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# Molecular and cellular determinants of healthy receptive and aged endometrium

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Institute of Clinical Medicine, University of Tartu, Estonia.

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# LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications:

I **Suhorutshenko, M.,** Kukushkina, V., Velthut-Meikas, A., Altmäe, S., Peters, M., Mägi, R., Krjutškov, K., Koel, M., Codoñer, F., Martinez-Blanch, J.F., Vilella, F., Simón, C., Salumets, A., Laisk, T. Endometrial receptivity revisited: endometrial transcriptome adjusted for tissue cellular heterogeneity. *Human Reproduction*. 2018; 33 (11), 2074−2086.

II Rekker, K., Altmäe, S., **Suhorutshenko, M.,** Peters, M., Martinez-Blanch, J.F., Codoner, F., Vilella, F., Simon, C., Salumets, A., Velthut-Meikas, A. A Two-Cohort RNA-seq Study Reveals Changes in Endometrial and Blood miRNome in Fertile and Infertile Women. *MDPI Genes*, 2018; 9 (12), 574.

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**Contribution of the author to the original publications:**

Paper I. Participated in designing the study, participated in sample collection, performed RNA sequencing (RNA-seq) and validation experiments, participated in RNA-seq data analysis and interpretation of the results, and wrote most parts of the manuscript.

Paper II. Participated in designing the study, participated in the sample collection, performed RNA-seq experiments, participated in the analysis and interpretation of data and wrote parts of the manuscript.

Paper III. Participated in designing the study, coordinated the sample collection and bioinformatic analysis, performed the RNA-seq and validation experiments, coordinated immunohistochemical analysis, participated in data analysis, performed data interpretation and wrote the manuscript.

# ABBREVIATIONS

| | |
|---|---|
| AcTubA | acetylated α-tubulin |
| AD | after deconvolution |
| AMA | advanced maternal age |
| ART | assisted reproductive techniques |
| AS | Asherman's syndrome |
| BB | basal body |
| BD | before deconvolution |
| BMI | body mass index |
| DAB | 3,3'-Diaminobenzidine |
| DE | differentially expressed |
| DEG | differentially expressed genes |
| E2 | oestrogen |
| EH | endometrial hyperplasia |
| EGF | epidermal growth factor |
| ER | endometrial receptivity |
| ERA | Endometrial Receptivity Array |
| ESE | early secretory endometrium |
| ESP | Spanish samples |
| EST | Estonian samples |
| FACS | fluorescence-activated cell sorting |
| FC | fold change |
| FGF | fibroblast growth factor |
| FSH | follicle stimulating hormone |
| GO | gene ontology |
| hCG | human chorionic gonadotropin |
| HRT | hormonal replacement therapy |
| IHC | immunohistochemistry |
| IL | interleukin |
| IVF | *in vitro* fertilisation |
| IPA | Ingenuity Pathway Analysis |
| IR | Implantation rate |
| LBR | live birth rate |
| LH | luteinizing hormone |
| lncRNA | long non-coding RNA |
| MCC | multiciliated cell |
| miRNA | microRNA |
| MR | miscarriage rate |
| MSE | mid-secretory endometrium |
| NK | natural killer cells |
| OD | oocyte donation |
| P4 | progesterone |
| PCA | principal component analysis |

| | |
|---|---|
| PCOS | polycystic ovary syndrome |
| PCR | polymerase chain reaction |
| PE | proliferative endometrium |
| PET | personalized embryo transfer |
| PGR | progesterone receptor |
| PGS | preimplantation genetic screening |
| PGT | preimplantation genetic test |
| PGT-A | preimplantation genetic testing for aneuploidies |
| PR | pregnancy rate |
| RIF | recurrent implantation failure |
| RT-qPCR | real-time quantitative polymerase chain reaction |
| SD | standard deviation |
| VEGF | vascular endothelial growth factor |
| WOI | window of implantation |
| YMA | young maternal age |

# 1. INTRODUCTION

Fertility rates are constantly decreasing in the developed countries, raising concerns for the sustainability of the modern society. While socio-economic reasons favour small families by choice, there is still a lot of families who wish to have children but cannot.

Major causes of infertility are associated with age and lifestyle, leading to poor ovarian reserve and sperm quality. This initiated the development the assisted reproductive techniques (ART) which allow the use of donor gametes or previously preserved cells and embryos. When donor oocyte is used, embryo is grown in the laboratory conditions until the blastocyst stage and then transferred into the uterus of a future mother. At this moment both embryo viability and uterine maturity are crucial to establish embryo implantation as a start point for a successful pregnancy. Endometrium or inner lining of the uterus is a monthly regenerating tissue, which growth and differentiation is supported by the ovarian hormones. Decades of previous research worldwide have produced knowledge of how endometrium is regulated, and which processes are essential to establish endometrial receptivity to embryo. This resulted in the development of histologic and molecular screening tests aiming to personalize infertility treatment by predicting the optimal day for embryo transfer in women experiencing implantation failure and early pregnancy loss. Unfortunately, none of the screening tests can predict endometrial receptivity with absolute accuracy and they all have their disadvantages.

One of the main disadvantages of the available endometrial tests available at clinics, is the invasiveness of the biopsy procedure required for endometrial sampling. This issue was addressed by an international Non-invasive Test for Endometrial Dysfunction (NOTED) consortium aiming to develop non-invasive endometrial diagnostic test. Blood, urine and saliva have also been studied to identify endometrial receptivity markers. The most promising results were obtained for uterine fluid samples, which represent a minimally invasive marker potential. In this thesis, we discuss the advantages and issues of endometrial testing and provide new knowledge that needs to be implemented.

Also, as most endometrial receptivity studies focus solely on women of reproductive age, the continuously rising maternal age emphasizes the importance of reproductive care for women of advanced age. With no clear consensus on whether age affects endometrial receptivity in pre-menopausal women, we attempted to explain the processes behind endometrial aging. Using the most advanced tissue and single-cell analysis techniques we address the specific changes and try to predict the possible onset of the age-related cellular changes. Our results rise concern that endometrial preparation protocols for in vitro fertilisation (IVF) may not be optimal for women of advanced maternal age, causing specific molecular and cellular changes in the endometrial epithelium, compromising endometrial receptivity and/or even woman’s health.

# 2. REVIEW OF LITERATURE

## 2.1. Epidemiology of female infertility

Declining birth rates is the major demographic problem in developed countries. In developed nations, a lower fertility rate is typically observed as a result of various lifestyle factors associated with economic prosperity. These factors include low mortality rates, the widespread availability of birth control methods, and the recognition of the potential financial burden that raising children can entail, such as housing and education expenses. Additionally, the pursuit of higher education and professional careers often leads women to delay starting a family until later in life (Nargund, 2009). On average, one of six couples fail to conceive naturally within twelve months of unprotected sexual intercourse, defined as 'infertility', and turn to a medical specialist seeking for a particular cause (Infertility prevalence estimates, 1990–2021. Geneva: World Health Organization; 2023). Infertility is ranked as the fifth highest serious global disability with a negative impact on the quality of life of women more than men (Zayed & El-Hadidy, 2020). This puts a lot of pressure on the medical community to find measures to help women struggle with fertility issues at the time when they decide to conceive.

The exact number of children born with the assistance of reproductive technologies worldwide can vary from year to year. However, according to a report published by the International Committee for Monitoring Assisted Reproductive Technologies in 2018, it is estimated that approximately 8 million children have been born globally through ART since their inception (Mancuso et al., 2018). In Estonia, 6% of children are born with the help of ART (Ravimiamet, 2022).

Women's health issues are accountable for almost one-third of all couples' infertility causes, the same proportion of infertility cases is caused by men's problems and in one-third of all cases both male and female reproductive health issues are detected. Although in nine out of ten cases, the reason for infertility is identified, there is approximately 10% of all infertile couples, for whom the cause of infertility remains unknown (Figure 1). For the past decades, a lot of effort has been put into unravelling mechanisms of unexplained infertility and developing methods to predict and manage this condition.

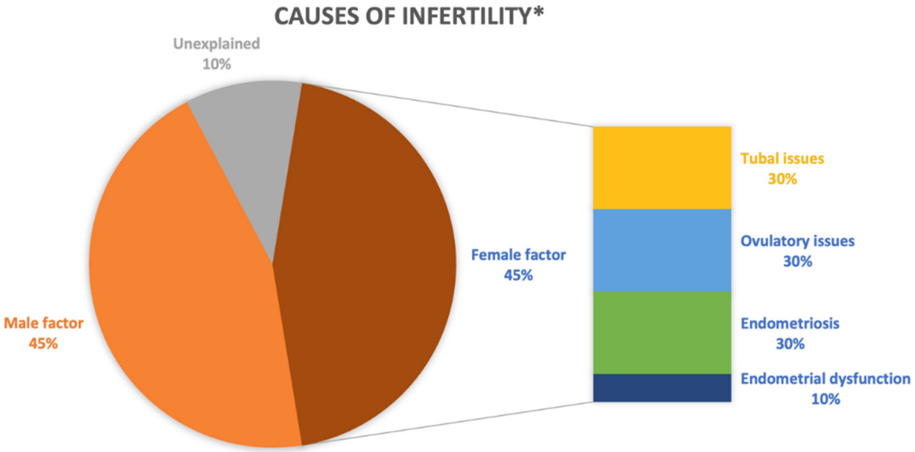


**Figure 1**. The causes of infertility. Male and female factor infertility are equally accountable for 90% of all infertility cases, leaving each tenth case of a couple's infertility with no identified cause. Common female infertility causes, such as tubal blockage, ovulatory dysfunction and endometriosis, are extensively studied and, in most cases, well-managed using modern ART. Endometrial dysfunction is believed to impact approx. 10% of all female infertility cases and, alongside embryo incompetency, can be the cause of implantation failure in women undergoing IVF. *The incidence of identified male and female factor infertility causes taken separately. In one third of all couple's infertility cases both male and female reproductive health issues are detected.

## 2.2. Causes of female infertility

Female infertility is most commonly caused by hormonal dysregulation of ovarian function, endometriosis, or endometrial and tubal pathologies (Figure 1). Ovulatory issues are usually caused by either polycystic ovary syndrome (PCOS), which is a metabolic-hormonal disorder with a genetic background (De Leo et al., 2016). PCOS is a common cause for primary infertility; or by a low or damaged ovarian reserve – a condition which is more associated with woman's age and environment and can be a common cause of secondary infertility (Kicińska et al., 2023). Tubal pathologies include the blockage of Fallopian tubes as a result of infection, but it can be also caused by previous mechanical damage to Fallopian tubes (e.g. ectopic pregnancy) (Ambildhuke et al., 2022). Tubal infertility is also the most common indication for IVF. Endometriosis is identified as an oestrogen-dependent condition characterized by retrograde menstruation, the presence of endometriotic lesions, and low life quality due to pelvic pain, dyspareunia and menorrhagia (Kuan et al., 2021). The impact of PCOS and endometriosis on female infertility is associated with the degree of disease progression and can easily remain undiagnosed.

## 2.3. Endometrium and its role in implantation

The endometrium is a complex uterine tissue that normally undergoes monthly cycles of proliferation, differentiation and degradation under the fine regulation of hypothalamus-pituitary-gonadal axis hormones (Aplin et al., 2008). The whole process is synchronized with the development of oocytes, fertilization, and migration from the ovary through the oviduct into the uterus. In non-conception cycles, the whole cycle ends with a programmed shedding of endometrial tissue together with the disposed oocyte. In the conception cycle, fertilization takes place in the oviduct within hours of ovulation (Figure 2). Once the fertilized oocyte attaches to the uterus the forming syncytiotrophoblast starts producing the hormone human chorionic gonadotropin (hCG) which supports the corpus luteum function and, together with rising P4, leads through the final decidualization of endometrium and placenta formation (Aplin et al., 2008; Koot et al., 2012). Now, since the successful implementation of IVF, the implantation of an embryo into the uterine tissue is one of the most crucial events in reproductive science (Aplin et al., 2008). For decades, the cases where fertilized oocyte fails to attach to the uterus have challenged couples, reproductive specialists, and scientists. Alongside most failed implantations being caused by embryo incompetence, there is a considerable number of cases where a healthy embryo was not accommodated by the uterus due to endometrial pathology (reviewed in Koot et al., 2012).

### 2.3.1. Menstrual cycle

In healthy reproductive-age women menstrual cycle lasts between 25–35 days. In an ideal 28-day non-conception cycle rising levels of pituitary hormones stimulate oestrogen production by ovaries, which stimulates follicle growth in the ovaries (Figure 2A). Simultaneously, ovarian oestrogen induces endometrial cells to proliferate and build up functional layer of the endometrial tissue. This part of the menstrual cycle is known as the follicular phase and the respective endometrial growth period is known as proliferative endometrium (PE). During this phase oestrogen (E2) peaks at 200–2000 pmol/L, and progesterone (P4) level is low (serum concentration <0.2 nmol/L) (Anckaert et al., 2021). Once the follicle reaches a certain size, normally 18–25 cm, the pituitary gland releases high levels of luteinizing hormone (LH, 8–72 IU/L) which takes place around day 14 of the normal menstrual cycle. This triggers the final maturation and the release of the oocyte from the ovary into the infundibulum of a Fallopian tube within 24–36 hours after the LH surge.

Ovulation starts the luteal phase, where an oocyte takes its journey towards fertilization. The empty follicle transforms into corpus luteum which starts to produce both E2 and P4, which lets endometrium enter its secretory phase. This causes the secondary rise of E2 that keeps building up endometrial tissue and the rise of P4 levels (13–46 nmol/L) to orchestrate the differentiation of endometrium into a gland-rich secretory epithelium, supported by round decidual stromal cells and numerous blood vessels. P4 is also responsible for the specific suppression and modulation of immune cell activity in the endometrial tissue, to let the embryo implant. These processes are combined into the term called endometrial receptivity, where all endometrial cells obtain specific molecular and phenotypical characteristics necessary to support implantation and further placentation (Aplin et al., 2008).

The period where P4 level rises is known as the early secretory phase (ESE), days 15–19 of the normal menstrual cycle. The endometrium is most receptive during the mid-secretory phase (MSE) corresponding to the days 20–24 of the normal menstrual cycle, which is referred to as the window of implantation (WOI). During this period, endometrium exhibits a specific molecular and histological profile: large epithelial glands filled with secrete, embedded in the oedematous stroma. No stromal mitosis occurs, the cells of the compact zone undergo pre-decidual change under the influence of P4, developing into polygonal cells with vesicular nuclei and abundant light cytoplasm with well-defined cell borders (Noyes et al., 1950). The epithelial cells have formed pinopodes (Martel et al., 1987). On a molecular level, the epithelial cells express transmembrane proteins (integrins, cadherins) that help the embryo interact with endometrial epithelium by mediating adhesion and invasion (Cha et al., 2012). If the implantation does not occur within ten days from LH surge, the P4 levels start decreasing as corpus luteum progresses into the corpus albicans, until P4 level reaches the concentration no longer able to support endometrial tissue. This kind of progesterone withdrawal induces hypoxia in the endometrial cells, which leads to the upregulation of inflammatory processes and collapse of endometrial tissue (Critchley et al., 2006). The shedding of blood-rich mucous functionalis is the reason for monthly flow.

To understand, how endometrium is monthly regenerated under the regulation of ovarian hormones, it is crucial to know how hormones mediate their action in endometrial cells. When oestrogen is diffused into the cells, it binds with a specific receptor dimer, oestrogen receptor alpha or beta homodimer, or oestrogen receptor alpha-beta heterodimer. Progesterone is bound to its own receptor, progesterone receptor (PGR) A or B. Activated by hormone binding the hormone-receptor complex moves to the nucleus where it binds hormone response element on DNA, which either promotes or inhibits the expression of affected genes. Oestrogen-responsive genes are mostly associated with growth and proliferation, whereas progesterone-response genes promote differentiation, decidualization and inhibit immune response (Tamm et al., 2009; Tamm-Rosenstein et al., 2013).

It is important to recognize, that only the type of endometrium that corresponds to the mid-secretory phase can accommodate the growing embryo. Because of this, the ability to identify WOI in patients has been one of the most important tasks in IVF.

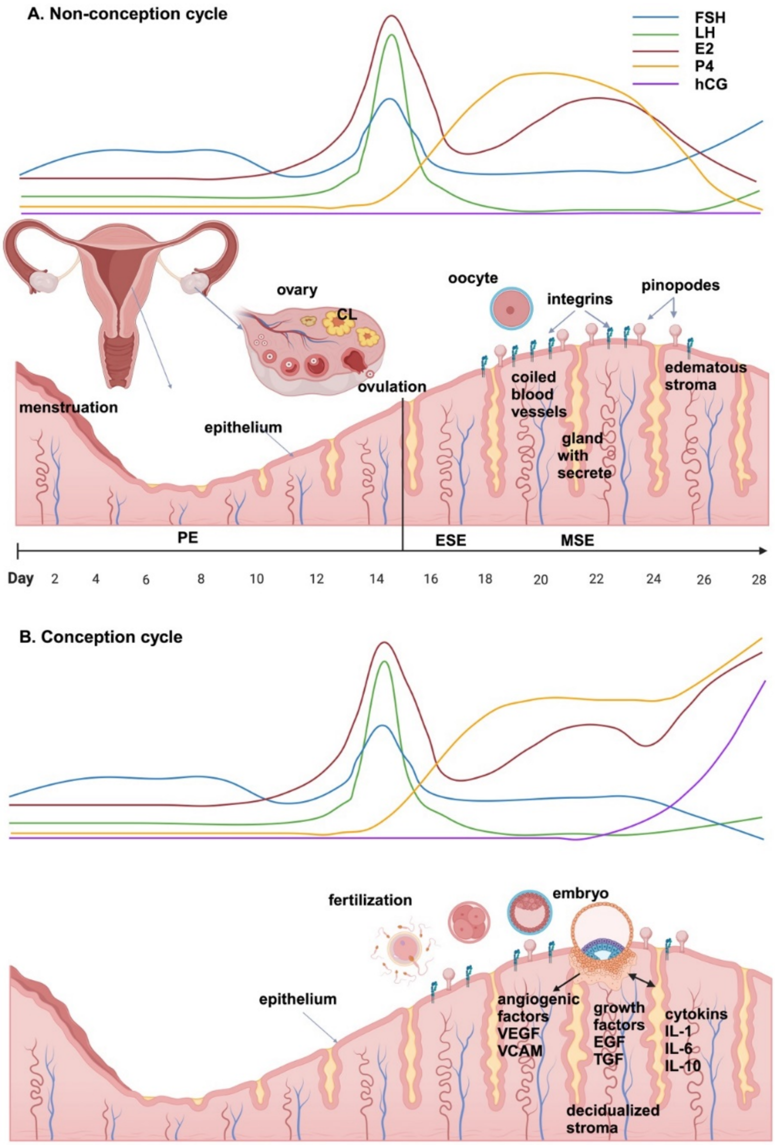


**Figure 2.** Endometrial changes under the influence of pituitary-gonadal hormones in non-conception and conception cycles. While in non-conception cycle the progesterone decline triggers tissue shedding, if fertilization takes place (conception cycle) the rising hCG levels support the maintenance of corpus luteum, the progesterone levels keep rising and endometrial tissue develops into a decidua. FSH – follicle stimulating hormone; LH – luteinizing hormone; E2 – oestrogen; P4 – progesterone; hCG – human chorionic gonadotropin; CL – corpus luteum; PE – proliferative endometrium; ESE – early secretory endometrium; MSE – mid-secretory endometrium. VEGF – vascular endothelial growth factor; VCAM – vascular cell adhesion molecule; EGF – epidermal growth factor; TGF – transforming growth factor, IL – interleukin. The image is created with BioRender.com

### 2.3.2. Implantation

The conception cycle starts also with a follicular phase synchronized with PE development (Figure 2B). When ovulation takes place the oocyte enters the Fallopian tube, where it meets the sperm (Psychoyos, 1986). The moment fertilization occurs the zygote starts dividing and moving along the oviduct toward the uterus, where it finally hatches out of the zona pellucida. The implantation takes place on the days 6–9 after fertilization (Navot et al., 1991). There are three distinct stages of this process that have to take place during this time for implantation to occur. Apposition is a process where the embryo establishes the first weak contact with the implantation site. During this stage, the inner cell mass inside the trophoblast layer is aligned closest to the decidua. The next stage, adhesion, is a stronger contact between the embryo and endometrial epithelium (reviewed in S.-M. Kim & Kim, 2017).

