DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS
331

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DENISS SÕRITSA

The impact of endometriosis and physical activity on female reproduction

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Institute of Clinical Medicine, University of Tartu, Estonia.

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1. **Sõritsa D**, Saare M, Laisk-Podar T, Peters M, Sõritsa A, Matt K, Karro H, Salumets A. Pregnancy rate in endometriosis patients according to the severity of the disease after using a combined approach of laparoscopy, GnRH agonist treatment and in vitro fertilization. Gynecol Obstet Invest. 2015; 79(1):34–9.
2. **Sõritsa D**, Mäestu E, Nuut M, Mäestu J, Migueles JH, Läänelaid S, Ehrenberg A, Sekavin A, Sõritsa A, Salumets A, Ortega FB, Altmäe S. Maternal physical activity and sedentary behaviour before and during in vitro fertilization treatment: a longitudinal study exploring the associations with controlled ovarian stimulation and pregnancy outcomes. J Assist Reprod Genet. 2020;37(8):1869–1881.
3. Saare M, Laisk T, Teder H, Paluoja P, Palta P, Koel M, Kirss F, Karro H, **Sõritsa D**, Salumets A, Krjutškov K, Peters M. A molecular tool for menstrual cycle phase dating of endometrial samples in endometriosis transcriptome studies. Biol Reprod. 2019;101(1):1–3.
4. Lavogina D, Samuel K, Lavrits A, Meltsov A, **Sõritsa D**, Kadastik Ü, Peters M, Rinken A, Salumets A. Chemosensitivity and chemoresistance in endometriosis – differences for ectopic versus eutopic cells. Reprod Biomed Online. 2019;39(4):556–568.

Author's personal contribution

Paper 1: Participating in the study design, data collection and analysis, writing the manuscript.

Paper 2: Participating in the study design, data collection (enrolling participants, taking informed consent, gathering clinical information) and writing the manuscript.

Paper 3: Participating in the study design, sample and data collection (enrolling participants, taking informed consent, gathering clinical information), interpretation of the results and assisting in manuscript preparation.

Paper 4: Participating in the sample and data collection (enrolling participants, taking informed consent, gathering clinical information), in conducting laboratory experiments (RNA extraction) and interpretation of the results.

ABBREVIATIONS

ART	–	assisted reproductive technology
ASRM	–	American Society for Reproductive Medicine
BMI	–	body mass index
COS	–	controlled ovarian stimulation
CPR	–	clinical pregnancy rate
DIE	–	deep infiltrating endometriosis
ecESC	–	ectopic endometrial stromal cells
euESC	–	eutopic endometrial stromal cells
EG	–	embryo glue
EI	–	endometrial injury
ER	–	endometrial receptivity
ES	–	early-secretory
ESHRE	–	European Society of Human Reproduction and Embryology
ET	–	embryo transfer
ICSI	–	intracytoplasmic sperm injection
IVF	–	in vitro fertilization
FET	–	frozen embryo transfer
FSH	–	follicle stimulating hormone
GnRHa	–	gonadotrophin releasing hormone agonists
GnRH	–	gonadotropin-releasing hormone
hCG	–	human chorionic gonadotrophin
LBR	–	live birth rate
LH	–	luteinizing hormone
LS	–	late-secretory
M	–	menstrual
MS	–	mid-secretory
MVPA	–	moderate-to-vigorous intensity physical activity
OHSS	–	ovarian hyperstimulation syndrome
OPR	–	ongoing pregnancy rate
P	–	proliferative
PA	–	physical activity
PCOS	–	polycystic ovary syndrome
PID	–	pelvic inflammatory disease
POI	–	premature ovarian insufficiency
PR	–	progesterone receptor
FSH	–	follicle stimulating hormone
rFSH	–	recombinant follicle stimulating hormone

PRL – prolactin
RIF – recurrent implantation failure
RM – recurrent miscarriage
STD – sexually transmitted diseases
TFI – tubal factor infertility
VMB – vaginal microbiome
WHO – World Health Organization
WOI – window of implantation

1. INTRODUCTION

It is known that the women's best reproductive age period lasts only till late 20s, so it seems to be appropriate to mention that "Women's fertility has the age", that is unfortunately a rather short period. Nowadays women are postponing the child-bearing, mainly due to the sociological factors, which leads to the decrease in oocytes numbers and increases the risk of infertility. The life-style factors, such as altered nutrition, smoking and alcohol consumption, drug addiction, stress, sleep disturbances, decreased physical activity (PA), as well as increasing environmental pollution have further negative impact on fertility. However, the causes of infertility may be equally shared by both partners and in 10–30% of cases the infertility reason remains unexplained. In addition to advanced age, the female infertility is most commonly caused by ovulatory disorders, followed by endometriosis, pelvic adhesions, tubal blockage, other tubal/uterine abnormalities and hyperprolactinemia. Chromosomal abnormalities and immunological disturbances can also cause infertility.

As mentioned, one of the frequent causes of infertility is endometriosis, a chronic benign gynaecological disease influencing negatively women`s quality of life (pain, loss of productivity, depression) in their reproductive age. It is not known exactly how endometriosis causes infertility, but it is assumed that deteriorated oocytes' quality or impaired endometrial receptivity (ER) may play an important role. Furthermore, endometriosis-associated infertility is poorly treatable and the treatment mainly consists of medical or surgical approaches or combination of both, and assisted reproductive technology (ART) e.g., in vitro fertilization (IVF) is frequently needed to solve the infertility issue. However, endometriosis is a heterogeneous disease and there are no unique treatment regimens suitable for all infertile endometriosis patients, emphasizing the need for further research to find the best infertility treatment options for different forms of disease.

Infertility treatment success depends on many factors, related to the cause of infertility, female and male age and gametes' and embryos' quality, ER and suitability of the chosen treatment regimen. However, the role of a lifestyle in fertility treatment outcomes should not be underestimated. The patients undergoing infertility treatment are usually very sensitive and ask plenty of questions regarding the IVF process and about factors that can influence its success. In addition to issues related to healthy eating and food supplements, smoking and medications used, patients are often interested in the impact of PA during the IVF treatment on the outcome of the procedure. According to the literature, there are many opinions regarding this subject. It is recommended to reduce PA as well as to continue the normal active life. However, most research on this topic is based on questionnaires and this affects the objectivity of the results. In order to bring more clarity to this issue and to come up with objective recommendations for the patients, studies with a different design that measure the actual PA and sedentary time with accelerometers are needed.

In addition to unsolved issues in endometriosis-related infertility, the molecular mechanisms in endometriosis pathogenesis are still unclear and need to be addressed to enhance the detection of proper new target molecules for diagnostics and treatment. Although transcriptomic and proteomic studies comparing composition of endometriotic lesions and endometrium have significantly broadened our knowledge on this topic, alternative approaches could provide additional insight into the behaviour of endometrial cells outside the uterus. For example, chemosensitivity studies could help to understand the cellular mechanisms behind the apoptosis resistance in endometriotic cells.

Coming from the need for new knowledge to provide recommendations for infertile patients undergoing IVF treatment and to find the best treatment options for endometriosis patients, the overall aim of the current study was to reveal the role of PA and endometriosis treatment options on IVF success. In addition, the evaluation of ER and elucidation of molecular alterations in endometriosis were undertaken to assess the mechanisms underlying endometriosis symptoms and etiology.

2. REVIEW OF LITERATURE

2.1. Female fertility

According to The International Glossary on Infertility and Fertility Care, 2017 (Zegers-Hochschild et al., 2017), fertility is defined as the capacity to establish a clinical pregnancy. There are many features that are related to women's fertility with age as the most important factor influencing spontaneous conception. In addition, several diseases, lifestyle and environmental factors play a considerable role in modulation of fertility status (Borght et al., 2018).

2.1.1. Female age-related fertility decline

Nowadays women tend to postpone the maternity and a large study involving 24 OECD countries demonstrated that the average age of mothers at first birth has raised by ∼4 years from 25 years in 1970 to 29 years in 2008 (Mills et al., 2011). In Estonia, the maternal age at the first birth has risen even more, from 22.7 years in 1992 to 29 years in 2020 (increase for a total of 6.3 years) (*Estonian Medical Birth Registry and Estonian Abortion Registry*, 2020). Postponement of parenthood, in turn, increases the risk of infertility, as spontaneous conception begins to decrease already by 25–30 years of age (Silvestris et al., 2019) and women aged 35–39 years have already 31% reduced fertility compared to women aged 20–24 years (Menken et al., 1986). Under natural conditions, if women start to try to get pregnant at age 30 years, 75% will succeed a conception ending in a live birth within 1 year, but the numbers decline to 66% and 44% for women at age 35 and 40 years, respectively (Templeton et al., 1996).

The highest culprit behind the decline in fecundity with increasing female age seems to be ovary-related: female germ cells are not replenished during life and there is a decline in the number of oocytes from birth to menopause, accompanied by the diminished quality of existing oocytes with age (Baird et al., 2005). The main issue of the decreasing number of oocytes due to the progressive follicular atresia is the increased risk for the concomitant infertility. There are about 6–7 million oocytes at 20 weeks of pregnancy in the female fetus and it decreases already to 1–2 million at birth. At the time of puberty, there are around 400,000 oocytes and only 25,000 is present at age 37. At the time of menopause, around the age 50 years, only 1,000 oocytes are left (Figure 1) (American College of Obstetricians and Gynecologists Committee on Gynecologic Practice and Practice Committee, 2014).

The quality of oocytes declines with age due to the oocyte cytoplasm quality decrease and increase in nuclear genome abnormalities (Vollenhoven et al., 2018). Therefore, there is a significantly increased risk of aneuploidy and spontaneous abortion for women postponing the maternity (Balasch et al., 2012).

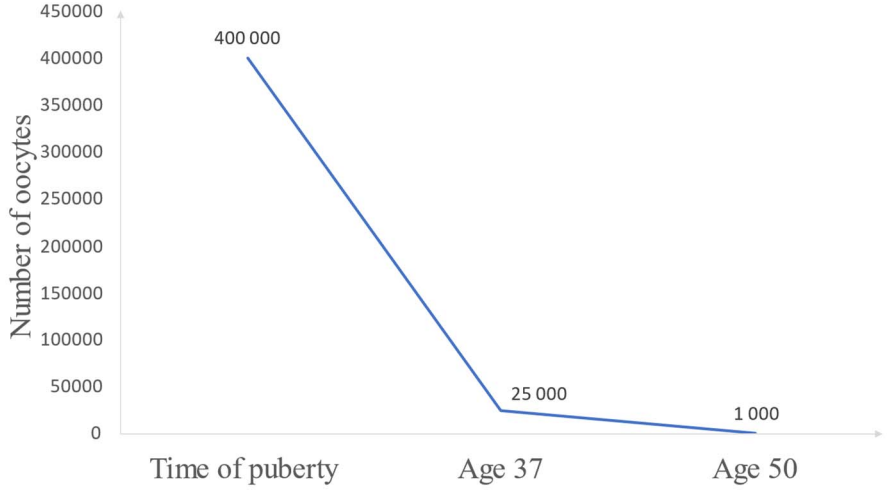

Figure 1. Female age-related decrease in a number of oocytes (American College of Obstetricians and Gynecologists Committee on Gynecologic Practice and Practice Committee, 2014).

Beside ovaries, the functions of other reproductive organs, e.g., uterus seem to be less affected by female age. It has been demonstrated that when using donor oocytes with controlled quality, the numbers of pregnancies and deliveries did not differ between women under or over 40 years (Navot et al., 1994). The other study revealed that oocyte donor age is the major determinant of pregnancy outcome success independent of recipient age (Wang et al., 2012), suggesting that the reproductive capacity of the uterus does not diminish with age. However, the ageing may still have some impact on the functional capacity of the human uterus as there is an evidence of the higher number of pregnancy complications in older reproductive age women (Baird et al., 2005).

A non-biological age-related determinant that may play a critical role in fecundity is sexual intercourse frequency (Stanford et al., 2007) that decreases by approximately 1% for every year increase in female age (Gaskins et al., 2016).

2.1.2. Follicular cycle and its impact on uterine endometrial lining cyclicity

The ovulatory menstrual cycle depends on the integrated actions of the hypothalamus, pituitary, ovary, and endometrium (Barbieri et al., 2014). As mentioned, ovaries contain at birth 1 to 2 million primordial follicles. The primordial follicle contains the oocyte surrounded by pregranulosa cells and develops into a primary follicle when the oocyte is surrounded by cuboidal granulosa cells. The oocytes in primordial follicles are arrested in Prophase I of the first meiotic division until the onset of puberty. At the onset of puberty, the gonadotrophic hormones will initiate the dormant folliculogenesis. However, even before the puberty, the

primordial follicles develop till the secondary follicle stage. During the first stages of folliculogenesis, the maturation of the primordial follicles is continued till the primary and secondary follicles. The granulosa cells divide and develop into multiple layers and theca layer is formed in the secondary follicles. The secondary follicles continue the development after the puberty and via the gonadotrophin stimulation, towards the antral stage follicles until the completion of the first meiotic division and the ovulation of the mature Meiosis II (MII) oocyte. However, the second meiotic division is only taking place when the MII oocyte is fertilized, resulting in the zygote. Although, during each menstrual cycle, a number of secondary follicles are recruited into the growth phase, only a single follicle becomes dominant and ovulates (Dorrington 1979). The follicular phase begins on the first day of menstruation and continues till day 14 (i.e., ovulation) of a typical 28-day cycle, followed by the luteal phase.

Ovulation is regulated by the dynamics of hormones. The pituitary gland is the major link between the brain and ovarian function. The hypothalamus releases gonadotropin-releasing hormone (GnRH) in a pulsatile manner. GnRH secretion stimulates the pituitary gland to secrete luteinizing hormone (LH) and follicle stimulating hormone (FSH). FSH is mostly needed to stimulate the development and final maturation of the follicles. In the ovarian follicle, LH stimulates theca cells to produce androstenedione that is converted to estradiol by the granulosa cells. A critical concentration of estradiol causes positive feedback in the hypothalamus, resulting in an increase in GnRH secretion and an LH surge, leading to the process of ovulation. After ovulation luteal phase begins, that lasts from day 14 to 28 of a typical menstrual cycle. In the luteal phase, both theca and granulosa cells of the follicle form the corpus luteum, which secretes progesterone (Barbieri 2014, Holesh et al., 2021). Progesterone is needed to prepare the endometrium for embryo implantation and to maintain the pregnancy until the placenta takes over progesterone production for the remainder of the pregnancy (Csapo et al., 1973). If fertilization fails, the corpus luteum undergoes luteolysis, and secretion of progesterone drops.

Endometrial tissue consists mainly of luminal and glandular epithelial cells and stromal cells with blood supply provided by the spiral arteries (Noyes et al., 1950). Simultaneously to the changes in the ovaries, several changes occur in the endometrium during the menstrual cycle. There are three phases of the menstrual cycle: the cycle starts with menstruation (days 0 to 4), follows by an estrogen-dominated preovulatory (proliferative) phase (days 5 to 13 days), and a postovulatory and progesterone-dominated secretory phase (days 15 to 28) (Figure 2). In the proliferative phase, estrogen that is produced by the granulosa cells of the developing follicle stimulates endometrial epithelial cell proliferation, gland growth, and vascularization of the glands. After ovulation that typically occurs on cycle day 14, the increasing concentration of progesterone results in secretory changes in the luminal cells, further gland development, decidualization of the stromal cells, and the development of spiral vessels (Barbieri et al., 2014).

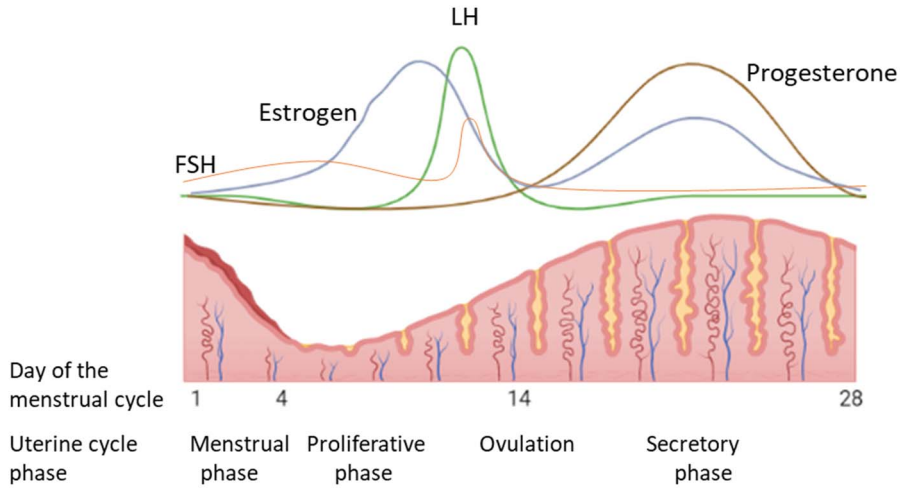

Figure 2. Endometrial changes during the menstrual cycle. Concentration curves of circulating hormones are indicated. The image is created with BioRender.com. FSH – follicle stimulating hormone; LH – luteinizing hormone.

The postovulatory or secretory phase begins after the cycle day 14 and is constituted by an early (ES), mid (MS), and late secretory (LS) phase. The ES phase is regulated by both estrogen and progesterone; the MS phase is regulated by progesterone alone and the LS phase is associated with progesterone withdrawal and, consequently, the collapse of the endometrial glands, constriction of the blood vessels, and the sloughing of the endometrium (menstruation) (Jabbour et al., 2006). The MS phase of the cycle is a critical period because changes at this time lead to the development of ER that is essential for the effective implantation of the embryo, also referred as the window of implantation. Consequently, MS phase is also called as receptive and ES phase as pre-receptive. In the MS phase of menstrual cycle, as a result of elevated levels of progesterone, endometrial stromal cells undergo decidualization. During the decidualization, the stromal cells transform into specialized secretory cells that provide both a nourishing and receptive cell microenvironment necessary for embryo implantation and subsequent placental development. During this process, stromal cells acquire a rounded morphology, and start expression of progesterone-dependent proteins and other biomolecules, including prolactin, glycogen, tissue factor, and insulin-like growth factor-binding protein 1 (IGFBP1) (Critchley et al., 2020). In the absence of pregnancy, with the demise of the corpus luteum, the menstruation occurs and the upper 2/3 layers of the endometrium are shed (Jabbour et al., 2006).

