RECEPTOR PATTERNS AND FUNCTION OF γδT CELLS DURING HEALTHY HUMAN PREGNANCY

Doctoral (PhD) thesis

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Pécs 2024

1 Introduction

In the mid-20th century, Medawar discovered similarities between an implanted embryo and an allograft transplant. He proposed the concept of the semi-allogenic fetus, where the offspring is hidden from the maternal immune system. Scientists have been working to understand the relationship between the maternal immune system and the embryo.

The maternal immune system recognizes the embryo. Unlike organ transplants, it's beneficial if the fetus's HLA signature differs from the mother's. The placenta acts as a buffer zone to prevent the child's antigens from directly contacting the maternal tissue. The maternal immune system supports implantation, defends against pathogens, and contributes to the initiation of labor.

It's important to note that the embryonic or fetal cells expressing class I and II HLA molecules are not in direct contact with maternal tissue. The classical HLA class I molecules, namely HLA-A, HLA-B, and HLA-C, play a pivotal role in the immune system's ability to discriminate between self and foreign. However, before the blastocyst implants into the decidua, it forms an outer layer called trophoblast, which primarily does not prefer to express classical but non-classical HLA molecules. This unique characteristic of the trophoblast is a key aspect of the immune response during pregnancy.

It is often assumed that the non-classical HLA molecules protect the trophoblast from being attacked. At the same time, paternal HLA-C, which is the only classical HLA I molecule that

is expressed on the trophoblast, might help recruit and activate local immune effector cells to promote tissue transformation and support placentation.

Pregnancy is a dynamic period in which immune reactions must be tightly orchestrated. While the early decidua is dominated by innate-like immune cells, adaptive immune effectors are the most prevalent immune cell population at term. Gamma/delta T cells, with their duality, appear to be the ideal mediator in this transition. Furthermore, they are well known to surveil the tissue integrity of frontiers between the organism and the environment. Studies reported a higher prevalence (10 % to 30 %) of γδT cells among decidual $CD3^+$ cells compared to the peripheral blood.

Peripheral blood γδT cells comprise 1 - 10 % of circulating T cells and are mainly $V\delta2^+$. When circulating through the intervillous spaces of the placenta, these cells can be in direct contact with the syncytiotrophoblast (ST). However, as the ST is void of MHC molecules, interactions between γδT and fetal tissues via the classical MHC-TCR pathway cannot occur. Yet as γδT cells are not MHC-restricted, they might be capable of recognizing antigens or communicating with the trophoblast by facilitating other mechanisms.

Villous and extravillous trophoblast express CD1d, potentially allowing antigen recognition via the γδTCR. However, other signals will determine the crosstalk between γδT cells and other participants in the microenvironment of the maternal-fetal interfaces. To date, the expression of essential

receptors targeting the unique HLA-profile of the extravillous trophoblast (EVT) (e.g., KIR, NKG2A/C or LILR) or other possible signaling-pathways have not been investigated in decidual γδT cells. Understanding the expression pattern of these critical molecules would allow an insight into the interplay between decidual γδT cells, the EVT, and other immune cells.

2 Aims

To date, basic physiological processes of immunoregulation during pregnancy are far from understood. We aimed to deepen the understanding of γδT cells and their role during pregnancy.

Our study was divided into two phases:

I. *Investigation of peripheral blood γδT cells during pregnancy.*
   a. Determination of the cytotoxic potential of peripheral blood γδT cells.
   b. Analyzing the correlation between the $CD56^{+}$ phenotype and the measured cytotoxic potential.

II. *Investigation of decidual γδT cells during early human pregnancy*
   a. Studying the capability to detect the trophoblast's unique HLA expression pattern.
   b. Exploring potential functions regarding pathogen clearance and implantation.

3 Materials and Methods

Mononuclear cells were isolated from peripheral blood samples (PBMC) or decidual samples (DMC). These were either frozen and later measured by flow cytometry or used for cell (co-)culture experiments.

In the first phase of our study, we investigated peripheral blood mononuclear cells from non-pregnant and pregnant women in the first, second, and third trimester of their pregnancy via flow cytometry for the following target molecules: TCRγδ, CD3, CD4, CD8, CD56, PD-1, CD107a.