On the trophoblast site, which is the outer layer of cells in the blastocyst that contributes to the formation of the placenta during early pregnancy, cell adhesion is essential for the attachment of the embryo to the uterine lining. This process involves specific adhesion molecules on the surface of trophoblast cells interacting with corresponding molecules on the surface of uterine cells. Cell adhesion molecules are proteins that play a crucial role in cell adhesion, which is the process by which cells interact and attach to each other (S.-M. Kim & Kim, 2017). One important group of cell adhesion molecules involved in this process is integrins. Integrins are transmembrane proteins present on both embryonic and endometrial cells that mediate cell-cell and cell-extracellular matrix adhesion. They play a crucial role in embryonic development and are involved in the attachment of trophoblast cells to the uterine wall during implantation (G. A. Johnson et al., 2023; Lessey et al., 1992). Additionally, other adhesion molecules such as selectins and cadherins may also be involved in the adhesion process at the trophoblast site (Nejatbakhsh et al., 2012; Rowlands, 2000). The interaction of these molecules helps to establish and maintain the necessary connections for the successful implantation of the embryo and the subsequent development of the placenta (Klentzeris et al., 1993; Nejatbakhsh et al., 2012).

Once adhesion is established, trophoblast cells undergo invasive behaviour to penetrate the endometrial tissue. The penetration of the trophoblast cells into the uterine lining is referred to as invasion. This invasion is a carefully regulated process to ensure that the embryo can access the maternal blood supply and establish a connection for nutrient exchange (S.-M. Kim & Kim, 2017). Invasive trophoblasts play a crucial role in the formation of the placenta, which is essential for supporting the developing embryo.

During implantation, several molecular cascades are activated to induce tissue modulation processes to support placentation (Figure 2B). Hormones activate growth factor signalling that regulated cell growth, proliferation and differentiation. For example, epidermal growth factor (EGF) and fibroblast growth factor (FGF) may influence the proliferation and differentiation of cells in the uterine lining, creating a suitable environment for embryo attachment and subsequent development (Cha et al., 2012; Krüssel et al., 2003). These growth factors also play roles in tissue repair and remodelling.

Angiogenic factors promote the growth and branching of blood vessels. Proper vascularization is necessary to support the growing embryo and establish connections for nutrient exchange. Vascular endothelial growth factor (VEGF) is a key angiogenic factor that plays a significant role in promoting the formation of blood vessels during implantation (Krüssel et al., 2003).

Cytokines are involved in the immune response and immune tolerance during implantation. They help modulate the maternal immune system to prevent rejection of the semi-allogeneic embryo. Some cytokines, such as interleukins (IL), participate in the complex interplay between the maternal immune system and the developing embryo (Figure 2B) (Dominguez et al., 2010).

## 2.4. Endometrial pathologies and the infertility of endometrial origin

There is no doubt that pathological endometrial growth and differentiation is a considerable reason for implantation failure. Healthy mature endometrium is characterized as sonographically measured 7–16 mm thick mucous lining during the expected WOI (Abdel Kader et al., 2016; Bergh & Navot, 1992; Mahutte et al., 2022). Most studies agree that at least 8 mm thick endometrium is required for optimal placentation and successful maintenance of pregnancy (Abdel Kader et al., 2016; Eftekhar et al., 2020; Hu et al., 2021; Shaodi et al., 2020; Yang et al., 2018). The endometrial thickness of less than 7 mm during the secretory phase and above 16 mm is considered pathological. Deficient endometrial growth is caused by the lack of ER activation in endometrial cells, as a result of either lack of oestrogen (e.g. post-menopause), or the disturbed ER activity. The excessive endometrial growth, known as endometrial hyperplasia, on the opposite, can be caused by elevated hormone activity by hormonal aberrations, during HRT, peri- and postmenopausal periods. Both deficient and excessive growth are associated with higher risk of miscarriage and implantation failure, due to the shift in the structure of endometrial tissue.

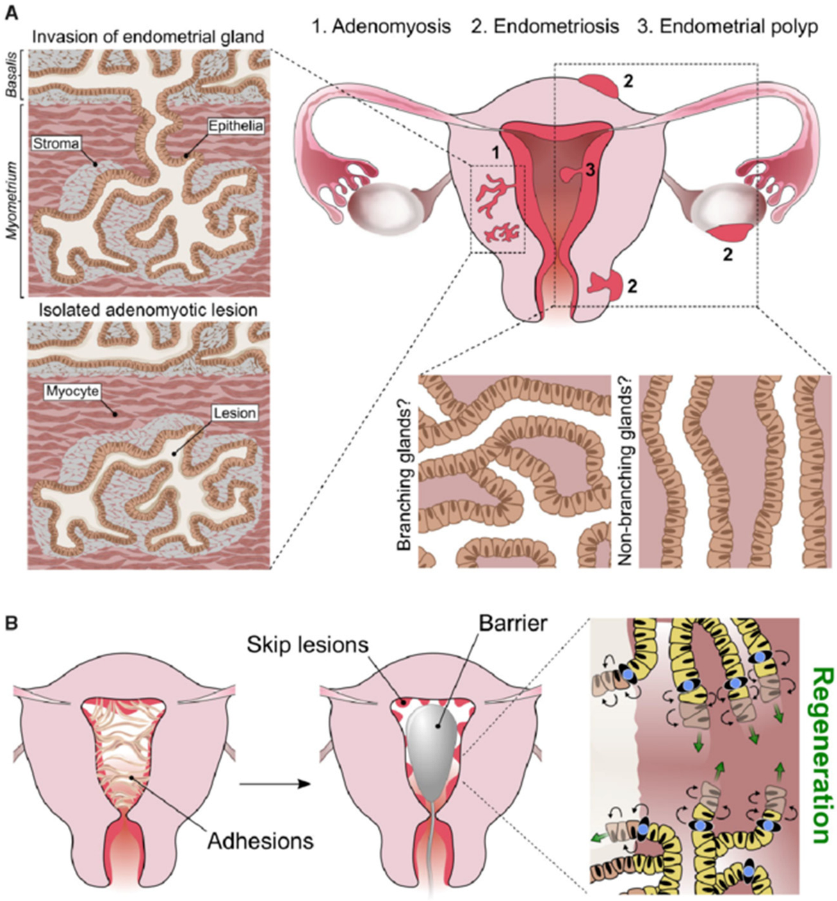


**Figure 3.** Endometrial pathologies associated with epithelial histoarchitecture. **A.** Colony-like structure of adenomyotic lesions containing epithelial glands with multiple branch points, proposed by Yamaguchi et al., 2021. Based on their origin, adenomyotic lesions may be connected directly to the endometrial basalis suggesting the eutopic glandular invasion of the myometrium, or isolated lesions. The glandular architecture of endometrial polyps and endometriotic lesions (including superficial, ovarian and deep-infiltrating endometriosis) remain to be elucidated. They may have a complex-branching or non-branching glandular conformation. **B.** Proposed model of endometrial regeneration in Asherman's syndrome; following surgical removal of intrauterine adhesions and barrier insertion to inhibit recurrence, basalis networks from neighbouring skip lesions are able to expand parallel to the myometrium in a horizontal plane by means of resident stem/progenitor cells, subsequently restoring the whole glandular element. Adapted from Tempest et al., 2022.

Sometimes endometrial tissue is found outside its physiological niche, causing such endometrial disorders as adenomyosis and endometriosis. Adenomyosis is defined by the growth of endometrial epithelial and stromal cells within the muscular wall of the uterus (myometrium) (Figure 3A). It can appear as both connected with basalis and as isolated lesions. There are several theories on how adenomyosis develops that include the invagination of endometrial basalis and the differentiation of adult endometrial stem cells within the myometrium (El Sabeh et al., 2021). Adenomyosis might contribute to infertility by distortion of uterine anatomy or causing inflammation (Harada et al., 2016), but the mechanisms are still not fully understood.

Endometriosis is an oestrogen-dependent reproductive health issue, characterized by the occurrence of endometrial tissue outside the uterus. Commonly, endometriotic lesions are discovered on ovaries, Fallopian tubes and in the peritoneum attached to other organs (Figure 3A). The most widely accepted theory of how endometrial tissue escapes uterine cavity is retrograde menstruation which causes the menstrual blood and tissue flow back through the fallopian tubes into the pelvic cavity (Kuan et al., 2021). Endometriosis of different stages may interfere with ovulation if attached to ovaries, though ovulation repression, it may cause anatomical changes, promote inflammation and hormonal imbalances of reproductive organs, contributing to both ovarian and uterine factor infertility. The inflammation of endometrial tissue, or endometritis, is most commonly caused by infection, which may disturb normal endometrial growth and differentiation, and alter the immune processes crucial for embryo tolerance. Adenomyosis, endometriosis and endometritis are relatively common endometrial disorders and can sometimes occur together.

Polyps and fibroids are mostly benign uterine tissue formations found in the uterine wall and cavity. Polyps are focal neoplasms that protrude into the uterine cavity from the surface epithelium (Figure 3A). Fibroids are muscular tumours that grow in the wall of the uterus but may also protrude into the uterine cavity. Each endometrial pathology has its own grade of impact on endometrial infertility. Some disorders like Asherman’s syndrome (AS), polyps and fibroids, impose a direct physical obstacle to embryo implantation, whereas inflammatory diseases (endometritis, endometriosis and adenomyosis) affect endometrial receptivity on the molecular and cellular level. The last ones, often undiagnosed, pose a substantial challenge and a continuous debate on their impact on woman’s fertility.

AS is a rare acquired gynaecological disorder characterized by a scar tissue bonding of uterine lining. Its prevalence varies between 2–20% in infertile patients. Usually caused by uterine ablation during surgery, or infection, damaged uterine wall develops intrauterine adhesions of avascular scar tissue, that may cause physical obstruction in the uterus and impede endometrial development. The treatment of AS includes the surgical removal of intrauterine adhesions and the insertion of a physical barrier to prevent the reoccurrence (Figure 3B). Modern management of AS also involves the successful application of endometrial CD133+ stem cell treatment, which highlights the tremendous potential of cell therapy in reproductive medicine (Santamaria et al., 2016).

### 2.4.1. Recurrent implantation failure

Endometrial pathologies do not necessarily constitute infertility, and vice versa – infertility of endometrial origin does not always form from a tangible pathology. Women with unexplained infertility are known to possess an endometrial transcriptomic profile significantly different from the one of young fertile women (Altmae et al., 2010). The term repeated or recurrent implantation failure (RIF) was introduced as a pathological condition of endometrial tissue that prevents a viable embryo from implanting, with no evident endometrial pathology at present. The biggest constraint in studying the origin of RIF since the beginning has been its definition. Early studies referred to RIF as an inability to achieve pregnancy after 1–2 good embryo transfers. As the general IVF success rate is around 30%, one would assume that implantation failure can be considered pathologic only after at least 3 unsuccessful embryo transfers. This all changed substantially when the preimplantation genetic screening (PGS) became popular after 2007 (Mastenbroek et al., 2007). PGS statistics showed that up to 60% of embryos that are considered viable based on their morphological analysis, may be genetically aberrant and therefore not able to achieve pregnancy (Fragouli et al., 2011). This put the previous results on the aetiology and occurrence of RIF under the question mark. In 2018, it was suggested that RIF is caused by a pathologic (approx. 20% of all RIF cases) or timely shifted WOI (25%), and in half of all cases it was a combination of both (Sebastian-Leon et al., 2018), whereas the occurrence of RIF of non-endometrial origin was below 5%. Two years ago, a consensus statement was issued postulating that up to 5% of infertile couples were likely to be suffering from RIF, if at least 3 tested euploid embryos had been transferred (Pirtea et al., 2023). This emphasized the importance of personalized approach to embryo transfer and encourages us to continue the development and improvement of endometrial receptivity tests. Notably, not only molecular, but also cellular composition changes can be potentially good predictors of endometrial disorders, as, for example, specific natural killer (NK) and PGR-positive epithelial cells were shown to be decreased in RIF (Lai et al., 2022).

To understand if a particular endometrial disorder is the only cause of a couple's infertility or if it just adds up to other factors (some of which may remain unidentified), molecular and cellular markers are implemented for endometrial analysis, that aim to both identify the health problem and predict the efficacy of infertility treatment.

## 2.5. The effect of woman’s age on endometrial health

Age is major fertility factor for women. Women’s reproductive age is considered between 15–49 years old, with the amount of women having children in their forties constantly rising worldwide (Lindh et al., 2022). The average age of mothers giving birth has increased due to a combination of factors including widespread contraception use and shifts in socio-economic and lifestyle dynamics. These dynamics encompass women’s educational and professional advancement, economic uncertainty in housing, unmarried cohabitation before having a first child, improved gender equality, and more. Paradoxically, the emergence of advanced ART has also played a role in motivating women to delay childbirth. Over the years, there has been a consistent upward trend in the average age of childbirth, with a yearly increase of about one year for each decade since 1970 across countries in the Organisation for Economic Co-operation and Development. In Europe, the fertility rate of women over 30 years old is constantly rising each decade (Figure 4). The term “advanced maternal age” initially referred to women older than 35 years old and was mostly associated with the decline of ovarian function. However, based on the recent literature, this threshold has been raised to 40 years, beyond which there is a substantial decline in fertility, even in IVF cycles (Bouzaglou et al., 2020; Shapiro et al., 2016).

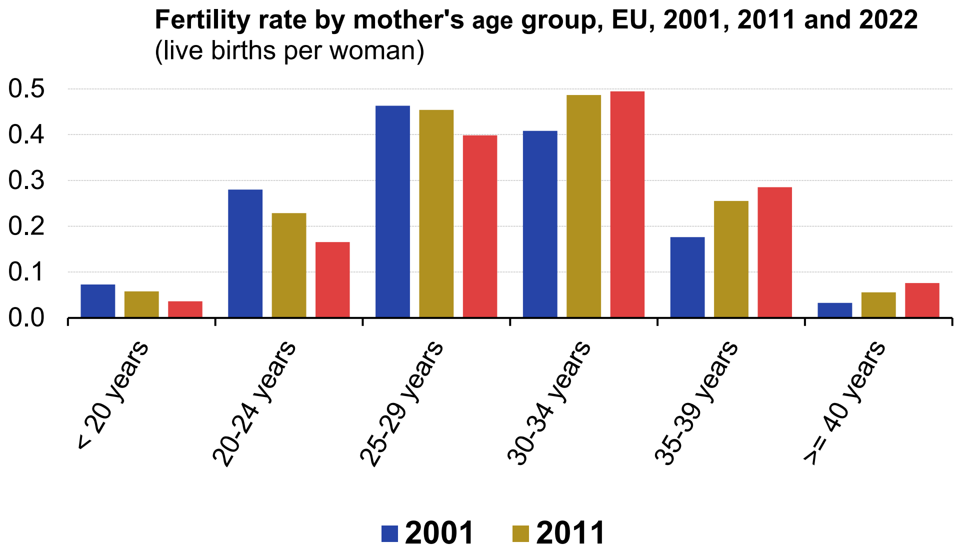


**Figure 4**. The fertility rate by mother’s age group is the sum of the single age fertility rates. Source: *Eurostat* (online data code: 10.2908/demo_frate). Y axis represents the number of live births per woman. Over the past twenty years, there have been a trend that the proportion of live births per woman is rising for women over 30 years old. During the last decade, the fertility rate in women over 30 has been higher than in younger women.

Increasing age is accountable for female infertility and IVF failure and is by far the most common factor for secondary infertility. The relationship between maternal age and the decline in oocyte quality as well as the occurrence of chromosomal abnormalities in oocytes and embryos is widely recognized, suggesting the rapid decline in oocyte quality around the age of 40 (Milán et al., 2010). The impact of endometrial aging on the implantation, miscarriage and live birth rates have been widely studied before producing somewhat controverting results. The overview of the clinical studies on endometrial aging, are presented in the Table 1. Early studies focusing on endometrial aging originate from 90-s to early 2000-s – the time before PGS of embryos was implemented. At that time, donor oocytes were used in HRT cycles to overcome the negative effect of oocyte quality on the IVF success (Abdalla et al., 1997; Flamigni et al., 1993; Navot et al., 1994). Since 2007, PGS was used to assess embryo quality before ET (Mastenbroek et al., 2007). Later studies using preimplantation genetic testing for aneuploidies (PGT-A) and bigger sample size suggested the later onset of endometrial aging (Table 1). We feel that this needs to be explained.

Although woman's age does not seem to directly influence endometrial health (Y.-F. Sun et al., 2020), it has been shown that the optimal endometrial thickness between 8–12 mm is associated with younger maternal age (Eftekhar et al., 2020). As a woman grows older her menstrual cycles tend to become shorter. One reason for that can be the reduced oestrogen level in the follicular phase, which results in shorter proliferative phase that produces insufficient endometrial growth (<7 mm) (Onogi et al., 2020). In ovulating women, as telomeres and telomerase activity decrease in granulosa cells with age, the lower luteal progesterone can be the result of decreased functional capabilities of corpus luteum (Butts et al., 2009). The other explanation of excessive oestradiol exposure and progesterone deficiency can be the fact that ovulations start reducing from late 30-s until they cease in early to mid-50-s, providing significant changes in the molecular mechanisms of endometrial maturation in women of advanced maternal age (Baerwald et al., 2018; Prior, 2005).

**Table 1.** The overview of the original studies between 1993 and 2023 aiming to evaluate the effect of age on endometrial receptivy and ability to maintain pregnancy to term. The effect of age on oocyte quality was eliminated by either oocyte donation from yourge donors, or by assessment of embryo caryotype using PGT. All women underwent HRT prior to fresh or frozen embryo transfer. AMA – advanced maternal age.

| Reference | Sample size | Method | AMA age (years) | Outcome in AMA |
|---|---|---|---|---|
| Abdalla et al., 1993 | 241 patients | Oocyte donation | ≥ 40 | Higher miscarriage rate (MR) |
| Flamigni et al., 1993 | 87 patients | Oocyte donation | > 40 | Decreased pregnancy (PR) and implantation rates (IR) |
| Yaron et al., 1993 | 458 cycles | Oocyte donation | ≥ 40 | Decreased PR ($p = 0.043$) |
| Balmaceda et al., 1994 | 258 cycles | Oocyte donation | > 40 | No association |
| Check et al., 1994 | 155 cycles | Oocyte donation | ≥ 40 | Lower PR and live birth rate (LBR) |
| Navot et al., 1994 | 102 cycles | Oocyte donation | ≥ 40 | No association |
| Sauer et al., 1994 | 192 patients | Oocyte donation | ≥ 40 | No association |
| Cano et al., 1995 | 35 patients | Oocyte donation | ≥ 40 | Higher MR ($p < 0.05$) |
| Legro et al., 1995 | 307 cycles | Oocyte donation | > 42 | No association |
| Borini et al., 1996 | 114 patients | Oocyte donation | ≥ 40 | Lower PR ($p = 0.01$) and IR (0.05) |
| Abdalla et al., 1997 | 104 patients | Oocyte donation | ≥ 40 | No association |
| Yaron et al., 1998 | 423 patients | Oocyte donation | > 40 | Decreased PR |
| Moomjy et al., 1999 | 370 patients | Oocyte donation | > 42 | Lower IR ($p = 0.002$) |
| Toner et al., 2002 | 17,339 cycles | Oocyte donation | ≥ 45 | Lower IR ($p = 0.008$), PR (0.036) and LBR (0.013) |
| Soares et al., 2005 | 3089 cycles | Oocyte donation | > 45 | Lower PR ($p = 0.045$) and IR (0.02), higher MR (0.03) |
| Gupta et al., 2012 | 270 patients | Oocyte donation | ≥ 40 | Lower ongoing pregnancy rate and IR ($p = 0.001$) |
| Gunnala et al., 2018 | 1078 patients | PGT-A | ≥ 45 | Lower PR ($p = 0.04$) |
| Sacchi et al., 2019 | 201 cycles | PGT-A | ≥ 38 | No association |
| Sanders et al., 2021 | 2464 cycles | PGT-A | ≥ 45 | LBR decreased 16.7% compared to the group <40 years |
| Z. Zheng et al., 2023 | 3,125 cycles | PGT-A | ≥ 40 | Decreasing trend of LBR |
| Barbakadze et al., 2024 | 320 patients | PGT-A and ERA | ≥ 35 | No difference when ERA test used |

It is still debatable what causes ovulation reduction with age. Early population-based studies show that the levels of follicle stimulating hormone (FSH) progressively increase during perimenopause (Burger et al., 1995, 1999) – a period which stands for advanced reproductive age, usually 2–8 years before actual menopause. It appears to take very little elevation of FSH, possibly caused by inconsistent suppression of ovarian oestradiol by inhibins (Welt et al., 1999), to produce high levels of oestradiol (>1800 pmol/L), endometrial thickening and hyperplasia (Djerassi et al., 1995). Insufficient endometrial thickness and endometrial hyperplasia can also develop during hormone replacement therapy (for example, ovulation induction with selective oestrogen antagonists or oestrogen replacement therapy in peri- and postmenopausal women) (Dehbashi et al., 2003; Epplein et al., 2009; Hawkins Bressler et al., 2021; Lethaby et al., 1999; van der Linden et al., 2015), where excessive oestrogen and poor progesterone either slows down or accelerates endometrial thickening, and result in early endometrial shedding reflected in atypically long or short menstrual cycles, no or heavy bleeding, and compromised endometrial receptivity.

