosis. Therefore, there is a need for new pharmacological targets to resolve inflammation and immune cell dysfunction that drive disease onset and progression. Current pharmacological options for symptom management are largely limited to hormonal therapies, analgesics and non-steroidal anti-inflammatory drugs (NSAID) (67). Unfortunately, these

treatments do not alleviate pain for all individuals and symptoms frequently recur after cessation.

In **Chapter 6**, we examined the role of Janus kinase/signal transducer and activator of transcription (JAK–STAT) signaling pathway as a potential therapeutic target. The JAK–STAT signaling pathway is activated by hormones, cytokines and growth factors (68) and is implicated in malignancies and autoimmune disorders (69–71). In our cohort, individuals with endometriosis had significantly higher levels of inflammatory proteins such as IL-6, and stimulation with inflammatory cytokines induced significantly increased levels of phosphorylated STAT3 (p-STAT3) in T cells from individuals with endometriosis compared to healthy controls. Notably, elevated p-STAT3 has also been detected in the eutopic endometrium of adenomyosis patients across both proliferative and secretory phases (72).

Preclinical studies using either human tissues or mouse models of endometriosis have explored the targeted inhibition of STAT3, including tofacitinib (JAK inhibitor) (73), Stattic (74), WP1066 (JAK2/STAT3 inhibitor) (75), Niclosamide (76), and Cryptotanshinone (77) and generally report reduced lesion growth. Consistent with these findings, short-term treatment in our cohort with the selective JAK1 inhibitor, Upadacitinib, reduced STAT3 phosphorylation to baseline. However, we need further evidence to determine how these treatments modulate inflammation, and immune function, and whether they translate into clinical benefit.

Finally, we investigated whether increased p-STAT3 influences T cell lineage commitment by analyzing FoxP3 and RorγT expression. Although p-STAT3 positively correlated with both markers, the data did not conclusively indicate a shift toward either the Treg or Th17 lineage. Overall, these findings support the JAK–STAT pathway as a promising therapeutic avenue in endometriosis, while underscoring the need for mechanistic studies and clinical trials to clarify efficacy, safety, and to identify which patient sub-group may benefit most.

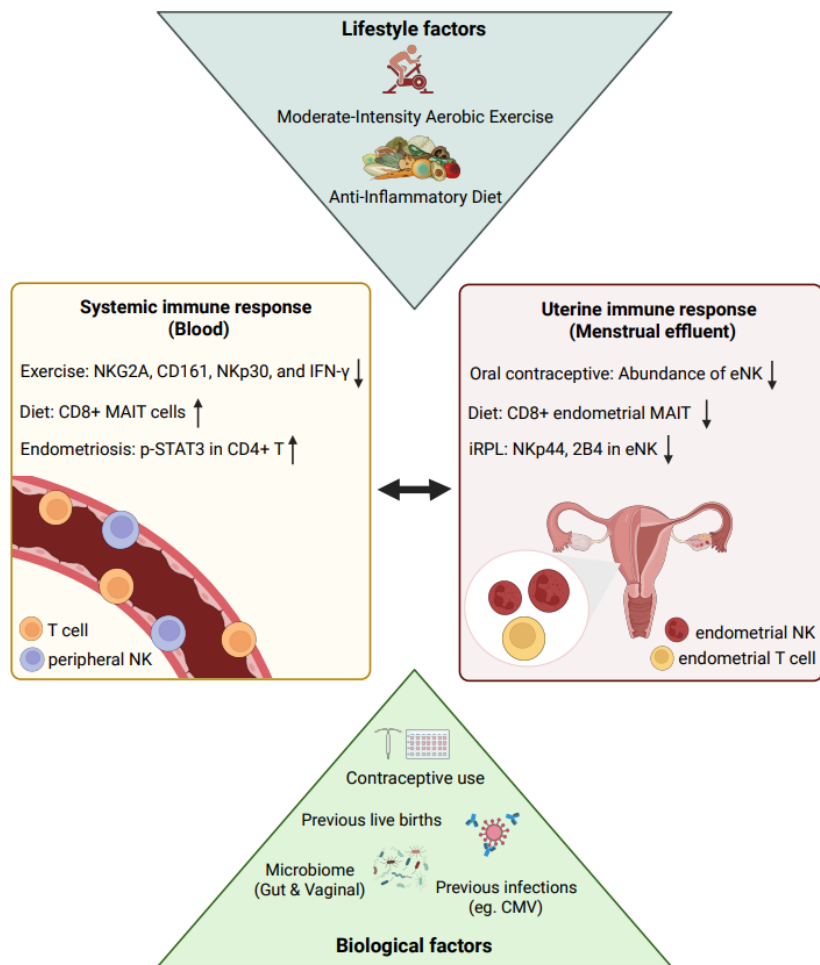


Figure 1. Lifestyle and biological drivers of systemic and uterine immune system. Lifestyle factors such as moderate-intensity aerobic exercise and an anti-inflammatory diet are managed by individuals and can differentially shape systemic (blood) and uterine (menstrual effluent) immune responses. Biological factors including previous infections (e.g., CMV), prior live births, contraceptive use, and the gut/vaginal microbiome set the baseline immune environment. Together, these factors contribute to individual variation in immune profiles relevant to reproductive health.

Concluding remarks and future perspectives

Despite years of research, curative treatments for reproductive health disorders such as endometriosis and RPL remain elusive, an important contrast to advances in other areas of medicine. Current treatment strategies are mainly focused on treating symptoms rather than addressing underlying causes. For example, women with endometriosis are often prescribed contraceptive pills to manage pain rather

than the disease itself. Diagnosis is frequently delayed, prolonging suffering and reduced quality of life. Similarly, RPL is often approached through trial-and-error therapies, with many women receiving similar therapies such as aspirin, antibiotics, or anti-inflammatory drugs regardless of the underlying mechanisms. This “one-size-fits-all” approach exposes patients to unnecessary treatments and frustration.

The findings of this thesis demonstrate that immune dysregulation plays a key role in the pathogenesis of reproductive health disorders and that lifestyle interventions, such as exercise and diet, can modulate immune responses. These interventions have the potential to serve as safe, accessible, and cost-effective adjunct therapies. Moreover, empowering women to take an active role in managing their health may improve not only physical symptoms but also mental well-being. As Aristotle once said: *“We are what we repeatedly do. Excellence, then, is not an act, but a habit.”* While building sustainable, healthy habits may not cure the disease, it can significantly improve quality of life, a meaningful and attainable goal.

Reproductive health disorders are inherently heterogeneous and complex, involving multiple biological systems. They should not be viewed as single diseases but as umbrella terms encompassing diverse subtypes with distinct underlying causes. Consequently, no single treatment is likely to be effective for all patients. Current research often focuses on isolated factors, such as hormones, genetics, the immune system, or the microbiome, without integrating them into a comprehensive framework. Advancing the field will require the development of integrated patient profiles that combine clinical and biological data including microbiome composition, hormonal status, metabolic markers, and gynecological history. Reliable diagnostic and prognostic tools are essential to classify patients and tailor treatments to their individual needs.

Looking ahead, addressing these disorders will require a multidisciplinary approach and close collaboration across clinical and research fields. Larger RCTs are needed to validate lifestyle interventions and explore their potential in combination with pharmacological treatments, a strategy currently investigated in oncology, where lifestyle interventions complement chemotherapy or immunotherapy. Ultimately, the goal is to move toward personalized medicine, in which each patient’s unique profile guides the choice for interventions, identifies the relevant biological pathways to target, and informs the timing of preventive and therapeutic measures. This integrated approach promises to improve diagnostic accuracy, reduce delays in effective treatment, and ultimately enhance the overall quality of life for women affected by these conditions.