In the second phase of our study, we focused on decidual mononuclear cells while using peripheral blood mononuclear cells as controls. Both decidual tissue samples and matched peripheral blood were obtained from healthy pregnant women undertaking an elective pregnancy termination during the first trimester. Here we investigated the following markers via flow cytometry: Perforin, Vδ2, TCRγδ, NKG2C, CD56, Vδ1, CD69, NKG2A, CD45, ILT2, KIR2DL4.

In the case of cell culture experiments of the second phase, non-touched γδT cells were isolated using the 'TCRγ/δ^+ T cell Isolation Kit' (Miltenyi Biotech) according to the manufacturer's instructions. Three human choriocarcinoma cell lines (JAr) were used as model tissues: A standard JAr cell line (HLA class I^-) and the two transfectants JArE (HLA-E^+) and $JArG_{1m}$ (HLA-G^+). The freshly isolated non-touched γδT cells were incubated with these cell lines or purified soluble HLA-E or HLA-G overnight. Supernatants were analyzed for cytokine secretion (IL-2, IL-4,

IL-10, IL-6, IL-17A, TNF-α, sFas, sFasL, IFN-γ, granzyme A, granzyme B, perforin, granulysin, Angiopoietin-2, BMP-9, EGF, Endoglin, Endothelin-1, FGF-1, FGF-2, Follistatin, G-CSF, HB-EGF, HGF, IL-8, Leptin, PLGF, VEGF-A, VEGF-C and VEGF-D) using bead-based assays.

4 Results

4.1 Phase I

4.1.1 A small subset of γδT cells shows CD56 positivity

We found a small population of $CD3^+$ lymphocytes, which was double-positive for γδTCR and CD56. We determined the prevalence of $CD56^+$ cells within the γδT cell population. $CD56^+$ cells among γδT cells were rare in non-pregnant women and during the 1st trimester. However, while the rate of $CD56^+$ cells in non-γδT cells significantly declined in the 2nd and 3rd trimesters, in γδT cells, the percentage of $CD56^+$ cells spiked in the 2nd trimester. From there, the rate of $CD56^+$ cells decreased marginally in the 3rd trimester.

4.1.2 $CD56^+$ γδT cells are predominantly double-negative for CD4 and CD8

To determine if the expression of CD56 on γδT cells has an impact on the functional aspects of this cell population, we analyzed the expression of CD4 and CD8 on $CD56^+$ compared to $CD56^-$ γδT cells. Here, we found significant differences in the prevalence of double negative ($CD4^-/CD8^-$) and $CD4^+$ cells between these two investigated subsets in all groups. The prevalence of double negative cells was permanently higher in $CD56^+$ γδT cells, while the CD4+ cells showed an inverse dominance.

Among $CD56^+$ γδT cells, the prevalence of $CD4^+$ cells was significantly higher in the 1st and 3rd trimester compared to the 2nd trimester and the non-pregnant control group. The

prevalence of $CD8^+$ cells was significantly higher among $CD56^+$ compared to $CD56^-$ γδT cells in non-pregnant samples as well as in the 1st and 2nd trimester of pregnancy. Furthermore, in both γδT cell subsets, the rate of $CD8^+$ cells was significantly lower in the 1st trimester compared to the non-pregnant control.

4.1.3 $CD56^+$ γδT cells show a robust cytotoxic potential

The potential for cytotoxic activity of γδT cells was determined by the surface expression of CD107a upon activation. Although the rate of $CD107a^+$ cells was significantly higher among $CD56^+$ γδT cells compared to the $CD56^-$ subset in all groups, this rate did not alter considerably during pregnancy. However, the percentage of $CD107a^+$ cells among $CD56^-$ γδT cells was significantly higher in pregnancy compared to the non-pregnant control.

For a better classification of the cytotoxic potential, we determined the CD107a-mean fluorescence intensity (MFI) of $CD107a^+$ cells. Here, the MFI of $CD107a^+$ cells was significantly higher in the $CD56^+$ γδT subpopulation in all sampled groups. In $CD56^+$ γδT cells, the CD107a-MFI was decreased during pregnancy compared to non-pregnant control. In pregnancy, the lowest CD107a-MFI was found in the 1st trimester; from there, it increased significantly to the 3rd trimester. The MFI of $CD56^-$ γδT cells did not alter during pregnancy.