We discussed above that endometrial growth and receptivity are orchestrated mainly by the cellular activity of two steroid hormones: oestrogen and progesterone. Their effect is mediated in reproductive tissue through their cellular receptors. Receptor activity in endometrial tissue may change with age even if the hormone levels are unaffected. Lower concentrations of oestrogen receptor in endometrial biopsy correlate significantly with implantation rates (Ohno, 1998) and can cause poor oestrogen sensitivity on peri-menopausal women. Aging also was reported to impact progesterone resistance in endometrial cells in mice (M.-Q. Li et al., 2017). One previous clinical study proposed that uterine aging may be compensated with higher administration of progesterone (Meldrum, 1993).

Uterine receptivity is also modulated by the embryo itself. During its development, the embryo secretes extracellular exosomes containing specific molecules uptaken by endometrial cells. Exosomes include genetic material, transcription factors and metabolites utilized by endometrial cells to induce endometrial development. As blastocyst exhibits a maternal age component, it has been shown that the content of extracellular exosomes secreted by trophectoderm cells changed significantly with maternal age (Ntostis et al., 2021). Trophectoderm samples from younger women expressed more genes associated with the reduction of oxidative stress, mitochondrial ATP synthesis and cholesterol biosynthesis, essential for implantation (Ntostis et al., 2021).

Although recent studies support the hypothesis that age affects endometrial function (Devesa-Peiro et al., 2022; Shirasuna & Iwata, 2017), to the date, it remains still unclear whether endometrial receptivity is compromised by the age alone, or it is a combination of processes and conditions, where each of them can be maintained separately in order to improve endometrial receptivity potential. Looking for the answer, whole-genome gene expression studies have proposed genes differentially expressed in aging endometrium. Animal studies showed the altered expression of genes associated with mitotic division, angiogenesis, cell migration and inflammation (Kawamura et al., 2021). Recent study on human gene expression in advanced maternal age endometrial samples revealed the changes in cell cycle progression, cell proliferation cilial formation and migration of ciliated cells in aged patients (Devesa-Peiro et al., 2022).

Gene expression is modulated by both transcription factor activity and DNA methylation pattern of hormone response elements in DNA. The methylation pattern itself is known to be affected by age and is even commonly used for prediction of the biological age of the samples (Horvath, 2013). Olesen and his colleagues showed that DNA methylation pattern mid-secretory endometrial samples correlates with human age and can be used as a potential marker for endometrial aging (Olesen et al., 2018). Genetic studies have proposed that mutations in common cancer genes in endometrial glandular epithelium accumulate with age (Moore et al., 2020), which can lead to neoplastic changes in the endometrium of advanced-age women. Our recent review summarised the knowledge collected to this day (Figure 5. adapted with permission from Pathare et al., 2023).

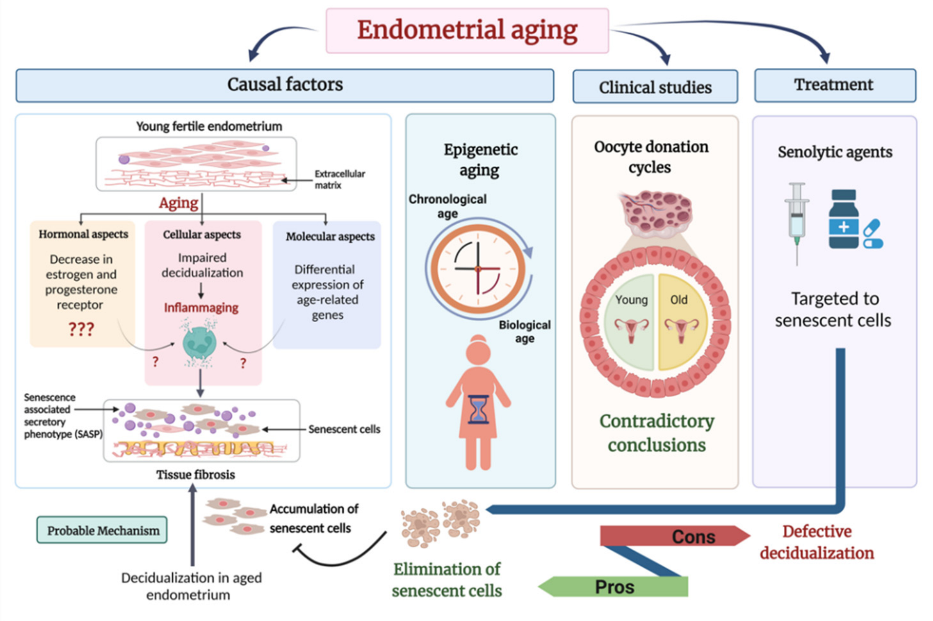


**Figure 5**. The overview of the proposed causes, research and treatment options for endometrial aging. The causes include hormone sensitivity, inflammatory background and altered gene expression in endometrial cells, as well as general epigenetic changes occurring with age. Controversial results regarding the influence of age on endometrial performance have been obtained from clinical studies analysing the treatment outcome in young vs older oocyte recipients, whereas molecular research is still ongoing. The senescence-associated changes in the endometrium could be reversed with senolytic agents. However, they were found to interfere with the normal decidualization process, which leaves us with no efficient and safe option to manage endometrial aging. Adapted with permission from Pathare et al., 2023.

A recent systematic review and meta-analysis evaluated the relationships between advanced maternal age (AMA) and uterine receptivity by combining the results of eighteen studies on oocyte donation (OD) cycles (Zhao et al., 2023). Significantly worse outcomes of clinical pregnancy rate (CPR), implantation rate (IR), miscarriage rate (MR) and live birth rate (LBR) in AMA groups emphasized the importance to explain the mechanisms behind the receptivity decline in AMA.

## 2.6. Diagnostic methods for endometrial disorders

To assess risks or confirm cases diagnostic markers are used. A biological marker can be any gene, protein, cell, molecule or even phenotypic feature that is strongly associated with biological condition and can help discriminate specific conditions from others in the search for the right diagnosis. Non-invasive approach is always the first choice in evaluating medical conditions. As for inner tissues, like endometrium, blood tests can provide some information about endometrial health. For example, measurements of anti-Müllerian hormone and inhibin B, can offer insights into ovarian and endometrial function (reviewed in Vujović et al., 2021).

Fertility assessment for women commonly starts with routine blood hormone level measures, ovulation testing with urine LH tests and ultrasonographic analysis of ovaries and endometrium during the luteal phase, usually on the day 20–21 of the normal menstrual cycle. If P4 levels are adequate or progestins are administered in HRT cycle, the endometrium exhibits specific features: thickness between 6–13 mm with no signs of uneven structures, such as polyps, leiomyomas (fibroids), hyperplasia or cancer (Momeni et al., 2011; Van Den Bosch et al., 2021). It was also established that blood P4 levels alone are not sufficient for the evaluation of endometrial maturation (Venetis et al., 2007). Magnetic resonance imaging can provide detailed images of the uterus and endometrium helping to identify structural abnormalities (Pintican et al., 2021). Because endometrial thickness ranges overlap between different stages of cyclic endometrial development, endometrial thickness alone is not a good predictor of endometrial receptivity (Momeni et al., 2011). Lately, 3D ultrasonography has become largely used to measure the uterine blood thickness and blood flow, as validated markers for uterine receptivity (M. Li et al., 2023).

Depending on the findings, further invasive procedures may be recommended to address specific issues. If implantation does not occur naturally or following hormonal stimulation, the endometrial biopsy analysis has been used to evaluate endometrial development since the early 50-s (Noyes et al., 1950). The analysis includes the presence or absence of specific histological markers corresponding to different developmental stages of endometrial tissue and can predict endometrial maturity with an accuracy of 4 days. Gland mitoses and pseudo-stratification of nuclei characterize the proliferative phase and are at their highest around ovulation, then decrease rapidly when active secretion begins. Basal vacuolization is seen during the early-secretory phase and active secretion and stromal oedema with no signs of stromal mitoses mark the mid-secretory endometrium. Pseudo-decidual reaction is evident during a late-secretory phase, and leukocytic infiltration marks the menstrual phase (Noyes et al., 1950). The accuracy of histological dating was critically revised in the late 90-s to early 2000-s (Murray et al., 2004) which instigated the rapid development of molecular analysis techniques. In 2007, 25 core receptivity genes were introduced based on microarray analysis as robust biomarkers of WOI (Horcajadas et al., 2007). Four years later, Endometrial Receptivity Array (ERA) test was launched to analyse the expression profile of 238 endometrial WOI markers as a promising diagnostic approach for personalized embryo transfer (PET) (Díaz-Gimeno et al., 2011). Since then, several endometrial receptivity tests have become available on the market, analysing different sets of endometrial receptivity markers. Several clinical studies then showed the effectiveness of PET after endometrial receptivity assessment (Díaz-Gimeno et al., 2013; Ohara et al., 2022; Ruiz-Alonso et al., 2013), whereas ERA test became the 'gold standard' of endometrial analysis during IVF. The main concern of the bulk tissue analysis was the fact that it only reflects the average expression of each marker across all endometrial cell types and the changes that occur only in rare cell types are eclipsed. In 2017, our group implemented RNA sequencing (RNA-seq) analysis of FACS-sorted endometrial epithelial and stromal cells, which manifested the differences in the expression of WOI markers in the dominant endometrial cell types (Altmäe et al., 2017).

### 2.6.1. Regulatory RNAs as potential molecular markers for endometrial receptivity

Early studies utilizing microarray technology reported protein-coding transcripts differentially expressed in endometrial tissue (Horcajadas et al., 2004). Although messenger RNA is responsible for the synthesis of respective proteins, the expression is also regulated by a variety of specific non-coding regulatory RNAs, such as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) (reviewed in Morris & Mattick, 2014). Their main function mechanism consists of complementary RNA binding resulting in its blockage (Faghihi & Wahlestedt, 2009). miRNAs are approximately 20–25 bp long, whereas lncRNAs are longer than 200 bp. Regulatory RNAs also interplay with each other: lncRNA can be the source of miRNAs, while miRNAs can target lncRNA for the production of phased small interfering RNAs (phasiRNAs) (Meng et al., 2021).

miRNAs can act within the cells they are produced in or be extracted into the circulatory system to target gene expression in other tissues. For this reason, miRNAs are often found useful non- or minimally invasive molecular markers for several gynaecological diseases, including endometriosis (Bendifallah et al., 2023; Moustafa et al., 2020) and different types of cancer (reviewed in Metcalf, 2024).

### 2.6.2. The search for non-invasive molecular markers for endometrial receptivity

The main limitation of endometrial molecular testing is the procedure to obtain endometrial sample. A speculum is inserted into the vagina as during the routine checkup and a local anaesthetic may be used to numb the cervix. Next, a thin, hollow tube called a Pipelle or a suction curette is introduced through the cervix into the uterine cavity. Several small samples of the endometrial tissue are then obtained by either rotating the Pipelle or creating suction with the suction curette. The healthcare provider may swab or scrape the uterus lining to ensure adequate sampling. Minor cramping, similar to menstrual cramps, may occur during or after the procedure. Minor health risks associated with endometrial sampling, such as bleeding and inflammation, which may compromise fertility within the subsequent menstrual cycle, which motivated researchers to seek for non-invasive markers for endometrial receptivity (Edgell et al., 2013). Blood and uterine fluid samples were analysed to identify specific biomarkers of WOI, and the results are somewhat controversial. Transcriptomic studies proposed a set of DE transcripts in the uterine fluid during WOI, confirming that the upregulation of many well-known receptivity genes, e.g. *PAEP*, *LIF*, *S100P*, *SPP1* and *GAST*, are promising non-invasive markers (Chan et al., 2013). The review by Salamonsen group raised issues regarding the validation of non-invasive transcriptomic markers for WOI (Edgell et al., 2013). Later the same group published the results of their proteomic analysis, showing that five proteins (PIGF, IL8, sGP130, sFlt and CSF3) were found elevated in the uterine fluid of mid-secretory endometrium of infertile women compared to fertile, where CSF3 appeared concentration ad glycosylation-dependent (Edgell et al., 2018). Our group reported using mass spectrometry technology that PGR, NNMT, SLC26A2 and LCN2 proteins from uterine fluid have a high predictive value of WOI (Kasvandik et al., 2020). Two independent transcriptomic analyses of circulating blood showed the higher levels of micro-RNAs (miRNAs) miR-31 and lower levels of Wnt/ß-catenin regulating micro RNA miR-200c, suggesting their non-invasive marker potential (Kresowik et al., 2014; Q. Zheng et al., 2017). Due to the different protein and transcript analysis methods, no consensus is still reached, and, to this date, no validated commercial non-invasive endometrial receptivity test available on the market.

## 2.7. The current management of endometrial disorders in young and advanced reproductive age women

Following the rise of endometrial receptivity testing and a constant debate on whether endometrial WOI timing is an important fertility factor causing RIF in normally ovulating women, it was suggested that WOI shift alone is accountable for at least one fifth of all endometrial disorders and more than half of endometrial issues included both WOI shift and pathological conditions (Sebastian-Leon et al., 2018). When endometrial receptivity was yet poorly understood, for many years, endometrial scratching was used to improve endometrial lining, with or without HRT, in women of young reproductive age. The procedure involved an intended ‘scratch’ of the uterine cavity to promote its improved regeneration. After many years of use, the procedure proved inefficient and was abandoned by most reproductive care physicians (Metwally et al., 2022).

In 90-s, advanced maternal age (AMA) was considered at least 40 years old which was associated with the ovarian decline (Zhao et al., 2023). Although ovarian function decreases rapidly in the early 40-s, the socioeconomic reasons and rapid advancement of IVF techniques revised our understanding of reproductive lifespan of women. Decades ago, multiple embryo transfer was the only option to increase fertility rate in older reproductive age women, for whom time was an essential negative factor for implantation success. This resulted in multiple pregnancies, which were a major risk for pregnancy complications, as reviewed in (Pinborg, 2005), promoting the use of PGT and personalised single-embryo transfer, where chances of implantation were improved using embryo and endometrial testing.

Successful use of HRT for endometrial preparation before embryo transfer in OD cycles since early 90-s provided controverting results on whether AMA affects endometrial receptivity (Abdalla et al., 1997; Borini et al., 1996; Check et al., 1995; Flamigni et al., 1993; Navot et al., 1994). Later studies focusing on endometrial aging demonstrated the significant drop of PR and IR from the age of 45 (Soares et al., 2005). Although it seemed that age was affecting endometrial health, it was shown that there is no difference in PR or live LBR between natural cycles and different endometrial preparation protocols in women of AMA (Liu et al., 2019). Recent molecular studies suggested the genes and pathways affected by woman’s age in the endometrial tissue and described specifically the accumulation of gene mutations in endometrial epithelium (Devesa-Peiro et al., 2022; Moore et al., 2020). However, there is still no good markers for the prediction of treatment outcome in advanced-age patients, which keeps this research going.

## 2.8. Rationale of the study

Female infertility is a global health issue, affecting the quality of life of couples. Its increasing trend highlights the continuous need for improved diagnostic and treatment approaches for female infertility. Whereas endometrial-factor infertility is responsible for a small proportion of infertility cases, it adds up to other causes and, paradoxically, being widely studied remains largely unexplained. One of the main reasons affecting the outcome of endometrial testing is the heterogenous nature of endometrial biopsy, and the invasiveness of the sample obtaining procedure. Over the past 15 years, the molecular mechanisms of endometrial receptivity have been studied in the bulk tissue and isolated endometrial cell populations aiming to explain crucial processes involved in embryo implantation on the uterine side. A set of robust transcriptomic markers evidently participating in the implantation process were identified and are routinely used for endometrial tissue analysis along with other robust uterine tissue assessment methods. However, it is still unknown how endometrial cellular composition affects the biopsy testing results and whether specific changes of dominant and rare cell type populations can predict endometrial health. Also, a debate on whether endometrial biopsy procedure required for tissue analysis is safe for the use in IVF cycles, has brought about the search for alternative, non-invasive endometrial receptivity markers. Some studies have proposed potential robust receptivity markers from samples of non-invasive origin, like urine, saliva, endometrial fluid, etc. However, none of them proved effective predicting endometrial maturation state.

Another important issue concerns the fact that endometrial receptivity studies analysed exclusively women of normal reproductive age (20–45 years), whereas the proportion of women of advanced reproductive age keeps constantly rising. It is unknown whether the endometrium exhibits specific molecular and cellular changes that occur with age, and whether they impact endometrial function. As most of AMA women undergo IVF with hormonal replacement therapy, often using oocytes from younger donors, and transcriptomic endometrial tests are commonly used to assess the effectiveness of hormonal treatment, it is important to understand whether endometrial preparation protocols initially developed for young women are equally effective and safe for AMA patients.

# 3. AIMS OF THE STUDY

The main goal of the study was to develop robust methods to assess endometrial receptivity.

Therefore, the specific aims were:

1. To identify significant and robust molecular and cellular biomarkers for the window of implantation.
2. To research non-invasive transcriptomic markers for endometrial receptivity and recurrent implantation failure from circulating blood.
3. To investigate the molecular and cellular profile of endometrium from women of advanced reproductive age and identify potential biomarkers for endometrial aging.

# 4. MATERIALS AND METHODS

## 4.1. Study participants

All studies were approved by the Research Ethics Committee of the University of Tartu (Tartu, Estonia: approval No. 22/M31 and 340–12), Ethical Clinical Research Committee of IVI Clinic (Valencia, Spain; No. 1201-C-094-CS) and the Ethics Committee of the Faculty of Biology of the Plovdiv University (Plovdiv, Bulgaria, No. 3/02.09.2019).

Reproductive-aged patients with unexplained infertility and healthy volunteers were recruited at Nova Vita and BioEximi fertility clinics (Tallinn, Estonia, Study I and II), IVI Clinic (Valencia, Spain, Study I and II) and Bulgarian Center for Woman's Health (Plovdiv, Bulgaria, Study III). All women donated the samples voluntarily and signed an informed consent. Anonymised samples were collected at the Competence Centre on Health Technologies (CCHT, Tartu, Estonia, Study III). The sample collection was carried out in accordance with all relevant regulations.

Blood hormonal levels were measured, and endometrial biopsy and blood samples were collected on days 1–3 and 7–9 after the LH surge of natural menstrual cycles (ESE and MSE, respectively), with no previous hormone administration during three months prior to sample collection. Recurrent implantation failure (RIF) group patients consisted of women diagnosed with primary or secondary unexplained infertility who had experienced at least three consecutive failed IVF cycles. The participants signed a written informed consent. Anonymised young and advanced reproductive age women's endometrial samples were collected in the hormonally induced cycles on the fifth day of progesterone treatment (P+5) by the CCHT (Study III) during routine endometrial receptivity assessment. The general characteristics of all study participants are presented in the Table 2**.**

In natural cycles (Study I and II), women performed a urine-based ovulation test to determine the luteinizing hormone (LH) surge (BabyTime® LH urine cassette, Israel). The day with a positive LH test is referred to as LH+0. In hormonally induced cycles (Study III), the first day of P4 administration is referred to as P+0. The progesterone was administered orally, vaginally or by injection. Endometrial samples were collected using Pipelle catheter (Laboratoire CCD, France). The effect of progesterone on the maturation of endometrial tissue was tested with endometrial receptivity assay of 68 common endometrial receptivity markers. All samples underwent histological evaluation according to Noye's criteria by a certified clinical pathologist. Following biopsy, all samples were immediately placed into RNA*later* solution (Ambion Inc., Texas, USA), frozen and stored at -86 until the analysis. Whole blood samples (Study II) were collected from Estonian and Spanish patients two different ways: PaxGene Blood RNA tubes (Qiagen, Germany) and tubes containing $K_2$-EDTA anticoagulant, respectively. Blood samples were frozen and stored at -86 until further use.