References

1. Feyaerts D, Benner M, van Cranenbroek B, van der Heijden OWH, Joosten I, van der Molen RG. Human uterine lymphocytes acquire a more experienced and tolerogenic phenotype during pregnancy. *Sci Rep*. 2017;7(1):2884.
2. van der Molen RG, Schutten JH, van Cranenbroek B, ter Meer M, Donckers J, Scholten RR, et al. Menstrual blood closely resembles the uterine immune micro-environment and is clearly distinct from peripheral blood. *Hum Reprod*. 2014;29(2):303-14.
3. Vomstein K, Egerup P, Kolte AM, Behrendt-Moller I, Boje AD, Bertelsen ML, et al. Biopsy-free profiling of the uterine immune system in patients with recurrent pregnancy loss and unexplained infertility. *Reprod Biomed Online*. 2023;47(2):103207.
4. Trundley A, Moffett A. Human uterine leukocytes and pregnancy. *Tissue Antigens*. 2004;63(1):1-12.
5. Hanna J, Goldman-Wohl D, Hamani Y, Avraham I, Greenfield C, Natanson-Yaron S, et al. Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. *Nat Med*. 2006;12(9):1065-74.
6. Lash GE, Schiessl B, Kirkley M, Innes BA, Cooper A, Searle RF, et al. Expression of angiogenic growth factors by uterine natural killer cells during early pregnancy. *J Leukoc Biol*. 2006;80(3):572-80.
7. Garmendia JV, De Sanctis JB. A Brief Analysis of Tissue-Resident NK Cells in Pregnancy and Endometrial Diseases: The Importance of Pharmacologic Modulation. *Immuno-Basel*. 2021;1(3):174-93.
8. Huhn O, Ivarsson MA, Gardner L, Hollinshead M, Stinchcombe JC, Chen P, et al. Distinctive phenotypes and functions of innate lymphoid cells in human decidua during early pregnancy. *Nat Commun*. 2020;11(1):381.
9. Wang F, Zhou Y, Fu B, Wu Y, Zhang R, Sun R, et al. Molecular signatures and transcriptional regulatory networks of human immature decidual NK and mature peripheral NK cells. *Eur J Immunol*. 2014;44(9):2771-84.
10. Björkstrom NK, Riese P, Heuts F, Andersson S, Fauriat C, Ivarsson MA, et al. Expression patterns of NKG2A, KIR, and CD57 define a process of CD56+ NK-cell differentiation uncoupled from NK-cell education. *Blood*. 2010;116(19):3853-64.
11. Lopez-Verges S, Milush JM, Pandey S, York VA, Arakawa-Hoyt J, Pircher H, et al. CD57 defines a functionally distinct population of mature NK cells in the human CD56dimCD16+ NK-cell subset. *Blood*. 2010;116(19):3865-74.
12. Taylor HS. Endometrial cells derived from donor stem cells in bone marrow transplant recipients. *JAMA*. 2004;292(1):81-5.
13. Lysiak JJ, Lala PK. In situ localization and characterization of bone marrow-derived cells in the decidua of normal murine pregnancy. *Biol Reprod*. 1992;47(4):603-13.
14. Mrozek E, Anderson P, Caligiuri MA. Role of interleukin-15 in the development of human CD56+ natural killer cells from CD34+ hematopoietic progenitor cells. *Blood*. 1996;87(7):2632-40.
15. Keskin DB, Allan DS, Rybalov B, Andzelm MM, Stern JN, Kopcow HD, et al. TGFbeta promotes conversion of CD16+ peripheral blood NK cells into CD16- NK cells with similarities to decidual NK cells. *Proc Natl Acad Sci U S A*. 2007;104(9):3378-83.
16. Strunz B, Bister J, Jonsson H, Filipovic I, Crona-Guterstam Y, Kvedaraite E, et al. Continuous human uterine NK cell differentiation in response to endometrial regeneration and pregnancy. *Sci Immunol*. 2021;6(56).
17. Li J, Xiao C, Li C, He J. Tissue-resident immune cells: from defining characteristics to roles in diseases. *Signal Transduct Target Ther*. 2025;10(1):12.
18. Flynn L, Byrne B, Carton J, Kelehan P, O'Herlihy C, O'Farrelly C. Menstrual cycle dependent fluctuations in NK and T-lymphocyte subsets from non-pregnant human endometrium. *Am J Reprod Immunol*. 2000;43(4):209-17.

19. Jabbour HN, Kelly RW, Fraser HM, Critchley HO. Endocrine regulation of menstruation. *Endocr Rev.* 2006;27(1):17-46.
20. Mor G, Cardenas I, Abrahams V, Guller S. Inflammation and pregnancy: the role of the immune system at the implantation site. *Ann N Y Acad Sci.* 2011;1221(1):80-7.
21. Tibbetts TA, Conneely OM, O'Malley BW. Progesterone via its receptor antagonizes the pro-inflammatory activity of estrogen in the mouse uterus. *Biol Reprod.* 1999;60(5):1158-65.
22. Park DW, Yang KM. Hormonal regulation of uterine chemokines and immune cells. *Clin Exp Reprod Med.* 2011;38(4):179-85.
23. Dunn CL, Critchley HO, Kelly RW. IL-15 regulation in human endometrial stromal cells. *J Clin Endocrinol Metab.* 2002;87(4):1898-901.
24. Okada H, Nakajima T, Sanezumi M, Ikuta A, Yasuda K, Kanzaki H. Progesterone enhances interleukin-15 production in human endometrial stromal cells in vitro. *J Clin Endocrinol Metab.* 2000;85(12):4765-70.
25. Bister J, Filipovic I, Sun D, Crona-Guterstam Y, Cornillet M, Ponzetta A, et al. Tissue-specific nonheritable influences drive endometrial immune system variation. *Sci Immunol.* 2024;9(94):eadj7168.
26. Arruvito L, Giulianelli S, Flores AC, Paladino N, Barboza M, Lanari C, et al. NK cells expressing a progesterone receptor are susceptible to progesterone-induced apoptosis. *J Immunol.* 2008;180(8):5746-53.
27. Guma M, Angulo A, Vilches C, Gomez-Lozano N, Malats N, Lopez-Botet M. Imprint of human cytomegalovirus infection on the NK cell receptor repertoire. *Blood.* 2004;104(12):3664-71.
28. Lopez-Vergès S, Milush JM, Schwartz BS, Pando MJ, Jarjoura J, York VA, et al. Expansion of a unique CD57+NKG2Chigh natural killer cell subset during acute human cytomegalovirus infection. *P Natl Acad Sci USA.* 2011;108(36):14725-32.
29. Kurioka A, Klenerman P, Willberg CB. Innate-like CD8+ T-cells and NK cells: converging functions and phenotypes. *Immunology.* 2018;154(4):547-56.
30. Kowalik TF, Yurochko AD, Rinehart CA, Lee CY, Huang ES. Productive infection of human endometrial stromal cells by human cytomegalovirus. *Virology.* 1994;202(1):247-57.
31. Pereira L, Maidji E, McDonagh S, Genbacev O, Fisher S. Human cytomegalovirus transmission from the uterus to the placenta correlates with the presence of pathogenic bacteria and maternal immunity. *J Virol.* 2003;77(24):13301-14.
32. Feyaerts D, van der Meer A, Joosten I, van der Molen RG. Selective expansion and CMV-dependency in pregnancy trained human endometrial NK cells. *Cell Mol Immunol.* 2019;16(4):410-1.
33. Gamliel M, Goldman-Wohl D, Isaacson B, Gur C, Stein N, Yamin R, et al. Trained Memory of Human Uterine NK Cells Enhances Their Function in Subsequent Pregnancies. *Immunity.* 2018;48(5):951-62 e5.
34. Feyaerts D, Benner M, Comitini G, Shadmanfar W, van der Heijden OWH, Joosten I, et al. NK cell receptor profiling of endometrial and decidual NK cells reveals pregnancy-induced adaptations. *Front Immunol.* 2024;15:1353556.
35. Kwak-Kim J, Gilman-Sachs A. Clinical implication of natural killer cells and reproduction. *Am J Reprod Immunol.* 2008;59(5):388-400.
36. Quenby S, Bates M, Doig T, Brewster J, Lewis-Jones DI, Johnson PM, et al. Pre-implantation endometrial leukocytes in women with recurrent miscarriage. *Hum Reprod.* 1999;14(9):2386-91.
37. Sfakianoudis K, Rapani A, Grigoriadis S, Pantou A, Maziotis E, Kokkini G, et al. The Role of Uterine Natural Killer Cells on Recurrent Miscarriage and Recurrent Implantation Failure: From Pathophysiology to Treatment. *Biomedicines.* 2021;9(10).