4.1.4 The rate of PD-1$^+$ cells is higher in the CD56$^+$ γδT cell subset

The inhibitory immune checkpoint molecule PD-1 is a potential regulator of cytotoxic function. To check its potential implication in the regulation of CD56$^+$ γδT cell cytotoxicity, we measured the surface expression of PD-1 on CD56$^+$ or CD56$^-$ γδT cells. Compared to CD56$^-$ γδT cells, PD1$^+$ cells were significantly more common in CD56$^+$ γδT cells at all measured time points. Within the CD56$^+$ γδT subset, the prevalence of PD-1$^+$ cells increased significantly in the first trimester and fell back to non-pregnant-level in the 2nd and 3rd trimesters. Among CD56$^-$ γδT cells, the prevalence of PD-1$^+$ cells was significantly higher in the 1st and 2nd trimesters compared to the non-pregnant control. No significant difference was detected regarding the MFI of PD-1.

4.1.5 The co-expression of PD-1 and CD107a on γδT cells correlates with their CD56 expression

For a better understanding of the impact of PD-1 within the cytotoxic γδT cells, we studied the expression of PD-1 on CD56$^+$/CD107a$^+$ and CD56$^-$/CD107a$^+$ γδT cells. Here, compared to the CD56$^-$ γδT subset, a significantly higher rate of cytotoxic CD56$^+$ γδT cells express PD-1 in the non-pregnant group and the 3rd-trimester pregnancy, whereas in the 1st trimester, we found the opposite result. The prevalence of PD-1$^+$ cells in the cytotoxic CD56$^+$ γδT subset was significantly lower in pregnancy than in the non-pregnant state. However, during pregnancy, the rate of PD-1$^+$ cells among the cytotoxic CD56$^+$ γδT cell subset did not

alter. Interestingly and in opposite to the cytotoxic $CD56^+$ γδT subset, the cytotoxic $CD56^-$ γδT subgroup showed a significant increase of PD-1^+ cells in the 1st trimester.

To determine whether PD-1 expression is related to the intensity of the cytotoxic potential, we finally analyzed the CD107a-MFI on PD-1^+ versus PD-1^- $CD56^+/CD107a^+$ and $CD56^-/CD107a^+$ γδT cells, respectively. Here, the CD107a-MFI-value of $CD56^+/CD107a^+$/PD-1^+ γδT cells was significantly higher in all groups compared with the $CD56^+/CD107a^+$/PD-1^- population. This correlation of a significantly higher CD107a-MFI was also significant for $CD56^-/CD107a^+$/PD-1^+ compared with $CD56^-/CD107a^+$/PD-1^- γδT cells. Here, among the $CD56^-/CD107a^+$/PD-1^+ cells, the CD107a-MFI was significantly lower in the 1st trimester compared to all other groups.

4.2 Phase II

4.2.1 Heterogeneity of peripheral and decidual γδT cells during early pregnancy

To characterize decidual γδT cells and compare them to their circulating counterparts, we utilized the downsampling plugin of FlowJo™ and concatenated previously gated γδT cell populations from DMCs and PBMCs. The tSNE algorithm defined separate clusters with minimal overlap assigned to decidual or peripheral blood γδT cells, respectively.

Due to the biological similarities between NK cells and γδT, we investigated the expression of CD56 on γδT cells. While

only $CD56^{dim}$ expression was detectable in peripheral blood γδT cell clusters, decidual γδT cells exhibited both $CD56^{dim}$ and $CD5^{6bright}$ phenotypes. Nevertheless, $CD56^{+}$ γδT cells are more prevalent in the decidua than in the periphery.

Classical γδT cell subsets were associated with distinct clusters. $Vδ1^{+}$ ($CD45^{+}TCRγδ^{+}Vδ1^{+}Vδ2^{-}$) cells were more prevalent in the decidua, while $Vδ2^{+}$ ($CD45^{+}TCRγδ^{+}Vδ1^{-}Vδ2^{+}$) cells were more common among circulating γδT cells. However, double-negative (DN, $CD45^{+}TCRγδ^{+}Vδ1^{-}Vδ2^{-}$) γδT cells were the most common in both decidual and peripheral blood.

4.2.2 Decidual γδT subsets express receptors that bind to HLA-E or HLA-G molecules

Using two flow cytometric panels, we investigated the prevalence and expression of HLA-E and HLA-G-binding receptors (NKG2C, NKG2A, and ILT2, KIR2DL4, respectively) on γδT cell subsets in the decidua and the matched peripheral blood. To estimate the expression intensity, we compared the median fluorescence intensity (normalized to the respective FMO) of all investigated receptors. While decidual DN γδT cells exhibited relatively high expression levels for all investigated receptors, decidual Vδ1+ cells showed a more focused expression of the activating NKG2C and the inhibiting ILT2. In contrast, decidual Vδ2+ cells expressed significantly more NKG2A on their cell surface.