**Table 2.** Characteristics of study participants and samples.

| | Study I | | Study II | | Study III | |
|---|---|---|---|---|---|---|
| **Study groups** | ESE | MSE | Fertile | RIF | YMA | AMA |
| **Participants (n)** | 35 | 35 | 39 | 38 | 12 | 12 |
| **Age ±SD** | EST: 30.2±3.4<br>ESP: 29.1±3.6* | | EST: 30.2±3.3<br>ESP: 29.4±3.6 | EST: 35.1±3.9<br>ESP: 36.7±3.3 | 25.17±1.23 | 47.92±1.16 |
| **BMI ±SD** | EST: 23.2±4.5<br>ESP: 23.2±2.9* | | EST: 23.1±4.2<br>ESP: 23.2±2.8 | EST: 22.3±2.3<br>ESP: 25.2±5.3 | 23.86±4.06 | 22.89±3.09 |
| **Cycle type** | natural* | | natural | natural | HRT | HRT |
| **Cycle day** | LH+1-3 | LH+7-9 | LH+1-3,<br>LH+7-9 | LH+7-9 | P+5 | P+5 |
| **Sample type** | endometria | endometria | endometria (n=78),<br>blood (n=78) | endometria (n=38),<br>blood (n=36) | endometria | endometria |
| **Analysis** | transcriptome | | miRNA | | transcriptome (n=12), miRNA (n=11), IHC (n=4) | |
| **Validation samples (n)** | 10 | 10 | 8 | 8 | HRT: 10 | HRT: 10 |
| | | | | | NC: YMA (6), IMA1 (6), IMA2 (6) AMA (6) | |
| **Total samples (n)** | **45** | **45** | **164** | **82** | **68** | |

* Study I compared the endometrial samples obtained at two time points from the same patients.

Age is presented as mean years ± standard deviation (SD), BMI is presented as mean kg/m2 ±SD, n – number of samples, EST – Estonian samples, ESP – Spanish samples, ESE – early secretory endometrial phase, MSE – mid-secretory endometrial phase, RIF – recurrent implantation failure, YMA – young maternal age, AMA – advanced maternal age, HRT – hormonal replacement therapy, LH – luteinizing hormone surge day, P – the first day of progesterone administration, IHC – immunohistochemistry, NC – natural cycles.

To associate age with endometrial transcriptome reflecting its receptivity status, endometrial transcriptomic profiles of 1249 anonymised mid-secretory samples collected on day P+5 from women aged between 20 and 60 years with assigned receptivity status: ‘pre-receptive’, ‘early receptive’, ‘receptive’, ‘late receptive’ and ‘post-receptive’. The profiles were categorised according to their classified receptivity status and compared between age groups of patients in their 20-s, 30-s, early 40-s, and over 45 years. The receptivity marker analysis was previously performed at CCHT as a part of routine endometrial assessment test (beREADY, (Meltsov et al., 2023). The Chi-squared test for trend in proportions was used to test whether the proportion of patients with any of the classified receptivity statuses changes linearly with age.

## 4.2. Gene expression analysis of endometrium and blood

### 4.2.1. RNA extraction from endometrial tissue and blood

To analyse gene expression, total RNA was first extracted from collected tissues. For endometrial whole-tissue transcriptome analysis, total RNA was extracted using 30 mg of the tissue was homogenized in the presence of QIAzol reagent (Qiagen, Germany) and mechanical lysis (0.5 mm diameter steel bead, TissueLyser LT, and processed using miRNeasy Mini kit (Qiagen), following the manufacturer’s protocol (Study I, II and III). miRNA fraction was enriched by adding a small RNA extraction step using RNeasy MinElute kit (Qiagen) according to manufacturer protocol (Study II). Blood RNA was extracted using PaxGene Blood RNA kit (Qiagen) in Estonia, and miRNeasy Mini Kit following purification with RNA Cleanup kit (Qiagen) in Spain (Study II). DNase I treatment was performed to eliminate DNA residues, and the quality of purified RNA was assessed by measuring concentration and RNA integrity.

### 4.2.2. RNA sequencing and data analysis

Following quality assessment, RNA was reverse transcribed and sequencing libraries were synthesized according to TruSeq Stranded mRNA technology (Study I and III, Illumina, USA) and TruSeq Small RNA sample preparation guide (Study II, Illumina, USA). The sequencing was carried out in Estonia and Spain in batches on Illumina HiSeq 2000/2500 for Study I and II, and NextSeq 550 in Netherlands (Study III). After quality control (QC), adaptor trimming and mapping, the fragments were counted and assembled fragment count matrices were compared to identify DEGs for each comparison.

For miRNA differential expression analysis, endometrial and blood miRNA-enriched samples (Table 2) were subjected to small RNA sequencing (Study II). Small RNA libraries were constructed following the TruSeq Small RNA Library Preparation Guide (Illumina, USA). As input, 1 µg of total RNA or small RNA fraction was used. Final purification step of 12-plex cDNA libraries was performed by manually selecting libraries corresponding to the length of inserted miRNAs (area between 145–160 bp) using gel electrophoresis (6% Novex TBE gels, Thermo Fischer, USA). Libraries were quantified and validated with Agilent 2100 Bioanalyzer (Agilent Technologies), normalised, pooled, used for cluster generation on the cBot and sequenced on HiSeq 2000/2500 with a configuration of 50 cycles single-end sequencing following manufacturer's instructions (Illumina) in Spain (ESP samples) and in Estonia (EST samples). Obtained small RNA reads passed QC and adapter trimming, and the data were deposited to GEO database under accession no GSE108966.

### 4.2.3. Differential expression analysis of endometrium during WOI

To identify DEGs and miRNAs associated with WOI, differential expression analyses were performed for total RNA reads ESE vs MSE (Study I and II); for RIF-specific genes the DEGs were compared between healthy and RIF groups from MSE phase (Study II). To identify circulating miRNAs that can predict WOI from a blood sample, expressed miRNAs were compared in groups between endometrium and blood. Age-associated genes were identified by comparing the expression profile of YMA with AMA group samples (Study III). Functional analysis of protein-coding genes was performed with g:Profiler and molecular interaction networks were conducted with Ingenuity Pathway analysis (IPA; Study I and III).

To ensure the significance of our findings, experiment batch correction, meta-analysis of samples from different experiments and multiple testing correction for the significance level were applied in all studies. A more detailed overview of the bioinformatic analysis for each study is described in the Study I, II and III. The bulk RNA-seq and deconvolution analyses were performed in collaboration with Viktorija Kukushkina (University of Tartu, Estonia) and Triin Laisk (University of Tartu, Estonia) for Study I. The single-cell RNA-seq analyses for Study III were driven by Darina Obukhova and Associate Professor Masoud Zamani Esteki (Maastricht University, Netherlands). miRNA analyses were curated by Agne Velthut-Meikas (Tallinn University of technology, Estonia; Study II) and Veselin Baev (University of Plovdiv, Bulgaria; Study III).

### 4.2.4. Tissue deconvolution analysis

Study I was the first study to utilize computational deconvolution to assess the proportions of endometrial cells in bulk tissue samples as an alternative to costly and time-consuming tissue dissociation techniques. To calculate the proportions of epithelial and stromal cells, the two dominant cell types of endometrial tissue, the gene expression profiles of isolated epithelial and stromal cell populations from Altmäe et al. (2017) were used as a reference. In Study III six endometrial cell type-specific gene expression profiles were available from the recently published single-cell transcriptomic atlas of human endometrial tissue (Wang et al., 2020), which allowed us to calculate the proportions of minor endometrial cell types, such as immune, endothelial cells and epithelial multiciliated cells (MCCs).

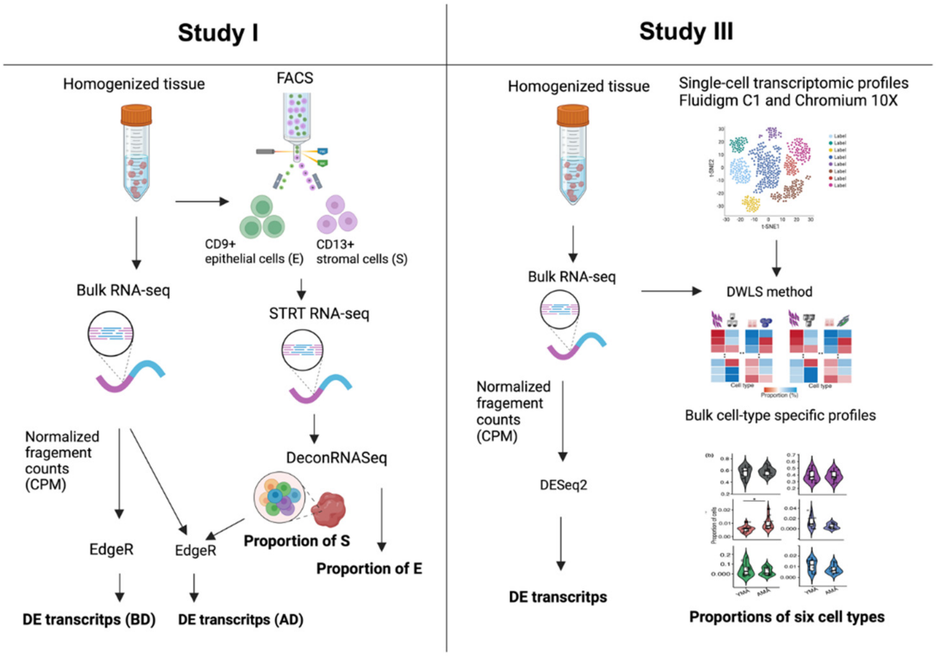


**Figure 6**. The overview of the computational deconvolution analysis. Here we describe two approaches to decompose the complex endometrial tissue using cell type-specific transcriptomic profiles. In Study I, only dominant cell types, FACS-sorted epithelial (E) and stromal (S) cell populations, were sequenced using the single-cell tagged reverse transcription (STRT) protocol with modifications. DeconRNASeq was used to calculate the proportions of E and S during ESE and MSE phases. The proportion of S was used as a covariate in differential expression (DE) analysis in combination with edgeR to adjust the transcriptomic profile of endometrial tissue for the differences in the proportions of epithelium and stroma between ESE and MSE. In Study III, single-cell transcriptomic profiles of six endometrial cell types were utilized from previously published dataset by Wang et al., 2020. The proportions of each cell type were calculated using dampened weighted least squares (DWLS) method from bulk transcriptomic profiles of young (YMA) and advanced maternal age (AMA) samples. The outcomes of both analyses are presented in bold. BD – before deconvolution, AD – after deconvolution. The image is created with BioRender.com

The overview of two types of deconvolution analysis is presented in Figure 6. Briefly, after mapping and fragment counting, the mixed tissue normalised expression levels are compared with normalised expression levels from pure cell fractions. In Study I, R package DeconRNASeq v.1.10.0 was used, which adopts a globally optimized non-negative decomposition algorithm through quadratic programming to estimate cell-type proportions in the heterogeneous tissue (Gong & Szustakowski, 2013). The transcriptomic profiles of bulk tissue were then adjusted to changes in the proportions of epithelium and stroma between ESE and MSE, by using the stromal cells transcript matrix obtained from deconvolution analysis as a covariate in the DE analysis using edgeR. Only stromal cell proportion was used as epithelial cells proportion was considered 1 – the proportion of stromal cells. In Study III, dampened weighted least squares method (*DWLS* R package v.0.1.0) was used as it shows high accuracy in rare cell types detection and the best performance among deconvolution methods that use single-cell RNA-seq data as input (Tsoucas et al., 2019). The results of the deconvolution analysis were then compared to the results of histological evaluation of endometrial samples. In Study I the proportions of epithelial cells were compared to the proportions obtained from manual epithelium and stroma compartments calculations and correlated with histological dating using Noye's criteria. In the Study III, the significantly altered proportions were validated with IHC staining analysis.

## 4.3. Histological analysis of endometrial tissue

The study focuses on the prediction of the most receptive phase of endometrial development and the factors that may affect it. To ensure the adequate assessment of the endometrial development stage under different physiological conditions, all endometrial samples underwent histological evaluation by a clinical pathologist. To perform this, microscope slides with tissue sections (4 µm, 3 sections per slide) were prepared at the Pathology Department of Tartu University Hospital (Tartu, Estonia) from formalin-fixed paraffin-embedded (FFPE) endometrial tissue sections using standard hematoxylin and eosin staining protocol (Fischer et al., 2008). The slides were scanned at East Tallinn Central Hospital, using a 3DHistech Panoramic Flash III 250 scanner (3DHistech, Budapest, Hungary) at a 20× magnification. Evaluation of cyclic endometrial changes was performed according to Noyes criteria (Noyes et al., 1950).

For the validation of deconvolution analysis, the histological evaluation of epithelial and stromal compartments of endometrial tissue during ESE and MSE was performed manually from hematoxylin and eosin-stained tissue (Study I). The size of the selected areas was converted to pixels, and the proportion of epithelial cells was calculated by dividing the area under epithelial cells (in pixels) to that of the entire specimen. The analysis included a total of 13 specimens (eight ESE and five MSE), as for some individuals, more than one image was available for evaluation. In case of multiple measurements for one biopsy, the average proportions were calculated across evaluations. The proportions obtained from histological analysis were then compared to the proportions calculated during deconvolution. To test the accuracy of the estimated proportions of stromal and epithelial cells resulting from deconvolution, we correlated the epithelial fraction estimates with estimated endometrial cycle day (corresponding to a 28-day cycle) from histological analysis. For this, we used the histological evaluation data for 18 paired samples (nine from ESE and nine from MSE phase) for which detailed dating information by clinical pathologist was available. More detailed description of histological analysis is found in Study I.

To visualize and quantify the expression of cellular senescence marker p16$^{INK4a}$ protein in endometrial tissue, FFPE tissue sections on slides were processed using the 3,3'-Diaminobenzidine (DAB)-based Master Polymer Plus Detection System IHC Kit (Master Diagnostica, Spain). Incubation with primary antibody was carried out overnight in a humidity chamber at 4°C. All steps were carried out according to manufacturer's protocol. All used antibodies, their clonality, dilutions and detailed workflow of IHC analysis is described in the Study III. Briefly, slides were scanned using Leica SCN 400 Slide Scanner (Leica Biosystems) with a maximum of 20x magnification objective and semi-quantitative analysis of three different areas of scanned sections was conducted with ImageJ package Fiji (v1.52e) (Schindelin et al., 2012) in relation to DAB signal intensity, which was measured separately for stromal, glandular epithelium and surface epithelium of endometrium. The Wilcoxon Mann-Whitney test was performed to determine statistical significance.

For the validation of MCC proportion obtained from the deconvolution analysis of endometrial tissue, the basal body (BB) staining was performed using cilium basal body specific protein LhS28 and multiciliated cells (MCCs) were visualised by staining acetylated α-tubulin (AcTubA) concentrated in the axonemes of endometrial epithelial cells, as previously described. The BB and MCC counting was performed blinded by two researchers using anti-LhS28- and anti-AcTubA-stained endometrial tissue slides and the average amounts of ciliated cells between two measurements were estimated. Unpaired sample student's t-test was applied to test statistical significance between the two groups (Study III).

## 4.4. Validation of RNA-seq using RT-qPCR

To validate the results of RNA-seq analysis, selected DE transcripts were quantified and then DE analysis was performed using RT-qPCR. For protein-coding and long non-coding RNA (lncRNA) genes (Study I and III), the total RNA was reverse-transcribed using RevertAid First Strand cDNA kit (Thermo Scientific, USA; Study I) or Maxima First Strand Synthesis kit (Thermo Scientific; Study III) and qPCR reaction was carried out with 20ng of template cDNA, 250ng of forward and reverse primer and 1x HOT FIREPol EvaGreen qPCR mix with ROX internal reference dye (Solis BioDyne, Estonia) on 7500 Fast Real-Time PCR System (Applied Biosystems, USA). The average levels of *GAPDH*, *TBP* and *SDHA* were used internal reference for RNA levels normalization.

For novel RIF-associated miRNA (chr2_4401) validation (Study II), custom TaqMan Small RNA assay (Thermo Fisher Scientific) was used with eight paired early secretory (ES) and mid-secretory (MS) endometrial samples from fertile women and eight MS endometrial samples from RIF women. The average levels of miR-151a-5p and miR-196b-5p were used as a reference for normalization, based on their stable expression levels according to the current small RNA sequencing data. Additionally, five paired endometrial stromal (CD13$^+$) and epithelial cell (CD9$^+$) samples (two from ESE and three from MSE phase), isolated by FACS in our previous study (Altmäe et al., 2017), were used to determine the cell type specificity of the novel miRNA. cDNA synthesis was conducted with TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) and quantitative real-time polymerase chain reaction (RT-qPCR) was performed with TaqMan Universal PCR Master Mix, No AmpErase UNG (Thermo Fisher).

The results of DE analysis were validated with RT-qPCR using validation samples obtained from patients other than study participants (Table 2). All real-time qPCR experiments were performed in duplicate. Gene expression fold changes (FC) were calculated according to $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Relative miRNA expression levels were compared between the studied groups by paired (ESE vs. MSE) or unpaired (fertile vs. RIF) two-tailed t-test and p-value $\leq 0.05$ was considered as significant. The oligonucleotides for validation of selected genes and miRNA are presented in Table 3. All analyses were performed blindly of their study group status.

**Table 3.** Oligonucleotides used for differential expression analysis in the study.

| Gene | Forward primer | Reverse primer | PrimerBank accession no* /Reference | Study |
|---|---|---|---|---|
| *MYOCD* | ccacctatggactcagccta | ctcagtggcgttgaagaagag | 226423888c3 | Study I |
| *LINC01320* | cctgtcatctccgatgcataa | ccccaggtcagagaatacgac | Suhorutshenko, M. et al., 2018 | Study I |
| *SLC8A1* | acaacatgcggcgattaagtc | gctctagcaattttgtcccca | 115529447c1 | Study I |
| *TRPC4* | aaaagcccacttggactgtt | gacctgtcgatgtgctgagag | 209863023c2 | Study I |
| *GGTA1P* | agaggagaccaaaggaaggaaa | ggattaaaccagtcccatagcc | 15824501a1 | Study I |
| *EML5* | ctgaacgagtgttggtagca | tcaaacgccaagcaagctatac | 291190807c2 | Study III |
| *ALDH3A1* | tgttctccagcaacgacaagg | agggcagagagtgcaaggt | 206597438c3 | Study III |
| *SPAG6* | gtgaagtgcgacattcttcca | tccacaatcgactattgcctga | 359718952c2 | Study III |
| *PPP1R1B* | caagtcgaagagacccaaaccc | gcctggttctcattcaaattgct | 111335057523c1 | Study III |
| *STC1* | gcaggaagagtgctacagcaag | cattccagcaggcttcggacaa | Aghajanova et al., 2016 | Study III |
| *TBX15* | atgtgattcgcaaagacttcagc | gataggccgtaactgtggtga | 55770859c3 | Study III |

* https://pga.mgh.harvard.edu/primerbank

# 5. RESULTS

## 5.1. The proportions of epithelium and stroma during ESE and MSE

To calculate the proportions of endometrial cell types, we utilized the modern method for analysis of gene expression in the cells of heterogenic tissues, single-cell RNA-seq in combination with the novel computational deconvolution analysis. In Study I, the computational deconvolution was first used to unravel the composition of endometrial tissue from the whole-tissue transcriptome. The main advantage of the deconvolution method is the possibility to accurately estimate cell type proportions using reference gene expression matrix of cell types of interest without the need to label, sort and perform RNA-seq for each separate experiment. Using the deconvolution method and gene expression matrix of endometrial CD9+ epithelial and CD13+ stromal cells from our previous work (Altmäe et al., 2017), it was identified that epithelial and stromal cell proportions shift between ESE and MSE. The results are presented in Figure 7.

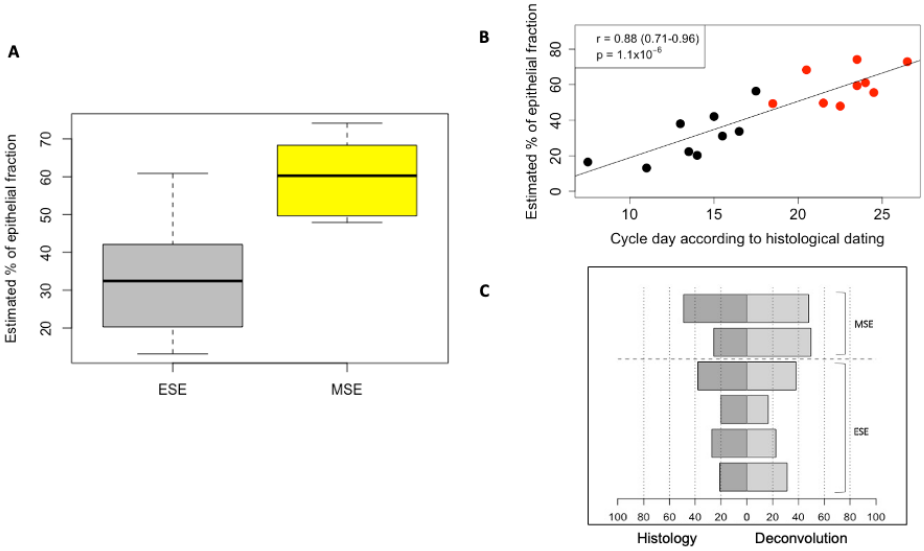


**Figure 7.** Tissue deconvolution results. A. Proportions of epithelial cells in biopsy estimated by computational deconvolution approach. B. Correlation between gene expression-estimated proportion of epithelial cells and menstrual cycle day predicted using Noyes' criteria for endometrial histological dating. Black dots – ESE samples, red dots – MSE samples, according to urine LH test. C. Comparison between epithelial cell proportions estimated using two different approaches, histological evaluation of epithelial fraction (dark grey) and proportions calculated based on cell-type specific gene expression patterns (light grey).