38. Seshadri S, Sunkara SK. Natural killer cells in female infertility and recurrent miscarriage: a systematic review and meta-analysis. *Hum Reprod Update*. 2014;20(3):429-38.
39. Tang AW, Alfrevic Z, Turner MA, Drury JA, Small R, Quenby S. A feasibility trial of screening women with idiopathic recurrent miscarriage for high uterine natural killer cell density and randomizing to prednisolone or placebo when pregnant. *Hum Reprod*. 2013;28(7):1743-52.
40. Hosseini S, Zarnani AH, Asgarian-Omran H, Vahedian-Dargahi Z, Eshraghian MR, Akbarzadeh-Pasha Z, et al. Comparative analysis of NK cell subsets in menstrual and peripheral blood of patients with unexplained recurrent spontaneous abortion and fertile subjects. *J Reprod Immunol*. 2014;103:9-17.
41. Benner M, Feysaerts D, Lopez-Rincon A, van der Heijden OWH, van der Hoorn ML, Joosten I, et al. A combination of immune cell types identified through ensemble machine learning strategy detects altered profile in recurrent pregnancy loss: a pilot study. *F S Sci*. 2022;3(2):166-73.
42. Marron K, Harrity C. Potential utility of a non-invasive menstrual blood immunophenotype analysis in reproductive medicine. *Reprod Fertil*. 2022;3(4):255-61.
43. McNerney ME, Lee KM, Kumar V. 2B4 (CD244) is a non-MHC binding receptor with multiple functions on natural killer cells and CD8+ T cells. *Mol Immunol*. 2005;42(4):489-94.
44. Sivori S, Parolini S, Falco M, Marcenaro E, Biassoni R, Bottino C, et al. 2B4 functions as a co-receptor in human NK cell activation. *Eur J Immunol*. 2000;30(3):787-93.
45. Vacca P, Pietra G, Falco M, Romeo E, Bottino C, Bellora F, et al. Analysis of natural killer cells isolated from human decidua: Evidence that 2B4 (CD244) functions as an inhibitory receptor and blocks NK-cell function. *Blood*. 2006;108(13):4078-85.
46. Lain KY, Catalano PM. Metabolic changes in pregnancy. *Clin Obstet Gynecol*. 2007;50(4):938-48.
47. Troiano NH. Physiologic and Hemodynamic Changes During Pregnancy. *AACN Adv Crit Care*. 2018;29(3):273-83.
48. La Verde M, Torella M, Ronsini C, Riemma G, Cobellis L, Marrapodi MM, et al. The association between fetal Doppler and uterine artery blood volume flow in term pregnancies: a pilot study. *Ultraschall Med*. 2024;45(2):184-9.
49. Lee JY, Lee M, Lee SK. Role of endometrial immune cells in implantation. *Clin Exp Reprod Med*. 2011;38(3):119-25.
50. Wang LL, Liu H, Zhao SJ, Shen L, Xie T, Luo J, et al. The metabolic landscape of decidua in recurrent pregnancy loss using a global metabolomics approach. *Placenta*. 2021;112:45-53.
51. Habets DHJ, Schiffer V, Kraneburg LPA, de Krom FJW, Gurtekin I, van Bree BE, et al. Preconceptional evaluation of women with recurrent pregnancy loss: the additional value of assessing vascular and metabolic status. *BMC Pregnancy Childbirth*. 2022;22(1):75.
52. Wagner MM, Jukema JW, Hermes W, le Cessie S, de Groot CJM, Bakker JA, et al. Assessment of novel cardiovascular biomarkers in women with a history of recurrent miscarriage. *Pregnancy Hypertens*. 2018;11:129-35.
53. Chastin SFM, Abaraogu U, Bourgois JG, Dall PM, Darnborough J, Duncan E, et al. Effects of Regular Physical Activity on the Immune System, Vaccination and Risk of Community-Acquired Infectious Disease in the General Population: Systematic Review and Meta-Analysis. *Sports Med*. 2021;51(8):1673-86.
54. Gustafson MP, Wheatley-Guy CM, Rosenthal AC, Gastineau DA, Katsanis E, Johnson BD, et al. Exercise and the immune system: taking steps to improve responses to cancer immunotherapy. *J Immunother Cancer*. 2021;9(7).
55. Scheffer DDL, Latini A. Exercise-induced immune system response: Anti-inflammatory status on peripheral and central organs. *Biochim Biophys Acta Mol Basis Dis*. 2020;1866(10):165823.
56. Idorn M, Hojman P. Exercise-Dependent Regulation of NK Cells in Cancer Protection. *Trends Mol Med*. 2016;22(7):565-77.
57. Quintana-Mendias E, Rodriguez-Villalobos JM, Gastelum-Arellanez A, Cervantes N, Carrasco-Legleu CE, Espino-Solis GP. The Effect of Acute Physical Exercise on Natural Killer Cells Populations and Cytokine Levels in Healthy Women. *Sports (Basel)*. 2023;11(10).