2.1.3. Impact of life-style factors on fertility

One of the life-style factors that can affect fertility is PA. However, the impact of PA on female fertility is a controversial topic. The sedentary lifestyle has been related to the increased risk of infertility and intensifying vigorous activity (but not moderate activity) has been associated with reduced relative risk of ovulatory infertility (Rich-Edwards et al., 2002). A recent meta-analysis indicated that PA may have beneficial effect on pregnancy rate in young adult women (Mena et al., 2019). On the contrary, other studies have demonstrated that increased frequency, duration and intensity of PA may raise the risk of infertility, and high frequency of PA has been associated with involuntary childlessness (Gudmundsdottir et al., 2009). Therefore, further research is required to determine the optimal parameters (intensity and duration) of PA that support the achievement of reproductive benefits.

Nutrition is an important factor affecting the ovarian function and thereby also female fertility. Both extremes, over- and underweight may be behind the ovulatory disorders (Frisch 1990). Overweight increases the risk of anovulation, lower assisted reproductive technology (ART) outcomes, miscarriage, gestational diabetes, hypertension, premature labor and preeclampsia (Chavarro et al., 2007; Dağ et al., 2015; Dean et al., 2014). Body mass index (BMI) already over 24 has been found to be a risk factor for subsequent ovulatory infertility (Rich-Edwards et al., 1994), whereas obese women were three times more likely to suffer infertility than women with a normal BMI. Altered nutrition also affects negatively the quality of embryos and implantation success (Bellver et al., 2007). Underweight causes amenorrhea and anovulation, increases risk of ovarian hyperstimulation syndrome (OHSS), lower ART outcomes, preterm birth as well as small-for-gestational age babies (Chavarro et al., 2007; Dean et al., 2014).

Smoking and alcohol consumption, a primary source of preventable risk factors, both affect female reproductive health. The negative effect of smoking on reproductive function in women is related to ovarian, oviductal and uterine functions, and has an impact on hormonal level (De Angelis et al., 2020). At ovarian level, smoking is associated with reduced ovarian reserve, which together with decreased oocytes' quality may lead to the development of early menopause. Oviductal function is impaired by affected oviductal smooth muscle contractility and smoking increases the risk of ectopic pregnancy. At uterine level, smoking has been associated to delayed implantation, mediated by reduced ER and impaired cytotrophoblast proliferation, migration and invasion. In addition, smoking is related to lower estrogens and progesterone and higher androgens levels. According to the literature, smoking is related to conception delay, higher risk of spontaneous miscarriage, preterm birth and lower birthweight, lower pregnancy rate from ART and drastic decrease of ovarian reserve (Rogers, 2009).

The effect of alcohol consumption on fertility is less clear. Some studies have shown negative relationship between ART outcomes and alcohol consumption (Klonoff-Cohen et al., 2004; Rossi et al., 2011), but other studies have not confirmed these findings (de Jong et al., 2014). However, alcohol consumption has

been related to the loss of menstrual cycle and ovulation regularity, increased level of hormones (LH, estradiol, testosterone), and diminished ovarian reserve (Emanuele et al., 2002). Alcohol abuse is also associated with health disorders like heart disease, hypertension, cancer, liver disease, gastrointestinal pathology, depression and anxiety, relationship issues and unemployment (Hammer et al., 2018) that all may indirectly affect reproductive functions. Drug addiction is related to a wide range of known detrimental effects on human health; however, the effect of the consumption of addictive drugs on female reproductive function and fertility has been scantly investigated (De Angelis et al., 2020). The impact of drug use on sexual behavior is more investigated and found to be associated to more risky behavior and unwanted pregnancy (Bosma-Bleeker et al., 2018; Zapata et al., 2008).

Furthermore, stress is one of the life-style factors that negatively affects the female fertility. Depression and anxiety may lead to altered oocyte maturation and lower fertilization rate (Hillary et al., 2004) and result in overall lower ART clinical outcomes (Purewal et al., 2018). It has been observed that decreasing hard-working time and increasing the relaxing period time may improve the pregnancy outcome (Domar, 1996). One of the very important factors influencing female fertility is the duration and quality of sleep as it is directly connected with emotional and physical well-being. The sleep disturbances can affect female fertility due to the irregular menstruation cycle that prolongs the time to conception, causing dysmenorrhea and increasing miscarriage possibility. The specific mechanism how fertility and sleep are connected is rather unclear (Kloss et al., 2015) but the major cause seems to be circadian misalignment, that is a problem in a shift work (Labyak et al., 2002; Mahoney, 2010). Circadian dysrhythmia may affect secretion of reproductive hormones such as LH, follicle FSH and prolactin (PRL) and therefore potentially interfere with fertility outcomes (Kloss et al., 2015).

2.1.4. Impact of environmental factors

The impact of environmental factors on the female fertility cannot be ignored. Continuous growth of the population creates a greater need for food, which in turn leads to increasing environmental pollution and raises the global annual emission rate and release of reprotoxic chemicals (Rzymski et al., 2015). Environmental contaminants, such as heavy metals may alter gene expression and lead to epigenetic changes (Arita et al., 2009). Epigenetics has been related to the pathogenesis of preeclampsia, intrauterine growth restriction and infertility due to the environmental exposure (Edwards et al., 2007; Pozharny et al., 2010). The widely-distributed environmental heavy metals pollutants, such as cadmium, lead and mercury have an adverse effect on the fertility due to their toxicity against all living organisms (Jang et al.,, 2011; Youness et al., 2012). Moreover, cadmium is considered to be a metalloestrogen capable of affecting estrogen and progesterone receptors (PR) functions (Rzymski et al., 2015) and higher mercury

concentration in female blood has been associated with lower fecundity (Cole et al., 2006). In addition, during pregnancy, the toxins may be transmitted from the pregnant woman to the developing fetus via the placenta (Lee et al., 2018).

The most studied chemical in association with female infertility (Pivonello et al., 2020) is bisphenol A, mainly found in the plastic bags, cans, and bottles (Konieczna et al., 2015). Bisphenol A represents the class of chemicals referred as the endocrine-disrupting chemicals that mimic, block, or interfere with hormones in the body's endocrine system. Bisphenol A can bind to estrogen, androgen, and thyroid receptors, and thereby detrimentally affect the reproductive system, fetal physiology and neonatal development (Rattan et al., 2017).

2.2. Causes of infertility

According to the World Health Organization (WHO) definition, clinical infertility is a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse (Zegers-Hochschild et al., 2009). Infertility is nowadays a common disease and serious social problem among people of reproductive age. Infertility is experienced by 13% to 15% of couples (48.5 million couples) worldwide (Kamel, 2010), and this number is steadily increasing with the trend to delay the time of first pregnancy in developed countries, and now also in developing countries. There are approximately 2 million new infertile couples per year and the numbers are increasing (Datta et al., 2016).

The infertility causes between female and male are distributed equally. It is reported that male infertility is responsible for 20–30%, female infertility for 20–35% of cases, and 25–40% of cases are related to both partners. The probability of unexplained infertility is 10–30% (Figure 3A) (ESHRE 2016; Quaas et al., 2008).

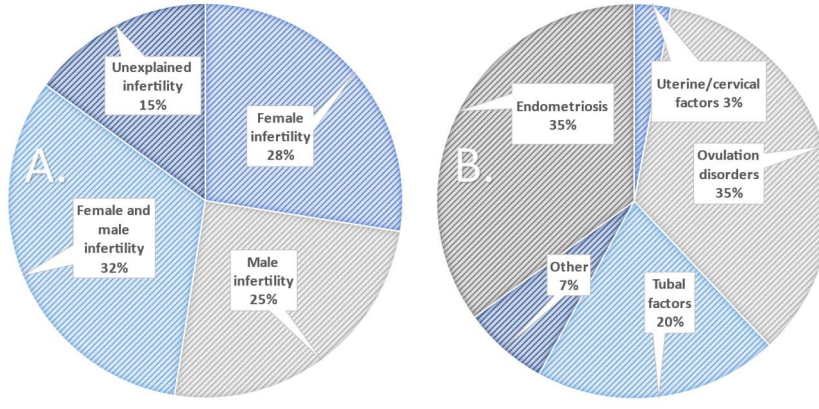

Figure 3. A. The range of distribution of infertility causes between females and males (ESHRE, 2016; Recent Advances in Medically Assisted Conception. Report of a WHO Scientific Group., 1992). **B.** Female causes of infertility (Riches et al., 2013).

The total number of women in reproductive age in Estonia is about 260,000. Therefore, there are probably over 10 thousand couples in our country who would like to conceive but cannot achieve pregnancy because of the reproductive health issues.

According to the large multinational WHO study (Recent Advances in Medically Assisted Conception. Report of a WHO Scientific Group 1992), female infertility is most commonly caused by ovulatory disorders, followed by endometriosis, pelvic adhesions, tubal blockage, other tubal/uterine abnormalities, and hyperprolactinemia (Figure 3B).

2.2.1. Ovulatory disorders

Ovulatory disorders are probably the main cause of infertility, divided according to the WHO Guidance into three categories (O'Flynn, 2014). Group I disorder, that makes up around 10% of ovulatory disorders, includes hypothalamic pituitary failure (hypothalamic amenorrhea or hypogonadotropic hypogonadism). In these diseases the secretion of the GnRH and gonadotropins are impaired at hypothalamic and hypophyseal levels, respectively.

Group II disorders that are the most frequent (around 85% of cases) include dysfunction of the hypothalamic-pituitary-ovarian axis, like polycystic ovary syndrome (PCOS). PCOS is the most common endocrinopathy affecting fertile age women, characterized by ovulatory and menstrual dysfunction, clinical or biochemical hyperandrogenism and polycystic ovary morphology. PCOS is often but not always accompanied with overweight (Mikhael et al., 2019). PCOS is also characterized by insulin resistance. The high insulin levels promote secretion of androgens from the ovaries and adrenal glands, leading to hyperandrogenism. Excess amounts of androgens lead to menstrual disturbances and infertility (Stadtmauer et al., 2002).

Group III involves premature ovarian insufficiency (POI) that occurs in around 5% of cases among the patients with ovulatory disorders. POI is a loss of ovarian function before the age of 40 years (O'Flynn 2014), caused by genetic, autoimmune, metabolic, iatrogenic, and infectious factors. For example, the surgical approaches on the ovaries, leading to the reduced ovarian tissue, have detrimental impact on the ovarian reserve, but in majority of diagnosed women, the etiology of POI is unknown (Welt 2008). One of the well-known genetic causes of POI is a partial or complete loss of one X chromosome in a 46,XX fetus, known as Turner syndrome (45,X), with a frequency of 1 in 2500 newborn females (Reindollar 2011).

2.2.2. Endometriosis

Endometriosis is defined as a disease characterised by the presence of endometrium-like epithelium and/or stroma outside the endometrium and myometrium, usually with an associated inflammatory process (Tomassetti et al., 2021) (Figure 4A-D). The most common method to diagnose endometriosis has been laparoscopy. However, as laparoscopy does not always visualize the presence of all microscopic lesions in the abdominal wall peritoneum (Khan et al., 2014), detailed anamnesis and symptoms of the patient have to be taken into account if endometriosis is suspected. In addition, endometriosis should be histologically confirmed by the presence of endometrioid glands and stroma (Figure 5). However, the new the European Society of Human Reproduction and Embryology (ESHRE) guidelines suggest that laparoscopy is only recommended in patients with negative imaging results and/or where empirical treatment was unsuccessful or inappropriate (Becker et al., 2022).

Endometriosis is a complex syndrome associated with estrogen-dependent benign chronic gynecological inflammatory processes that influence female health negatively by causing abdominopelvic pain and infertility (Eskenazi et al., 1997). Endometriosis affects continually or intermittently women from the teenage years up to menopause (Brawn et al., 2014), and it is estimated that around 200 million women around the world, that is 10% of reproductive age women, are suffering from this disease (Rogers et al., 2009). Among infertile women, endometriosis frequency is up to 50% (Giudice 2010).

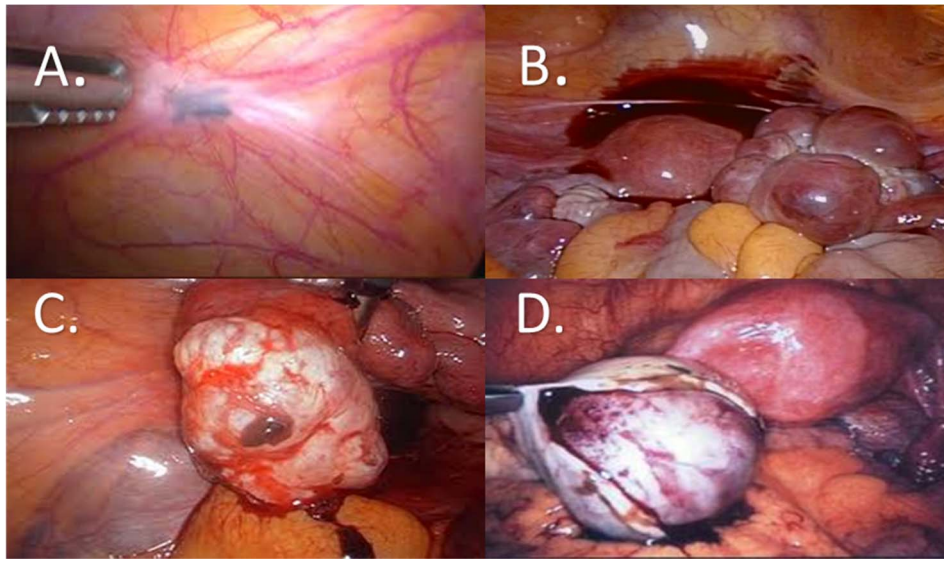

Figure 4. Laparoscopic visualization of endometriosis. **A.** Peritoneal lesion; **B.** Menstrual blood in the abdominal cavity; **C.** Endometriotic lesion on the ovary; **D.** Ovarian endometrioma. The photographs made by the author of the Thesis (Elite Clinic, Estonia).

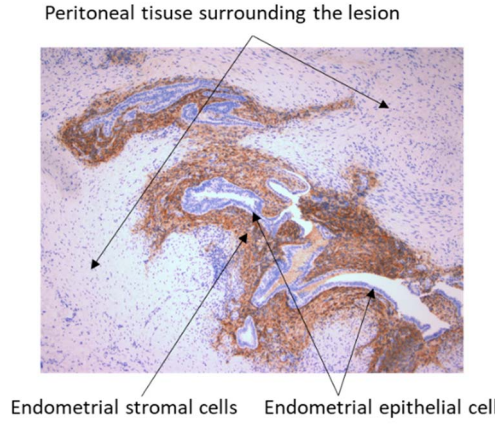

Figure 5. Photomicrograph of peritoneal endometriotic lesion. Section of *ligamentum sacrouterina*, 10×magnification, 10 µm section stained with haematoxylin and for CD10. Brown staining indicates CD10 positive endometrial stromal cells. Image courtesy of Dr. M. Saare from the Institute of Clinical Medicine, University of Tartu.

In women undergoing abdominal surgery for chronic pelvic pain or infertility, endometriosis represents one of the most common pelvic pathologies (Apostolopoulos et al., 2016). The pelvic endometriosis symptoms are pain during menstruation and intercourse, infertility, or continuous pelvic pain, which impact detrimentally women`s social, academic, professional, and economic potential. The cyclic pelvic pain and knowledge of infertility could cause depression and anxiety (Chen et al., 2016). However, because of the nonspecific symptoms, the disease is underdiagnosed and the delay of diagnosis between the beginning of the complaints and the accurate diagnosis is around 7 years (Rogers et al., 2013). The etiology of endometriosis is believed to be the retrograde menstruation while endometrial cells in menstrual blood migrate through the fallopian tubes to the abdominal cavity (Figure 4B), where they attach on the pelvic peritoneal surfaces, rectovaginal area as well as on ovaries (Figure 4C). The endometriotic lesions may also be found on the bladder, rectum, appendix, ureters, abdominal wall and skin, pelvic nerves, diaphragm, and in rare occasions on pleura, lungs and even on the brain (Davis et al., 2017). Women, who do not have menstrual periods and have never had ovulation followed by menstrual bleeding, usually do not have endometriosis (Bulun et al., 2019).

Three different types of endometriosis have been described based on the location and invasive nature of lesions. Superficial peritoneal lesions are mainly located on the peritoneum and peritoneal cavity organs, and are colored dark-brown, black, blue, red, or are present as white fibrotic areas. Endometriomas are ovarian cysts filled with dark-brown blood and covered by endometrial tissue and the cyst wall (Figure 4D). In case of deep infiltrating endometriosis (DIE), the endometriotic cells penetrate fibromuscular tissue deeper than 5 mm and form nodules (Hsu et al., 2010; Klemmt et al., 2017). The severity of endometriosis is classified according to the American Society for Reproductive Medicine (ASRM)

into four stages (I-minimal, II-mild, III-moderate, IV-severe) (Canis et al., 1997). Women with minimal-mild endometriosis have superficial lesions and mild adhesions. In case of moderate-severe disease, superficial lesions may be present, but also ovarian endometriomas are included and adhesions are more severe. Extraperitoneal endometriosis, as well as DIE are both considered as a severe disease. However, the symptoms of endometriosis frequently do not correlate with the severity of disease, which again leads to the delay of diagnosis.

There is no definite cure for endometriosis. The therapeutic options are suppression of ovulation and decreasing the level of estradiol and overcoming progesterone resistance. Estradiol is necessary for endometriotic lesion sticking to peritoneum, lesion survival, angiogenesis, and inflammatory substances production, such as cytokines, prostaglandins, and growth factors. Therefore, ovarian suppression by menopause or aromatase inhibition that both block estradiol production, lead to regression of the disease, and amelioration of the symptoms, such as pain. Endometriotic tissue responds poorly to progesterone or its agonists because of the lack of PR in endometriotic stromal cells, which produce locally large amount of progesterone. However, use of synthetic antiprogestin like mifepristone and ulipristal acetate, which bind to PR with higher affinity, may help to suppress endometriosis symptoms (Bulun et al., 2019). The suppression of ovulation with long-term hormonal treatment is the main way of management in case of endometriosis-associated pelvic pain (Vercellini et al., 2014).