## 5.2. The effect of cellular heterogeneity on the transcriptomic profile of endometrial tissue

The transcriptomic analysis of endometrial tissue is only informative if it truly reflects the molecular processes taking place in the endometrial cells and can be used to identify the differences of interest. Therefore, any possible physiological and technical bias can be an issue in the endometrial tissue analysis.

In Study I, we aimed to analyse the transcriptomic changes in human endometrium between ESE and MSE stages of endometrial maturation during the menstrual cycle. Total RNA-seq was used to analyse 70 endometrial samples and the results are presented in Figure 8. When the expressed transcripts were compared between the two stages, it resulted in 3591 DEGs (Figure 8A).

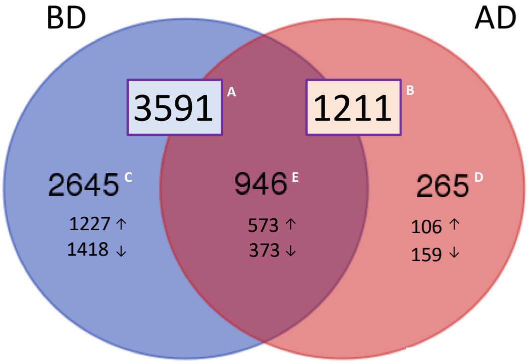


**Figure 8**. Numbers of differentially expressed genes (DEGs) before and after adjustment to cellular composition was performed. The significant transcripts were identified from meta-analysis of transcriptomic profiles of endometrial tissue samples from different groups (Bonferroni p-value $< 2.79 \times 10^{-6}$). A. Transcripts identified in whole endometrial tissue (before deconvolution, BD; n=3,591) B. Transcripts identified in cellular heterogeneity-adjusted expression profiles (after deconvolution, AD; n=1,211). C. Transcripts significant only in whole-tissue transcriptome and non-significant after deconvolution (blue, n=2,645) were considered genes, whose expression change in whole-tissue may be derived from variation in cell types proportions. D. Transcripts significant only in after tissue deconvolution (pink, n=265) were considered genes whose expression change may be eclipsed in mid-secretory endometrium due to fluctuations in cell type proportions, leading to underestimation of molecular processes and pathways involved in endometrial maturation. E. Transcripts significant in both approaches (purple, n=946) are considered potential WOI markers.

Deconvolution analysis using CD9+ epithelial and CD13+ stromal cells population-specific transcriptomic profiles from ESE and MSE identified endometrial and stromal cell proportions during ESE and MSE. This was found to affect the endometrial transcriptomic profile used for the assessment of endometrial receptivity. After the transcriptome was adjusted to stromal cell proportion variation between two phases, differential expression analysis resulted in 1211 DEGs (Figure 8B). A total of 946 (78%) of these genes were identified as DEGs before the adjustment to cellular composition (Figure 8E), and 265 genes (22%) were new, which means the least did not come out significant during the previous whole-tissue analysis (Figure 8D). It also appeared that only 34% of the genes from whole-tissue analysis (Figure 8A) remained significant after the adjustment to tissue cellular composition, whereas the remaining 2645 genes (66%) upraised from the artifact of tissue cellular heterogeneity and might not be truly up- or downregulated in endometrial biopsy during WOI (Figure 8C).

The genes, that exhibited significant expression changes between ESE and MSE in both whole-tissue (BD) and stromal cell proportion-adjusted transcriptomes (AD), were characterized as genes with true expression change. The top highly differentially expressed and top significant genes are presented in the outer and inner circle the circos plot (Figure 9) with 26 of these genes found in both circles. These genes are likely to play an important role in the endometrial maturation for embryo implantation and possess the highest marker potential for true assessment of WOI.

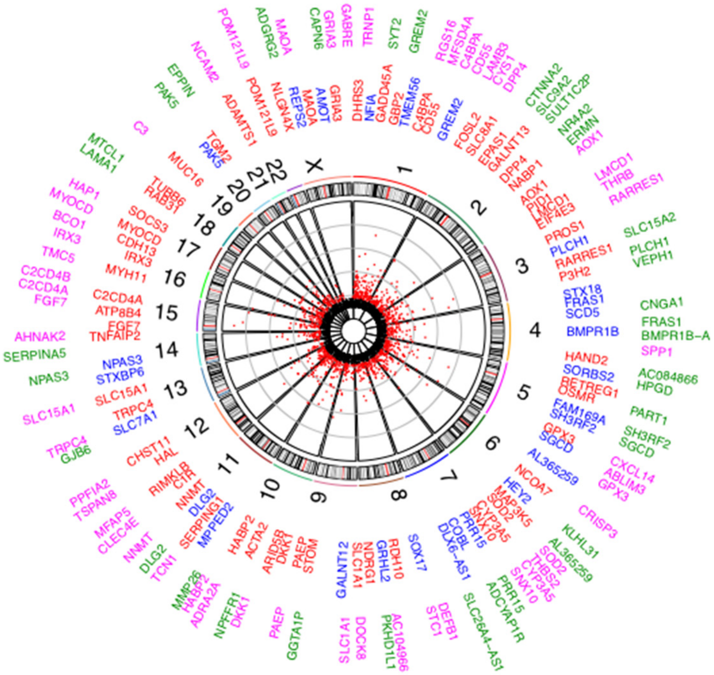


**Figure 9**. Top 90 differentially expressed genes (DEGs) between early secretory (ESE) and mid-secretory (MSE) endometrial samples. Scatterplot represents DEGs significant after deconvolution (Bonferroni p-value $<2.79 \times 10^{-6}$). Numbers represent chromosomes. Inner circle gene names contain top significant DEGs (red – up-regulated and blue – down-regulated in MSE, respectively), while outer circle contains DEGs with the highest expression rate fold change between ESE and MSE (pink – up-regulated and green – down-regulated in MSE, respectively).

Adjusted endometrial transcriptome between ESE and MSE revealed that several genes previously used for assessment of endometrial receptivity, such as *OLFM4* and *POSTN*, can be affected by the fluctuations of cellular composition during the transition to the receptive state. The Table 4 summarizes the top DE genes, whose expression change occurred unsignificant after adjustment for tissue cellular heterogeneity. The study also identified a set of novel genes, such as long non-coding RNA LINC01320, whose role in the endometrial cyclic maturation must be further confirmed.

**Table 4**. Top significant DEGs in endometrial whole-tissue samples, that remained sub-significant after adjustment to tissue cellular composition, arranged by fold change (FC). P-value—Bonferroni P-value.

| Gene | Name | FC | P-value |
|---|---|---|---|
| *OLFM4** | Olfactomedin 4 | -15.8 | $9.02x10^{-33}$ |
| *LEFTY1* | Left-right determination factor 1 | 13.35 | $6.02x10^{-35}$ |
| *LHFPL* | LHFPL tetraspan subfamily member 3 | 14.04 | $1.04x10^{-30}$ |
| *BRINP1* | BMP/retinoic acid inducible neural specific 1 | 12.58 | $4.87x10^{-23}$ |
| *POSTN** | Periostin | -12.48 | $5.11x10^{-39}$ |
| *SLC47A1* | Solute carrier family 47 member 1 | -11.87 | $7.01x10^{-44}$ |
| *FGF1* | Fibroblast growth factor 1 | -10.97 | $2.1x10^{-49}$ |
| *GABRA3* | Gamma-aminobutyric acid type A receptor alpha3 subunit | -9.52 | $1.83x10^{-52}$ |
| *COL17A1* | Collagen type XVII alpha-1 chain | 8.9 | $4.96x10^{-30}$ |
| *TMEM154* | Transmembrane protein 154 | 8.68 | $2.42x10^{-40}$ |

*genes utilized for assessment of endometrial receptivity from biopsy.

## 5.3. miRNA expression in MSE and blood of RIF patients

In the search for non-invasive endometrial biomarkers, endometrium and blood were compared. In Study II, to identify ESE and MSE-specific miRNAs, 116 endometrial samples from Estonia (EST) and Spain (ESP) were included for miRNA data analysis (Table 2). The analysis identified 615 and 624 miRNAs from endometrial samples among the EST cohort and ESP cohort, respectively (CPM of at least 1 in 75% of samples within a sample group of ES or MS of the fertile group, or RIF). The most highly expressed miRNAs in endometrial tissues from both cohorts were miR-10b-5p, miR-10a-5p and miR-27b-3p. Among fertile women, 91 DE endometrial miRNAs were confirmed from MSE vs ESE (Figure 10A) in both EST and ESP. The comparison of MS endometrial samples from RIF patients to fertile women in EST and ESP datasets revealed 21 DE miRNAs (Figure 10B).

miR-424-5p was the only DE miRNA present in both comparisons—between the ES and MS endometria of fertile women as well as between fertile women and RIF patients. Interestingly, while down-regulated in the MSE of fertile women (average FC = −1.87 between the two cohorts), it was up-regulated in RIF patients' samples from the same phase (average FC = 1.74). When predicting miR-424-5p targets from our mRNA dataset, the detected targets genes were involved in different canonical pathways important in MS endometrial functions such as glucocorticoid, insulin receptor, axonal guidance and interleukins signalling.

Further, blood samples were analysed to identify the respective miRNAs. Altogether, 65 blood samples from EST and 49 samples from ESP validation cohort were included for miRNA data analysis (Table 2). Using two different miRNA isolation techniques, 305 miRNAs were detected from blood samples among EST cohort and 710 miRNAs among the independent ESP cohort. Possibly due to the differences in sample treatment protocols between the EST and ESP, the most abundant miRNAs in blood varied between the two cohorts: miR-486-5p, miR-92a-3p and miR-451a demonstrated the highest read counts among EST blood samples, while miR-26a-5p, miR-191-5p and miR-181a-5p exhibited the highest expression levels among ESP samples.

No DE miRNAs were detected between blood samples corresponding to ES and MS cycle phase in fertile women either in the EST dataset or in the validation ESP dataset (Figure 10C). The comparison of blood samples corresponding to MS cycle phase from fertile and RIF women revealed that miR-30a-5p was significantly up-regulated among RIF patients in EST cohort (FC = 3.0, FDR = 0.01); and the difference was also confirmed in ESP cohort (FC = 1.9, FDR = 0.03) (Figure 10D).

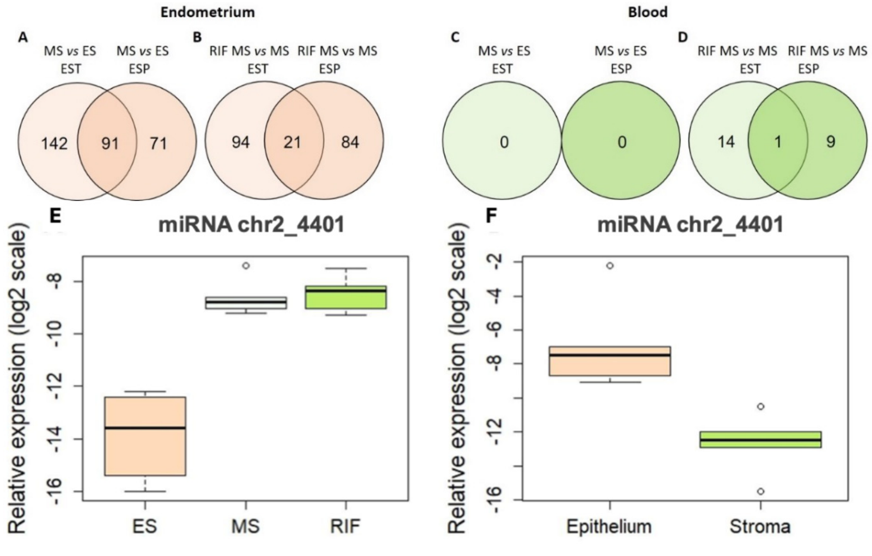


**Figure 10**. The numbers of differentially expressed (DE) miRNAs in Estonian samples and validation cohort from Spain presented as four comparisons. A. DE miRNAs between MS and ES phase endometrial samples of fertile women. B. DE miRNAs between MS phase endometrial samples from infertile recurrent implantation failure (RIF) patient and fertile women. C. DE miRNAs between MS and ES phase blood samples of fertile women. D. DE miRNAs between MS phase blood samples from RIF patients and fertile women. E. Validation of the novel miRNA chr2_4401 by quantitative real-time poly-

merase chain reaction (RT-qPCR) using paired ES and MS samples (n = 8). miRNA expression level of chr2_4401 changes between ES and MS endometria of fertile women, and MS endometria of RIF patients (n = 8). The relative miRNA expression levels (ΔCt) in the endometrium were 37-fold higher in MS compared to ES samples (p = 0.0001). No differences between MS samples of fertile and RIF patients were observed. F. Relative expression level differences in epithelial (n = 5) and stromal (n = 5) fractions. The relative miRNA levels (ΔCt) were 55-fold higher in epithelial cells (CD9+) compared to stromal cells (CD13+) (p = 0.003). ES - early secretory phase; MS – mid-secretory phase. EST – Estonian samples; ESP – Spanish samples; RIF – recurrent implantation failure; ES – early secretory phase; MS – mid-secretory phase. Adapted from Study II.

Out of 18 novel miRNAs, identified from endometrial and blood samples, chr2_1900 (sequence: aucugaaauuugaaauggucc) and chr16_22077 (aggcuaggcugggccacag) were detected only from MS endometrial samples (from 7 and 4 out of 73 MS samples, respectively) and chr2_4401 (gaacacugaaguuaauggcug) was found from the majority (75.3%; 55/73) of MS endometrial samples and only from 2.7% (1/37) of ES endometrial samples. chr14_10307 (ucugagcccuguucucccuagg) was uniquely determined from blood samples of 16.7% RIF patients (4 out of 24 RIF blood samples). We further focused on the most promising novel miRNA chr2_4401, detected from most MS samples. Validation analysis by RT-qPCR confirmed the differential expression of chr2_4401 showing that the level of this novel miRNA was 37-fold higher (p = 0.0001) in MS compared to ES endometrium in fertile women with no statistically significant differences in the expression levels of chr2_4401 between the MS endometrial samples from fertile and RIF women (Figure 10E). Cell type-specific expression analysis showed very low levels of chr2_4401 in endometrial stromal cells, but 55-fold upregulation in epithelial cells was detected (p = 0.003, Figure 10F).

The structure of miRNA chr2_4401 identified in revealed sequence similarity with five previously well-described miRNAs miR-141-3p, miR-200a-3p, miR-200b-3p, miR-200c-3p and miR-429.

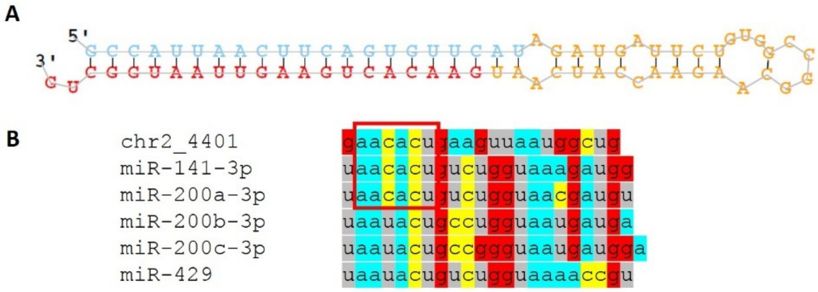


**Figure 11**. The structure of the novel miRNA chr2_4401 overexpressed in MSE endometrium. A. The hairpin structure of chr2_4401 miRNA precursor predicted by miRDeep2. B. Sequence alignment between chr2_4401 and five miR-200 family miRNAs. The seed region (nucleotides 2–7, red square) of chr2_4401 is identical with miR-141-3p and miR-200a-3p.

# 5.4. Endometrial transcriptome of advanced reproductive age women

Endometrial receptivity assays are developed to identify WOI in women of reproductive age, characterized by an age range between twenties and early forties. Little knowledge is available on the molecular profile of endometrial tissue in late forties, which is a rapidly increasing segment of ART patients. To focus on age-specific changes in the genome-wide transcriptomic analysis of endometrial tissue, we included only patients with assessed endometrial receptivity.

Initially, we compared the proportions of receptivity statuses of 1249 endometrial transcriptomic profiles assessed with beREADY algorithm (Meltsov et al., 2023) based on the targeted gene expression of 67 endometrial receptivity markers. As illustrated in the Figure 12, the proportion of 'receptive' endometrial samples appeared significantly lower in women of age over 45 compared to women in their 20-s.

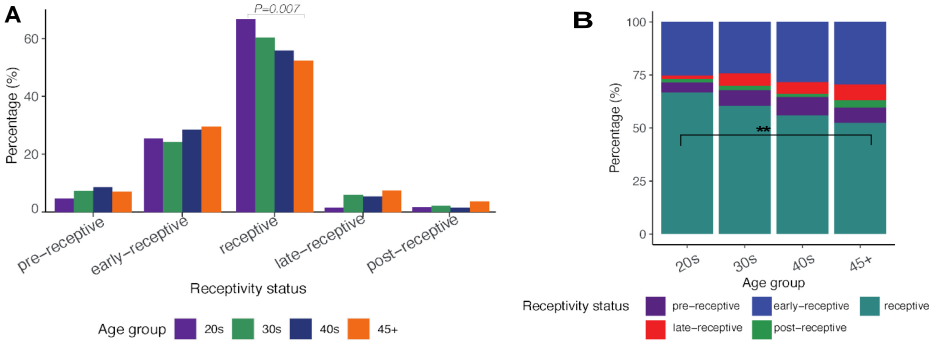


**Figure 12.** The results on the analysis of 1249 endometrial receptivity reports based on the beREADY algorithm. A. The percentage of samples classified as 'pre-receptive', 'early receptive', 'receptive', 'late receptive' and 'post-receptive' in four age groups, arranged by receptivity status. The proportion of 'receptive' samples was significantly lower in women older than 45 years, compared to women aged 20-29. Chi-squared test p-value = 0.007. B. The proportions of receptivity status, arranged by age. ** Chi-squared test p-value < 0.01.

Endometrial samples, characterised as 'receptive', were then assigned to two groups of patients according to age, advanced (AMA, age >45, n=12) and young (YMA, age <20, n=12) and whole transcriptome sequencing was applied (Table 2).

### 5.4.1. AMA-associated DEGs

The study aimed to describe the specific molecules and molecular pathways in the endometrium, which are affected by a woman's age. To test this, whole-transcriptome sequencing of endometrial tissue samples from women of advanced reproductive age was applied and compared to those obtained from young women. As a result, 491 DEGs were identified as significantly dysregulated in AMA endometrium, as summarized in Figure 13. The chord plot representing the top AMA genes and their respective biological processes is presented in the Figure 13A. When DEGs were subjected to functional analysis, it appeared that the most significantly enriched pathways are associated with cilium and motile cilia structures and functions, such as cilium (GO:0005929, p-value = $2.42\text{x}10^{-20}$), cilium movement (GO:0003341, p-value = $4.74\text{x}10^{-17}$), axoneme (p-value = $2.0\text{x}10^{-17}$), ciliary plasm (GO:0097014, p-value = $2.4\text{x}10^{-17}$). The results of GO analysis of AMA genes are presented in the Figure 13B. Pathway analysis suggested five significant molecular interaction networks involved in the processes of endometrial aging, based on AMA DEGs. Networks with the highest rating score were involved in cellular motility and cell cycle progression and suggested the central role of p16-senescence marker encoded by *CDKN2A* gene, one of the top AMA genes (Figure 13A). Also, several top-upregulated DEGs (*TBX15*, *EML5*, *KLK3*) interact directly with Histone H3, the biosynthesis and modification of which is strongly related to senescence in human cells (Ivanov et al., 2013; O'Sullivan et al., 2010). Immunohistochemical analysis of p16INK4a protein showed cytoplasmic accumulation of p16 and confirmed its higher expression in luminal (p=0.04) and glandular epithelial compartments of endometrium (p=0.02).