58. Bigley AB, Rezvani K, Chew C, Sekine T, Pistillo M, Crucian B, et al. Acute exercise preferentially redeploys NK-cells with a highly-differentiated phenotype and augments cytotoxicity against lymphoma and multiple myeloma target cells. *Brain Behav Immun*. 2014;39:160-71.
59. Hanson ED, Sakkal S, Que S, Cho E, Spielmann G, Kadife E, et al. Natural killer cell mobilization and egress following acute exercise in men with prostate cancer. *Exp Physiol*. 2020;105(9):1524-39.
60. Nieman DC, Miller AR, Henson DA, Warren BJ, Gusewitch G, Johnson RL, et al. Effects of high- vs moderate-intensity exercise on natural killer cell activity. *Med Sci Sports Exerc*. 1993;25(10):1126-34.
61. Rumpf C, Proschinger S, Schenk A, Bloch W, Lampit A, Javelle F, et al. The Effect of Acute Physical Exercise on NK-Cell Cytolytic Activity: A Systematic Review and Meta-Analysis. *Sports Med*. 2021;51(3):519-30.
62. Coletta AM, Agha NH, Baker FL, Niemiro GM, Mylabathula PL, Brewster AM, et al. The impact of high-intensity interval exercise training on NK-cell function and circulating myokines for breast cancer prevention among women at high risk for breast cancer. *Breast Cancer Res Treat*. 2021;187(2):407-16.
63. Li YR, Shen X, Zhu Y, Lyu Z, Yang L. The microbiota shapes the life trajectory of mucosal-associated invariant T cells. *Trends Microbiol*. 2025;33(8):903-19.
64. El Morr Y, Furstenheim M, Mestdagh M, Franciskiewicz K, Salou M, Morvan C, et al. MAIT cells monitor intestinal dysbiosis and contribute to host protection during colitis. *Sci Immunol*. 2024;9(96):eadi8954.
65. Jabeen MF, Hinks TSC. MAIT cells and the microbiome. *Front Immunol*. 2023;14:1127588.
66. Bister J, Crona Guterstam Y, Strunz B, Dumitrescu B, Haij Bhattarai K, Ozenci V, et al. Human endometrial MAIT cells are transiently tissue resident and respond to *Neisseria gonorrhoeae*. *Mucosal Immunol*. 2021;14(2):357-65.
67. Garcia-Izquierdo L, Marin-Sanchez P, Garcia-Penarrubia P, Martinez-Esparza M. New Potential Pharmacological Options for Endometriosis Associated Pain. *Int J Mol Sci*. 2024;25(13).
68. Darnell JE, Jr. STATs and gene regulation. *Science*. 1997;277(5332):1630-5.
69. Villarino AV, Kanno Y, O'Shea JJ. Mechanisms and consequences of Jak-STAT signaling in the immune system. *Nat Immunol*. 2017;18(4):374-84.
70. Banerjee S, Biehl A, Gadina M, Hasni S, Schwartz DM. Erratum to: JAK-STAT Signaling as a Target for Inflammatory and Autoimmune Diseases: Current and Future Prospects. *Drugs*. 2017;77(11):1261.
71. O'Shea JJ, Schwartz DM, Villarino AV, Gadina M, McInnes IB, Laurence A. The JAK-STAT pathway: impact on human disease and therapeutic intervention. *Annu Rev Med*. 2015;66:311-28.
72. Hiraoka T, Hirota Y, Aikawa S, Iida R, Ishizawa C, Kaku T, et al. Constant Activation of STAT3 Contributes to the Development of Adenomyosis in Females. *Endocrinology*. 2022;163(5).
73. Kotlyar AM, Mamillapalli R, Flores VA, Taylor HS. Tofacitinib alters STAT3 signaling and leads to endometriosis lesion regression. *Mol Hum Reprod*. 2021;27(4).
74. Inui H, Kawakita T, Murayama M, Nakagawa T, Sasada H, Shinohara A, et al. Effects of STAT Inhibitors in Mouse Models of Endometriosis. *Reprod Sci*. 2023;30(8):2449-56.
75. Hu Y, Chen H, Jin L, Chi X, Zhao J, Cao Q. Hypomethylation of IL6ST promotes development of endometriosis by activating JAK2/STAT3 signaling pathway. *PLoS One*. 2025;20(1):e0317569.
76. Prather GR, MacLean JA, 2nd, Shi M, Boadu DK, Paquet M, Hayashi K. Niclosamide As a Potential Nonsteroidal Therapy for Endometriosis That Preserves Reproductive Function in an Experimental Mouse Model. *Biol Reprod*. 2016;95(4):76.
77. Xie L, Zhong Y, Chen Y, Wang Y, Xian P, Liu S, et al. Cryptotanshinone alleviates immunosuppression in endometriosis by targeting MDSCs through JAK2/STAT3 pathway. *Phytomedicine*. 2025;136:156227.



CHAPTER 9

Nederlandse Samenvatting

Reproductieve aandoeningen vormen wereldwijd een grote bedreiging voor de gezondheid en het welzijn van vrouwen. Twee veelvoorkomende voorbeelden zijn endometriose en herhaalde miskramen ('recurrent pregnancy loss', RPL). Endometriose treft ongeveer 10% van vrouwen in de vruchtbare leeftijd en gaat vaak gepaard met hevige menstruatiepijn, chronische bekkenpijn en verminderde vruchtbaarheid. RPL wordt gedefinieerd als het verlies van twee of meer zwangerschappen vóór 24 weken zwangerschap. In ongeveer de helft van de gevallen wordt geen duidelijke oorzaak gevonden, dit wordt aangeduid als idiopathische RPL (iRPL). Ondanks de hoge prevalentie en de grote impact op het leven van vrouwen, zijn er nog maar weinig effectieve behandelingen beschikbaar.

Een belangrijke overeenkomst tussen deze twee aandoeningen is de ontregeling van het immuunsysteem en de chronische ontstekingsreactie. In de baarmoeder wordt normaal een delicate balans gehandhaafd tussen enerzijds de afweer tegen ziekmakende organismen (pathogenen) en anderzijds de noodzakelijke tolerantie voor het half-lichaamsvreemde embryo. Het in stand houden van deze tolerantie is voornamelijk afhankelijk van drie typen immuuncellen: de natural killercellen (NK-cellen), T-cellen en macrofagen. Wanneer de functie of activiteit van deze cellen wordt verstoord, kan dit leiden tot een ontregeling van de immunologische omgeving in de baarmoeder, met als gevolg verminderde kans op innesteling of implantatie, miskramen en de ontwikkeling van endometriose.

De huidige behandelingen voor deze aandoeningen zijn vaak ontoereikend. Bij iRPL ontbreekt een duidelijk aangrijpingspunt voor therapie, waardoor vrouwen vaak zijn aangewezen op behandelingen waarvan de effectiviteit niet is aangetoond, zoals progesteron, aspirine, glucocorticoïden of leukocytenimmunotherapie. Voor endometriose richten de gangbare behandelingen waaronder 'non-steroidal anti-inflammatory drugs' (NSAID's), oftewel niet-steroïde ontstekingsremmers en hormonale therapieën zich voornamelijk op symptoombestrijding. Deze middelen verlichten de chronische pijn meestal slechts tijdelijk, de klachten keren vaak terug na het stoppen van de therapie en het effect bij ernstige vormen van de ziekte is beperkt. Chirurgische ingrepen kunnen de pijnklachten eveneens tijdelijk verlichten, vaak komen de klachten terug. Dit benadrukt de noodzaak voor alternatieve, meer doeltreffende behandelstrategieën.

In dit proefschrift wordt onderzocht in welke mate leefstijlinterventies, zoals regelmatige lichaamsbeweging en een ontstekingsremmend dieet, invloed hebben op het immuunsysteem en respectievelijk het krijgen van iRPL en de pijnklachten bij endometriose. Het uitgangspunt is dat leefstijl een laagdrempelige, niet-belastende

en patiëntgerichte manier kan zijn om het immuunsysteem te beïnvloeden, om daarmee zowel de kwaliteit van leven als het verloop van de zwangerschap te verbeteren.

Onderzoeksofzet en resultaten

Allereerst is onderzocht wat de invloed is van hormonale voorbehoedsmiddelen op de samenstelling van immuuncellen in de baarmoeder van gezonde vrouwen. Hieruit bleek dat het gebruik van hormonale anticonceptie inderdaad veranderingen teweeg kan brengen in deze immuuncelpopulaties, wat belangrijk is bij de interpretatie van immuun-gerelateerde gegevens in vruchtbaarheidsonderzoek.