To find new therapeutic targets and non-hormonal treatment options for endometriosis, disease pathophysiology should be better understood. Therefore, it is necessary to discover the molecular alterations occurring in endometrial cells in ectopic locations. Hull et al. (Hull et al., 2008) have identified alterations in key pathways (cellular injury, inflammation, tissue remodelling and cellular proliferation) that are active in the molecular interactions between ectopic endometrial tissue and peritoneal tissue. As taken together by Klemmt and Starzinski-Powitz (Klemmt et al., 2017), the components of canonical WNT/β-catenin signalling pathway, which is essential for renewal and controlled proliferation of somatic stem cells in several tissues, including endometrium, are often found to be dysregulated in endometriosis. A proteomic study of peritoneal endometriotic stromal cells revealed extensive metabolic reprogramming and alterations in protein levels involved in cellular invasiveness and adhesiveness, apoptosis and immune function (Kasvandik et al., 2016).

Apoptosis (Harada et al., 2004) is the process of programmed cell death that occurs in endometrium every menstrual cycle to eliminate senescent endometrial cells during the menstruation. In normal endometrium, the level of apoptotic proteins is elevated during the late secretory phase but there is evidence that in the endometrium of endometriosis patients, apoptosis is inhibited. Consequently, the endometrial cells survive and implant in ectopic sites. Even if some genes, such as *BCL-2*, *SRC-1* and *FAS* have been proposed to play a role in the aberrant apoptosis resistance in endometriosis (Reis et al., 2013; Harada et al., 2004), it is still not completely clear, what mechanisms are behind the hampered expression of apoptotic proteins. However, as apoptotic pathways may represent promising

targets for the development of novel therapeutic options against endometriosis (Kapoor et al., 2021), it would be important to look more closely at these mechanisms. One possibility to elucidate the mechanisms involved in aberrant cellular mechanisms e.g., apoptosis in endometriosis, is to use in vitro assay methods to screen cytotoxic compounds for the potential to induce apoptosis in eutopic and ectopic cells.

2.2.3. Pelvic adhesions and tubal blockade

One of the frequent causes of infertility is pelvic infection resulting from sexually transmitted diseases (STD). STDs alter fertility by causing inflammation and adhesions in the fallopian tubes (salpingitis) and pelvic inflammatory disease (PID), both of which may end with the tubal factor infertility (TFI) (Tsevat et al., 2017). *Chlamydia trachomatis* is the most common pathogen which causes PID and TFI as its infection is frequently asymptomatic and may therefore stay untreated (Papp et al., 2014). It has been shown that after the anamnesis of *C. trachomatis* infection the spontaneous pregnancy rate is lower and the risks for embryo implantation failure and ectopic pregnancy are higher (Coppus et al., 2011). The second most common STD-causing microbe is *Neisseria gonorrhoeae*, that is also in majority cases asymptomatic and causes damage to the fallopian tubes which ends with TFI (Tsevat et al., 2017). *Mycoplasma genitalium* is also well known sexually transmitted infection. It causes also tubal damage and infertility but not as severely as *C. trachomatis* and *N. gonorrhoeae*. *Trichomonas vaginalis* is understudied sexually transmitted infection that causes also infertility but not as harmfully as *C. trachomatis*. Although, together with *C. trachomatis*, the risk of severe upper genital tract infection is higher (Moodley et al., 2002).

2.2.4. Uterine abnormalities

The exact frequency of congenital uterine anomalies is unknown (Hassan et al., 2010). The anatomical abnormalities of the uterus such as Mullerian pathologies, myomas, polyps, adhesions, which are usually asymptomatic and undiagnosed, may be behind the infertility, as well as endometrial pathology due to previous trauma, deteriorated uterine blood flow or thin endometrium (Bashiri et al., 2018). The endometrium-related issues in thickness, vascularity, morphology and receptivity are frequently followed by implantation failure (Taylor et al., 2008). Even the congenital absence of endometrium has been demonstrated (Berker et al., 2008).

The relationship between uterine anomalies and adverse pregnancy outcome, most commonly because of the recurrent miscarriage (RM), is well known. It is important to understand the possible negative effect of the uterine anomaly to the following conception, and if the impact is detrimental, to find out whether and which type of treatment could resolve the issue (Hassan et al., 2010). For example,

the RM after IVF has been shown in female with uterine septa with the prevalence of around 10% (Dicker et al., 1996). The surgical removal of the uterine septa improves the IVF outcome (Hassan et al., 2010). Endometrial polyps and myomas may cause implantation failure due to the impaired uterine receptivity and the surgical removal of them could provide the promising result in improving the implantation rate (Mouhayar et al., 2017; Vlahos et al., 2017).

2.2.5. Other reasons of infertility

The chromosomal abnormalities, with a prevalence about 2%, may be the infertility cause. The most frequent abnormalities are related to chromosomal translocations, but also deletions, inversions and mosaicism have been described in association with infertility (Bashiri et al., 2018). Therefore, karyotyping should be recommended to nulliparous female with a long history of infertility (De Sutter et al., 2012).

The immunological disturbances could not be underestimated as a possible cause of female infertility. Increased levels of T helper 2 cells are characteristic to pregnancy and elevated T helper 1 immune response may lead to rejection of embryos (Nakagawa 2015). The autoimmune antibodies like anti-nuclear antibodies, anti-phospholipid antibodies, and anti-cardiolipin antibodies are related to reproductive failure (Deroux et al., 2017). It is reported that the hereditary thrombophilia (Azem et al., 2004) as well as decreased level of leukemia inhibitory factor (Seli et al., 2005) could be involved in recurrent implantation failure (RIF). As mentioned above, male infertility forms a significant part of the overall infertility that has to be taken into account when considering the couple's fertility. The male infertility is associated mainly with genetic causes, endocrine diseases and malignancies. Chromosomal abnormalities, Y-chromosome microdeletions, several Y-chromosomal genes' specific mutations or deletions are the most prevalent genetic causes of azoospermia (Miyamoto et al., 2012). Bacterial infections and inflammations are another cause of male infertility, and although the precise pathogenesis is not completely clear, they have a detrimental impact on the semen assessment parameters (Gimenes et al., 2014). In about 50% of cases the male infertility cause remains unknown (Stojanov et al., 2018).

2.3. Infertility treatment

The involuntary childlessness is a very stressful condition for a couple. Therefore, many couples are seeking for a help to overcome the infertility. The treatment strategy depends on the cause and duration of infertility and an individual approach plan is required for every couple. There are three main types of infertility treatments: treatment with medicines, surgical approaches, and ART.

The medical approaches mainly consist of infections treatment, regulation of hormones to restore the normal uterine/endometrial cyclicity, induction of

ovulation and support of early pregnancy. The hormones, antibiotics and vitamins are mainly used for medical treatment (Venturella et al., 2019). The surgical treatment means restoration of the anatomy with removal of adhesions, tumors, malformations and endometriotic lesions, permeability check of fallopian tubes, ovarian drilling for ovulation induction in PCOS patients, and preservation of fertility in cancer patients (National Institute for Health and Care Excellence, 2013; Szamatowicz et al., 2020). If these approaches do not give the desired result, the ART treatment is a way to follow. The combination of previously mentioned approaches is often the way to achieve the pregnancy.

2.3.1. In vitro fertilization

In vitro fertilization is a well-known ART. The pioneer of IVF is the British scientist Sir Robert Geoffrey Edwards, who successfully performed an IVF cycle, which resulted in the birth of the world's first IVF-baby in 1978 (BBC News, 1978) and more than 9 million IVF-babies have been born worldwide since then. The first IVF-baby in Baltic countries was born in Estonia in 1995.

In 1997 and 1998, the first IVF-babies were born in Latvia and Lithuania, respectively. Nowadays, we have six centers in Estonia which perform around 3000 ART cycles per year with the pregnancy rate of around 30% per embryo transfer (ET), which is similar to the success rate of other European countries (Calhaz–Jorge et al., 2017). As a result, around 4.0–5.0% (ca 600 babies per year) of all babies born in Estonia are conceived by ART (Figure 6) (Wyns et al., 2020). Estonia together with Austria, Belgium, Denmark and Slovenia are the leading countries in Europe on the availability of ART per million of population (ESHRE, 2016).

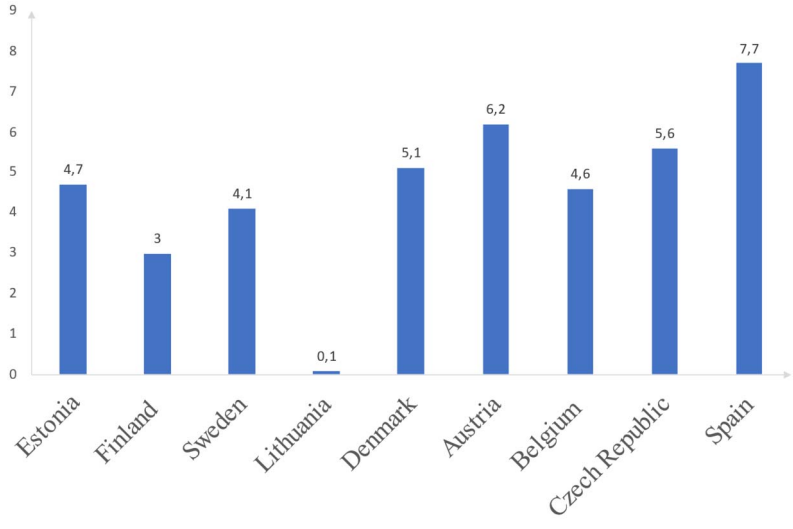

Figure 6. ART infants per national births (%) in 2016 (Wyns et al., 2020).

IVF is the procedure where oocytes are fertilized by sperms outside of the female body (Figure 7). The hyperstimulation of ovaries is performed mainly by recombinant follicle stimulating hormone (rFSH) administration for approximately 10–12 days. The oocytes are retrieved with the needle under the control of ultrasonography and anesthesia.

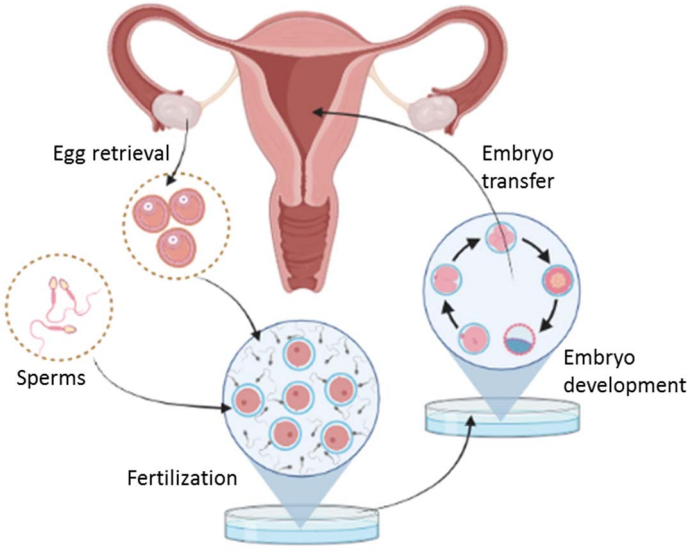

Figure 7. Schematic of basic steps involved in the in vitro fertilization process. The image is created with BioRender.com.

On the day of oocyte retrieval, the male partner provides semen, and the fertilization procedure is selected based on the quality of semen and previous infertility history of the couple. In traditional IVF technology, the oocytes are fertilized/inseminated by sperms spontaneously on a dish. In case of intracytoplasmic sperm injection (ICSI) technology, performed mainly due to the male infertility, the sperm is directly injected into the oocyte under the microscope control. The treatment success is similar in both procedures (ESHRE, 2016). If fertilization has been successful, the development of embryos is followed in the laboratory from 2 to 7 days. The embryo transfer is usually performed on the day 3 or day 5 using the highest morphological quality 4- to 8- cell embryos or blastocysts, respectively. In average, two embryos are transferred in Estonia and 1.81 embryos in Europe (ESHRE, 2016). The pregnancy test will be performed 10 to 13 days later. When the pregnancy test is positive, the clinical pregnancy will be defined by ultrasonography investigation 2 to 3 weeks later.

After the hyperstimulation of the ovaries and fertilization of oocytes, and the transfer of the best embryos, some good-quality embryos usually are left for freezing on the day of ET for the latter use. If frozen embryo transfer (FET) is used, embryos are thawed and are transferred without ovarian hyperstimulation.

In Estonia, frozen embryos may be preserved and used for 7 years after IVF that provides possibility to preserve the fertility.

Due to the ovarian hyperstimulation the size of the ovaries may increase, which in turn may cause discomfort and even some abdominal pain. Bleeding and infection may occur during and after the oocyte retrieval, that are usually easily solved. According to ESHRE fact sheet, the most common complication of IVF is the OHSS, when the ovaries overreact to the rFSH dosage and the fluid accumulates in the abdominal cavity, which in turn cause imbalance of electrolytes, abdominal pain, breathing difficulty, nausea and diarrhea. The incidence rate of OHSS is about 0.2% (ESHRE 2016).

2.3.2. Factors affecting IVF success

IVF is now widely used assisted reproductive technology in the world. However, there are several factors that affect the success of IVF.

The main factor that determines the IVF outcome is female age. It has been shown that the decrease in IVF outcome starts already in women aged 35, and the duration of infertility and the higher number of previous unsuccessful IVF cycles have additional negative effect (Nelson et al., 2011). According to the Centers for Disease Control and Prevention, American Society for Reproductive Medicine Society for Assisted Reproductive Technology, in year 2010, the IVF results using fresh embryos from non-donor eggs were almost 10% higher among women under 35 years compared to those aged 35–37 years (accordingly 41.5% vs 31.9%). The pregnancy success rates declined further with the increasing age of women, being 22.1% for women aged 38–40 years, 12.4% for women aged 41–42 years, 5% for women aged 43–44 years, and 1% for women older than 44 years (Figure 8) (ART Surveillance and Research Team 2010). According to the ESHRE ART fact sheet the majority of women who undergo IVF treatments are aged between 30 and 39 (ESHRE 2016).

TFI, male factor infertility or combination of different causes of infertility are also negative influencers of IVF outcome, whereas diagnosis of endometriosis was related to lower oocyte yield (Bhattacharya et al., 2013). The insemination method seems also to play a role in case of male factor infertility as some studies have shown that performance of ICSI was associated with higher delivery rates compared to IVF (Elizur et al., 2005). On the other hand, others have reported that even if ICSI ensures better fertilization rate, the delivery rate is higher for IVF treatment if embryos are achieved for transfer (Bhattacharya et al., 2013). ESHRE has reported that ICSI is the most common fertilization technique, which has been used in around 75% of all treatments worldwide comparing with 25% of IVF use (ESHRE 2016). Furthermore, the embryo quality plays an important role in achieving pregnancy. It is reported that live birth rate is statistically significantly higher in top quality embryo transfers (Veleva et al., 2013).

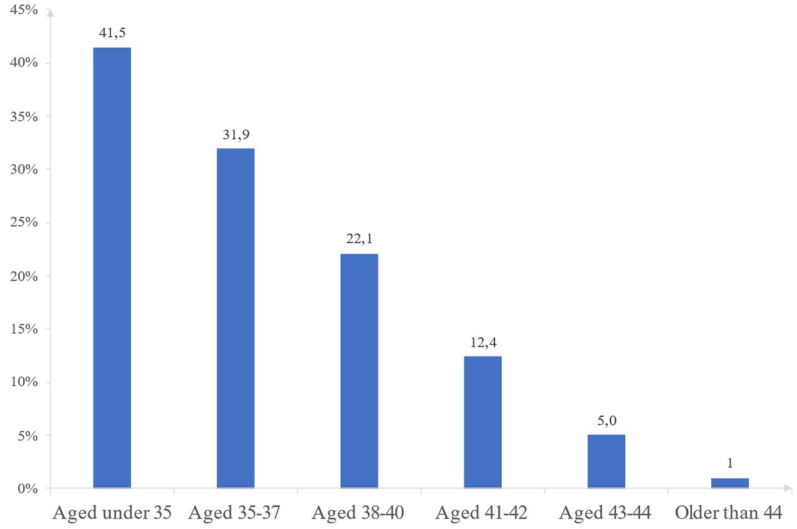

Figure 8. The pregnancy success rates decline with the increasing age of women (ART Surveillance and Research Team 2010).

The highest delivery rate has been reported with the 10–14 retrieved oocytes and with two transferred embryos (Elizur et al., 2005). However, increasing trend in the world is to transfer one embryo to avoid multiple pregnancies, and according to the Agency of Medicines in Estonia, since 2018 one embryo has been transferred in more than 50% cycles in Estonia (Ravimiamet 2021).

Ovarian stimulation protocols and other medical interventions may have an impact on ART outcome. For example, the luteal support with progesterone supplementation plays an important role in IVF outcome, due to the decreased level of progesterone during ovarian hyperstimulation (Hutchinson-Williams et al., 1989), LH release inhibition (Fauser et al., 2003) and the disruption of the corpora lutea associated with oocyte retrieval (Garcia et al., 1981). Although the exact duration of progesterone supplementation remains controversial, it may be discontinued as soon as the first positive human chorionic gonadotrophin (hCG) test is achieved (Watters et al., 2020). The endometrial injury (EI), prednisolone and adhesive compound called embryo glue (EG) may be beneficial in patients with RIF. The significantly higher ongoing pregnancy rate (OPR) and live birth rate (LBR) were demonstrated in women with two or more implantation failures after EI (Nastri et al., 2015). The usefulness of EI has been extensively discussed but the very recent meta-analysis suggested that EI may still be beneficial for younger patients with few previous failed cycles (Nahshon et al., 2020). The exact mechanisms how EI may enhance implantation, are still unclear but it is assumed that EI causes an inflammatory process in the endometrium and induces a immunological response that plays a major role in implantation (Santos-Ribeiro et al., 2017). The use of prednisolone is beneficial on RIF and RM (Dan et al., 2015; Fawzy et al., 2014). Prednisolone is an immunomodulatory agent that suppresses immune responses, has positive impact on the treatment of autoimmune disorders, embryo

implantation, and the development of early pregnancy and IVF outcomes (Abdolmohammadi-Vahid et al., 2016; Robertson et al., 2016).

The use of EG, culture media with added hyaluronic acid, that bridges implanting embryo with endometrial surface, demonstrated higher LBRs. However, as the use of EG was also shown to be related to the higher incidence of multiple pregnancies, it's use should be discussed with a patient if more than one embryo is planned to be transferred (Bontekoe et al., 2014).