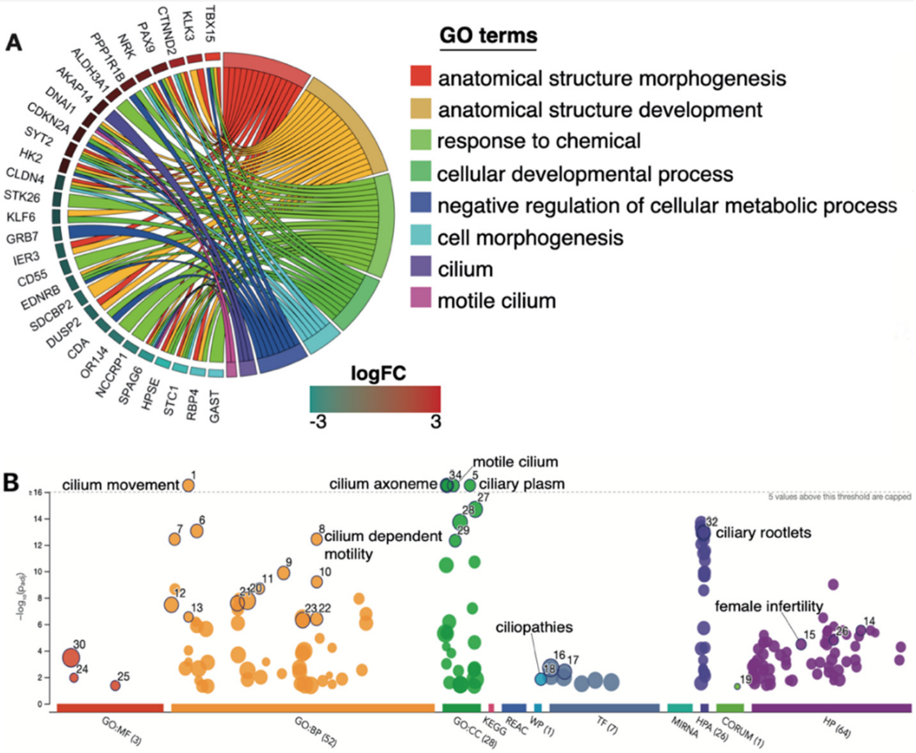


**Figure 13.** The effect of age on endometrial transcriptome. A. Genes dysregulated in the endometrium of women of advanced maternal age, and their respective biological functions, FC > 1, Benjamin-Hochberg adjusted p-value < 0.05. B. Functional enrichment of 491 AMA DEGs by g:Profiler identified biological process (BP) ‘cilium movement’, and cellular components (CC) ‘cilium’ and ‘motile cilium’ as most enriched GO terms. Y-axis represents the significance level of the enrichment in -$\log_{10}$(P adj).

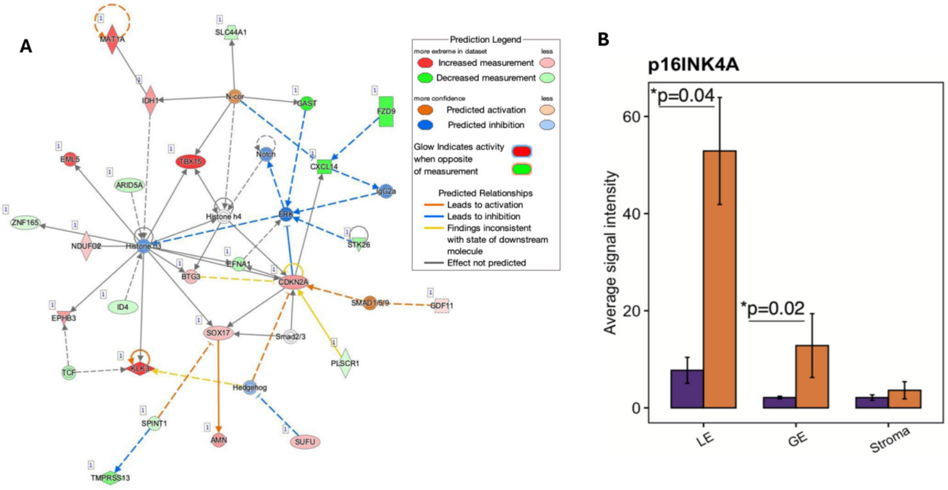


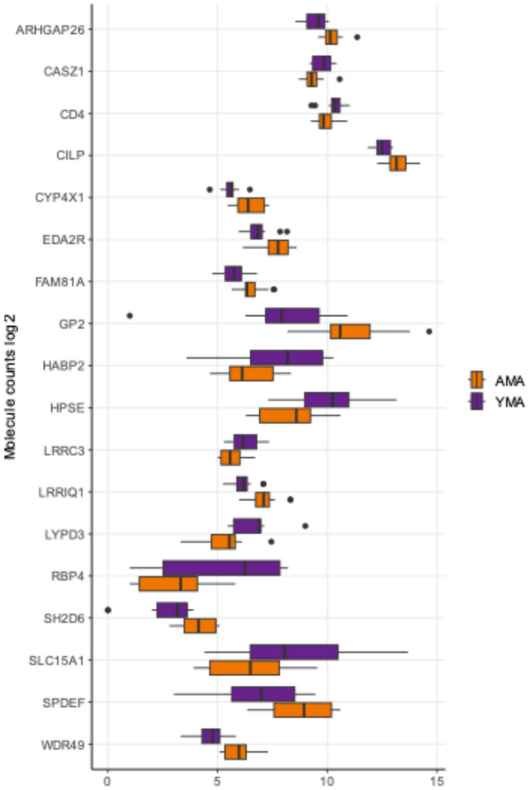
**Figure 14**. The expression of senescence-associated genes in endometrium of AMA. A. The molecular interaction network (score 35) identified by IPA suggests the central role of CDKN2A (p16) and its interaction with ERK, Notch and Hedgehog signaling pathways in AMA endometrium. Several top-upregulated genes (*TBX15*, *EML5*, *KLK3*) interact directly with Histone H3, the occupancy of which is strongly related to aging. B. $p16^{INK4a}$ protein expression in endometrial cellular compartments, identified by immunohistochemistry from YMA patients and AMA patients. p16 exhibited significantly higher expression in LE and GE (Wilcoxon rank-sum test $p < 0.05$).

To understand, whether age-associated changes may impact endometrial receptivity, we compared AMA transcriptomic profile with our previously reported endometrial receptivity-associated changes. A total of 101 DEGs, identified in Study I as potential biomarkers for endometrial receptivity, were oppositely regulated in AMA endometrium. Genes with highest expression change are shown in Table 5.

**Table 5**. DEGs associated with WOI and dysregulated in AMA. Bold – genes presented in Figure 9 as genes with the highest receptivity marker potential. *long non-coding RNA1320 identified as a new potential endometrial receptivity marker (Study I). logFC – logarithmic fold change; Bonferroni p-value – meta-analysis Bonferroni adjusted p-value; B-H p-value – Benjamini-Hochberg FDR; baseMean – average transcript count.

| MSE vs ESE (Study I) | | | AMA vs YMA (Study III) | | |
|---|---|---|---|---|---|
| Gene name | logFC | p-value | logFC | B-H p-value | baseMean |
| *EML5* | **-1,4** | 4,61E-13 | **1,8** | 0,0110 | 143,5 |
| ***GALNT12*** | **-2,5** | 5,59E-100 | **1,2** | 0,0008 | 216,9 |
| ***SYT2*** | **-2,2** | 3,39E-34 | **1,0** | 0,0088 | 421,9 |
| *IDH1* | **-1,2** | 3,73E-16 | **1,0** | 0,0061 | 21840,9 |
| *XK* | **-1,5** | 6,45E-31 | **1,0** | 0,0037 | 84,2 |
| *SIAH3* | **-2,0** | 2,55E-49 | **1,0** | 0,0433 | 362,9 |
| ***ARID5B*** | **2,0** | 1,67E-90 | **-0,9** | 0,0004 | 2935,8 |
| ***GABRE*** | **2,2** | 5,21E-67 | **-0,9** | 0,0085 | 1016,0 |
| *MET* | **2,8** | 2,61E-98 | **-1,0** | 0,0226 | 4021,9 |
| *LINC01320** | **3,7** | 2,77E-80 | **-1,0** | 0,0353 | 2925,7 |
| *KLF6* | **2,2** | 1,86E-93 | **-1,1** | 0,0002 | 7600,1 |
| *FAM84B* | **2,8** | 1,53E-106 | **-1,1** | 5,42E-05 | 4391,6 |
| ***LAMB3*** | **5,1** | 6,06E-87 | **-1,1** | 0,0167 | 646,1 |
| *IER3* | **3,2** | 4,16E-58 | **-1,2** | 0,0009 | 374,9 |
| ***CD55*** | **4,4** | 2,15E-128 | **-1,2** | 4,43E-05 | 4340,8 |
| *EDNRB* | **2,8** | 5,70E-74 | **-1,2** | 0,0011 | 747,6 |
| ***MFSD4A*** | **6,1** | 4,80E-87 | **-1,2** | 0,0031 | 2157,5 |
| ***SLC1A1*** | **4,5** | 3,91E-228 | **-1,3** | 0,0154 | 1349,5 |
| ***RIMKLB*** | **4,0** | 6,73E-136 | **-1,3** | 2,42E-05 | 7302,4 |
| *INHBB* | **2,3** | 1,98E-29 | **-1,4** | 0,0075 | 151,1 |
| ***CYS1*** | **2,6** | 2,50E-27 | **-1,5** | 0,0377 | 29,6 |
| ***HABP2*** | **5,1** | 4,25E-157 | **-1,5** | 0,0133 | 226,0 |
| ***C2CD4B*** | **4,6** | 4,86E-95 | **-1,5** | 1,80E-08 | 648,4 |
| ***C2CD4A*** | **7,5** | 3,82E-160 | **-1,6** | 5,34E-06 | 2272,8 |
| *NCMAP* | **3,1** | 8,36E-37 | **-1,6** | 0,0122 | 272,2 |
| ***HAP1*** | **6,4** | 2,96E-72 | **-1,9** | 0,0207 | 1350,9 |
| ***SLC15A1*** | **6,8** | 6,00E-180 | **-2,0** | 0,0278 | 549,0 |
| ***CXCL14*** | **7,0** | 3,50E-93 | **-2,0** | 0,0063 | 2322,9 |
| *HPSE* | **2,9** | 4,06E-51 | **-2,1** | 0,0031 | 1067,1 |
| ***STC1*** | **4,2** | 2,48E-58 | **-2,3** | 0,0017 | 4551,4 |

When comparing the AMA DEGs with WOI-associated genes from natural cycles, it's crucial to consider that AMA samples are procured during hormonally induced cycles, commonly utilized for in IVF in older women. This is done to mitigate the impact of natural hormonal fluctuations between cycles and to ensure adequate endometrial growth by the time of embryo transfer. Nonetheless, previous research has demonstrated distinct variations in the transcriptomic profile of the endometrium between natural and artificially induced cycles (Altmäe et al., 2016). To ascertain the influence of age on gene expression in hormonally stimulated endometrium, we compared the transcriptome of AMA samples with the set of 18 genes that undergo alterations in HRT endometrium in contrast to normal cycles, according to the above-mentioned study. This comparison revealed that AMA affects the genes associated with hormonal response (Figure 15).



|  | Fold change |  |  |
|---|---|---|---|
| **Gene** | HRT* | RIF* | AMA |
| **LRRC3** | 104,67 |  | -1,79 |
| **SH2D6** | 42,49 |  | 2,06 |
| **WDR49** | 37,08 |  | 2,25 |
| **CASZ1** | 18,38 |  | -1,50 |
| **EDA2R** | 14,09 |  | 1,71 |
| **CYP4X1** | 9,34 |  | 1,89 |
| **ARHGAP26** | 5,13 |  | 1,53 |
| **CILP** | 4,79 |  | 1,51 |
| **FAM81A** | 3,43 |  | 1,60 |
| **RBP4** | -4,06 |  | -6,14 |
| **SLC15A1** | -4,61 |  | -3,96 |
| **LYPD3** | -4,83 |  | -2,27 |
| **GP2** | -5,03 |  | 5,75 |
| **HPSE** | -7,48 |  | -4,31 |
| **CD4** | -11,32 |  | -1,44 |
| **LRRIQ1** | -13,75 |  | 1,89 |
| **HABP2** |  | -4,98 | -2,82 |
| **SPDEF** |  | 10,73 | 3,97 |

**Figure 15**. Age-sensitive genes, associated with hormonal replacement therapy (HRT) and recurrent implantation failure (RIF). *Fold change for HRT and RIF group was calculated in comparison with natural cycles by Altmäe and colleagues (Altmäe et al., 2016), the expression change of these genes in AMA samples suggests age-associated impaired hormone response. X-axis represents the relative expression of DEGs.

### 5.4.2. Proportions of epithelial MCCs in AMA endometrium

The results obtained from deconvolution analysis utilizing transcriptomic profiles of six endometrial cell types agrees with the results Study I, where the proportions of epithelial cells dominate over stromal fibroblasts during MSE. The significant results are presented in Figure 16. Comparison of cell type proportions between YMA and AMA identified the higher proportion of ciliated epithelial cells in

AMA group (Figure 16A). If the proportions of ciliated epithelial cells in the young group varied between 0.1% and 1.2% in some samples of AMA group women, the proportions on ciliated epithelial cells were as high as more than 2% of all cells (Figure 16B). This reveals the higher variation of ciliated cells proportions occurring with age, which may be a consequence of age-associated hormone or cell-cycle signalling.

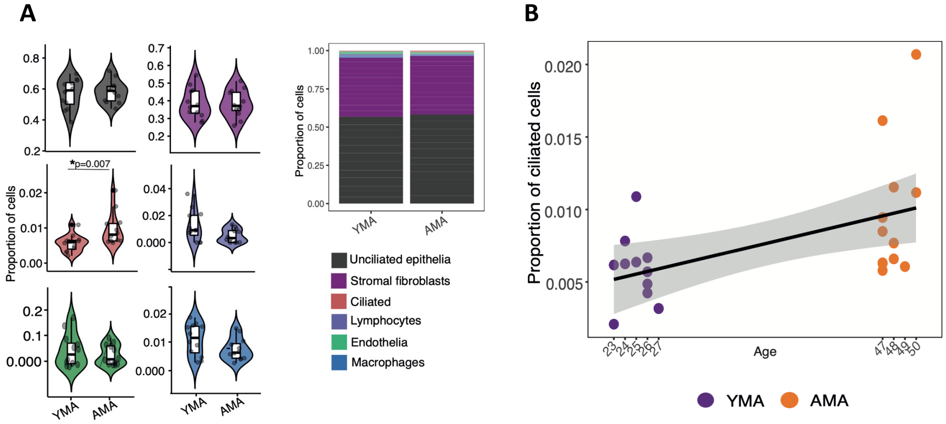


**Figure 16.** Proportions of six endometrial cell types in young and advanced reproductive age women. The proportions were calculated from RNA-seq data using deconvolution analysis and Fluidigm C1 single-cell gene expression data from Wang *et al.,* 2020 as a reference. The samples represent endometrial tissue during MSE (days 20-24 of natural menstrual cycle). A. As the single-cell dataset included distinct transcriptomic profiles for epithelial multiciliated cells (MCC), the deconvolution analysis identified a significant shift in the proportion of MCC in the advanced maternal age group (AMA), p-value 0.007. B. The proportion of MCCs in study samples arranged by age reveals a higher variation of MCC proportion in AMA group.

To validate the findings obtained from *in silico* analysis of cell type proportions, we applied IHC analysis which allows to visualize and manually count the ciliated cells in both groups. Both basal body staining using anti-LhS28 antibody and cilia staining with an antibody against acetylated α-tubulin (AcTubA) confirmed the higher occurrence of ciliated cells in the endometrium of AMA, compared to YMA. The antibody staining and result of the analysis is presented in the Figure 17. AMA group had also higher variation of ciliated cell amounts between samples (Figure 17E and J).

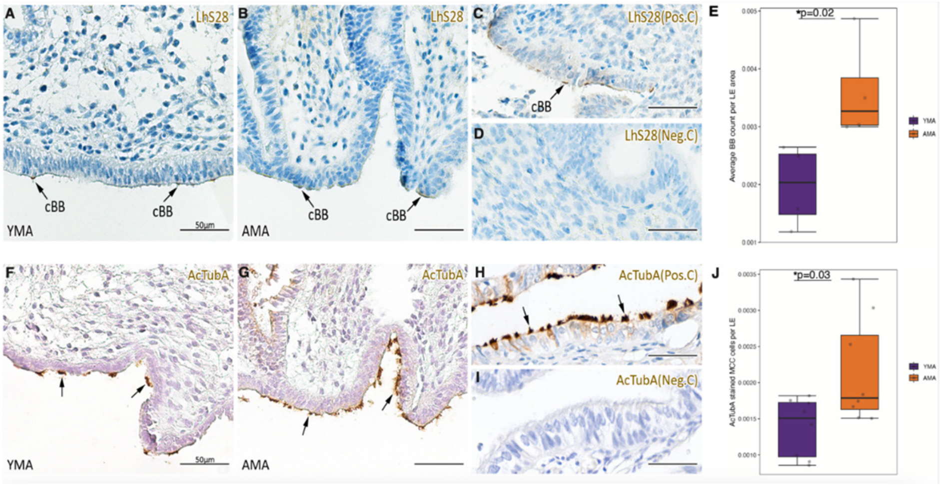


**Figure 17**. Immunohistochemical staining and analysis of endometrial epithelial multiciliated cells (MCCs) in samples from young and advanced maternal age group women. A. Basal body staining using LhS28 antibody in endometrial tissue of young maternal age (YMA), B. LhS28 staining in advanced maternal age (AMA) sample, C. LhS28 staining in the endometrium on day 16 of natural cycle (positive control of endometrial tissue sample from fertile YMA woman). D. A negative control for LhS28 staining, where primary antibody was omitted. E. Relative basal body (BB) count per luminal epithelium (LE) area, calculated based on LhS28 staining in human endometrium, showed significantly higher numbers of BBs in AMA group, according to independent sample student's t-test, $p = 0.02$. F. Motile cilia staining using acetylated α-tubulin (AcTubA) antibody in endometrial tissue of YMA. G. AcTubA staining in AMA sample. H. Fallopian tube sample was used as a positive control for AcTubA. I. negative control for AcTubA, where primary antibody was omitted. J. Relative MCC count per luminal epithelium (LE) area, calculated based on AcTubA staining in human endometrium, showed significantly higher numbers of MCCs in AMA group, according to independent sample student's t-test, $p = 0.03$.

### 5.4.3. The expression of cilia-associated genes

To dispute whether aging only changes the overall proportions of ciliated cells or possibly affects their phenotype altering specific cilia development pathways, we analysed identified cilia-associated genes from the whole DEG set. Of 491 DEGs, 93 were confirmed expressed in ciliated cells and previously described in relation with ciliogenesis and motile cilial function. The genes are expressed in all parts of ciliated cells, from nucleus to axonemal structures, as depicted the Figure 18, which means that no specific parts of cilial structure are affected by aging. The cilia specific genes showed low expression rates compared to other DEGs, stemming from low amount of MCCs in endometrial tissue (Figure 16A).

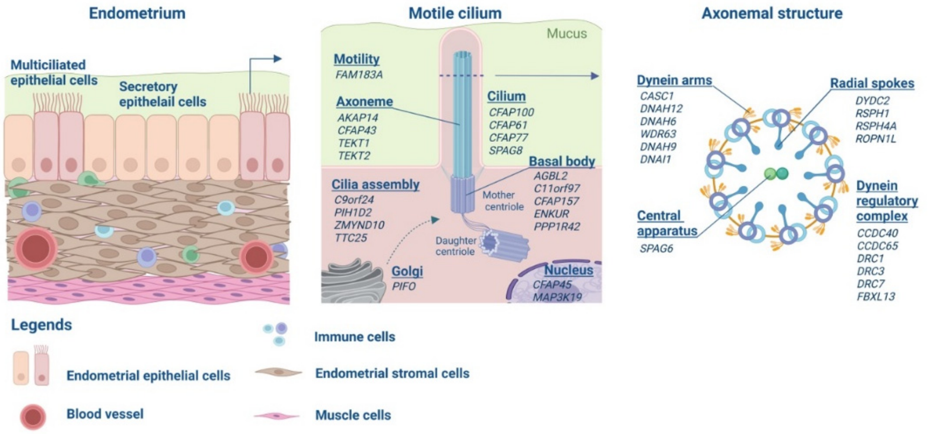


**Figure 18**. Structure of endometrial multiciliated cells (MCCs) and functions of motile cilia affected by woman's advanced age. The gene names represent DE genes in the advanced maternal age (AMA) endometrium (Benjamin and Hochberg (BH) adjusted p-value < 0.05) and are associated with motile cilia. Figure was created using BioRender.com.