The prevalence of $NKG2C^{+}$ cells was generally higher among decidual γδT cells. However, this difference reached the

significance level only in the Vδ2$^+$ and DN subsets. Furthermore, NKG2C positivity was significantly more common in the Vδ1$^+$ subset compared to the Vδ2$^+$ subset. Likewise, cells expressing the inhibitory counterpart NKG2A were more prevalent in the decidua. While the percentage of NKG2A$^+$ Vδ2$^+$ cells did not differ between decidua and peripheral blood, a significantly higher proportion of DN γδT cells expresses NKG2A and NKG2C in the decidua.

The inhibitory, HLA-G-binding ILT2 was commonly expressed by γδT cells independently of their origin. Generally, ILT2$^+$ cells were less prevalent in the Vδ2$^+$ subsets. However, when focusing on the prevalence of ILT2$^+$ cells within each γδT cell subset, significantly fewer decidual DN γδT expressed ILT2 than their peripheral blood counterpart. The HLA-G-binding KIR2DL4 was expressed by the majority of γδT cells.

4.2.3 Decidual γδT cells secrete trophoblastotropic cytokines

To determine the functional consequences of the HLA-E or HLA-G recognition by γδT cells, we incubated purified γδT cells with soluble HLA-E or -G (sHLA-E/-G). Furthermore, we utilized human choriocarcinoma cell lines (JAr) transfected with HLA-E or HLA-G1m to investigate more complex interactions of membrane-bound HLA-E or -G (mHLA-E/-G).

Vascular transformation by the trophoblast and the local immune environment is crucial to establishing a healthy placenta during early pregnancy. Therefore, we analyzed the collected cell

co-culture supernatants for potential angiogenic cytokines and growth factors. When comparing peripheral blood to decidual γδT cells without HLA molecules, we found significantly higher levels of granulocyte colony stimulating factor (G-CSF) produced by the decidual ones. Furthermore, decidual γδT cells produced fibroblast growth factor (FGF)-2, whereas no FGF-2 was detected in the wells of peripheral γδT cells. On the other hand, peripheral blood γδT cells produce small amounts of epidermal growth factor (EGF), which was not detected in the wells of decidual samples. While the production of most measured cytokines was not influenced by the presence or absence of HLA-E or -G molecules, incubating mHLA-G with γδT cells, independently from their origin, increased the measured leptin concentrations. Additionally, we detected elevated concentrations of follistatin when incubating peripheral blood γδT cells with mHLA-E.

4.2.4 Decidual γδT cells are potent producers of cytotoxic mediators

γδT cells act as first responders in the mucosal defense against pathogens and many frontiers between the body and its environment. Therefore, we also analyzed the intracellular perforin expression to determine each γδT cell subset's cytotoxic potential in the decidua.

Upon interaction with HLA-E or HLA-G, NKG2C, NKG2A, ILT2, and KIR2DL4 are potential regulators of the cytotoxic capability of immune cells. Investigating the perforin content of the different NK receptor-expressing decidual γδT cell

populations, we found that the expression of NKG2C and ILT2 was associated with significantly higher levels of intracellular perforin in all decidual subsets. The expression of NKG2A, however, correlated only in the Vδ1+ and DN γδT subset with higher levels of intracellular perforin. A similar, significant relation between KIR2DL4 expression and perforin content was only detectable in the DN γδT cell subset.

To determine if this hypothetical relationship between cytotoxicity and the expression of HLA class I binding receptors has functional consequences, we analyzed the secretion of typical NK cell cytokines and cytotoxicity-related soluble molecules after exposure to sHLA-E/sHLA-G or mHLA-E/mHLA-G. However, the measured perforin concentration did not differ significantly. In addition, we found that decidual γδT cells secrete excessive amounts of granulysin and high levels of interferon-γ (IFN-γ).