The vaginal microbiome (VMB) plays an essential role in the female fertility. A normal VBM, that includes various species of *Lactobacillus*, is associated with a good pregnancy outcome and absence of infections. The *Lactobacillus* species produce lactic acid in the vagina, that lowers vaginal pH and creates unsuitable environment for the pathogenic bacteria growth (García-Velasco et al., 2017). The constitution of VMB affects also IVF outcome and the data suggests that a balanced, *Lactobacillus*-dominated microbiota is associated with a successful IVF outcome (Al-Nasiry et al., 2020). For example, it has been demonstrated that low percentage of *Lactobacillus* in the vaginal sample has a negative effect on embryo implantation success. However, the higher abundance of *L. crispatus* affected the pregnancy rate detrimentally, and women with a low *L. crispatus* abundance had considerably higher chance to become pregnant after the first fresh ET (Koedooder et al., 2019).

The PA is a relevant factor affecting IVF success. In a recent meta-analysis, Rao et al. concluded that PA before ART raises the clinical pregnancy and LBR. However, no impact of PA on the implantation and miscarriage rate was shown (Rao et al., 2018). The other study demonstrated that there was no need to diminish normal PA after ET and bed rest did not improve the success rates of IVF (Cozzolino et al., 2019). Further, PA programs have been suggested for IVF-pregnant women to diminish the risk of gestational diabetes and fpreeclampsia (Charkamyani et al., 2019). It is well known that maternal PA as well as smoking, weight, and nutrition have an impact on female fertility (Gaskins et al., 2016). Fortunately, it seems that PA level of people in Estonia is improving as we can see many people doing sport and investing in the sporting appliances. Although, there are still many people who are inactive due to their sedentary lifestyle, social or financial reasons. It is described that PA could be as helpful as other clinical interventions used for improving reproductive outcome, but the type, intensity, frequency, and duration of optimal PA to keep the good reproductive health are still unclear (Mena et al., 2019). It is important to support individual life-style management, to motivate women to be continuously active and keep BMI in a normal range. There are, however, no specific PA-related recommendations for women planning to conceive or women undergoing IVF.

The endometrial receptivity (ER), defined by a limited period of time (known as the window of implantation, WOI) when the endometrium is opportune for embryo adhesion and the subsequent attachment and invasion processes (Wilcox et al., 1999), is a tremendously important aspect of IVF success ensuring the implantation and subsequent pregnancy. The acute inflammation, influx of leukocytes and production of local inflammatory mediators(Critchley et al.,

1999), is also required for successful implantation while chronic inflammation is harmful and may cause infertility (Lessey et al., 2017). Furthermore, if there is an asynchrony between the embryonic and endometrial development and endometrium is not in an optimal receptivity phase at the moment of ET or FET, the procedure may fail. In the past, the endometrial histological appearance was evaluated according to the criteria described by Noyes et al. (Noyes et al., 1975) for dating the receptivity status of endometrial tissue. Nowadays, several molecular tests have been developed that enable determination of the gene transcription pattern changes occurring in endometrial tissue/cells during the acquisition of receptivity (Enciso et al., 2018; Ruiz-Alonso et al., 2013). The usefulness of these tests to determine the most suitable day for embryo transfer is highly debated (Bassil et al., 2018; Cozzolino et al., 2020; Messaoudi et al., 2019) but the very recent multicenter randomized controlled trial indicated that adjustment of embryo transfer time according to receptivity test resulted in significantly improved pregnancy, implantation and cumulative LBR (Simón et al., 2020).

ER is influenced negatively by endometriosis over the alteration of normal endometrial function (Vannuccini et al., 2016) and referring to progesterone resistance and estrogen dominance that are believed to occur in endometriosis (Fox et al., 2016). It was shown that if transferring the same sibling donor oocytes into the women with or without endometriosis, implantation, clinical pregnancy rate (CPR), OPR as well as LBR were lower in patients with endometriosis (Prapas et al., 2012). The lower pregnancy outcome in women with endometriosis has been related to the endometrial stromal cell decidualization defects (Minici et al., 2008). However, another study demonstrated that if confirmed euploid embryos were used for transfer, the IVF outcomes were similar between endometriosis patients compared with patients undergoing treatment for male factor infertility, suggesting that ER was not affected (Bishop et al., 2020). The similarities between endometrial gene transcription pattern of endometriosis patients and controls during the WOI further confirmed that molecular changes in endometrium are probably not the main reason of endometriosis-related infertility (Da Broi et al., 2019).

2.4. Endometriosis-associated infertility treatment

The treatment of endometriosis-associated infertility is a challenging task, as the reasons behind this phenomenon are not completely understood (Da Broi 2016). However, the reasons could be related to deteriorated quality of the oocytes, or lower number of retrieved oocytes, which lead to the decreased IVF outcome (Rolla 2019). On the other hand, if the embryo quality is satisfactory, endometriosis has no impact on the embryo transfer in IVF in patients under 35 years of age (Chauffour et al., 2016). Basically, treatment of endometriosis and its accompanying symptoms, including infertility, needs special consideration and individual approach to every patient (Johnson and Hummelshoj 2013). The treatment consists mainly of surgical approaches, hormonal medication and IVF

or their combination (Sõritsa et al., 2015). The purpose of surgery, that is mainly laparoscopy, is to remove all visible endometriotic lesions and adhesions, and to restore the initial pelvic anatomy. The laparoscopy should be performed by skilled surgeons to avoid the recurrence of endometriosis due to poor surgery skills or incomplete lesion removal. It is known that surgery of endometriomas influences harmfully the ovarian reserve, therefore it is important to avoid the removal of excessive ovarian tissue that surrounds endometriomas. Although, there is still controversy on surgery for endometriomas and the research is ongoing (Somigliana 2018), it has been found that surgery (i.e. the removal of lesions) increases the IVF outcome in infertile women with endometriosis (Cesana et al., 2017). According to the literature, there is almost 50% chance of spontaneous pregnancy during 1–2 years after curative treatment of endometriosis if fallopian tubes are functional and male partner semen analysis is normal (Vercellini et al., 2006).

The surgical treatment of endometriosis remains the best approach, by removing the endometriotic lesions and adhesions, and improving the probability of subsequent spontaneous pregnancy (Abbott, 2017; Nesbitt-Hawes et al., 2015). However, fertility preservation by oocyte, embryo or ovarian tissue cryopreservation should be discussed with the patient prior to surgery and the surgery should be performed by skilled surgeon to avoid the damage of ovaries. Several studies have shown a lower ovarian reserve, lower Anti-Müllerian hormone concentration and lower antral follicle count in women with endometriosis, particularly in case of bilateral endometriomas (Nezhat et al., 1991; Nieweglowska et al., 2015) and surgical treatment of endometriomas may further reduce Anti-Müllerian hormone level. Nowadays, the European guidelines recommend to preserve oocytes before the ovarian surgery (Garcia-Fernandez and García-Velasco, 2020) but there is not enough evidence to propose preoperative oocyte cryopreservation to prevent endometriosis-associated infertility in women with deep lesions or having only small endometriomas without any additional infertility risk factors (Pluchino et al., 2020).

The benefit of surgery to IVF outcome is closely related to the different stages of the endometriosis, previous history of infertility and the age of the patient. The curative surgery is frequently beneficial to conceive in case of minimal and mild stages of endometriosis. The IVF is often required in the presence of moderate and severe stages of endometriosis. However, there are no randomized controlled studies investigating the impact of surgery in moderate to severe stage of endometriosis-associated infertility comparing with medical treatment or expectant management (Angioni et al., 2015). In case of DIE, according to the last guideline of endometriosis, the effectiveness of surgical approach on the reproductive outcome is unclear due to the lack of research (ESHRE Endometriosis Guideline Development Group 2022). The IVF outcome after repeated surgery is usually low (Rolla 2019).

The medical treatment options of endometriosis consist of gonadotrophin releasing hormone agonists (GnRHa), progestins, oral contraceptives, or aromatase inhibitors, with a purpose to suppress the follicle growth, which leads to anovulation and amenorrhea. The low level of estradiol and reduced uterine blood flow

in turn lead to suppression of endometriotic lesions growth (Vercellini et al., 2018). The GnRHa therapy is a well-known treatment option of endometriosis, lowering the estradiol levels by down-regulating GnRH receptors, but unfortunately causing also unfavorable side effects, like hot flushes, hyperhidrosis, vaginal dryness, headache, decreased libido, mood changes, and sleep disturbances (Giudice and Kao 2004). In the last few years, the new oral GnRH antagonist was introduced for the treatment of endometriosis with less side effects due to uncomplete suppression of estradiol (Taylor et al., 2017). Also, the plant-synthesized natural remedy Resveratrol is reported to be effective treatment for endometriosis due to its anti-oxidative, anti-inflammatory, and anti-angiogenic impacts (Schwartz et al., 2020).

The benefit of pre- or postoperative treatment GnRH is still uncertain. The preoperative treatment could reduce the inflammation as well as the growth of some small lesions. The postoperative medical treatment could reduce the recurrence rate but also postpone the desired conception, due to the suppressed folliculogenesis (Tanbo and Fedorcsak 2017). On the other hand, as surgical removal of pelvic endometriotic tissue, even by the most skilled surgeons, will not be curative, postoperative treatment before conception may be useful. Three- to six-month administration of GnRH analogues before initiation of IVF has shown positive impact on the CPR and LBR (Sallam et al., 2006). Also, the patients with surgically diagnosed endometriosis, pretreated with GnRH analogues for 3 months before IVF, had better pregnancy rates (Surrey et al., 2002). However, some studies have shown that there was no improvement in CPR in infertile patients with endometriosis after 3-months treatment with GnRHa before IVF (Kaponis et al., 2020; Rodríguez-Tárrega et al., 2020). Therefore, the benefit of postoperative GnRHa treatment for the long-term pituitary downregulation in women with endometriosis-associated infertility is still uncertain (Bulletin 2000). Also the recent Cochrane Review confirms the ambiguity on this topic (Georgiou et al., 2019).

2.5. Summary of the literature review

Infertility is a global health issue affecting increasing numbers of couples worldwide. Large efforts have been done to find out which factors affect the couples' fertility and which conditions may lead to the infertility. Over the past decades, ART has rapidly evolved and the number of children conceived through IVF is growing. However, the IVF success rate is still far from satisfying, warranting further studies to determine biological and lifestyle factors influencing infertility treatment outcome and to find the best approaches to shorten the time to successful pregnancy. For example, the effect of PA on IVF outcome has been scantly studied and further research is needed to recommend optimal levels of PA for women undergoing IVF treatment.

One of the frequent reasons of female infertility is a benign gynecological disease endometriosis. Despite of extensive studies, the factors behind endometriosis-related infertility are not completely understood. Endometriosis-associated infertility is poorly treatable and needs further investigations to find the most effective individual treatment approach for every patient. Alterations in endometrial gene expression pattern in women with endometriosis have been proposed as one possible cause of infertility, but the results of different studies are contradictory. Moreover, even if endometriosis research has demonstrated that multiple genes and molecular pathways are differently expressed in diseased cells, more detailed mapping of changes is necessary to broaden our understanding of the disease mechanisms and to find molecular targets, which could be specifically used to destroy endometriotic cells in lesions.

3. AIMS OF THE STUDY

The general aim of the study was to acquire additional knowledge about the endometriosis-related infertility and the factors that influence the infertility treatment outcomes.

The specific aims of the study were:

1. To evaluate the effects of combined treatment approaches (laparoscopy, GnRHa therapy and IVF) on endometriosis-associated infertility in different stages of endometriosis.
2. To analyse the impact of physical activity on the IVF outcome.
3. To reveal alterations in expression of endometrial receptivity genes of endometriosis patients and to use the same gene panel to specify the menstrual cycle phase of endometrial samples.
4. To find molecular alterations in endometriotic cells by implementing cytotoxic compounds' sensitivity testing.

4. MATERIALS AND METHODS

The Research Ethics Committee of University of Tartu approved the study protocols and the informed consent forms (approval numbers 191T-8, 227/T-7 and 276/M-13). All participants signed a written informed consent form.

4.1. Study participants

Altogether, 235 endometriosis patients (Studies I, III and IV), 101 infertile women (Study II) and 33 healthy individuals (Study III) participated in the studies presented in this thesis (Table 1). Patients undergoing laparoscopy at the Tartu University Hospital Women's Clinic (Studies III and IV) in the years 2011–2018 and patients with endometriosis-associated infertility undergoing laparoscopy for curative purposes as a part of combined treatment at Elite Clinic between 2005–2008 (Tartu, Estonia, Study I) were recruited into the study. All enrolled patients were in reproductive age. The presence of endometriosis was confirmed during the laparoscopy by visual inspection and following histological examination of endometriotic lesions. The severity of endometriosis was determined according to the ASRM (1996) revised classification system. Only women with no hormonal treatment for at least 3 months before surgery were enrolled in the Studies III and IV. Endometrial tissue samples and peritoneal endometriotic lesions were collected from women with histologically confirmed endometriosis.

Table 1. General characteristics of the study participants.

	Endometriosis patients				Infertile women	Healthy women
	Study I		Study III	Study IV	Study II	Study III
	Group I	Group II				
N of patients	121	58	45	11	101	33
Age in years, mean±SD	33.2±4.4	32.5±4.4	31±4.7	31.1±5.9	33.5±4.1	32±5.1
BMI, mean±SD	22.4±2.9	21.5±2.6	22±3.5	22.8±2.9	23.9±4.4	23±3.6
Endometriosis stage (n)	I–II (121)	III–IV (58)	I–II (25) III–IV (20)	I (7) III (4)	NA	NA
Duration of infertility, mean±SD	6.1 ± 3.3	6.3 ± 3.6	NA	NA	4.6±3.5	NA

SD – standard deviation; NA – not applicable

The participants of Study II were infertile women in reproductive age, entering IVF/ICSI treatment cycle and receiving fresh ET at Elite Clinic or Tartu University Hospital's Women's Clinic in the years 2013–2016. IVF treatment cycles with donor oocytes were excluded. The main reasons for infertility treatment were male factor, unexplained infertility, TFI, advanced female age, or no partner (70.3% of cases, n=71), infertility because of PCOS (including male factor infertility and PCOS or tubal factor and PCOS) and infertility due to endometriosis (including women with male factor infertility and endometriosis) were present in 6.9% (n=7) and 22.8% (n=23) of cases, respectively.

4.2. Infertility treatment (Studies I and II)

In Study I, the patients who had undergone curative laparoscopy and were eligible for IVF, or who were found to have adenomyosis or moderate to severe endometriosis, were postoperatively given a GnRHa, either Diphereline (Ipsen Pharma Biotech, France) or Zoladex (AstraZeneca UK, Ltd., UK). The first dose of GnRHa was administered on the first postoperative day, and each following dose was administered after every 28 days for 3–6 months. In patients scheduled for IVF, GnRHa treatment and pituitary suppression were continued by IVF without waiting for a menstrual period. In FET program, after GnRH treatment and pituitary suppression, endometrium was prepared using oestrogens. Natural conception was recommended for couples with patent fallopian tubes and normal sperm quality. Some patients received GnRHa treatment but did not undergo IVF treatment because they spontaneously achieved pregnancy prior to the scheduled IVF treatment. IVF and ICSI were performed according to standard protocols. Pregnancy was documented by the presence of gestational sac(s) at 6–7 weeks of gestation, with miscarriages occurring between the detection of a pregnancy and the 22nd week of gestation.

In Study II, IVF treatment was conducted using GnRH antagonist (28%) or agonist (72%) protocol. All patients started rFSH (1560.7 ± 575.0 IU) (Gonal F, Merck Serono, Italy; or Bemfola, Finox Biotech AG, Switzerland) injections on day 2–7 of menses, continuing daily for 9 ± 2–3 days until 3 follicles achieved 18 mm of diameter, and hCG (Merck Serono, Italy) was administered. The controlled ovarian stimulation (COS) follow-up included 3–4 ultrasound assessments of endometrium and follicular growth. Final follicular maturation was achieved using 250 mcg of hCG followed by oocyte retrieval 36 h later. Patients who received IVF (n = 39) or ICSI (n = 62) were both included. On average 1.5 (±0.6) embryos were transferred after COS on days 2 to 5 (n = 59 and 42, respectively). A serum pregnancy test, referred to as positive hCG, was performed on 14 ± 2 days after ET and considered to be positive if β-hCG > 10 mLU/mL. The ultrasound evaluation for defining clinical pregnancy was performed 6 weeks after oocyte retrieval and was considered positive in the presence of at least one intrauterine gestational sac and cardiac activity on ultrasound.

4.3. Assessment of PA and sedentary behaviour (Study II)

For the objective measurement of PA and sedentary time the Uniaxial accelerometer GT1M (ActiGraph LLC, FL, USA) (Figure 9A) was placed on participant waist (Figure 9B).

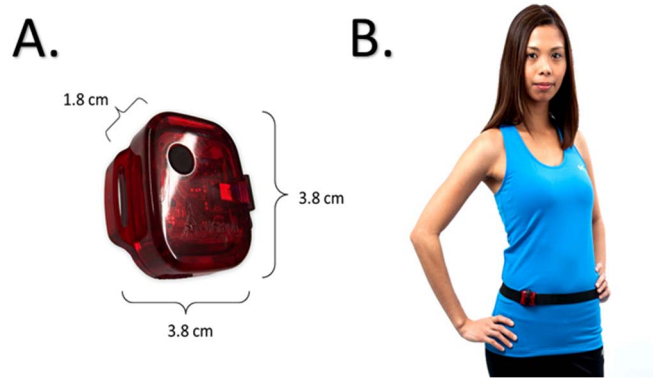

Figure 9 A. Uniaxial accelerometer GT1M (ActiGraph LLC, FL, USA); **B.** Participant wearing the accelerometer on the waist. The photograph made by Signe Altmäe from the Instituto de Investigación Biosanitaria ibs. Granada, Spain.

PA and sedentary behavior were measured for 14 consecutive days at each of the three time points as follows: i) before IVF treatment; ii) during IVF at the implantation time (immediately after embryo transfer); and iii) after positive pregnancy test. Patients, enrolled in the study, did not get any recommendations for PA during infertility treatment. The statistical assessment of PA and sedentary behavior were made by partners from University of Granada, Spain. The analyses of power calculation showed that the study group of 100 women with an alpha error of 5% and a power of 90% would be enough to detect an association of an effect size of 10% variance explained of the study outcomes. Accelerometer data was processed by using ActiLife version 6.10.2 (ActiGraph LLC, FL, USA) software. The analysis was performed among women wearing the accelerometer for at least 10 h/day for 4 or more days (N=98). Women were divided into different groups according to the intensity of PA and different stages of energy expenditure, usually measured as metabolic equivalents.