## 5.4.4. miRNA expression in AMA endometrium

To understand, how the gene expression is regulated with age, we utilized the miRNA sequencing to compare the miRNomes of young and advanced maternal age endometria. miRNA miR-34c and miR-34b and their isomiRs target *MET* and *EML5* genes, that are receptivity genes, dysregulated in AMA (for the genes, see Table 5).

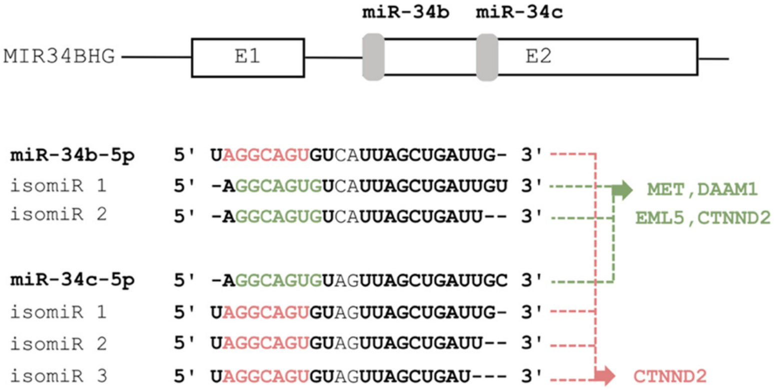


**Figure 19.** The genomic localization of differentially expressed miRNAs associated with cilia and the target genes. miR-34b and miR-34c are localized to a single cluster that resides in the host gene MIR34BHG on chromosome 11. The target genes for miR-34b and miR-34c and their isomiRs are predicted with the miRDB database according to their sequence, which is depicted in colour.

### 5.4.5. The timeline of endometrial aging

To understand how endometrial gene expression changes over the woman's lifespan, the transcriptomes of 24 additional endometrial samples from women aged between 20– 50 years obtained in were analysed in four age groups (marked as YMA, IMA1, IMA2 and YMA). The expression of previously identified age-associated genes was pairwise compared among four all age groups. The Figure 20 shows how AMA genes changed at different ages (Bonferroni adjusted $p < 0.05$). Most of the age-associated changes occurred in the late thirties and early forties (Figure 20A), suggesting the putative onset of endometrial aging. However, the cilia-associated genes *ERICH3*, *SNTN* and *WDR38* were upregulated significant between the twenties and early thirties (IMA1 vs YMA), suggesting the early onset of the cellular changes. The expression of *TBX15* gene, most highly differentially expressed in AMA group (Table 5), changed gradually over the years and correlated positively with a woman's age ($R = 0.64$, $p = 0.00071$) (Figure 20B). Interestingly, no steroid hormone receptor genes showed significant changes between age groups.

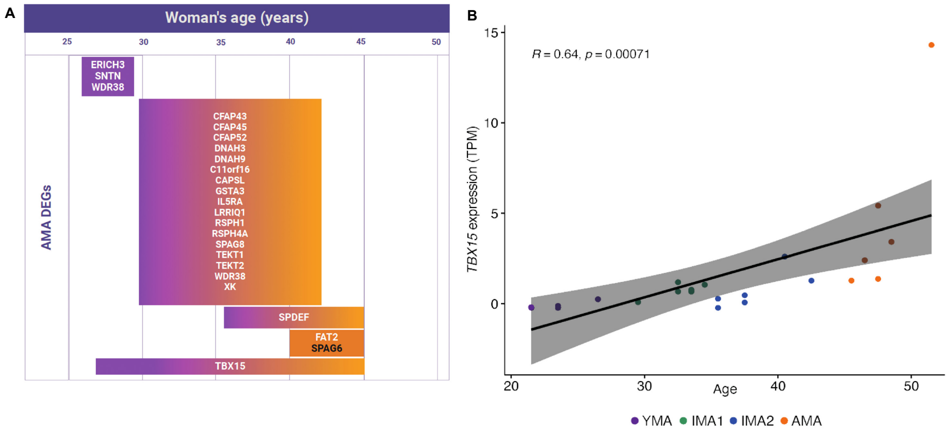


**Figure 20.** A. The pairwise comparisons among four age groups (young maternal age (YMA), intermediate maternal age groups 1 and 2 (IMA1, IMA2) and advanced maternal age (AMA)) revealed the possible onset of specific AMA-related gene expression changes (Bonferroni adj p-value < 0.05). The upregulated genes in groups with higher age are presented in white and the down-regulated genes are presented in black, which corresponded to the direction of gene expression changes in the study group. B. Gene *TBX15*, which participates in the epithelial-to-mesenchymal transition of epithelial cells, showed a positive correlation with age of the women (R=0.64, p-value = 0.00071).

# 6. DISCUSSION

## 6.1. It takes three 'to tango'

Development of a baby starts with a fertilization process, that requires genetically healthy oocyte and sperm. The forming embryo needs a fertile ground to support its nutrient needs during growing. Not only all three are crucial for the establishment of pregnancy, but they also need to be synchronised with each other (B. Sun & Yeh, 2022). Since the first successful IVF treatment in 1977 (M. H. Johnson, 2011), uterine mucous lining or endometrium has attracted attention to understand how its growth and function is regulated on molecular level. Gathered knowledge helps to explain and better manage conditions where endometrium may be the limiting factor. Endometrial thickness, the expression of surface proteins and specific transcriptomic profile were all correlated with natural pregnancy rate, live birth and IVF outcomes (reviewed in Aghajanova et al., 2008; Cavagna & Mantese, 2003). It was postulated that both the thickness and the expression of specific proteins can predict the ability of uterus to support embryo implantation and growth, if embryo quality is accurately assessed. For this reason, personalised approach to infertility management involves semen, oocyte/embryo and endometrial testing (Barratt et al., 2022; Cornelisse et al., 2020; Lemseffer et al., 2022; Meltsov et al., 2023).

## 6.2. Endometrial transcriptomic profiling as a tool for personalized embryo transfer

The term 'personalized' refers to the fact that intra-individual variation can undermine the accuracy of approaches developed using statistical methods. As histological dating allows to predict endometrial growth phase with an accuracy of 3-4 days, it does not predict the actual window of implantation (WOI). Studies agree that WOI has a distinct transcriptomic signature in response to adequate hormonal stimulation, corresponding to the mid-secretory phase of endometrial cycle, and it can be used to predict the time of maximal endometrial receptivity (Altmäe et al., 2017; Díaz-Gimeno et al., 2011; Horcajadas et al., 2007). Screening tests based on the transcriptomic profile of healthy mid-secretory endometrium started developing using qPCR and micro-array technology many years before RNA sequencing became widely affordable (Díaz-Gimeno et al., 2011; Bhagwat et al., 2013; Enciso et al., 2018). But the main limitation was not the method itself, but the fact that the whole-tissue biopsy contained of several cell types, that have different transcriptomic changes under hormone fluctuations. Previously, in one of our studies we have shown that two major endometrial cell types, epithelial and stromal, have different transcriptomic signatures between early and mid-secretory phases (Altmäe et al., 2017) emphasising the need to explore possible changes in the cellular proportion between the phases.

In our study, we confirmed the hypothesis that the proportions of cell type populations vary during the menstrual cycle by showing the significant change of epithelial and stromal cell proportions between early and mid-secretory phases. To test this, we used a novel computational method known as deconvolution, which allows to assess the proportions of dominant and rare cell types using quantitative data, such as gene expression or methylation. Our study was the first to utilize deconvolution for the analysis of endometrial tissue, and with our study we validated the fact that deconvolution can be successfully used for the accurate estimation of cell type proportions in reproductive tissues, as an alternative to the costly cell sorting procedures. Since then, this method have been widely used in reproductive science (Bruno et al., 2023; Bunis et al., 2022; Dinh et al., 2021; Garcia-Alonso et al., 2021).

As an additional outcome of our study, we demonstrated how the changes of two dominant cell types, epithelial and stromal, affect the transcriptomic profile of endometrial tissue, and may lead to inaccurate receptivity assessment. For this purpose, we adjusted the whole-tissue transcriptome to the changes of proportions of epithelial and stromal cells between ESE and MSE, which resulted in a list of DEGs. For example, olfactomedin 4 (*OLFN4*) and periostin (*POSTN*) genes, downregulated in MSE and commonly used in endometrial receptivity tests (Díaz-Gimeno et al., 2011), were not significant after the adjustment, which suggests that the expression changes of these genes may derive from the alteration of cell type proportions. This result helps to improve the transcriptomic screening tests to avoid misinterpretation of gene expression changes caused by the intra-individual variation in endometrial cell proportions. Also, with the help of deconvolution we confirmed the core genes differentially expressed in the mid-secretory endometrium as the most prominent endometrial receptivity markers. Among these are core receptivity markers, such as *PAEP*, *LIF*, *GPX3*, *C4BPA*, *CD55*, *S100P*, *LAMB3*, *DPP4*, *STC1*, and others. The expression of these genes was not only strongly changed, but it was also highly significant, supporting the central role of these genes' regulation in endometrial preparation for embryo implantation. Novel DEGs, that appeared significant after the adjustment for tissue cellular heterogeneity, showed the changes that were eclipsed due to the fluctuations in proportions of epithelial and stromal compartment. These results were further carried over to the improvement of the local endometrial receptivity test developed using home-tailored Targeted Allele Counting by sequencing (TAC-seq) technology (Meltsov et al., 2023).

Our study was also first to report a set of regulatory RNA molecules that are differentially expressed in endometrium during WOI and, therefore, potentially may modulate endometrial receptivity. Among these was long non-coding RNA 1320 (LINC01320), which appeared to be one of the top novel endometrial receptivity genes, based on our results, and have once been reported in endometrial transcriptome during WOI (Sigurgeirsson et al., 2017). The putative role of LINC01320 in regulating genes differentially expressed in epithelial and stromal cells, is depicted in the Figure 21.

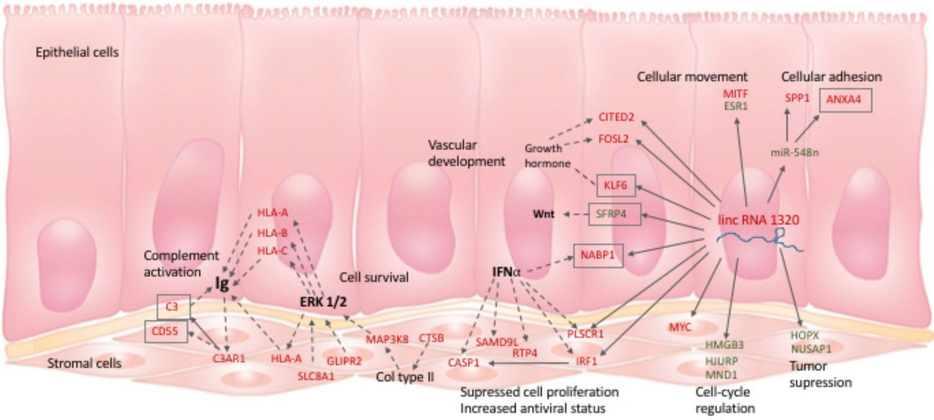


**Figure 21**. The cell-type specific activation of genes, differentially expressed during WOI, identified in our study. The genes depicted in the figure remained significant after adjustment for tissue cellular heterogeneity. Long non-coding RNA 1320 has a putative regulatory effect on many receptivity-associated genes, affecting the movement and adhesion of epithelial cells and/or the cell-cycle regulation and cell proliferation. Red - upregulation, green – downregulation.

Of all miRNAs identified in endometrial tissue, miRNA chr2_4401 was upregulated in MSE, suggesting its specific WOI marker potential. However, the expression of chr2_4401 was also higher in epithelial cells compared to stromal, which hints to the fact that its upregulation in the bulk tissue may also be positively affected by the epithelium-stroma shift identified as a result of the deconvolution analysis.

## 6.3. Non-invasive tools for endometrial receptivity

Endometrial biopsy is not a routine procedure during early fertility assessment due to its considerable invasiveness. Biopsy can cause discomfort and bleeding to women undergoing examination and this may impact her sexual life and ability to get pregnant in current menstrual cycle. So, once the transcriptomic signature of WOI endometrium was affirmed, the main concern remained the fact that to perform the endometrial screening test to any patient that might benefit from PET we would need a strong indication to take the risks of the invasive biopsy procedure. It was then immediately clear that a good endometrial receptivity screening test should be able to predict endometrial receptivity though a urine or a blood sample. As previously circulating miRNAs (found in the bloodstream) were known to be good predictors of various health conditions (reviewed in Ho et al., 2022), we, as a part of the NOTED consortium, took an attempt to identify endometrial receptivity markers from woman's blood.

As a results of the gene-expression profiling of paired endometrial and blood samples, it became clear that no endometrial receptivity markers are found in the bloodstream. No non-invasive markers were reported from blood, urine nor saliva samples. Several markers candidates were suggested from uterine fluid (Chan et al., 2013; Salamonsen et al., 2013; Edgell et al., 2018; Von Grothusen et al., 2022), however, the ultrasonic analysis remains the only clinically validated non-invasive method for assessment of endometrial receptivity (Dechaud et al., 2008; M. Li et al., 2023).

## 6.4. Endometrium of women of advanced reproductive age

The current study emphasizes the essence of clinical attention to the long-term aging process in human endometrium. Our study described the accumulation of p16-positive senescent cells in AMA samples, which is a sign of increased cellular senescence processes in endometrial epithelium. Previously positively correlated with age, p16-accumulating epithelial cells were also associated with higher miscarriage risk (Parvanov et al., 2021). $p16^{INK4a}$ is an important cell cycle regulator that inhibits the activation of CDK4/6, resulting in cell cycle arrest at the G1 phase. Senescent cells cease proliferation and secrete factors that initiate the senescence-associated secretory phenotype, which includes events that recruit immune cells and clear senescent cells for tissue regeneration (Brighton et al., 2017; Teissier et al., 2022). However, if the elimination of senescent cells does not occur, senescent cells accumulate and lead to cancer or tissue aging (Baker et al., 2016; Lujambio, 2016). The localization of p16 in the cytoplasm of endometrial epithelia indicated the increased accumulation of non-malignant senescent cells in AMA endometrium. Our results also comply with the previously shown higher levels of senescence-associated β-galactosidase activity in endometrial samples of women over 45 (Laser et al., 2010). It is unclear, whether excessive epithelial senescence affects receptivity, but it was previously shown that transition to the receptive state requires extensive gene expression activation in epithelial cells (Wang et al., 2020). While p16 could be a possible therapeutic target in epithelial cells, on the other hand, the cellular senescence is an important modulator of stromal decidualization, which should not be disturbed (Brighton et al., 2017).

While the senescence-accumulating epithelial cells appeared to be mostly secretory, our deconvolution analysis using cell type-specific gene expression profiles from six endometrial cell populations showed a significantly higher proportion of multiciliated epithelial cells in the AMA group, confirmed by IHC (Figure 16 and 17). MCCs are known to be a rare epithelial cell subtype (1–2%) and characterised by a specific transcriptomic profile (Garcia-Alonso et al., 2021; Wang et al., 2020). These cells develop motile cilia – microtubule-based cellular structures with 9+2 axonemal organisation and accessory structures that enable cilia movement. In the female reproductive tract, MCCs move around mucus and support embryo journey along the epithelial surface. Ciliogenesis is driven, although not exclusively, by oestrogen and through the inhibition of Notch signalling (Haider et al., 2019), and regulated by miR34/449 family microRNAs

(Chevalier et al., 2015; Marcet et al., 2011; Song et al., 2014). miRNA analysis also confirmed the upregulation of cilia-regulating miRNAs and their target genes in AMA women (Figure 19), supporting our results.

## 6.5. Relevance of findings in context of clinical reproductive care

One has to be cautious calling endometrium 'receptive' solely based on expression of core genes differentially expressed during mid-secretory phase. Some studies have postulated that WOI shift alone is a minor cause of endometrial dysfunction. Most causes may stay unidentified due to the extensive use of PET based on WOI genes. For this reason, ideal endometrial receptivity test would consider a various set of endometrial markers, both transcriptomic, ultrasonic and cellular. Also, existing transcriptomic tests need to be critically revised and updated to the new knowledge on how endometrium is regulated. For example, our study of the endometrial transcriptomic profile adjusted to tissue cellular heterogeneity helped us to improve the development of local endometrial receptivity test based on targeted gene expression profiling of endometrial tissue (Meltsov et al., 2023). And, although we did not provide the promising non-invasive markers for endometrial receptivity, we have introduced new miRNAs as putative regulators of endometrial gene expression.

Endometrial testing is used in clinics for decision making on the day of embryo transfer and the optimal response to treatment. Younger patients are often advised to use natural cycles, while in advanced-age women HRT is preferred to overcome the effect of natural hormone fluctuations concomitant with aging. Aging organism displays changes in different cells and tissues at different time points and for a long time the endometrium was believed to be intact of aging until menopause. Recently, molecular aging of endometrial tissue was introduced (Devesa-Peiro et al., 2022) and our results confirm that endometrial aging deserves clinical attention. Understanding when and how aging processes are initiated and regulated in reproductive tissues is crucial for both infertility management and the risk assessment of reproductive care. Till what age endometrium remains receptive, if supported by hormone implementation, is a long-lasting debate with many confounders. Our study describes the specific molecular and cellular processes that may impact endometrial ability to support implantation. Translating this knowledge into the clinical practice, the adjustment of reproductive care protocols according to the specific needs of advanced age patients may help to improve infertility treatment outcomes.

But endometrial aging does not only pose challenges for women willing to conceive – it must also be considered as a risk factor of endometrial preparation protocols. If oestrogen-responsive endometrial hyperplasia is associated with age (J. H. Kim et al., 2015), then it has to be taken into the account before starting HRT to avoid elevated endometrial cancer risks. Our study on endometrial aging supports the hypothesis that some women in their late forties may experience mild manifestation of endometrial hyperplasia and need to be tested for it to avoid side effects of hormonal use. MCC abundance is associated with hyperplasia and can be a promising early marker for hyperplastic processes compared to endometrial thickness and gland-stroma ratio, which are used for already developed hyperplasia.

## 6.6. Limitations of our study

This research was conducted with the best knowledge available to date. While the study's major strengths lie in the novelty of the methods and findings, the small sample size typically used in pioneer studies may limit their clinical significance, which means the findings need to be validated on large cohorts. We must also acknowledge the fact that no follow up can be done for the infertile patients participating in the study anonymously, leaving us unaware of their parity status after the study was performed. As for the advanced maternal age women, the new data of the cellular and molecular shifts does not provide evidence that their endometrial receptivity is compromised. Also, it is not clear, whether the endometrial multiciliated epithelial cells of older women are different from those of younger patients, or it is only their amount that changes, and this needs to be clarified with the future studies.

## 6.7. Future perspectives

Endometrial transcriptomic tests analyse different sets of endometrial receptivity markers and prove to be differently efficient. Most of the tests have been developed and applied using microarray techniques. During past decade Illumina sequencing has evolved into a routine gene expression tool while the whole-transcriptome sequencing has become affordable for many clinical labs. This has raised the main concern of endometrial transcriptomic tests: why do we analyse a specific set of markers if we have whole transcriptome at our service? With our analyses we have shown that full transcriptome may predict not only the expression changes of specific transcriptomic markers but even precisely estimate the changes in cellular composition. This leads to a whole new era in endometrial testing where a single biopsy analysis may unravel a lot of molecular and cellular changes, that would provide information for decision making during infertility and peri-menopause treatment.

Although meta-analyses of clinical studies on personalized PET using transcriptomic endometrial receptivity tests questions their efficacy, there is a lot of data supporting their diagnostic potential (Garcia-Velasco et al., 2023). Our study shades light to the aspects of transcriptomic testing of endometrial biopsy, that were never addressed before. As clinical studies continue to prove, that endometrial receptivity to embryo should not be underestimated, we believe that the results of our study will lead to a more accurate endometrial screening in future, helping to improve the live birth rates in both young and advanced-age patients.

# 7. CONCLUSION

This doctoral thesis aimed to provide new knowledge on how endometrial growth and function are regulated in young and advanced-age patients. In our study we applied the newest gene expression analysis methods, like single-cell RNA sequencing, and introduced computational tissue decomposition, as a prominent method to look closely at molecular and cellular changes of the cycling endometrium. We were able to show that gene expression profiling of whole tissue sample is not optimal to explain endometrial receptivity, because it can be easily affected by the cellular composition of endometrial biopsy. We identified the differentially expressed genes (DEGs) with the true expression change, as well as the DEGs significant due to the fluctuations in cell type proportions and eclipsed by them. This helped to elaborate the development of local endometrial receptivity screening test from endometrial biopsy.