Vervolgens is bij vrouwen met iRPL gekeken naar de expressie van verschillende receptoren op hun NK-cellen. De resultaten laten zien dat twee factoren hierbij een belangrijke rol spelen, namelijk het verloop van eerdere zwangerschappen en de aanwezigheid van antistoffen tegen cytomegalovirus in het bloed. Deze bevindingen benadrukken het belang van een zorgvuldige karakterisering van patiëntprofielen bij het interpreteren van immuunafwijkingen bij RPL.

Daarna werd een interventiestudie uitgevoerd naar de effecten van matig-intensieve duurtraining bij vrouwen met iRPL. De resultaten lieten zien dat lichaamsbeweging een verschillend effect heeft op NK-cellen in het bloed (systemisch) vergeleken met die in het baarmoederslijmvlies (uterien). Dit benadrukt dat de activiteit van het immuunsysteem in het perifere bloed niet altijd representatief is voor de lokale immuunactiviteit in de baarmoeder.

In een daaropvolgende studie werd onderzocht hoe een specifieke signaalroute tussen immuuncellen, de JAK-STAT-signaalroute, betrokken is bij ontstekingsprocessen en T-celactivatie bij vrouwen met endometriose. De bevindingen laten zien dat deze signaalroute overactief is bij endometriose, wat suggereert dat remming van deze route (bijvoorbeeld met zogeheten JAK-remmers) een mogelijk therapeutisch aangrijpingspunt zou kunnen zijn.

Ten slotte werd in een klinische studie het effect onderzocht van een twaalf weken durend ontstekingsremmend dieet bij vrouwen met endometriose. Dit dieet leidde tot een duidelijke vermindering van pijnklachten en een verbetering van de kwaliteit van leven. Daarnaast werden veranderingen waargenomen in de frequentie van specifieke, atypische T-celpopulaties in het baarmoederslijmvlies.

Ook werden effecten gevonden op het microbioom van de darm en de vagina, wat suggereert dat voeding een invloed kan hebben op zowel het systemische als het lokale immuunsysteem.

Conclusie

Dit proefschrift laat zien dat leefstijlinterventies, zowel op het gebied van lichaamsbeweging als voeding, het immuunsysteem en ontstekingsprocessen kunnen beïnvloeden bij aandoeningen die de vruchtbaarheid en zwangerschap raken. De resultaten tonen bovendien aan dat dergelijke interventies niet alleen laagdrempelig en veilig zijn, maar ook kunnen bijdragen aan een groter gevoel van eigen regie en zelfmanagement bij patiënten. Daarmee bieden ze een veelbelovend alternatief of belangrijke aanvulling op bestaande behandelingen voor vrouwen met endometriose en irPL.

Appendix

Research data management

This thesis is based on research involving human participants and existing data from published studies, all conducted in compliance with relevant national and international legislation and regulations, ethical guidelines, codes of conduct and Radboudumc policy. The privacy of the participants, involved in these studies, was protected through pseudonymization. The pseudonymization key was stored securely on a restricted-access network drive, available only authorized project members, and kept separately from the research data. Written informed consent was obtained from all participants for the collection and processing of their data for this research project.

For the study described in chapter 2, menstrual effluent samples were obtained from healthy control participants. The raw and analysed dataset has been added to the "*Investigating endometrial immune cells in contraceptive users*" collection [DOI: <https://doi.org/10.34973/gcxv-ex07>] Research Documentation Collection (RDC) in the Radboud Data Repository, with closed access. Additionally, the aggregated data from chapter 2 have been published open access in the American Journal of Reproductive Immunology (<https://doi.org/10.1111/aji.70145>). All data can be accessed using standard, widely available software.

In chapter 3, peripheral and menstrual effluent samples from healthy controls and women with recurrent pregnancy loss were analysed. All participants provided written informed consent in compliance with the Dutch Medical Research involving Human Subject Act (Commissie Mensgebonden Onderzoek Arnhem-Nijmegen, Nr 2017-3256). The raw and analysed data of the research performed in this study were added to the "*Unexplained recurrent pregnancy loss*" collection [DOI: <https://doi.org/10.34973/pwc0-j489>] of the RDC, to which access is closed.

In chapter 5, menstrual effluent and peripheral blood samples were used from women with recurrent pregnancy loss participating in the HMove study. This clinical trial was performed between 2021-2025 in collaboration with Maastricht University Medical Center. This study was conducted in accordance with the Declaration of Helsinki, the Medical Research Involving Human Subjects Act, and the guidelines for Good Clinical Practice (GCP). Approval of this study was obtained from the Dutch Central Committee on Research Involving Human Subjects (CCMO, NL77307.091.21) and the local ethics committees of the Radboudumc and Maastricht UMC+. Written informed consent was obtained from all participants. The results of this study have been published in *Frontier in Immunology* (<https://doi.org/10.3389/fimm.2025.1542111>).

org/10.3389/fimmu.2025.1602939). The corresponding dataset is available in the "*Aerobic exercise intervention in recurrent pregnancy loss*" collection [DOI: <https://doi.org/10.34973/eax7-2a18>] in RDC, with closed access.

In chapter 6 and 7, peripheral blood and menstrual blood samples from individuals with endometriosis and healthy controls were analysed. These samples were obtained as part of the AIDEN study, a prospective, exploratory feasibility study conducted at the Radboudumc between February 2023 and June 2024. Ethical approval was granted by the regional ethics committee 'METC Oost-Nederland' in the Netherlands (reference number: 2022-13650) and the study was registered at the EU Clinical Trials Register (reference number EUdRACT 2022-13492). The dataset from this study is available in the "*Anti-inflammatory diet intervention in endometriosis*" collection [DOI: <https://doi.org/10.34973/qrwt-tc04>] in RDC of the Radboud Data Repository, to which access is closed.

Data from chapters 2-7 were also stored and analyzed on the department server. Clinical data used in chapter 5, 6, and 7 were stored in Castor EDC and are only accessible by project members working at the Radboudumc. These secure storage options safeguard the availability, integrity and confidentiality of the data. Paper (hardcopy) data is stored in cabinets on the department.

PhD portfolio of Aysel Gurbanova

Department: Laboratory of Medical Immunology
PhD period: **01-10-2021/01-10-2025**
PhD Supervisor(s): **Marien de Jonge**
PhD Co-supervisor(s): **Dr. Renate van der Molen, Dr. Tess Meuleman, Dr. Lotte Wieten**

Training activities	Hours
Courses	
Radboudumc - In the lead - Radboudumc introduction for PhD candidates (2023)	12.00
Radboudumc - General Radboudumc introduction for research personnel (2023)	9.00
RU - Design and Illustration (2023)	26.00
RU - Essential skills to manage your PhD project (2023)	24.00
RU - Writing Scientific Articles (2023)	96.00
RU - Entrepreneurship and Innovation for PhD-students (2023)	84.00
Career Development for PhD candidates & PostDocs 'The next step in my career' (2025)	24.00
Radboudumc - Scientific integrity (2025)	20.00
Seminars	
Placental Biology Course (2022) - Oral presentation	40.00
Conferences	
European Society of Reproductive Immunology (2022) - Poster presentation	40.00
NVVI-2022 (2022) - Oral and poster presentation	16.00
PhD Retreat 2023 (2023) - Poster presentation	8.00
International Society of Reproductive Immunology (ISIR)-2023 (2023) - Poster presentation	40.00
Joint Belgian-Dutch Immunology Meeting (2023) - Poster presentation	16.00
European Society of Reproductive Immunology (ESRI)-2024 (2024) - Oral presentation	40.00
NVVI-2024 (2024) - Poster presentation	16.00
Dutch Nutritional Immunology Conference (2025) - Poster presentation	8.00
16th World Congress on Endometriosis (2025) - Poster presentation	40.00
Other	
Chair of the PhD Council (2024)	72.00
Teaching activities	
Supervision of Internships	
Supervision of a master student (2022)	36.00
Supervision of a master student (2023)	36.00
Supervision of a master student (2024)	36.00
Total	739.00



Acknowledgements

I think it is time to write my acknowledgements to the people who made it possible for me to complete my PhD. Writing this chapter is especially difficult because it feels like the end of an important period in my life. To be honest, I do not know what the next chapter will look like, but I do know that this one has been challenging and full of growth, opportunities, and learning.