4.4. In vitro cell culture experiments (Study IV)

In vitro cell culture experiments were performed by Darja Lavõgina, PhD (University of Tartu, Institute of Chemistry). Briefly, stromal cells were isolated from peritoneal endometriotic lesions (ectopic endometrial stromal cells, ecESC) and endometrial tissues (eutopic endometrial stromal cells, euESC) according to the previously published protocol (Kasvandik et al., 2016). The isolated stromal cells

were cultured for 5–6 passages in DMEM/Ham's F-12 medium supplemented with 10% fetal bovine serum (FBS; Capricorn, Ebsdorfergrund, Germany) and a mixture of penicillin, streptomycin and amphotericin B (Capricorn, Ebsdorfergrund, Germany) at 37°C in 5% CO2 in an incubator.

For necrosis/late apoptosis assay euESC and ecESC were seeded onto 96-well plates at a density of 4000–6000 cells per well and after cultivating the cells for 24 h, the medium was exchanged and dilution series of 14 compounds targeting pro-survival enzymes, cytoskeleton proteins, the proteasome and the cell repair machinery were added (see TABLE 1 in Study IV); the final volume per well was 110 µl, and the concentration of DMSO in the treated wells was ≤0.1% by volume. For each plate, each concentration of each compound to be tested was represented in duplicate; the controls (10% DMSO and 0.1% DMSO) were represented in sextuplicate. In 22 hours, the medium was removed and 1 µmol/l Sytox Blue solution in PBS was added. The plates were placed into a multimode reader and incubated for 10 min at 37°C, and the fluorescence intensity was measured (excitation 430 nm, emission 480 nm, monochromator, top optics, gain 90; area scan mode 5 × 5, read height 2.5 mm, with lid).

The viability assay was performed directly after the necrosis/late apoptosis assay using the same plates. The solution of Sytox Blue was replaced by 50 µmol/l resazurin solution in PBS. The absorbance was measured by the multimode reader (570 nm and 600 nm, monochromator; kinetic mode with a reading taken every 15 min for 2 h, read height 8.5 mm, with lid). Next, resazurin solution was replaced by fresh sterile DMEM/Ham's F-12 medium supplemented with FBS, and the cells were incubated for 24 h at 37°C in 5% CO2 in a humidified incubator. Finally, the viability assay was performed again (without the preceding necrosis/late apoptosis assay).

4.5. RNA extraction and detection of gene expression (Studies III and IV)

Total RNA was extracted from both endometrial tissue biopsies and *in vitro* cultured cells using RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNase I treatment was performed using the DNA-free DNA removal kit (Invitrogen, Carlsbad, CA, USA). A 2200 TapeStation system in conjunction with RNA ScreenTape (Agilent Technologies, Palo Alto, CA, USA) was used to determine the quality and quantity of purified RNA.

Before quantitative real-time PCR (qRT-PCR), DNase treated RNA samples were converted to cDNA using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA), and qRT-PCR was performed using 1 × HOT FIREPol EvaGreen qPCR Mix Plus (ROX) (Solis BioDyne, Tartu, Estonia) in 7500 Fast Real-Time PCR System (Applied Biosystems). ACTB was used as a reference gene. Primer sequences are available in the publication (Study IV).

For whole transcriptome sequencing of cultured endometrial (n=3) and endometriotic (n=3) stromal cells (Study IV), RNA from two technical replicates was pooled together. cDNA was synthesized as previously described (Teder et al., 2018) and converted to the next-generation sequencing library using a Nextera XT Library Prep kit (Illumina, San Diego, CA, USA).

For large-scale detection of 57 ER genes (Study III), TAC-seq methodology was used (Teder et al., 2018). SDHA, CYC1, TBP and HMBS were utilized as reference genes. Briefly, mRNA was converted to cDNA, hybridized with specific oligonucleotide probes and converted to next generation sequencing library. Libraries were sequenced with Illumina NextSeq 500 high output 75 cycles kit (Illumina, San Diego, CA, USA).

4.6. Statistical analysis

In study I, the unpaired t test, Wilcoxon's rank sum test and the χ 2 test were applied to compare the groups. Logistic regression analysis adjusted for age, body mass index (BMI), presence of adenomyosis and severity of endometriosis was used to assess pregnancy and delivery outcomes in different study groups.

In study II, associations of baseline PA/sedentary variables with oocytes and embryos obtained after hormonal stimulation were examined using linear regression models, after adjustment for a set of potential confounders selected based on existing clinical knowledge: accelerometer registered time, age, BMI, educational level, smoking, infertility diagnosis, infertility duration, and the amount of FSH administered. The light PA (the mean accelerometer counts per minute (cpm) 100–1951), moderate PA (1952–5724 cpm), vigorous PA (> 5724 cpm), and sedentary time (< 100 cpm) (Freedson et al., 1998) were measured, and using binary logistic regression models after adjustment for the same set of confounders, the associations of PA/sedentary levels at different time points (i.e., baseline, after embryo transfer, and after positive serum hCG pregnancy test) or changes from baseline to implantation period with different pregnancy outcomes (i.e., positive hCG, clinical pregnancy, and live birth) were examined. Additionally, the women were asked to fill questionnaire regarding the spent duration of screen time (TV watching, DVD, computer) during the last 5 work days and total screen time spent during the last non-work days (e.g., weekend). The isotemporal concept analysis was performed to measure the total accelerometer registered waking time (the accelerometer was removed during sleep and exposure to water). Furthermore, one-way analysis of covariance model was used to measure whether PA/sedentary levels at the three different time points varied between women who became pregnant and those who did not (positive hCG, positive clinical pregnancy, and live birth).

In Study III, raw sequencing reads were processed and analysed by Priit Paluoja, MSc (University of Tartu, Institute of Computer Science) as described in (Teder et al., 2018). Each sample was normalized using geometric mean of four housekeeper genes (CYC1, HMBS, SDHA and TBP) expression level using

psych (version 1.7.8) package in R (version 3.2.2). TAC-seq data was processed with open-source software (https://github.com/cchtEE/TAC-seq-data-analysis).

In Study IV, raw sequencing reads were processed and analysed by Alvin Meltsov, MSc (University of Tartu, Institute of Molecular and Cellular Biology) as described in the publication. The 2-ΔΔCt method (Livak and Schmittgen, 2001) was used for calculating the relative expression and to determine mRNA expression fold changes.

5. RESULTS

5.1. The effect of infertility treatment approaches on pregnancy and delivery rates of endometriosis patients with different disease severity (Study I)

To analyze the effect of combined approaches on endometriosis patients' infertility treatment results, the retrospectively collected data of 179 infertile women, who had undergone curative laparoscopy, were analyzed. Only the data of women whose complete pregnancy follow-up results, including both natural and assisted conceptions and deliveries, were used for analysis. The patients were firstly divided into two groups according to their endometriosis severity: 121 patients in group I with minimal-mild (stage I–II) and 58 patients in group II with moderate-severe (stage III–IV) endometriosis. Further, the patients in both groups were divided according to the admitted GnRHa treatment status (with and without GnRHa treatment) after laparoscopy (Figure 10). There were no patients without GnRHa treatment in group II and the average duration of postoperative GnRHa treatment after surgery was longer in this group compared to group I (4.9 ±1.6 vs. 4.3 ±1.4 months, p=0.02). Also, the use of IVF treatment was significantly higher among group II patients compared to group I patients with GnRHa treatment (89.7% vs. 75.0%, p=0.03), being the lowest (71.1%) among stage I–II patients receiving no GnRHa therapy.

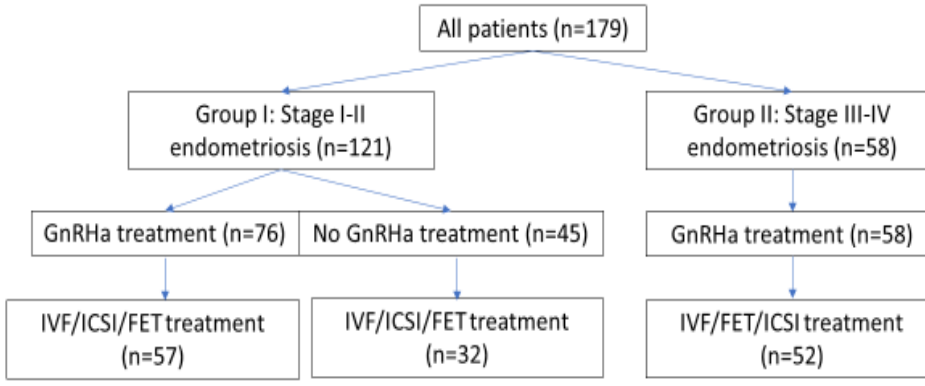

Figure 10. The treatment groups of endometriosis patients.

The results of different treatment approaches are given in Table 2. The cumulative pregnancy rate for all IVF/ICSI/FET cycles was calculated for patients participating in the IVF program until December 2009. The study results showed the beneficial effect of combined treatment on endometriosis-associated infertility. The number of retrieved oocytes was statistically significantly higher among women with stage I–II endometriosis compared to those with stage III–IV disease (p=0.008, Table 2), but the overall CPR and delivery rates separately for ART

and natural pregnancies and combined for all pregnancies did not differ significantly neither between the patients with and without GnRHa treatment inside the group I nor between groups I and II (Table 2). The total pregnancy rate per all patients in both groups was 66.5% (n=119), including women who had conceived spontaneously (18.4%, n=33), or through IVF (48.0%, n=86). Patients with minimal to mild endometriosis (66.9%, n=81) conceived similarly to patients with moderate to severe endometriosis (65.5%, n=38). The miscarriage and delivery rates were also similar in both groups. Seventy-one (58.7%) patients with minimal to mild endometriosis and 30 (51.7%) patients with moderate to severe endometriosis gave birth (Table 2).

Table 2. Results of treatments of endometriosis patients with infertility

Characteristic	**Group I**			**Group II**
	GnRHa treatment after laparoscopy			
	GnRHa+/−	**GnRHa+**	**GnRHa−**	**GnRHa+**
No of patients (%)	121	76 (62.8)	45 (37.2)	58
Total no of IVF cycles	142	89	53	71
IVF cycles per IVF patients	1.6	1.6	1.7	1.4
Total no of FET cycles	15	13	2	8
FET cycles per IVF patients	0.2	0.2	0.1	0.2
Retrieved oocytes (mean ±SD)	10.1±5.8*	9.7±5.5	10.7±6.4	7.4±4.3*
IVF/FET cumulative pregnancies per IVF patients (%)**	57 (64.0)	37 (64.9)	20 (62.5)	29 (55.8)
Conceived during I–II IVF attempt per all IVF/FET pregnancies (%)**	51 (89.5)	33 (89.2)	18 (90.0)	26 (89.7)
Spontaneous pregnancies per all patients (%)**	24 (19.8)	16 (21.1)	8 (17.8)	9 (15.5)
The number of total pregnancies per all patients (%)**	81 (66.9)	53 (69.7)	28 (62.2)	38 (65.5)
The number of total miscarriages per all pregnancies (%)	10 (12.3)	6 (11.3)	4 (14.3)	8 (21.1)
The number of total deliveries per all patients (%)	71 (58.7)	47 (61.8)	24 (53.3)	30 (51.7)

GnRHa+/− patients with and without GnRHa treatment. Groups marked with *are statistically significantly different ($p<0.05$); **for clinical pregnancies confirmed by ultrasound scan. SD – standard deviation

5.2. The effect of maternal physical activity during the IVF procedure on the treatment outcome (Study II)

5.2.1. The PA parameters of study participants before and after IVF treatment

To assess the true impact of maternal PA and sedentary behavior before and during IVF procedure on the treatment outcomes, the activity parameters of 101 women were registered three times by accelerometry at: a) 1–6 months before IVF (baseline activity), b) immediately after embryo transfer and c) after positive serum pregnancy test (Figure 11). The patients were highly motivated to participate, so that the minimum requirement for the data to be included in the analyses (time of detected PA for 4 days with 10 or more accelerometer wearing hours per day) was achieved in case of 98 participants. In 88 out of 98 women, the PA data were registered for 10 days or more. After IVF treatment, 43.6% of participants had a positive hCG test, 39.6% had clinical pregnancy and 28.7% of women gave live birth.

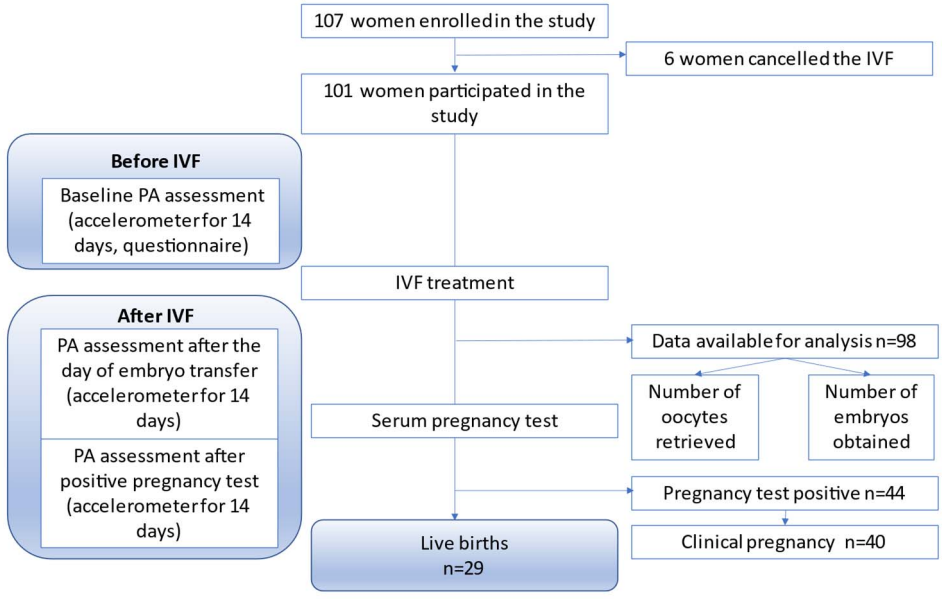

Figure 11. Study design for PA and IVF study. PA – physical activity.

The results of the study revealed that compared to the baseline activity levels, the women significantly altered their PA after starting the IVF treatment. Particularly after ET, the women significantly increased sedentary time ($p < 0.001$) and reduced their PA in all measured PA categories. Time spent both on light and vigorous PA, and also number of steps per day were significantly decreased after ET (Figure 12).

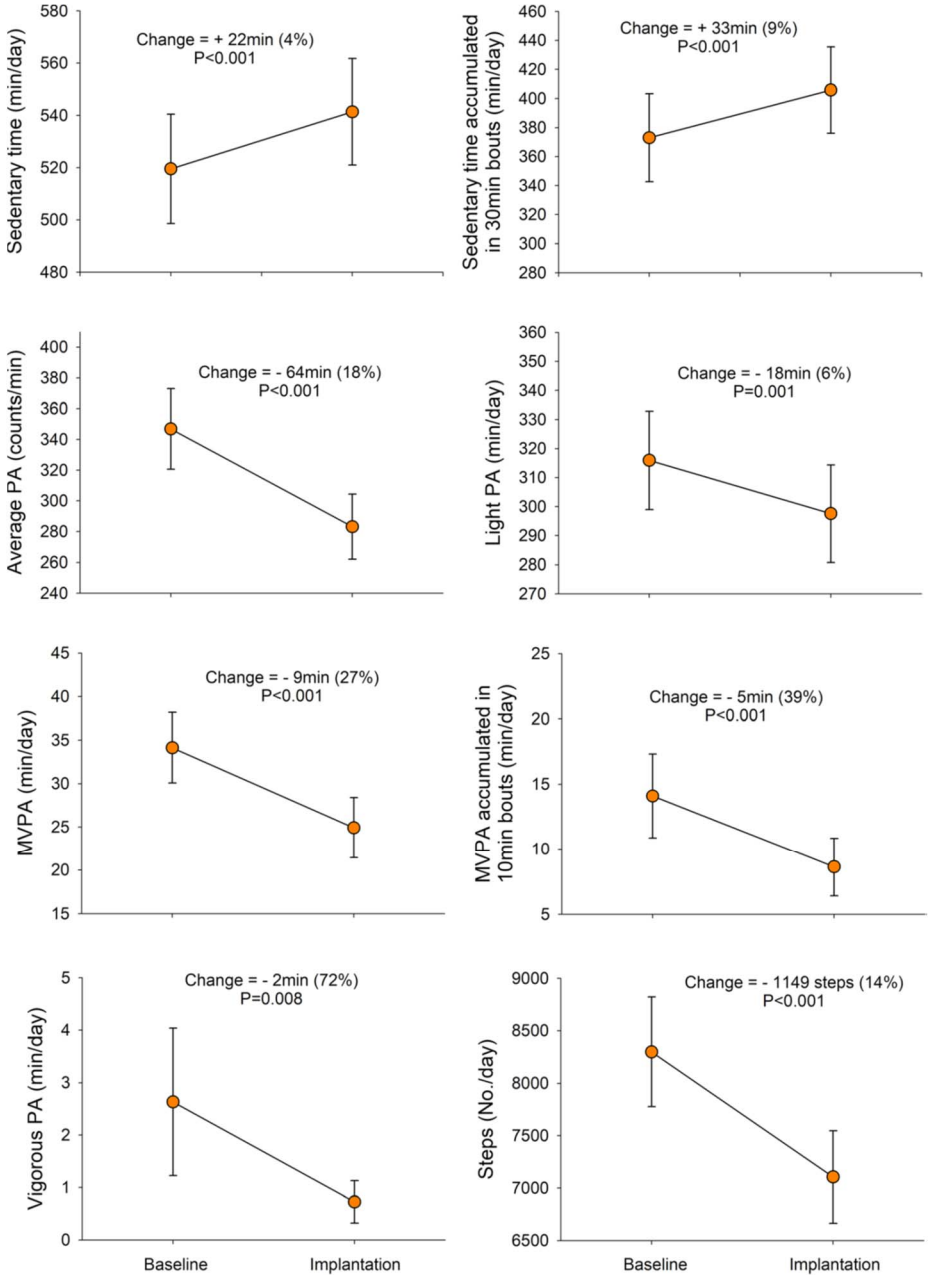

Figure 12. Changes in physical activity and sedentary behaviour from baseline levels to the infertility treatment procedure, at the period of embryo implantation (14 days registered after embryo transfer) (N = 79 women with valid accelerometer data at both time points). PA, physical activity; MVPA, moderate-to-vigorous intensity physical activity. The "bout" is the period of time e.g. a sedentary time bout is the consecutive period of time with less than 100 cpm. MVPA was calculated by summing the time spent at moderate and vigorous PA. P values report the significance of the intra-subject changes tested by repeated measured analysis of variance (ANOVA). The ranges (min. and max.) of the scale used in the Y axes are based on the 25 and 75 percentiles of the present study sample.