5 Theses

1. About half of NKT-like ($CD3^+/CD56^+$) cells express a γδT cell receptor.
2. The $CD56^+$ population of γδT cells expands during the 2nd and 3rd trimesters of pregnancy.
3. $CD56^+$ γδT cells maintain predominantly $CD4^-/CD8^-$ or $CD8^+$ phenotypes during pregnancy.
4. Peripheral blood $CD56^+$ cells represent the cytotoxic fraction of γδT cells.
5. Regarding the potential regulation of γδT cells' cytotoxicity, the prevalence of PD-1^+ peripheral blood γδT cells is increased during the first trimester. This trend is more dominant in more cytotoxic $CD56^+$ γδT cells.
6. Interestingly, in the whole peripheral blood γδT cell population, cytotoxic potential and PD-1 expression are not correlated.
7. $V\delta1^+$, $V\delta2^+$, and DN γδT cells are present in the decidua during early pregnancy. Previous flow cytometric studies neglected the largest DN γδT cell population.
8. All decidual γδT cell subsets harbor cells expressing receptors for HLA-E or HLA-G.
9. Decidual γδT cells secrete G-CSF and FGF-2 independently of HLA-E and HLA-G.
10. The presence of mHLA-G and γδT cells increases measured leptin concentrations.

6 Conclusions

Our research on γδT cells during human pregnancy sheds light on their role in immune regulation at various maternal-fetal interfaces. Initially focusing on peripheral blood γδT cells derived from the three trimesters of healthy human pregnancy, we described some basic features of the $CD56^+$ subset of γδT cells, their CD4/CD8 phenotype and cytotoxic character. Than we explored the potential of an interaction with fetal tissue, particularly the syncytiotrophoblast expressing PD-L1/PD-L2, which is the natural ligand of PD-1 immune checkpoint molecule. Although PD-1 expression on γδT cells varied throughout pregnancy, it could probably control the cytotoxicity of $CD56^+$ γδT cells, but appears to have a minor impact, when looking at the whole peripheral blood γδT population.

During early pregnancy, γδT cells exhibit diverse functions and interactions in the decidua. Three major subsets - $V\delta1^+$, $V\delta2^+$, and DN γδT cells - were identified, where DN γδT cells potentially representing $V\delta3^+$ cells. The decidual γδT cells express NK receptors and may interact with non-classical HLA molecules like HLA-E and HLA-G, which could modulate their function.

Decidual γδT cells might contribute to angiogenic factor production, aiding trophoblast invasion and nurturing the placental environment. HLA-G expression correlates with leptin secretion, suggesting interdependent regulation mechanisms. Despite intracellular perforin expression, no immediate effects of

HLA-E or HLA-G were observed, indicating rather potential long-term consequences.

Overall, γδT cells play crucial roles in immune regulation and pathogen defense at various stages of pregnancy, interacting with fetal tissue and responding to environmental cues. Understanding these interactions provides insights into pregnancy complications and potential therapeutic targets for infertility.

7 Publications

7.1 Contributions to Scientific Journals

Total cumulative impact factor: ***44.759***

Total citations: ***28,*** H-index: ***3***

Cumulative impact factor of articles associated with this PhD thesis (7.1.1): ***16.087***

7.1.1 Publications Related to the Thesis

1. **Nörenberg J**, Vida P, Bösmeier I, Forro B, Nörenberg A, Buda A, Simon D, Erdo-Bonyar S, Jakso P, Kovacs K, Miko E, Berki T, Mezosi E, Barakonyi A. Decidual γδT cells of early human pregnancy produce angiogenic and immunomodulatory proteins while also possessing cytotoxic potential. *Front. Immunol.* doi: 10.3389/fimmu.2024.1382424
   Impact factor: 7.300, Q1

2. **Nörenberg J**, Jakso P, Barakonyi A. Gamma/Delta T Cells in the Course of Healthy Human Pregnancy: Cytotoxic Potential and the Tendency of CD8 Expression Make CD56+ γδT Cells a Unique Lymphocyte Subset. *Front. Immunol.* 2021 Feb 2;11:596489. doi: 10.3389/fimmu.2020.596489. eCollection 2020. PMID: 33603738
   Impact Factor: 8.787, Q1

7.1.2 Publications not related to the Thesis

1. Simon D, Erdo-Bonyar S, Böröcz K, Balazs N, Badawy A, Bajnok A, **Nörenberg J**, Sereny-Litvai T, Varnagy A, Kovacs K, Hantosi E, Mezosi E, Nemeth P, Berki T. Altered Levels of Natural Autoantibodies against Heat Shock Proteins in Pregnant Women with Hashimoto's Thyroiditis *Int. J. Mol. Sci.* 2024 Jan, 25(3), 1423; doi: 10.3390/ijms25031423
   Impact factor 5.600, Q1