Doing a PhD did not only improve my scientific skills; it also shaped my personal development and changed the way I see the world and myself. What once seemed like a distant dream has now become reality, and along the way I realized that what truly matters in the end is not the dream itself, but the journey and the people you meet during that journey. So here, I would like to take you back through these past years and introduce the people who made my PhD journey meaningful, fun, and memorable.

Almost five years ago, one of my friends was doing an internship at the Department of Medical Immunology at Radboudumc. She told me that there was a supervisor looking for a master's student to work together on writing a PhD proposal. I have always liked taking opportunities that come my way, so I decided to contact this supervisor, **Renate**. I still remember our first meeting in the LMI meeting room. I was immediately impressed by the topic and the project, but even more by how welcoming and enthusiastic she was. We decided to write the proposal together and went through several rounds of selection. Unfortunately, we did not receive the funding. I was disappointed, because the topic had already sparked something in me. Helping women and understanding female biology was not only intellectually interesting but also personally meaningful to me. I wanted to contribute to a field that is still not studied enough. Because of that, I decided to stay in touch with Renate in the hope of a future PhD opportunity, even though there was not enough funding at the time. During the following year, I worked as a research assistant, which is when I met my promoter, **Marien**.

Renate and **Marien** always treated me as a scientist who was responsible for her own project, while at the same time making it clear that this was a learning process and that I was here to grow. **Renate**, I deeply appreciate your enthusiasm for the field and your courage to take chances, build connections, and somehow make things work. I remember periods when obtaining funding was difficult, and I truly admire that you never gave up. You kept pursuing what you believed in and made things happen even during challenging times. I also remember you driving me to

conferences, introducing me to new people and professors, and always supporting me and believing in my abilities. Both of you created a space where I could grow as an independent scientist and where my ideas were welcomed and valued. At the end of my year as a research assistant, I was almost losing hope of continuing in academia, but you managed to find funding for my PhD. I will always be grateful for the effort you put into giving me the chance to make my PhD dream a reality. **Marien**, I will always remember you walking through the corridor with a big smile on your face, greeting me and asking how my project was going. Whenever I doubted myself, you both reminded me that I was doing well and that uncertainty is simply part of the process.

During my PhD, I worked on two main projects. One of them was the HMove project, where we aimed to help women with recurrent pregnancy loss through an aerobic exercise intervention in collaboration with Maastricht UMC. I still remember our first joint meeting, where I met the rest of my supervision team. Our meetings were never only about science; after long discussions, we would often go to dinner together and celebrate our milestones and achievements. I was lucky not only to have two supervisors, but also several additional supervisors, including **Tess**, **Lotte**, **Salwan**, **Denise**, and **Marc**. I learned so much from their expertise and truly appreciate their support throughout my PhD.

Tess gave me a fresh perspective on what patients go through, and working together helped me better understand the challenges these women face. **Lotte** has a unique teaching style which is very constructive and always providing thoughtful and useful feedback. Her positive attitude encouraged me to stay optimistic about my progress and the outcomes of our research. **Salwan** and **Marc**, as gynecologists, often brought valuable ideas from the perspective of patient care, which was very eye-opening. They also helped me learn how to communicate my science in a clearer and more accessible way for a broader audience. **Denise** was always very welcoming and supportive. She helped me greatly with the clinical aspects of the study, and her dedication and work ethic were a great example for me.

And the person who carried half of the burden of this project on her shoulders was **Amber**. We were rowing the same boat, and honestly, without her help I would not have reached the port on time. Her support played a huge role in completing this project and achieving meaningful outcomes. Her attention to detail and organizational skills were the yin to my chaotic yang. We went through the same

challenges and shared the same disappointments whenever a participant dropped out. Since we started our PhDs at the same time, I had the privilege of observing her growth as a scientist. I am very proud of your growth and of the talented scientist you are becoming.

My second PhD project was AIDEN, where we conducted an anti-inflammatory diet intervention to help women with endometriosis. This project was carried out in collaboration with the Gynecology Department, and I worked closely with **Annemiek, Janneke, and Emma. Annemiek**, I really appreciate your calm and positive approach to science. It was inspiring to see how much you care about your patients and how strongly you are motivated to improve their quality of life. **Janneke**, you were always very supportive and provided thoughtful and critical feedback. It was also a lot of fun to spend time outside of work during Lowlands Science, where I got to know you as a very friendly and fun person.

Emma, I must admit that when we first met, I was not sure whether we would get along. I thought we had very different personalities. And yet, here we are, having spent three weeks traveling across Australia together from coast to coast, 24/7, and having an amazing time. That trip allowed me to see a completely different side of you, and I must say I learned a lot from you. Your ambition, work ethic, and organizational skills will always be an example for me. Exploring nature, enjoying great food together, and even thrifting in Australia are memories I will always cherish. I also loved how you would pick clothes for me because you thought I would like them. During that time we learned a lot about each other and became very close. I truly appreciate all the effort you put into the AIDEN project and the support you gave me throughout it.

I would like to acknowledge the people of **LMI** who made it such a pleasant place to work. Everyone was incredibly friendly and welcoming, and I am grateful to have met such amazing people. **Dimitry, Lilly, Jeroen, Hans Jacobs, and Evelien**, thank you for the constructive feedback during LMI meetings, as well as for the fun conversations and drinks during NVVI meetings and team-building activities. **Arnold**, thank you for your help with my NK cell research. I could always come to you with my NK cell-related questions. Your positive energy and attitude will always be remembered. **Hans Koenen** and **Ruben**, I truly appreciate your help with my phosphoflow research. You always provided new perspectives and brought different angles to my ideas that I had not considered before. **Xuehui, Lucille, Myrthe, and Bart**, thank you for always being available to answer my questions and for your willingness to help me with my research.