5.2.2. The impact of PA on COS and pregnancy outcomes

The analysis showed that the baseline PA and sedentary time are associated with COS outcome. The mean number of retrieved oocytes and obtained embryos in the study group were 11.6 (± 7.1) and 6.6 (± 4.5), respectively. The both parameters were positively associated (both $p < 0.05$) with higher PA, while sedentary time affected these parameters negatively. Also, women with high self-reported screen time obtained 4.7 oocytes ($p=0.005$) and 2.8 embryos ($p = 0.008$) less, compared to women with low-medium screen time. Isotemporal analysis suggested that when woman increased her daily light PA for 1.5 hour and reduced her daily sedentary time for 1.5 hour, she could receive 1.8 oocytes ($p = 0.03$) and 1.2 embryos ($p = 0.03$) more during IVF treatment (Figure 13). The analysis of relationships between PA/sedentary time and IVF outcomes revealed no significant impact neither on positive hCG, CPR and LBR nor on the early pregnancy establishment and risk of miscarriage.

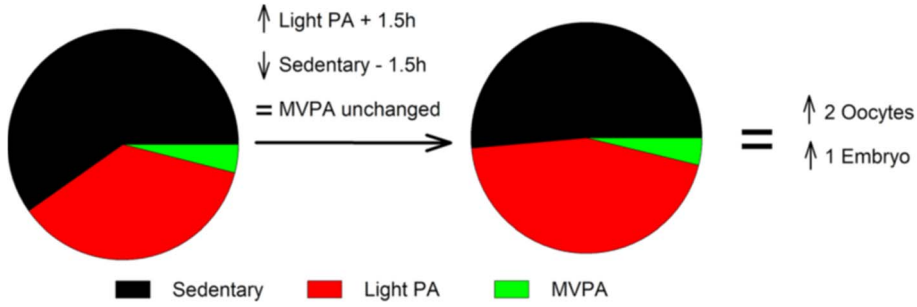

Figure 13. Graphical illustration of the accelerometer isotemporal analyses.

5.3. The expression pattern of endometrial receptivity-involved genes in women with and without endometriosis and as a tool for precise dating of archived endometrial samples (Study III)

To uncover whether genes enabling ER determination are alternatively expressed in women with (n=45) and without endometriosis (n=33, women who have undergone laparoscopy because of symptoms of suggestive of endometriosis and proved to be disease-free), we determined the gene expression levels of a panel of 57 known receptivity genes (Altmäe et al., 2017). The results showed no clear segregation between women with and without endometriosis, suggesting similar gene expression in both groups independent of diagnosis (Figure 14).

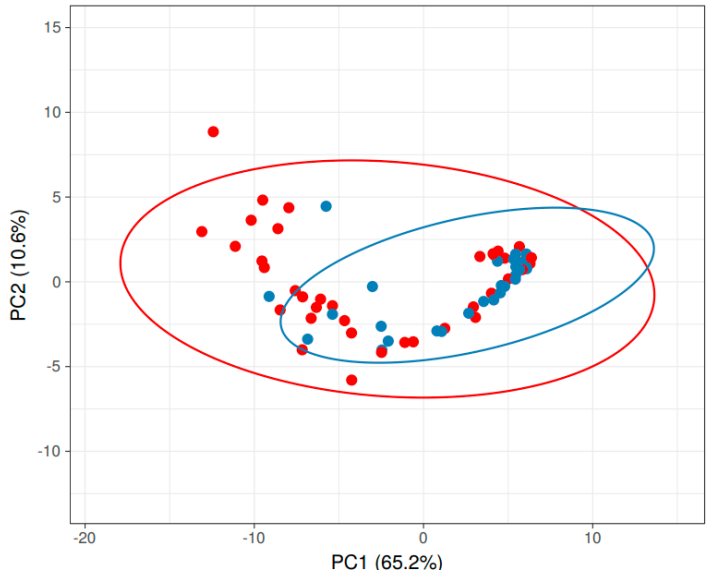

Figure 14. Principal Component Analysis plot of normalized RNA sequencing data of 57 ER genes in women with (red circles) and without (blue circles) endometriosis. The plot was generated using ClustVis web tool (biit.cs.ut.ee/clustvis/).

The endometrial tissue samples in both groups were collected in different phases throughout the menstrual cycle according to the self-reported menstrual cycle days: M (cycle days 1–5, n=4), P (cycle days 6–14, n=17), ES (cycle days 15–18, n=19), MS (cycle days 19–23, n=19), and LS (cycle days 24–28, n=19). We found that if to take the sample collection day into account, the expression pattern of these 57 genes divided the samples roughly into four distinct groups (Figure 15A). All endometrial samples from P phase clustered together and a subset of LS phase samples (n = 8) formed a distinct cluster; however, several samples from LS phase (n = 8) were more similar to MS samples and two LS samples grouped together with M phase samples. Furthermore, there was no clear segregation of ES and MS samples that formed one diffuse cluster. This indicated that self-reported menstrual cycle day does not allow reliable distinction between samples from these adjacent phases, especially not those where there is a transition from pre-receptive (ES) to a receptive (MS) phase and where the length of the cycle therefore plays an important role. According to the available clinical data, all women in our study had a regular menstrual cycle with the duration of 24–35 days but the exact length of the particular cycle when the samples were collected, was not recorded.

The expression pattern of the same 57 genes was also determined in 54 paired endometrial samples from 27 healthy parous women, collected at the histologically and biochemically (predicted from the LH peak in urine) confirmed pre-receptive (ES) and receptive (MS) cycle phases (described in (Suhorutshenko et al., 2018)). Based on the expression data of these samples, a machine learning

support vector machine model for discrimination of ES and MS phase samples was established and applied to segregate the studied self-reported ES and MS samples. After this adjustment, 4 out of 19 ES phase samples were re-classified as MS samples and 9 MS samples were re-classified as ES samples (Figure 15B), showing that molecular profiling helped to assign the endometrial samples from adjacent phases correctly even without precise chronological dating.

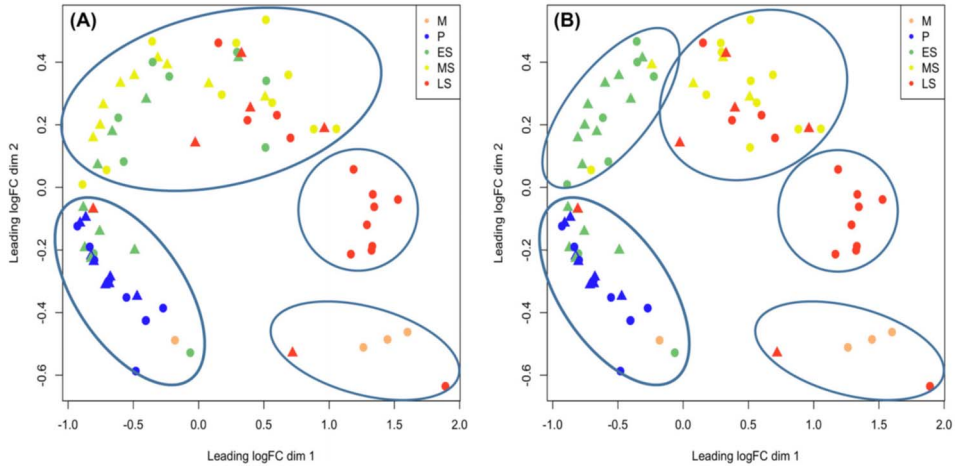

Figure 15. Multidimensional scaling plot of normalized RNA sequencing data of 57 ER genes in women with and without endometriosis. (A) Clustering analysis of RNA sequencing data. (B) Clustering after applying support vector machine classifier to ES and MS phase samples. P – proliferative, ES – early secretory, MS – mid-secretory, LS – late secretory, M – menstrual phase endometrial samples. Triangles represent women without endometriosis and circles mark women with endometriosis.

5.4. The impact of doxorubicin on endometrial and endometriotic stromal cells' gene expression (Study IV)

We hypothesized that when we find compound(s) that affect/reduce viability of ectopic cells more than eutopic cells that would help us to determine alterations in molecular pathways involved in endometriosis development. For this purpose, cytotoxic effects of 14 compounds that target pro-survival enzymes, cytoskeleton proteins, the proteasome and the cell repair machinery (Table 1 in publication of Study IV) were determined in 11 paired samples of euESCs versus ecESCs from peritoneal lesions. The results showed that most of the compounds (GSK690693, CYC116, ARC-775, Bortezomib, MMAE) affected the viability of euESCs more strongly compared to ecESCs ($P \leq 0.05$). SGI-1776 reduced the viability of both cell-types significantly ($P \leq 0.001$) but the difference of the effect between euESCs and ecESCs was nonsignificant ($P > 0.05$). The only compound found with more pronounced effect on ectopic cells ($P \leq 0.001$) was doxorubicin.

Thereafter, to find out which genes and pathways are affected by doxorubicin in euESC and ecESC, large-scale mRNA sequencing was performed after 24 h incubation of cells from three patients with 2 μmol/l doxorubicin or 0.1% DMSO control. The effect of doxorubicin on ectopic cells was stronger at the concentration of 10 μmol/l but as the number of surviving cells for mRNA analysis was very low, we decided to conduct RNA sequencing using treatment of cells with 2 μmol/l doxorubicin. The analysis of mRNA sequencing showed that treatment with doxorubicin affected the expression of larger number of genes in euESC compared to ecESCs (4009 vs. 249, respectively). Several genes that had a higher expression in control euESC relative to control ecESC (*MMP1/3/10, PENK, PTN* and *GRP*) or in control ecESC relative to control euESC (*ESM1, IL33* and *PTX3*) also showed greater expression in the same cell type following treatment with doxorubicin. Furthermore, treatment with doxorubicin resulted in a reduced expression of several genes in euESC (e.g., *DUSP1/10* and *BARD1*) as well as in ecESC (e.g., *DKK1, HAS2*) relative to the control cells of the same type. However, the expression of some genes (such as histone cluster 1 and 2 family members *HIST1H2AE, HIST1H2BK* and *HIST2H2AA4*) in euESC increased upon treatment with doxorubicin relative to control cells, but there was no significant increase in gene expression in toxin-treated ecESC relative to the control treatment (Table 3).

The differential expression of two genes that are known to be involved in chemoresistance to doxorubicin (*PTN* and *HSPA2*) was also validated by qRT-PCR. The gene encoding cyclooxygenase 2 (*PTGS2*) was also chosen for the validation, despite its' slightly higher adjusted p-value than considered significant (Padj = 0.07). However, the product of this gene is known to protect cells against doxorubicin-induced apoptosis. The qRT-PCR validation confirmed significantly higher expression of *PTN* in both control and doxorubicin-treated euESC versus the corresponding ecESC (both $P < 0.05$), and significantly higher expression of *HSPA2* in control euESC versus ecESC ($P = 0.05$). In addition, doxorubicin treatment elevated the level of *PTN* and *HSPA2* in eutopic and ectopic stromal cells compared to the respective control cells (both $P < 0.05$). Furthermore, analysis showed significantly higher expression of *PTGS2* in both control and doxorubicin-treated ecESC versus respective euESCs (both $P < 0.05$).

Table 3. Genes showing significantly different expression in control and toxin-treated euESC and ecESC by mRNA sequencing. Only genes with cut-off values of log_2FC less than or equal to 4 or ≥+4 are shown.

Comparison[a]		**Gene names and log_2FC values**[b,c]
euESC control vs ecESC control	Higher expression in euESC	*MMP12* (8.4), ***MMP10*** (8.0), ***MMP3*** (8.0), *TFAP2C* (7.4), ***RGCC*** (6.8), ***HTR2B*** (6.4), ***GRP*** (6.4), ***DIO2*** (5.7), ***MMP1*** (5.5), *RBP1* (4.9), ***CARD16*** (4.8), *LEPR* (4.8), *PRDM1* (4.7), *CTSK* (4.6), *HSPA2* (4.6), *NID1* (4.6), *GCNT4* (4.5), *PLAU* (4.5), ***PENK*** (4.5), *PTN* (4.4), *IFI6* (4.2), *SEMA5A* (4.1), *AREG* (4.0), *NPY1R* (4.0)
	Higher expression in ecESC	*GIPC2* (–9.7), ***PTX3*** (–9.0), *EFEMP1* (–6.1), ***IL33*** (–6.0), *SFRP4* (–4.5), *PPP1R3C* (–4.3), ***ESM1*** (–4.0)
euESC control vs euESC + toxin	Higher expression in control treatment	***HTR2B*** (8.0), *CCDC107* (7.0), *ING3* (6.4), ***BARD1*** (6.2), *CARNMT1* (5.9), *KRT19* (5.8), *TUBA1A* (5.3), ***DIO2*** (5.2), *PAN3* (5.1), *DUSP1* (4.9), *PKIG* (4.9), *PBK* (4.9), *UTP18* (4.8), *CEMIP* (4.7), *SLC5A3* (4.5), *CITED2* (4.5), *CTGF* (4.4), *SASS6* (4.1), *DUSP10* (4.1), *NOP10* (4.1)
	Higher expression in toxin treatment	*HIST1H2AE* (–7.0), *INSYN2* (–6.7), *TMEFF2* (–6.0), *HIST1H2BPS2* (–5.2), *HIST1H2BK* (–5.0), *HIST2H2AA4* (–4.8), *CXCL3* (–4.7)
ecESC control vs ecESC + toxin	Higher expression in control treatment	*HAS2* (6.9), *MRPL14* (5.0), ***CARD16*** (4.5), *DKK1* (4.0)
euESC + toxin vs ecESC + toxin	Higher expression in euESC	***GRP*** (7.3), ***MMP3*** (7.1), ***MMP10*** (6.1), *PTN* (5.1), ***RGCC*** (4.7), *IFITM1* (4.5), *SOX11* (4.3), ***MMP1*** (4.2), *PENK* (4.1)
	Higher expression in ecESC	***ESM1*** (–6.2), *TFPI2* (–5.3), ***PTX3*** (–4.9), *IL33* (–4.4), ***BARD1*** (–4.1)

[a]Control treatment: 24 h incubation in growth medium containing 0.1% DMSO; toxin treatment: 24 h incubation in growth medium containing 2 µmol/l doxorubicin. [b]The binary logarithm of the fold change of averages is shown in brackets; n = 3. Negative values indicate higher expression in ectopic cells (for euESC versus ecESC comparisons) or in doxorubicin-treated cells (for treatment comparisons). [c]Genes that are listed under more than one comparison in the table are shown in bold. ecESC, ectopic endometrial stromal cell; euESC, eutopic endometrial stromal cell; log_2FC, binary logarithm of fold change of averages.

6. DISCUSSION

6.1. The effect of endometriosis treatment on IVF outcome

It is still unclear how endometriosis affects fertility. The well-known ASRM classification of endometriosis severity does not correlate with the infertility status and patients with milder stages of endometriosis may have infertility issues while patients with severe stages of endometriosis may still be fertile (Garcia-Fernandez and García-Velasco 2020). Our results showed that also IVF outcome among surgically treated patients was not statistically significantly different between patients with different stages of endometriosis. However, our study revealed statistically significantly smaller number of retrieved oocytes in women with moderate-severe stage of endometriosis compared to minimal-mild endometriosis. It is well known that endometriosis affects negatively the number of retrieved oocytes (Pacchiarotti et al., 2020; Borges et al., 2015) but it has also been shown that less oocytes were retrieved from women with moderate-severe stage of endometriosis compared to those with minimal-mild stage disease (Coccia et al., 2011). Similar ovulation rate has been observed in young women ovary, regardless the presence of the endometrioma (Maggiore et al., 2015). However, among women with more severe stages of endometriosis, the number of retrieved oocytes was reduced in women who had endometriomas compared to those with peritoneal disease (Li et al., 2020). According to this data, both disease severity and involvement of lesions in ovaries affect the number of oocytes available for fertilization.

Endometriosis-associated infertility treatment may be the combination of surgery, hormonal therapy and IVF. The treatment has to be individualized according to the previous history of infertility, patient's age and the stage of endometriosis, but it is often difficult to choose the right approach due to the different manifestation of endometriosis symptoms (Vercellini et al., 1996). The largely used ASRM classification of disease severity may not work for all patients due to different pattern and nature of symptoms (The Practice Committee of the American Society for Reproductive Medicine 2012; Zeng et al., 2014). Patients with the same disease stage often have different symptoms with variable severity. In our opinion, combined treatment should be used especially in the more severe forms of endometriosis, although its superiority should still be proven. Nowadays, it is recommended to use the more precise Endometriosis Fertility Index classification, that considers in addition to the stage of endometriosis also the function of the fallopian tubes, fimbria, and ovaries, the age of the patient, the duration of infertility, and if the patient has had prior pregnancies (Adamson and Pasta 2010).

Similarly to our study, others have shown the benefit of curative laparoscopy in case of endometriosis-associated infertility with the similar pregnancy rate in different stages of endometriosis (Vercellini et al., 2006). For example, a recent retrospective study with a follow-up period of four to 14 years demonstrated that

the excision of lesions in moderate or severe endometriosis resulted in a high postoperative pregnancy rate of 65.8%, leading to considerable relief of symptoms and to the low rate of recurrency (Schippert et al., 2020). On the other hand, IVI group in Spain demonstrated that conservative surgery on ovarian endometriomas before ART did not increase the probability of pregnancy, but might increase the costs and time to pregnancy, reducing the ovarian reserve and increasing the risks arising from the surgery (Garcia-Velasco et al., 2004).

The GnRHa treatment is well used in the therapy of endometriosis, especially in case when adenomyosis is also found (Lin et al., 2000; Pouly et al., 2007). In the mild stages of endometriosis, the use on GnRHa is questionable (Bulletti et al., 2010). In our study the GnRHa was used in mild stage of endometriosis mainly when adenomyosis was detected with transvaginal ultrasound. Also, the GnRHa postoperative treatment was used to block the menstruation before following IVF in cases when endometrial pathology was defined during hysteroscopy. In patients with moderate to severe stage of endometriosis, with the frequently presented adenomyosis, the GnRHa treatment after laparoscopy is beneficial on achieving pregnancy (Lin et al., 2000; Pouly et al., 2007). However, a large study of over 900 patients showed that even if women were pre-treated with GnRHa, the presence of adenomyosis had a negative effect on reproductive outcome in IVF cycles (Sharma et al., 2019). In our study, we found that the outcome of infertility treatment among patients with different stages of endometriosis was similar between those treated or untreated with GnRHa. A prospective randomized controlled trial including 450 women with endometriosis showed similar results, an overall pregnancy rate was comparable among GnRHa alone, surgical laparoscopy alone, and the combination of the treatments (Alkatout et al., 2013).