2. Erdo-Bonyar S, Simon D, Bajnok A, **Nörenberg J**, Litvai T, Varnagy A, Kovacs K, Hantosi E, Mezosi E, Berki T. Physiological Changes in the Levels of Anti-Cytokine Autoantibodies in Early Pregnancy Are Missing in Pregnant Women with Hashimoto's Thyroiditis. *J. Immunol. Res.* 2023 Aug 25:2023:5221658. doi: 10.1155/2023/5221658. eCollection 2023. PMID: 37663050
   Impact factor: 4.100, Q1

3. Bajnok A, Sereny-Litvai T, Temesfoi V, **Nörenberg J**, Herczeg R, Kaposi A, Berki T, Mezosi E. An Optimized Flow Cytometric Method to Demonstrate the Differentiation Stage-Dependent Ca2+ Flux Responses of Peripheral Human B Cells. *Int. J. Mol. Sci.* 2023 May 22;24(10):9107. doi: 10.3390/ijms24109107. PMID: 37240453
   Impact Factor: 5.600, Q1

4. Sereny-Litvai T, Bajnok A, Temesfoi V, **Nörenberg J**, Pham-Dobor G, Kaposi A, Varnagy A, Kovacs K, Pentek S, Koszegi T, Mezosi E, Berki T. B cells from anti-thyroid antibody positive, infertile women show hyper-reactivity to BCR stimulation. *Front Immunol.* 2022 Oct 25:13:1039166. doi: 10.3389/fimmu.2022.1039166. eCollection 2022. PMID: 36389812
   Impact Factor: 7.300, Q1

5. **Nörenberg J**, Meggyes M, Jakso P, Miko E, Barakonyi A. TIM-3 and TIM-1 Could Regulate Decidual gamma-delta TCR Bright T Cells during Murine Pregnancy. *J. Immunol. Res.* 2019 May 20:2019:3836942. doi: 10.1155/2019/3836942. eCollection 2019. PMID: 31236420
   Impact Factor: 3.327, Q1
6. Meggyes M, Szereday L, Jakso P, Bogar B, Bogdan A, **Nörenberg J**, Miko E, Barakonyi A. Expansion of CD4 phenotype among CD160 receptor-expressing lymphocytes in murine pregnancy. *Am. J. Reprod. Immunol.* 2017 Dec;78(6). doi: 10.1111/aji.12745. Epub 2017 Sep 16. PMID: 28921767
   Impact Factor: 2.745, Q1

7.2 Contributions to Scientific Conferences

1. Poster presentation: Nörenberg J. et al. Exploring the interactions between decidual γδT cells and non-classical HLA molecules expressed by the extravillous trophoblast. 18th International Congress of Immunology (Cape Town, South Africa, 2023)

2. Invited speaker: Nörenberg J. et al. Different γδT cell population and their possible role in the maintenance of pregnancy. 16th International Medical Postgraduate Conference at Charles University (Hradec Králové, Czech Republic, 2019)

3. Poster presentation: Nörenberg J. et al. Flow cytometric analysis of gamma-delta T cells in spleen and placenta during murine pregnancy. 34th Congress of the International Society for Advancement of Cytometry (Vancouver, Canada, 2019)

4. Poster presentation: Nörenberg, J. et al. Characteristics of gamma/delta T cells at the feto-maternal interface of murine pregnancy. European Congress of Immunology (Amsterdam, Netherlands, 2018)

8 Acknowledgements

I am deeply thankful for the tremendous support I have received from my colleagues and loved ones throughout this project. I want to extend a special thanks to Dr. Alíz Barakonyi, my supervisor, who provided invaluable guidance during my PhD project, and to my wife, Dr. Anna Nörenberg, who has been a constant source of encouragement and inspiration throughout the ups and downs of the past few years. I would also like to express my gratitude to Prof. Emese Mezősi for her support and mentorship.

This project would not have been possible without the contributions of my co-authors, whose work and constructive feedback were critical to its success. Additionally, I am grateful to the courageous women who participated in this study despite their personal challenges.

Finally, I would like to thank my family and friends for their unwavering love, understanding, and support.