I would also like to thank our research technicians. Without your help, I would have had a very difficult time in the lab. **Manon, Annemijn, and Lois**, thank you for your help with sample processing and for patiently showing me countless times where every reagent was stored, even though I kept forgetting. Your work is incredibly valuable to all the PhD students in our department. **Bram**, initially I was hesitant to approach you for help, but after a few meetings I realized that you are genuinely willing to help and are also a very fun person to talk to. Your knowledge and experience with laboratory techniques and data analysis were always impressive to me. Whenever I encountered difficulties, I knew you would be able to help. **Marc**, thank you so much for all your help with technical issues. I cannot remember how many times I knocked on your door with small questions or problems. You were always helpful and positive, and you made even the most complicated technical problems seem easy to solve. Outside of work, we would often go for drinks with the whole lab, and you were always there with us. **Fred**, I truly enjoyed your positive energy in the office. You would often bring organic food as gifts and invite us to your home. I really appreciate how much you care about others, your work, and especially your dogs. **Christa**, thank you for showing me countless times how to upload samples in Labguru, even though I somehow managed to get an error every time. Even though the platform could be quite frustrating, you made it much easier to deal with. **Esther van Rijssen**, thank you for your help with my phosphoflow project and for sharing your knowledge with me. Your calm attitude always had a positive influence on me. **Esther Fasse**, thank you for regularly checking in on us to see whether we had any issues in the lab and for always being supportive. **Sandra**, walking through the main corridor and seeing you there smiling always brought a lot of positivity to my day. Thank you for arranging last-minute meetings for me despite the impossibly busy schedules of my supervisors. **Mirjam**, I truly enjoyed our conversations about breathwork and meditation. Even though we never managed to organize a team meditation activity, I have always admired your calm and positive presence. I would also like to mention **Muriel**, who no longer works at LMI. When I was a research assistant, you were immensely helpful in securing funding for my PhD. Thank you for encouraging me to grow and explore my opportunities.

During my PhD, I had the opportunity to supervise a group of students, and that experience taught me a great deal about mentorship and about setting a good example for others. I would like to thank my students **Imke, Kleopatra, Claudia, and Battice** for giving me the opportunity to improve my skills as a mentor. In the beginning, it was quite challenging because I often questioned whether I knew enough to guide a student. However, with time and practice, I realized that I could



learn just as much from the experience. I even had the opportunity to hand my first student, Imke, her master's diploma, which was a very proud moment for me. Seeing her now pursue a PhD makes me even prouder. I hope you all achieve your academic and personal goals.

For four years, I shared my office with twelve other PhD candidates who truly understood the struggles and challenges that came with doing a PhD. We all started around the same time, and during the first years we would take long breaks together and play card games, which was always a lot of fun. Towards the end of our PhDs, I could sense the growing stress in the office, as everyone became deeply invested in finishing their own research.

Anna, your sense of fashion, positivity, hard work, and discipline have always been very inspiring to me. We often talked about our experiences in Stockholm, and knowing that you will move there for your postdoc makes me very happy and proud of you. I truly see you becoming a great scientist. **Judith**, I really admire your openness and your willingness to challenge yourself by running marathons and completing the 4Daagse. At the same time, you know how to enjoy life and you have a very calming personality. Thank you for your support throughout my PhD. **Suus**, you have always inspired me to bake more. I still remember the taste of the delicious bread and pastries you made for us. I am also proud of how you push yourself to run long distances and continuously challenge yourself. **Janeri**, I always saw your work-life balance as something to aspire to. For me it sometimes seemed almost unreal that someone could manage both a successful PhD and a well-balanced life. You always encouraged me to run more, and I really enjoyed the races we did together. Bringing Luna to our activities always added extra joy. I love seeing you build such a peaceful and fulfilling life. **Geert**, I already knew you a little from my master's program, but I was not sure what to expect when we started working together. As I got to know you better, I realized what a kind heart you have. Your sense of humor is something you should always treasure. I still think you could work a little less for your own sake, but I really admire your enthusiasm and ambition for the future. I truly believe you will achieve all your goals. **Loutje**, you brought a lot of new energy to the office when you joined. I remember the party on the boat during the 4Daagse, dancing with everyone and forming a huge group with many strangers. I really hope you enjoy your PhD and make the most of it. Always keep your positive energy and great sense of humor. **Vera**, since we started our PhDs around the same time, we could easily relate to each other's struggles. Even during the most challenging times, you always managed to stay calm. I truly believe that you will successfully finish your PhD. You are very strong and deserve

the best in life. **Milou**, when you joined the lab I was already finishing up my work, so we did not have much time to get to know each other. Still, I really appreciated the effort you put into connecting with everyone in the office. And thank you for the “towel” gift—I absolutely loved the idea. **Rienke**, in the beginning it took me a while to understand your sense of humor, but over time I started to appreciate it more and more. Thank you for joining our parties even though it was not always your style. I truly hope you enjoyed them. Your kindness and willingness to help others is something I have always admired. I am very happy to see you building a life with Gui—you two are a wonderful example of love, partnership, and most importantly friendship.

Gui, I always enjoyed our banter and discussions about the most random topics. In the beginning, you sometimes got on my nerves, hahaha, but as time passed I learned how to handle our debates better. You are not only my colleague but also a very good friend. We would take the train all the way to Amsterdam to go raving together, and during those trips we would often talk about life. It was always so much fun to see you dancing and enjoying yourself. Your energy in the lab is irreplaceable. With your jokes, random facts, and simply your presence, you brought so much entertainment and fun to the office. I feel like a proud sister seeing you build a home with Rienke and grow in your career. I wish you both so much happiness and love.

Ezgi, I really appreciated your help when I first started working in the lab. Because of you, I felt much more comfortable in the beginning. I could always rely on your knowledge and your willingness to go out of your way to help me. I also truly value the deep conversations we had about our childhoods, families, dating lives, and the challenges of doing a PhD. I remember coming to the lab during weekends and seeing you working incredibly hard. Even though I sometimes thought we could have had a better work–life balance back then, it was still nice to see you there and talk about the latest person I was dating, haha. Over the past years, I think we have both matured a lot and have come to understand ourselves better. I am happy to see how we have grown. I wish we had spent more time together outside of work, but that is something we can still make happen.

Sam, mijn taalmaatje. Het is best moeilijk om dit in het Nederlands te schrijven. Maar vandaag spreek ik Nederlands dankzij jou. Ik ben begonnen mijn Nederlands te verbeteren door met jou te praten. Bedankt dat je me altijd aanmoedigt om uit mijn comfortzone te stappen. Je hebt zo’n unieke persoonlijkheid. Ik geniet echt van onze gesprekken. Je geeft me altijd een frisse en praktische kijk op dingen en

herinnert me eraan dat het leven niet zo ingewikkeld is. Ik geniet echt van onze chai latte pauzes waar jij altijd piano voor me speelde, en dat jij en Judo me uitnodigden voor een diner en zo lekker kookten. Ik ben blij dat ik je vriend mag zijn en dat jij altijd jezelf blijft.

Charbel, Jaimy, and Layla, thank you for the great time in the PhD Council. It was wonderful to step outside the day-to-day work of my PhD and organize events and activities together with you. Your energy for helping others and your enthusiasm for creating positive change for PhD candidates are truly inspiring.

Conchi, Solane, and Hatice, thank you, girls, for always being supportive and listening to me without judgment. Each of you has such a unique personality, and I learn something different from every one of you. I am truly impressed by your strength and the effort you put into making the best of your lives. I deeply appreciate having you in my life.

Leonardo and Benard, I am so glad that we built such a strong friendship through the Dutch course. I really enjoy our conversations about life, going to the sauna together, and getting burgers afterwards. It is always refreshing to hear your perspectives on life, and I often leave our meetings feeling more energized and happier. I am very happy to see both of you thriving in your relationships and careers.

Laura, girlie, I am so happy for our friendship. You have added an extra layer of joy to my social life. It is always so much fun to go out together, enjoy music, and practice yoga. I love your authentic, bubbly, and adventurous personality. We have bonded not only over our shared interests but also over our experiences and challenges during the PhD and discussions about our future careers. I truly believe that a great career and a fulfilling life are waiting for us. I hope we both keep our spark and sense of adventure in life.