In general, data on the utility of GnRHa therapy in improving IVF outcomes are conflicting. Some studies have reported no useful effect of GnRHa treatment to IVF outcome (Kaponis et al., 2020; Rodríguez-Tárrega et al., 2020) or it has even been proposed that ovarian reserve may decrease due to the treatment time before IVF, and also the higher rFSH units are necessary for hyperstimulation of the ovaries after GnRHa postoperative treatment. On the other hand, some studies have demonstrated significantly improved IVF outcome in women with postoperative GnRHa treatment before IVF (Sallam et al., 2006; Surrey et al., 2002). The benefit of GnRHa treatment to CPR and LBR was also shown in a group of endometriosis patients with RIF (Zhong et al., 2021). A recent meta-analysis demonstrated that for endometriosis patients with milder disease stages, long and ultra-long GnRHa protocols achieve the same clinical outcome but for patients with severe endometriosis stages, the GnRHa ultra-long protocol can improve pregnancy outcomes (Cao et al., 2020). One study has compared the effect of GnRHa and GnRH antagonist treatments on IVF success rate of endometriosis patients and revealed that regardless of the disease severity, CPR and LBR were not significantly affected by the type of GnRH analog used (Drakopoulos et al., 2018).

Some studies have also shown that treatment of infertile women planning IVF after laparoscopic surgery of ovarian endometriomas with an oral progestin dienogest resulted in improved CPR and delivery rates compared to GnRHa treatment, suggesting that dienogest may be more efficient treatment option (Muller et al., 2017) but another study showed the opposite results in patients with severe endometriosis (Tamura et al., 2019). However, it has to be mentioned that the numbers of patients participating in these studies were modest and no definitive conclusions can be drawn. Furthermore, a recent meta-analysis concluded that dienogest treatment has no advantage in improving pregnancy rates compared to other treatments, including GnRHa therapy (Liu et al., 2021).

A comprehensive systematic review and network meta-analysis was conducted to find the best treatment strategy for infertile women with laparoscopic proven endometriosis (Hodgson et al., 2020). The study included 2,245 women and the results showed that compared with placebo, only surgical laparoscopy alone or GnRHa alone were significantly associated with higher odds of clinical pregnancy. Surprisingly, the combination of laparoscopy and GnRHa treatment did not show consistent positive effect on the pregnancy rate (Hodgson et al., 2020), however, it has to be pointed out that only natural pregnancies were considered and the assisted reproduction treatments were excluded.

Limitations and strengths. As a limitation of our study, we have to mention the absence of a group with severe endometriosis without postoperative GnRHa treatment, so it was not possible to evaluate the effect of GnRHa treatment within this group. This is because of the known benefit of GnRHa treatment in severe stages on endometriosis (Pouly et al., 2007; Sallam et al., 2006). Although, it is important to underline, that all procedures were performed by a single gynecologist, which makes the results more consistent and reliable.

In conclusion, in view of all the above, it can be said that it is still unclear what would be the best treatment strategy for infertility in endometriosis. Based on our and other's results, with similar pregnancy and delivery rates in endometriosis patients with/without GnRHa treatment and followed by IVF, the GnRHa treatment seems to be not advocated for patients with less severe forms of endometriosis. However, in our opinion, the combined treatment should be recommended to the women with long history of endometriosis-associated infertility, especially in case of several previous unsuccessful IVF cycles.

6.2. The effect of PA on IVF outcome

According to our experience in clinical work, women reduce their PA during infertility treatment. The main reason for this is discomfort due to medication that may cause increase of the ovaries after hyperstimulation and fluid accumulation into abdomen after oocyte retrieval. In practice, we have noticed that the PA level is lower in women with higher BMI; however, the majority of the Study II participants had a normal BMI. The results of Study II confirmed that infertile women undergoing IVF significantly reduced their PA levels and increased sedentary

time while entering the treatment cycle. This result, as well as finding no association of PA and IVF outcome, is in accordance with the previous report by Evenson et al. (Evenson et al., 2014). Also, in a very recent study (Läänelaid et al., 2021), the PA levels of both men and women seeking for infertility treatment were measured and the results confirmed no significant associations between the time spent on PA and positive pregnancy test or LBR. However, this study also revealed that couples who became spontaneously pregnant after entering the study were physically more active than those needing ART, supporting the positive effect of PA on fertility.

As the result of this study, we would like to recommend to infertile patients to increase PA and decrease sedentary time, because our isotemporal analysis predicted two more oocytes and one more embryo to women increasing PA for 1.5 hours and decreasing sedentary time for 1.5 hours per day. If considering the 101 patients of Study II from whom approximately 1,200 oocytes were obtained, that would mean 200 more oocytes and nearly 10 more babies. The positive influence of the PA on the BMI and thereby in turn on the reproductive system is also a well-known fact (Redman 2006).

Furthermore, it has been proposed that many subfertile couples could benefit from other pre-conception lifestyle interventions implemented before fertility treatment. The use of a modeled impact of lifestyle interventions on fertility outcomes showed a reduction in the number required ART treatments per couple to achieve a successful ongoing pregnancy, accompanied with a considerable cost savings (Steegers-Theunissen et al., 2020).

Recently, even a specific lifestyle program PreLiFe, that provides online coaching on PA, diet and mindfulness based stress reduction for IVF-couples, has been developed (Boedt et al., 2021). Currently, a randomized trial is ongoing to reveal whether the PreLiFe-program effectively improves lifestyle and IVF-success rates.

Several mechanisms have been proposed how PA could affect female fertility: it may have impact on the ovarian function by regulation of estrogens and other steroid hormones production over the hypothalamic-pituitary-ovarian-axis (Tworoger et al., 2007); PA can influence reproductive function via possibility to regulate energy balance affecting BMI, which plays important role in IVF outcome (Redman, 2006); may have effect on the inflammation and lipid profiles (Sorensen et al., 2003) and increased expression of antioxidant enzymes throughout the body have been demonstrated with moderate PA levels (Gomez-Cabrera et al., 2008). PA may also have an effect on fertility through positive effect on the insulin sensitization, which is important in ovulation induction via better clomiphene citrate response of ovaries (Palomba et al., 2010) and by reducing the stress level and anxiety, which both influence negatively IVF outcome (Frederiksen et al., 2015). However, extremely high levels of PA could result in big weight loss owing to the relative energy deficiency due to active sporting, absence of ovulation and amenorrhea, which in turn may lead to infertility (Wise et al., 2012). Unfortunately, there is an absence of guidelines regarding the normal range of PA, which is optimal for fertility.

Limitations and strengths. The accelerometer used in the study required removal while in the water; therefore, water-related activities were not recorded. Also, despite the inclusion of a broad set of potential confounders in this study, there is always the possibility that other confounding factors not included in the analyses could influence the results. The strengths of our study are that PA and sedentary behaviour were measured objectively using accelerometry for 14 consecutive days during three critical periods. Also, we included only fresh embryo transfers, and measured PA before any infertility treatment and 4 weeks during the treatment, which enabled us to closely observe the periods of embryo implantation and early pregnancy establishment.

In conclusion, to our knowledge, this is the first study with objectively measured PA and sedentary time before, during and after IVF treatment. PA seems to affect ovarian stimulation outcomes in IVF treatment but does not affect the embryo implantation and early pregnancy establishment processes. Based on our study results, the patients receiving embryos in IVF program should continue their normal lifestyle without reducing PA levels. However, increased PA and decreased sedentary time may result in more oocytes and embryos available for IVF that may improve the cumulative pregnancy rate from all fresh and frozen ETs. Unfortunately, we could not analyse cumulative pregnancy rate as we included only fresh ET cycles and excluded FET cycles from the analysis.

6.3. The endometrial receptivity genes expression pattern is not disturbed in endometriosis patients but allows to determine the menstrual cycle phase of archived endometrial samples

We discovered that expression of 57 ER marker-genes was similar both in laparoscopically confirmed endometriosis patients and in women without endometriosis, suggesting that altered gene expression of ER determinants is probably not the main reason of endometriosis-related infertility. The issue of endometrial defects involvement in endometriosis patients' infertility has been actively disputed and no common position has been taken. Some authorities declare endometrium is a primary barrier to implantation in women with endometriosis because of inflammatory processes and altered signalling pathways associated with endometrial cells proliferation and survival (Lessey and Kim 2017). The other authors show that according to comprehensive transcriptomic data, the ER of endometriosis patients is not affected (Miravet-Valenciano et al., 2017) that is in accordance with our results.

Endometrial tissue gene expression studies are frequently performed to detect which genes may be involved in endometrium-related pathologies, e.g., endometriosis. For study purposes, endometrial samples are usually collected and classified according to the self-reported menstrual cycle day. However, due to the variation of the normal cycles' length between 24 and 35 days, it is difficult to discriminate the samples from adjacent cycle phases to determine the exact cycle

phase, and to distinguish the ovulatory cycles from anovulatory cycles (Wideman et al., 2013). Many genes in endometrium are expressed under the strict control of steroid hormones which levels differ considerably during the menstrual cycle; therefore, comparing gene expression of incorrectly classified endometrial samples may cause biased results and cycle phase-specific differences will be detected instead of disease-specific alterations. It has been shown that the global transcriptional profile of the endometrial tissues is concordant with the histological evaluation enabling endometrial cycle stage prediction (Ponnampalam et al., 2004), but now we demonstrated that using expression data of only 57 ER related genes is sufficient to address the same issue and it enables to classify most of the endometrial samples according to the menstrual cycle phase. Such kind of molecular tool is beneficial to specify the precise menstrual cycle phase of already collected endometrial samples with self-reported menstrual cycle day and also of endometrial samples from uncertain cycle phases in transcriptomic studies to facilitate the discovery of true disease-related markers and avoid false-positive findings.

Strengths and limitations. The strength of the study is that we analyzed a large number of endometrial tissue samples that were collected throughout the menstrual cycle and included all phases of the cycle. However, it has to be mentioned that the approach is less sensitive for precise classification of samples collected during the transition of the menstrual cycle phases, a phenomenon that can be explained by the fact that menstrual cycle is a continuum without strict boarders between the adjacent phases (Ponnampalam et al., 2004).

In conclusion, the results of the Study III revealed that the molecular tool, consisting of a panel of 57 well-described ER genes, is useful to identify menstrual cycle phases of endometrial tissue samples from uncertain cycle phases of women with and without endometriosis. In addition, our results confirmed that ER is not disturbed in endometriosis.

6.4. Doxorubicin influences the gene expression of endometrial and endometriotic lesions stromal cells differently

To survive, implant and develop endometriotic lesions in the abdominal cavity, endometrial cells in ectopic locations must avoid cell death and have high resistance to apoptosis. Impaired apoptosis of ectopic and eutopic endometrial tissue from patients with endometriosis compared to endometrial tissue from control subjects has been shown in several studies (Imai et al., 2000; Nasu et al., 2011). The results of drug-induced apoptosis studies have demonstrated higher resistance of ectopic cells to staurosporine (Watanabe et al., 2009; Izawa et al., 2006) but there are no reports about chemicals influencing more ectopic cells. The proteasome-targeting compound bortezomib has been shown to reduce the size of endometriotic implants in rats (Celik et al., 2008), yet no studies of bortezomib in euESC from women with endometriosis have been reported; and in our study,

treatment with bortezomib had significantly greater negative effect on the viability of euESCs than ecESCs. The exact mechanisms behind these processes are still unclear and we hypothesized that finding compounds that affect more ectopic than eutopic cells may help to elucidate the role of apoptosis-associated molecules in the pathogenesis of endometriosis. However, our results showed that out of 14 tested compounds only two reduced the cellular viability of ecESCs more significantly compared to euESCs. Whereas the effect of staurosporine was concentration-dependent: 5 μmol/l staurosporine caused greater cellular death in ecESC, 0.2 μmol/l staurosporine was more effective in euESC. Doxorubicin was the only compound that had consistently greater impact on the viability of ectopic cells and therefore we decided to investigate further how this compound affects gene expression of ectopic and eutopic stromal cells.

Doxorubicin has antimitotic and cytotoxic activity through a number of proposed mechanisms, by: forming complexes with DNA by intercalation between base pairs, disrupting topoisomerase-II-mediated DNA repair, and generating free radicals that cause damage to cellular membranes, DNA and proteins. This compound is routinely used in the treatment of several cancers including breast, lung, gastric, ovarian, and thyroid cancers but the major limitation for the use is cardiotoxicity (Thorn et al., 2011). Doxorubicin has also been used in the treatment of endometrial cancer (Chitcholtan et al., 2012; Byron et al., 2012), which increased our interest to investigate the impact of this compound on endometrial and endometriotic cells.

The large-scale transcriptome analysis revealed sets of genes with significantly higher expression in eu- relative to ecESCs or in ec- relative to euESCs, irrespective of the treatment conditions (Padj < 0.05, log2FC of –4 or less or +4 or more). It was postulated that these sets might reflect variations in survival strategies in eutopic and ectopic endometrium, because it is likely that after 24 h of treatment of cells with 2 μmol/l doxorubicin, the isolated mRNA profile was characteristic of the population of survivors. Interestingly, we saw that the treated versus control cells yielded in excess of 10 times more significantly differentially expressed genes in the case of euESC than ecESC. This difference originates probably from the large interpatient variation of gene expression in the ecESC group, being explained by the heterogeneity of lesions originating from the different locations. All three lesions that were used for sequencing, were superficial peritoneal lesions but two of them originated from women with stage I endometriosis and were located on the *excavatio vesicouterina*, and one lesion originated from *ligamentum latum* of more severe form of endometriosis.

In euESC, among other genes, several members of the matrix metalloproteinase (*MMP*) family, precursor for the endogenous opioid peptides, preproenkephalin (*PENK*), and pleiotrophin (*PTN*) were significantly higher expressed in both control and doxorubicin-treated euESC versus ecESC. *MMP*s, *PENK* and *PTN* have previously been linked to endometriosis, showing significantly higher expression in eutopic endometrium from women with endometriosis relative to healthy controls, or lower expression in ectopic than eutopic tissue (Chung et al., 2002; Burney et al., 2007; Kobayashi et al., 2012), thus

pointing to their possible role in initiating peritoneal invasion. Furthermore, PTN has been reported to promote chemoresistance to doxorubicin in several cancers, including osteosarcoma and breast cancer (Huang et al., 2018; Wu et al., 2017). Therefore, it can be suggested that the lower expression of *PTN* in untreated ectopic cells is one of the factors responsible for the higher chemosensitivity of this cell type to doxorubicin – although it should be considered that the viability of euESC was also significantly affected by doxorubicin treatment.

Another gene, Heat shock-related 70 kDa protein 2 (*HSPA2*) was also more highly expressed in eutopic than ectopic cells. *HSPA2* protects cells from the cytotoxic and growth-inhibiting effects of doxorubicin by several mechanisms, including binding misfolded or damaged proteins and enabling these proteins to acquire proper folding, and controlling the duration of cell cycle arrest (Karlseder et al., 1996). According to the qRT-PCR data, the drug treatment enhanced the expression of *HSPA2* in ecESC (average fold change 4.5), suggesting a response to the toxic effect; however, as the initial expression of *HSPA2* in untreated cells was much lower in ectopic then eutopic cells (average fold change –11.8), the expression was still less than that of the eutopic cells.

The gene encoding cyclooxygenase 2 (*PTGS2*) was also chosen for the validation taking into account its' possible role in endometriosis development (Lai et al., 2019). Endometriotic lesions have higher PTGS2 expression compared with the normal endometrium which leads to high cell proliferation, a low level of apoptosis, high invasion, angiogenesis, and infertility. PTGS2 has also been explored in the context of management of endometriosis-related pain (Cobellis et al., 2004). Furthermore, PTGS2 has been shown to protect cells against doxorubicin-induced apoptosis, albeit in the context of tissues other than endometrium (Singh et al., 2008; Puhlmann et al., 2005). The latter observation indirectly confirms the hypothesis that the mRNA profile identified for doxorubicin-treated euESC and ecESC reflects the corresponding cellular survival strategies. The fact that the viability of ecESC was severely affected by doxorubicin treatment indicates that the major chemoresistance-ensuring molecules that contribute to the survival of ectopic cells under DNA damage and ROS-triggered conditions of stress might be less efficient than those in eutopic tissue.

Strengths and limitations. The strengths of the study are that we used a large panel of 14 compounds to find compounds that preferentially affect ectopic cells and utlized a large-scale mRNA sequencing to reveal all potential genes responsible for the pronounced effect of doxorubicine in ectopic cells. This study has also some limitations: first, only stromal cells were studied; however, endometrium contains also other cell types, e.g. epithelial cells that may be involved in unique patterns of signalling and cellular interactions. Second, as only ESCs isolated from superficial peritoneal lesions were investigated, the observed results may not necessarily reflect the effects of toxins in other types of lesions, i.e. endometriomas and deep infiltrating lesions.

In conclusion, mRNA sequencing results confirmed differential gene expression between euESCs and ecESCs and highlighted the genes that may be responsible for the altered characteristics of the endometrial cells in ectopic locations.

7. CONCLUSIONS

Based on the results of the current thesis, following conclusions can be made:

1. Combined treatment approach may be used for endometriosis-associated infertility. Individual approach is needed for every infertile woman. Pregnancy and delivery rates at different stages of endometriosis were not affected by the different approaches used for infertility treatment, with >60% and >50% of patients conceived and delivered a baby, respectively, in both groups of minimal-to-mild and moderate-to-severe forms of endometriosis. The usefulness of GnRHa treatment for endometriosis patients with minimal-to-mild forms is questionable and deserves further studies.
2. PA and sedentary levels did not influence embryo implantation in IVF; however, physically more active women obtained higher number of oocytes and embryos after COS. Based on our study results, the patients receiving embryos in IVF program should continue their normal lifestyle without reducing PA levels.
3. ER genes' expression is not affected in women with endometriosis. However, the expression levels of the well-known ER genes enable to determine the endometrial biopsy collection time throughout the menstrual cycle.
4. Endometrial cells in distinct locations, i.e., eutopic and ectopic locations, react differently to the treatment with cytotoxic compounds, a phenomenon that may be related to the altered expression of several genes involved in endometriosis pathogenesis.