Further on, after the whole cellular atlas of endometrial tissue became available, we were able to identify the changes in rare cell types associated with advanced reproductive age. Utilizing RNA sequencing to analyse both protein coding and regulatory RNA molecules, we showed that miRNome of circulating blood does not reflect the changes in the endometrial tissue of healthy and infertile patients. Overall, based on the results presented in the thesis, the following conclusions can be made:

I. The proportions of dominant endometrial cell types (epithelial and stromal) change between pre-receptive and receptive state, which affect the expression levels of endometrial receptivity markers in the bulk tissue and should be accounted for during endometrial evaluation based on the transcriptomic markers of whole tissue.
II. Although miRNAs, e.g. chr2_4401, can be potentially effective in predicting endometrial receptive state, no miRNA markers are found in the circulating blood.
III. Endometrium undergoes specific molecular changes with age, resulting in higher proportion of senescent epithelial and multiciliated cells, which may affect the receptive capacity of endometrium.

In summary, the results encourage us to move further with the development of specific and universal endometrial screening tests for young and advanced-age patients, which would allow to discriminate for the possible causes compromising implantation on the endometrial side.

# SUMMARY IN ESTONIAN

## Molekulaarsed ja rakulised muutused retseptiivses endomeetriumis ja naise vanuse mõju

Sündimuse langus on arenenud riikide suurim demograafiline probleem. Arenenud riikides täheldatakse tavaliselt madalamat sündimust erinevate elustiili tegurite tõttu, mis on seotud sotsiaalmajandusliku olukorraga. Vananev ühiskond, rasestumisvastaste meetodite laialdane kasutus, laste kasvatamisega kaasnev koormus ning eneseteostuse soov tihti sunnib naised pere loomist edasi lükkama (Nargund, 2009). Keskmiselt üks kuuest paarist ei rasestu loomulikul teel ühe aasta jooksul ja ning pöördub reproduktiivarsti poole otsides viljatuse põhjust. Viljatus on maailmas viies suurim tõsine puue, millel on negatiivne mõju paaride elukvaliteedile.

Naisepoolne viljatus võib olla ovariaalne (munarakkude reservi puudulikkus), tubaarne (viljastumist takistav munajuha patoloogia), või emakapoolne, ehk endomeetriumi puudulikkus. Selleks et munajuhas või labori katseklaasis viljastunud munarakk saaks jätkata oma arengut emakas, peab endomeetrium olema molekulaarselt ja histoloogiliselt sünkroniseeritud embrüo kasvuga, ning sellist endomeetriumi valmisolekut embrüo pesastumiseks kutsutakse retseptiivsuseks. Endomeetrium on kõige retseptiivsem menstruaaltsükli luteaalfaasi keskel, 20.-24. päeval, mida peetakse ‘implantatsiooniaknaks’. Et hinnata endomeetriumi küpsust ja ajastada viljatusravi, kasutavad arstid erinevaid endomeetriumi ultraheli, histoloogilisi ja molekulaarseid markereid. Viimasel aastakümnel on hoogu saanud endomeetriumi transkriptoomil põhinevad testid, mis kasutavad geeniekspressiooni markereid endomeetriumi biopsia dateerimiseks. Need testid võimaldavad tuvastada võimaliku implantatsiooniakna nihet, mis on põhjustatud loomulike hormoonide või hormoonasendusravi tundlikkuse muutusest. Eestis on saadaval lisaks rahvusvaheliselt tunnustatud ERA testile ka kohalik beREADY endomeetriumi skriiningtest.

Kliinilised uuringud on näidanud, et implantatsiooniakna nihke osakaal endomeetriumist tingitud viljatuse põhjustest on alla 5%. Enamik põhjuseid võib jääda tuvastamata endomeetriumi transkriptoomitestide metoodika tõttu, mis ei arvesta tsüklilisi rakulisi muutusi biopsia koostises. Oma uuringus kasutasime uuemaid geeniekspressiooni analüüsimeetodeid, nagu ühe-raku RNA sekveneerimine, ja tutvustasime bioinformaatilist koe dekonvolutsiooni kui silmapaistvat meetodit, et uurida tsükliliselt regenereeriva endomeetriumi molekulaarseid ja rakulisi muutusi. Oma töös näitasime, et täiskoe geeniekspressiooniprofiil ei ole endomeetriumi retseptiivsuse määramiseks optimaalne, kui ta ei arvesta endomeetriumi biopsia muutuvat rakulist koostist. Tuvastasime olulised ekspressioonimarkerid, mille muutus oli implantatsiooniakna ajal tõene, ning geenid mille avaldumine täiskoes sõltub oluliselt rakuproportsioonide kõikumisest. Terve retseptiivse endomeetriumi transkriptoomiuuring, mis võimaldas kohandada koe geeniekspressiooniprofiili rakkude heterogeensusele, aitas parandada uudse endomeetriumi retseptiivsuse testi väljatöötamist (Meltsov et al., 2023). Ja kuigi me ei leidnud verest potentsiaalselt mitteinvasiivseid endomeetriumi markereid, tuvastasime me uued endomeetriumi geeniekspressiooni regulaatormolekulid nagu pikad mittekodeerivad RNAd (lncRNA) ja mikro-RNAd (miRNA).

Endomeetriumi testimist kasutatakse kliinikutes optimaalse embrüo siirdamise päeva ja hormoonasendusravi (HAR) efektiivsuse määramiseks. Noorematel patsientidel soovitatakse sageli kasutada loomulikku tsüklit, samas kui kõrges eas naistel eelistatakse HAR, et ületada vananemisega kaasnevate loomulike hormoonide kõikumiste mõju. Vananev organism ilmutab spetsiifilisi muutusi erinevates rakkudes ja kudedes erinevatel ajahetkedel, ning pikemat aega arvati, et endomeetrium ei vanane enne menopausi. Hiljuti kirjeldati esmakordselt endomeetriumi vananemisega seotud molekulaarseid muutusi (Devesa-Peiro et al., 2022) ja ka meie töö tulemused kinnitavad, et endomeetriumi vananemine väärib kliinilist tähelepanu.

Arusaam, millal ja kuidas vananemisprotsesse reproduktiivkudedes käivitatakse ja reguleeritakse, on ülioluline viljatusravi efektiivsuse ja riskide hindamiseks. Mis vanuseni endomeetrium jääb retseptiivseks, kui seda toetada hormoonraviga, on olnud pikaajaline debatt. Meie uuring kirjeldab spetsiifilisi molekulaarseid ja rakulisi protsesse, mis võivad mõjutada endomeetriumi võimet toetada implantatsiooni. Nende teadmiste ülekandmine kliinilisse praktikasse võib aidata kohandada endomeetriumi ettevalmistamise protokolle vastavalt vanemate patsientide spetsiifilistele vajadustele. Meie töös leitud rakulised muutused võivad toimida eduka markerina endomeetriumi vananemisprotsesside hindamiseks.

Endomeetriumi vananemine ei sea väljakutseid ainult naistele, kes soovivad rasestuda – seda tuleb pidada ka endomeetriumi ettevalmistusprotokollide riskiteguriks. Kui östrogeenile reageeriv endomeetriumi hüperplaasia on seotud vanusega (Kim et al., 2015), tuleb seda enne HAR alustamist arvesse võtta, et vältida suurenenud riski endomeetriumi vähi tekkeks. Meie uuring endomeetriumi vananemise kohta toetab hüpoteesi, et mõnel hilistes neljakümnendates naistel võib esineda endomeetriumi hüperplaasia kerge ilming ja neid tuleb selle suhtes testida, et vältida hormoonravi kõrvaltoimeid. Ripsrakkude arvukus on seotud hüperplaasiaga ja võib olla paljulubav varane hüperplastiliste protsesside marker võrreldes endomeetriumi paksuse ja näärme-strooma suhtega, mida kasutatakse juba väljakujunenud hüperplaasia korral.

Kokkuvõtteks võib töös esitatud tulemuste põhjal teha järgmised järeldused:

I. Dominantsete endomeetriumi rakutüüpide (epiteel- ja stroomarakkude) proportsioonid muutuvad retseptsioonieelse ja vastuvõtliku seisundi vahel, mis mõjutavad endomeetriumi vastuvõtumarkerite ekspressioonitaset koes ja mida tuleks arvesse võtta endomeetriumi hindamisel, tuginedes rakkude transkriptoomilistele markeritele.

II. Kuigi miRNA-d, nt. chr2_4401, võib olla potentsiaalselt efektiivne marker endomeetriumi retseptiivsuse ennustamisel, ei leidu veres sobilikke tsirkuleerivaid miRNA markereid.

III. Endomeetriumis toimuvad vanusega spetsiifilised molekulaarsed muutused, mille tulemuseks on vananevate epiteeli- ja ripsrakkude suurem osakaal, mis võib mõjutada endomeetriumi vastuvõtlikkust embrüole.

Tulemused julgustavad meid jätkama spetsiifiliste ja universaalsete endomeetriumi sõeltestide väljatöötamisega noortele ja kõrges eas patsientidele, mis võimaldaksid eristada võimalike endomeetriumipoolset viljakust ohustavaid põhjuseid ja pakkuda personaalseid ravivõimalusi.

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I use the opportunity to thank my scientific mentor Dr Karin Rosenstein, who should take credit for my interest, motivation and continuous progress in the reproductive research. You taught me most of the techniques, and I will always keep saying that I have learnt from the best.

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# PUBLICATIONS

# CURRICULUM VITAE

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## Education

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|---|---|
| 2014–2024 | PhD candidate, University of Tartu, Institute of Clinical Medicine |
| 2012–2013 | Tallinn University of Technology, Department of Gene Technology, Master’s Degree (*cum laude*) |
| 2003–2010 | University of Tartu, Institute of Molecular and Cell Biology, Chair of Evolutionary Biology, Bachelor's Degree |

## Institutions and positions

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| 2017–... | Celvia CC AS (Competence Centre on Health Technologies), researcher/project manager |
| 2018–2019 | University of Tartu, Faculty of Medicine, Institute of Clinical Medicine, junior research fellow of clinical medicine |
| 2013–2017 | Tallinn University, Institute of Mathematics and Natural Sciences, laboratory specialist |
| 2013–2015 | Competence Centre on Health Technologies, researcher |
| 2010–2013 | Tallinn University of Technology, Faculty of Science, Centre for Biology of Integrated Systems, laboratory specialist |
| 2009–2010 | Estonian University of Life Sciences, laboratory technician |
| 2009 | Icosagen Cell Factory OÜ, trainee |
| 2006 | University of Oulu (Finland), Faculty of Science, Department of Plant Genetics, laboratory assistant |
| 2006 | University of Tartu, Faculty of Biology and Geography, Institute of Molecular and Cell Biology, laboratory assistant |

## R&D related managerial and administrative work

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| 2023–... | Member of Estonian Society of Embryology and Reproduction |
| 2013−... | Member of European Society of Human Reproduction and Embryology (ESHRE) |
| 2012−... | Member of Estonian Biochemical Society |
| 2012−... | Member of Estonian Society of Human Genetics |

## Academic degrees

**Marina Suhorutšenko**, Master’s Degree (*cum laude*), 2013, (sup) Madis Metsis; Karin Rosenstein; Jaak Simm. Gene Regulation by Natural Modulators of AhR: Their Role in Male Infertility and Prostate Cancer., Tallinn University of Technology Faculty of Science, Department of Gene Technology.

## Honours & awards

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| 2023 | Award for Best New Investigator Poster Presentation, 70th Annual Scientific Meeting, Society for Reproductive Investigation (SRI) |
| 2022 | Basic Science Award for Poster Presentation, 38th Annual Meeting of European Society of Human Reproduction and Embryology (ESHRE) |
| 2017 | Andreas and Elmerice Traksi Scholarship, University of Tartu Foundation |

## Projects in progress

PRG1076 “The role of endometrium as a major factor affecting female fertility and pregnancy outcome” (1.01.2021−31.12.2025); Principal Investigator: Helle Karro; University of Tartu, Faculty of Medicine, Institute of Clinical Medicine; Financier: Estonian Research Council; Financing: 558 900 EUR.

## Completed projects

IUT34-16 “Basic and translational approaches for female reproductive medicine: continuum from oocyte maturation to delivery” (1.01.2015−31.12.2020); Principal Investigator: Helle Karro; University of Tartu, Faculty of Medicine, Institute of Clinical Medicine; Financier: Estonian Research Council; Financing: 466 800 EUR.

EU41564 (EU41564) “Non-invasive Tests for Endometrial Dysfunction (NOTED) – NOVEL CLINICAL PERSPECTIVES FOR INFERTILITY AND ENDOMETRIOSIS DIAGNOSTICS” (1.12.2011−31.12.2014); Principal Investigator: Andres Salumets; Competence Centre on Health Technologies (partner); Financier: Enterprise Estonia; Financing: 847 363 EUR.

## Supervised dissertations

Arina Laanemets, Master's student, (sup) **Marina Loid;** Tambet Tõnissoo; Keiu Kask. The Analysis of Endometrial Epithelial Ciliated Cells in Young and Advanced Maternal Age Women (2024), University of Tartu, Faculty of Science and Technology, Institute of Molecular and Cell Biology.

Merili Peltser, Bachelor’s student, (sup) **Marina Suhorutšenko;** Inderk Tenno. Steroidhormoonide mõju geeniekspressioonile endomeetriumi stroomarakkudes polütsüstilise munasarja sündroomiga patsientidel (2018), University of Tartu, Faculty of Science and Technology, Institute of Molecular and Cell Biology.

Liis Mägi, Bachelor’s student, (sup) **Marina Suhorutšenko;** Margit Nõukas. Naisepoolne viljatus: geeniregulatsiooni roll endomeetriumi vastuvõtlikkuse kujunemisel (2015), University of Tartu, Faculty of Science and Technology, Institute of Molecular and Cell Biology.

## Publications

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Tamm, K.; **Suhorutšenko, M.**; Talving, E.; Kaljas, A.; Metsis, M. (2012). Suguhormoonide östradiooli ja progesterooni koespetsiifiline roll inimese endomeetriumis ja rinnanäärmes. *Eesti Arst*, 91 (4), 182−189.

Tamm, K.; **Suhorutshenko, M.**; Rõõm, M.; Simm, J.; Metsis, M. (2012). The Tissue Specific Role of Estrogen and Progesterone in Human Endometrium and Mammary Gland. In: Abduljabbar, H. (Ed.). *Steroids – Basic Science. (35−64). InTech – Open Access Publisher. (Steroid hormones).* DOI: 10.5772/25815.

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| 2014–2024 | PhD kandidaat, Tartu Ülikool, Kliinilise meditsiini instituut |
| 2012–2013 | Tallinna Tehnikaülikool, Geenitehnoloogia instituut, magistrikraad (*cum laude*) |
| 2003–2010 | Tartu Ülikool, Molekulaar- ja Rakubioloogia Instituut, bakalaureusekraad |

**Asutused ja positsioonid**

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| 2017–... | Celvia CC AS (Tervisetehnoloogiate Arenduskeskus AS), teadur/projekti koordinaator. |
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| 2010–2013 | Tallinna Tehnikaülikool, Loodusteaduste instituut, Integreeritud süsteemide bioloogia keskus, laborispetsialist. |
| 2009–2010 | Eesti Maaülikool, laboritehnik. |
| 2009 | Icosagen Cell Factory OÜ, praktikant. |
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| 2023–... | Eesti Viljatusravi ja Embrüoloogi Seltsi liige |
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**Akadeemilised kraadid**

**Marina Suhorutšenko,** Master's Degree (*cum laude*), 2013, (sup) Madis Metsis; Karin Rosenstein; Jaak Simm. Gene Regulation by Natural Modulators of AhR: Their Role in Male Infertility and Prostate Cancer., Tallinna Tehnikaülikool, Geenitehnoloogia instituut.

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| 2017 | Andreas and Elmerice Traksi stipendium, Tartu Ülikooli Fond. |
| 2014 | Euroopa Biokeemiliste Seltside Föderatsiooni noore teadlase stipendium. |

## Jooksvad projektid

PRG1076 “The role of endometrium as a major factor affecting female fertility and pregnancy outcome” (1.01.2021−31.12.2025); Principal Investigator: Helle Karro; University of Tartu, Faculty of Medicine, Institute of Clinical Medicine; Financier: Estonian Research Council; Financing: 558 900 EUR.

## Täidetud projektid

IUT34-16 “Basic and translational approaches for female reproductive medicine: continuum from oocyte maturation to delivery” (1.01.2015−31.12.2020); Principal Investigator: Helle Karro; University of Tartu, Faculty of Medicine, Institute of Clinical Medicine; Financier: Estonian Research Council; Financing: 466 800 EUR.

EU41564 (EU41564) “Non-invasive Tests for Endometrial Dysfunction (NOTED) – NOVEL CLINICAL PERSPECTIVES FOR INFERTILITY AND ENDOMETRIOSIS DIAGNOSTICS” (1.12.2011−31.12.2014); Principal Investigator: Andres Salumets; Competence Centre on Health Technologies (partner); Financier: Enterprise Estonia; Financing: 847 363 EUR.

## Juhendatud väitekirjad

Arina Laanemets, magistrant, (juh) **Marina Loid**; Tambet Tõnissoo; Keiu Kask. Epiteeli ripsrakud noorte ja vanemate reproduktiiveas naiste endomeetriumis: immunohistoloogiline analüüs ja uue analüüsimetoodika arendus (2024), University of Tartu, Faculty of Science and Technology, Institute of Molecular and Cell Biology.

Merili Peltser, bakalaureus, (juh) **Marina Suhorutšenko**; Indrek Teino. Steroidhormoonide mõju geeniekspressioonile endomeetriumi stroomarakkudes polütsüstilise munasarja sündroomiga patsientidel (2018), University of Tartu, Faculty of Science and Technology, Institute of Molecular and Cell Biology.

Liis Mägi, bakalaureus, (juh) **Marina Suhorutšenko**; Margit Nõukas. Naisepoolne viljatus: geeniregulatsiooni roll endomeetriumi vastuvõtlikkuse kujunemisel (2015), University of Tartu, Faculty of Science and Technology, Institute of Molecular and Cell Biology.

## Publikatsioonid

**Loid, M.**; Obukhova, D.; Kask, K.; Meltsov, A.; Derks, K.; Altmäe, S.; Saare, M.; Peters, M.; Minajeva, A.; Adler, P.; Krjutškov, K.; Zamani Esteki, M.; Salumets, A (2024). Aging promotes accumulation of senescent and multiciliated cells in human endometrial epithelium. *Human Reproducution Open,* 2024(3), hoae048. DOI: 10.1093/hropen/hoae048.

Pathare, A.D.S.; **Loid, M.**; Saare, M.; Gidlöf, S.B.; Zamani Esteki, M.; Acharya, G.; Peters, M.; Salumets, A. (2023). Endometrial receptivity in women of advanced age: an underrated factor in infertility. *Human Reproduction Update*, dmad019. DOI:10.1093/humupd/dmad019.

**Loid, M.**; Obukhova, D.; Derks, K.; Meltsov, A.; Kask, K.; Altmäe, S.; Saare, M.; Peters, M.; Esteki, M.Z.; Salumets, A. (2023). Endometrium Matters. The Endometrial Transcriptome of Advanced Reproductive Age Patients Reveals Changes in the Epithelial Cell Development. *Reproductive Sciences*, Vol. 30, Supplement 1: 256A. 70th Annual Meeting of Society for Reproductive Investigation (SRI), March 21-25, 2023. Brisbane, Austraalia.

**Loid,** M.; Obukhova, D.; Derks, K.; Meltsov, A.; Keiu Kask, Altmäe, S.; Saare, M.; Peters, M.; Zamani Esteki, M.; Salumets, A. (2022). Does endometrium age? The endometrial transcriptome of advanced reproductive age patients reveals the signs of cellular ageing, altered immune response and compromised receptivity. *Human Reproduction,* 37, Issue Supplement 1: *38th Annual Meeting, European Society of Human Reproduction and Embryology. Oxford University Press,* deac104.074. Milan, Itaalia. DOI: 10.1093/humrep/deac104.074.

Khatun, M.; Meltsov, A.; Lavogina, D.; **Loid, M.**; Kask, K.; Arffman, R.K.; Rossi, H.-R.; Lättekivi, F.; Jääger, K.; Krjutškov, K.; Rinken, A.; Salumets, A.; Piltonen, T.T. (2021). Decidualized endometrial stromal cells present with altered androgen response in PCOS. *Scientific Reports*, 11 (16287). DOI: 10.1038/s41598-021-95705-0.

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