Nadine, I met you towards the end of my PhD, and it felt like such a fortunate coincidence. I am so happy that you became my flatmate. You brought so much happiness, positivity, and fun into my life. Thank you for your kindness and for always reminding me to be kind to myself as well.

Begüm, biz uzun yıllardır tanışıyoruz fakat son 4–5 yılda hayat bizi gerçekten çok daha yakınlaştırdı. Bunu tanıştığım herkese söylüyorum: senden çok şey öğrendim. Seninle arkadaş olmak hayatımda birçok şeyi değiştirdi; kendimle olan ilişkimi,

diğer insanlarla olan ilişkimi ve hayata bakış açımı. Seninle birlikte hayattan zevk almayı, kendimi sevmeyi ve kendimi daha iyi anlamayı öğrenmeye başladım. Bu son yıllarda her zor anımda yanımda olduğun için sana çok teşekkür ederim. Hayatıma iyi ki girdin ve iyi ki benim arkadaşısın. Umarım ne kadar uzak yaşarsak yaşayalım arkadaşlığımız her zaman devam eder. Senin için hayatta en güzel şeyleri diliyorum, çünkü bunu gerçekten hak ediyorsun.

Aarti, we have known each other for more than six years now, and I truly feel that we have grown up together. I deeply appreciate all the effort you have put into our friendship. You and **Thijs** have become like a second family to me. Thank you for always inviting me to your home during holidays, for giving me advice about love and relationships, and for supporting me through difficult times. I will never forget the moment you came to pick me up when I was not feeling well. Our friendship and the memories we have created together mean so much to me. I often tell people that your relationship with Thijs is a true example of a healthy and loving partnership. It makes me very happy to see you building such a strong foundation for your future together. Thank you also for the beautiful artworks that made this thesis a million times better. Your creativity and kindness are truly inspiring.

Laurien, I cannot fully express in words how much gratitude and love I have for you. Your kind heart is what makes you so special. We became closer through our 4Daagse training walks, where we spent hours walking and talking about life, sharing our problems, and supporting each other. With you, I always felt seen, heard, and understood. We have so much in common, and I feel incredibly lucky to have a friend like you in my life. As I write this, so many memories come to mind. We have experienced so much together: going to raves and festivals and dancing from dawn to dusk, walking the 4Daagse where you literally dragged me through the last days so I could finish (without you, I truly would not have made it), traveling together, crying together, celebrating together, and taking long walks through the city center. My personal growth is greatly connected to your support. Whenever I needed help, you were always the first person there for me. Seeing you build a home together with **Aaron** is incredibly inspiring, and it also reminds me how much we have both grown over the years. It truly makes me happy to see you two together. You have always reminded me of who I am and what I am capable of. I admire how open you are to new experiences. You see the world from such a unique perspective and always manage to find the positive side, even on difficult days. I am so happy that you are my friend.



Validəynlərimə və **qardaşıma**, xaricdə yaşamaq və alim olmaq arzusunda mənə dəstək olduğunuz üçün təşəkkür edirəm. İlhamınız, dəstəyiniz və cəsarətiniz olmadan bu gün olduğum yerdə ola bilməzdim. **Mama**, bu günə qədər xatırlayıram ki, xaricdə yaşamaq sənin arzun idi və zamanla mənim arzum oldu. Həmişə məni xaricə getməyə təşviq edirdin. Bəzən bu arzumı gerçəkləşdirdiyim üçün özümü günahkar hiss edirəm, çünki bu, bir çox başqa şeylərdən vaz keçmək demək idi. Amma bəlkə də mənim böyüyüb müstəqil bir qadın olmağım üçün lazım olan məsafə məhz bu idi, hər vəziyyətlə başa çıxmağı bacaran. Bəzən o qız olmağı darıxıram; səhər məni oyandırdığın, üzümü yuduğun və məni şahzadə kimi geyindirdiyin zamanları. Düşünürəm ki, heç kim mənə o qədər qayğı və sevgi verə bilməz. Ümid edirəm ki, mənim sevgimi siz də hiss edirsən, baxmayaraq ki, sizdən uzaqda yaşayıram. **Papa**, sən mənim ürəyimdə xüsusi yerə maliksən. Həmişə bacarıqlarıma, gücümə və dözümlülüyümə inanmısan. Məncə, bunların hamısını səndən öyrəndim. Kaş sənənlə daha çox vaxt keçirə bilsəm və bağımızı daha da dərinləşdirə bilsəm. Sən və mama arzularımızı gerçəkləşdirmək üçün çox şeydən keçdiniz. Bunun üçün size həmişə minnətdar olacağam.

These past four years have not only been about conducting research. They have also given me a deeper perspective on what it means to be a woman and on the fascinating complexity of female biology. During my PhD, I realized how extraordinary the female body truly is. The ability to grow a new human being in the womb is, from an immunological perspective, nothing short of a marvel yet it is often perceived as something completely ordinary, even expected. I would like to acknowledge all the women who struggle with different reproductive health disorders, yet continue to live their lives, work on themselves, and try to manage their symptoms despite the limited knowledge and treatment options that are often available. The lack of awareness in this area is difficult to witness not only as a scientist, but also as a woman who hopes to build a family and have children one day. It is painful to see how female biology has been misunderstood, neglected, or even used against women throughout history. That is why I feel proud to have worked on a project with wonderful women and men who are committed to bringing more awareness to this field and advancing women's health. I sincerely hope that this work can contribute, even in a small way, to the growing efforts to better understand reproductive health and ultimately improve the lives of women everywhere.



Curriculum Vitae

Born in Baku, Azerbaijan, Aysel Gurbanova developed an early fascination with biology and human health, which led her to pursue a BSc in Molecular Biology & Genetics at Bilkent University in Turkey. During her undergraduate studies, she completed internships that provided hands-on experience in molecular research, laying the foundation for her scientific career.

She continued her education at Radboud University, completing an MSc in Biomedical Sciences, where she further developed her skills in experimental design, clinical research, and data analysis. During this time, she gained experience in laboratory techniques and contributed to collaborative research projects that deepened her interest in translational science. For her first master internship, she worked in the laboratory of Prof. Mihai Netea, investigating the training of macrophages with the IL-4 cytokine. For her second internship, she joined Prof. Niklas Björkström at the Karolinska Institute, applying 29-color high-dimensional flow cytometry to study surgical trauma-related immune signatures in patients after colorectal cancer surgery.

Following her master's, Aysel pursued a PhD in Medical Immunology at Radboudumc, focusing on immune mechanisms in reproductive health, particularly recurrent pregnancy loss and endometriosis. She designed and managed clinical studies, conducted systematic literature reviews, and authored multiple first-author publications. In parallel, she supervised MSc students and actively contributed to multidisciplinary collaborations.

Beyond her research, Aysel has demonstrated leadership and engagement within the academic community. As Chair of the Radboudumc PhD Council, she represented over 500 PhD candidates, organized career events, and fostered collaborations with industry partners. She also served on the Research & Innovation Council, contributing to institutional strategy and policy.

Aysel combined her scientific expertise with entrepreneurial thinking by participating in the Mercator Launch Venture Track, where she developed WombTrack, a concept aimed at improving women's health through innovative solutions. Fluent in English, Dutch, Turkish, and Azerbaijani, she has presented her work at international conferences, received travel grants, and earned a Cambridge CTR scholarship. She is now aiming to work with companies that focus on improving women's healthcare.





9 789465 152479 >