In conclusion, the results of these studies have broadened our knowledge of how endometriosis and PA affect the effectiveness of infertility treatment. In addition, our basic scientific findings confirmed differences in gene expression between the endometrium and endometriotic lesions and could help refine future endometriosis research.

8. PRACTICAL IMPLICATIONS

1. Both the severity of endometriosis and previous infertility should be considered when planning infertility treatment. Patients with suspected endometriosis and with several previous unsuccessful IVF attempts should undergo combined treatment of endometriosis.
2. Patients undergoing infertility treatment should also be counselled on lifestyle factors during treatment. Women should be advised to continue their normal active lifestyle or at least not to reduce their physical activity.
3. As the menstrual cycle significantly affects the expression of endometrial genes, this should definitely be taken into account in endometrial molecular studies. When using archived endometrial biopsies for research, it should first be determined at what phase they have been collected. Based on our studies, for example, an endometrial receptivity test with a limited number of genes can be used for this purpose.
4. Further research is needed to investigate the molecular mechanisms of endometriosis. To find new target genes, a comparison of endometrial and endometriotic lesions' gene expression has already been exhausted without providing the results usable in practice and new directions are urgently needed. One possible strategy is to treat the endometrial cells from different locations with different chemical compounds to find altered key pathways and bring us closer to the molecular causes of this disease.

9. SUMMARY IN ESTONIAN

Endometrioosi ja kehalise aktiivsuse mõju naiste viljakusele

Tänapäeval lükatakse laste saamist edasi, kuna soovitakse esmalt teha karjääri, saavutada majanduslik kindlustatus ja leida sobiv elukaaslane. Paraku on naise kõige viljakamad eluaastad kahekümnendate esimene pool ja 35.–39. eluaastaks langeb naise viljakus juba pea kolmandiku võrra. Naise vanuse tõustes väheneb nii munarakkude arv kui nende kvaliteet, mis mõlemad suurendavad riski viljatuse tekkeks. Viljakust mõjutavad negatiivselt ka eluviisi tegurid, nagu ebatervislik toitumine, söömishäired, stress, suitsetamine, alkoholi liigtarvitamine, vähenenud kehaline aktiivsus ja suurenev keskkonnareostus. Lisaks vanusest tingitud viljakuse langusele on naisepoolse viljatuse sagedasemateks põhjusteks ovulatsioonihäired, endometrioos, munajuhade ja emakaga seotud haigused ning hüperprolaktineemia. Viljatust võivad põhjustada ka kromosoomimuutused ja immunoloogilised probleemid.

Üheks sagedaseks naisepoolse viljatuse põhjuseks on endometrioos, krooniline günekoloogiline haigus, mis mõjutab negatiivselt nii naiste tervist kui ka üleüldist elukvaliteeti. Endometrioosi korral migreeruvad emaka limaskesta sarnased rakud kõhuõõnde, kinnituvad seal ja hakkavad kolletena kasvama. Kõige sagedamini leiduvad endometrioosi kolded väikese vaagna sidekoel, kõhukelmel, munasarjadel, aga ka sooltel, emakakaelal, emaka lihaskihis, kusepõiel või operatsiooniarmides. Endometrioosi esineb umbes 10% viljakas eas naistel ja kuni 50% viljatutel naistel. Kuidas täpselt endometrioos viljatust põhjustab ei ole teada, aga arvatakse, et rolli võivad mängida nii munarakkude halvenenud kvaliteet kui ka häirunud endomeetriumi retseptiivsus. Endometrioosiga seotud viljatuse ravi on keeruline ja koosneb peamiselt kirurgilisest ravist, hormonaalsest ravist või nende kombinatsioonist ning sageli on vajalik kasutada ka *in vitro* viljastamise (IVF) protseduuri. Kuna endometrioos on väga mitmekesise haiguspildiga, ei ole ka kõigile patsientidele sobivat universaalset raviskeemi ja seetõttu on vajalikud edasised uuringud parimate ravivõimaluste leidmiseks. Uute endometrioosi diagnoosimise- ja ravivõimaluste leidmist takistavad ka ebapiisavad teadmised selle haiguse patogeneesi molekulaarsetest mehhanismidest.

Viljatuse ravi edukus IVF abil oleneb nii viljatuse põhjusest, partnerite vanusest, sugurakkude ja embrüote kvaliteedist, endomeetriumi retseptiivsusest kui ka valitud raviskeemi sobivusest. Endomeetriumi retseptiivsus on oluline aspekt rasestumisel ja sellega seotud geenide ekspressiooni uurimine on märkimisväärse tähtsusega. Samuti ei tohi alahinnata elustiili mõju ravi edukusele ja patsiente tuleb nõustada nii raviaegse toitumise, suitsetamise, ravimite tarbimise kui ka füüsilise aktiivsuse mõjude suhtes. Viimase faktori suhtes ei ole üheseid seisukohti, on nii soovitusi füüsilist aktiivsust vähendada kui ka jätkata normaalset aktiivset elustiili. Objektiivsete soovituste andmiseks on väga oluline teostada uuringuid kasutades näiteks otsest füüsilise aktiivsuse mõõtmist aktseleromeetriga ja mitte tugineda ainult inimeste ütlusepõhistele andmetele.

Uuringu eesmärk

Antud doktoritöö põhieesmärk oli välja selgitada endometrioosi erinevate raviskeemide ja füüsilise aktiivsuse mõju IVF ravi edukusele viljatusravi läbivatel patsientidel ning hinnata endomeetriumi retseptiivsust ja molekulaarsete muutuste ulatust endometrioosi korral.

Uuringu alameesmärgid on:

1. Analüüsida kombineeritud ravi (laparoskoopia, GnRHa ja IVF) tõhusust endometrioosiga seotud viljatuse ravis erineva raskusastmega haiguse korral.
2. Hinnata objektiivselt mõõdetud füüsilise aktiivsuse mõju IVF ravi tulemustele.
3. Hinnata, kas endometrioosi korral on endomeetriumi retseptiivsuse geenide ekspressioonimuster muutunud ja kasutada sama geenipaneeli endomeetriumi koeproovide menstruaaltsükli faasi täpsustamiseks.
4. Selgitada välja endometrioosi kolletes toimunud molekulaarseid muutusi, kasutades selleks tsütotoksiliste ühendite tundlikkuse testimist.

Materjal ja metoodika

Uuringud on kooskõlastanud Tartu Ülikooli inimuuringute eetika komitee (kooskõlastused 191T-8, 227/T-7 ja 276/M-13) ja uuringusse värvatud naised allkirjastasid informeeritud nõusoleku vormi.

Uuringus osalenud patsiendid

Retrospektiivses uuringus I osales 179 Elite kliinikus kirurgiliselt ja histoloogiliselt kinnitatud endometrioosi diagnoosiga patsienti. Tuginedes Ameerika Reproduktiivmeditsiini klassifikatsioonile (1996) jaotati patsiendid sõltuvalt endometrioosi raskusastmest gruppidesse. Uuringus I jaotusid patsiendid: grupp 1 – minimaalne-kerge, I–II astme endometrioos – 121 patsienti ja grupp 2 – mõõdukas-raske, III–IV astme endometrioos – 58 patsienti. Grupp 1 ja grupp 2 patsiendid jaotati omakorda vastavalt GnRHa ravi järgi, kas GnRHa ravi toimus või mitte. Grupp 2-s olid kõik patsiendid saanud GnRHa ravi. Ravi järgselt sai patsient vastavalt operatsiooni leiule, varasemale anamneesile ja seemnerakkude analüüsi tulemusele soovituse rasestumiseks spontaanselt või IVF teel. Uuringus osalenud patsiente jälgiti kuni sünnituseni.

Uuringus II osales 101 reproduktiivses eas viljatuse diagnoosiga naist, kes läbisid IVF viljatusravi protseduuri Elite kliinikus või Tartu Ülikooli Kliinikumi (TÜK) naistekliinikus. Embrüo siirdamine toimus teisel kuni viiendal päeval. Rasedust diagnoositi inimese koorioni gonadotropiini (hCG) analüüsi abil 14±2 päeva peale siirdamist. Positiivse hCG tulemuse korral kontrolliti kliinilise raseduse olemasolu ultraheli abil 4–5 nädalat peale munarakkude punktsiooni. Füüsilist aktiivsust mõõdeti ActiGraph GT2X+ aktselerомeetri ehk väikese vööl kantava

seadeldise abil, mis registreerib kandja kõik liikumisintensiivsused. Mõõtmisi teostati kolmel korral 14 järjestikuse päeva jooksul – enne kehavälist viljastamist, selle ajal ja juhul kui naine rasestus, siis ka kaks nädalat peale rasestumist. Analüüsis kasutati nende naiste andmeid, kes kandsid aktselerомeetrit vähemalt 10 tundi päevas neli või rohkem päeva (N=98). Naistel paluti täita ka küsimustik n.ö. ekraaniaja kohta (televisioon, DVD, arvuti kasutamine) viimase viie tööpäeva kohta ja eraldi nädalavahetuse kohta.

Uuringus III osales 45 endometrioosiga ja 33 endometrioosita ning uuringus IV 11 endometrioosiga patsienti, kes värvati TÜK naistekliinikus endometrioosi kahtluse või viljatuse tõttu laparoskoopilisele operatsioonile suunatud naiste hulgast.

Uuringus III osales 25 I–II astme ja 20 III–IV astme endometrioosiga naist. Uuringus IV osales 7 I astme ja 4 III astme endometrioosiga patsienti. Kõik uuringus osalenud naised olid reproduktiivses eas (vanus 18–42 aastat) ja polnud vähemalt kolm kuud enne operatsiooni saanud hormoonravi. Kõikidelt värvatud patsientidelt uuringutes III ja IV koguti operatsiooni käigus Pipelle kogumiskateetrit kasutades endomeetriumi biopsia ja endometrioosiga patsientidelt koguti lisaks operatsiooni käigus eemaldatud kolded (uuring IV).

In vitro rakukultuuri katsed (uuring IV)

Strooma rakud eraldati endomeetriumi ja endometrioosi kollete biopsiamaterjalist ja kultiveeriti 5–6 passaaži DMEM/Ham's F-12 söötmes koos 10% FBS, penitsilliini, streptomütsiini ja amfoteritsiin B-ga inkubaatoris 37 °C juures 5% CO2 tingimustes. Nii endomeetriumist kui endometrioosi kolletest eraldatud kultiveeritud strooma rakkudele lisati 14 erinevat tsütotoksilist ühendit (uuring IV, Tabel 1 vastavas publikatsioonis) ja teostati nekroosi/apoptoosi ja rakkude elulemuse määramise katsed.

RNA eraldamine ja geeniekspressiooni määramine (uuringud III ja IV)

Totaalne RNA eraldati endomeetriumi koest ja kultiveeritud strooma rakkudest kasutades kommertsiaalset komplekti RNeasy Mini kit. DNase I töötlus viidi läbi kasutades komplekti DNA-free DNA removal kit. Eraldatud RNA kontsentratsioon ja puhtus mõõdeti 2200 TapeStation süsteemiga. Kvantitatiivne reaal-aja PCR viidi läbi kasutades 7500 Fast Real-Time PCR System aparatuuri.

Endomeetriumi ja endometrioosi kollete kultiveeritud rakkude kogu transkriptoomi sekveneerimiseks loodi raamatukogud kasutades Nextera XT Library Prep komplekti (uuring IV) ja endomeetriumi retseptiivsust näitavate geenide tasemete tuvastamine viidi läbi kasutades TAC-seq tehnoloogiat (uuring III). Sekveneerimise tulemusel saadud lugemid analüüsiti Priit Paluoja, MSc (Tartu Ülikool, arvutiteaduse instituut) või Alvin Meltsov, MSc (Tartu Ülikool, Molekulaar- ja rakubioloogia instituut) poolt.

Tulemused

Antud doktoritöö raames tehtud uuringute tulemused võib kokku võtta järgnevalt:

1. Retrospektiivse uuringu tulemused näitasid, et mõõduka-raske astme endometrioosiga patsiendid (grupp II) on saanud rohkem postoperatiivset GnRHa ravi (p=0,021) kui kergema astme endometrioosiga patsiendid (grupp I) ja üle 75% nendest patsientidest läbisid IVF ravi. Kõikidest uuritavatest rasestus kokku 119 naist (66.5%), kellest 33 (18.4%) rasestus spontaanselt ja 86 (48.0%) IVF abil. Kuigi kergema endometrioosi raskusastmega patsiendid rasestusid sagedamini spontaanselt ja raskema endometrioosi raskusastmega patsiendid rasestusid sagedamini IVF abil, siis vastavad erinevused ei olnud statistiliselt olulised. Samas saadi kergema astme endometrioosiga naistelt rohkem munarakke IVF tsüklis, võrreldes raskema astme haigusega naistega (p=0,008). Üldine rasestumise, sünnitamise ja raseduste katkemiste protsent ei erinenud oluliselt ei grupi I siseselt sõltuvalt GnRHa ravi saamisest ega ka gruppide vahel.
2. Füüsilise aktiivsuse ja viljatusravi tulemuslikkuse uuringust selgus, et naised vähendavad oluliselt oma füüsilist aktiivsust kehavälise viljastamise ajal. Samas leidsime, et füüsilise aktiivsuse määr ei olnud seotud rasestumisega edukusega. Küll aga õnnestus füüsiliselt aktiivsematel naistel munasarjade stimulatsiooni järgselt saada rohkem munarakke (+2) ja embrüoid (+1) võrreldes nendega, kes vähendasid viljatusravi ajal oma füüsilist aktiivsust.
3. Endomeetriumi retseptiivsuse geenide avaldumise uuringust selgus, et nende geenide ekspressioon on endometrioosiga ja endometrioosita naistel sarnane ja kõige enam mõjutatud naise menstruaaltsükli faasist endomeetriumi koe kogumise ajal. Koeproovid jaotusid retseptiivsuse geenide avaldumise taseme järgi üsna selgelt menstruaaltsükli spetsiifilistesse gruppidesse (menstruaalfaas, proliferatiivne, vara-, kesk- ja hilissekretoorne). Vara- ja kesksekretoorsed proovid ei eristunud omavahel selgelt, sest nende faaside üleminek sõltub menstruaaltsükli kestvusest ja ütlusepõhise menstruaaltsükli päeva järgi ei ole võimalik seda väga täpselt määrata. Samal põhjusel klasterdusid mitmed hilissekretoorses faasis kogutud proovid koos menstruaalfaasi või kesksekretoorse faasi proovidega. Masinõppe tugivektorklassifitseerija mudeli abil, mis oli treenitud eristama vara- ja kesksekretoorseid endomeetriumi proove, oli võimalik eristada ka nende kahe kõrvutise faasi endomeetriumi biopsiaid.
4. Tsütotoksilised ühendid mõjutavad endomeetriumi stroomarakkude elulemust oluliselt rohkem võrreldes endometrioosikolde rakkudega. Neljateistkümnest kasutatud ühendist ainult üks, doksorubitsiin vähendas kolde stroomarakkude elulemust oluliselt tugevamalt võrreldes endomeetriumi rakkudega. Doksorubitsiiniga töötlemine mõjutas erinevalt ka endomeetriumi ja endometrioosikollete stroomarakkude geeniekspressiooni, nii et endomeetriumi rakkudes oli võrreldes koldega toimunud tunduvalt rohkem geenide avaldumise muutusi

(vastavalt 4009 ja 249). Doksorubitsiini mõjul toimus mitmete geenide ekspressiooni vähenemine (näiteks *DKK1, HAS2*) nii endomeetriumi kui kolde rakkudes, aga näiteks *HIST1H2AE, HIST1H2BK* ja *HIST2H2AA4* ekspressioon tõusis doksorubitsiini mõjul endomeetriumi rakkudes, samas kui kolderakkudes nende geenide avaldumine ei muutunud. Kolme geeni korral (*PTN*, *HSPA2* ja *PTGS2*) kinnitati geeniekspressiooni muutused reaal-aja PCR meetodil.

Järeldused

1. Erineva raskusastme endometrioosiga naiste viljatusravis kasutatud strateegia, kas ainult laparoskoopiline kollete eemaldamine või laparoskoopia kombineeritult GnRHa raviga, ei mõjutanud oluliselt rasestumise ja sünnituseni jõudmise määra loomuliku ja IVF rasestumise korral. Viie aasta jooksul rasestus mõlemas grupis enam kui 60% naistest ja sünnitas lapse enam kui 50% naistest.
2. Viljatusravi läbivate naiste üldine kehaline aktiivsus IVF ravi ajal mõjutab positiivselt munasarja stimulatsiooni tulemusi, aga ei mõjuta embrüo implantatsiooni ega rasestumise tõenäosust. Seega võib soovitada IVF ravi läbivatel naistel ravi ajal füüsilist aktiivsust mitte vähendada, vaid jätkata oma tavapärase aktiivse eluviisiga.
3. Endomeetriumi retseptiivsusega seotud geenide ekspressioon ei erine endometrioosiga ja endometrioosita naistel. Samade geenide ekspressiooniprofiil võimaldab määrata varem kogutud endomeetriumi biopsiate menstruaaltsükli faasi. Antud tulemus on väga oluline arhiveeritud endomeetriumi proovide kasutamisel teadustöös.
4. Endomeetriumi rakud on tsütotoksilistele ühenditele palju tundlikumad kui endometrioosi kolde rakud. Kogu transkriptoomi sekveneerimise tulemused kinnitasid erinevat geeniekspressiooni endomeetriumi ja peritoneaalsete endometrioosi kollete rakkude vahel ja tõi esile geenid, mis võivad olla vastutavad endomeetriumi rakkude muutunud omaduste eest emakavälistes kolletes. Kolderakkude suurem tundlikkus doksorubitsiini suhtes tuleneb ilmselt teatud geenide juba algselt madalamast avaldumistasemest, mis vähendab resistentsust doksorubitsiinile.

Kokkuvõtteks võib öelda, et meie uuringute tulemustel on oluline kliiniline väljund, sest need aitavad otsustada endometrioosiga naistele sobiva viljatusravi strateegiate valimisel ja võimaldavad anda teaduspõhiseid elustiili soovitusi viljatusravi läbivatele naistele. Meie tulemused viivad sammu lähemale ka endometrioosiga seotud teaduslike probleemide lahendamisele, samas tuleb tõdeda, et selles vallas on vaja teha veel palju edasist uurimistööd, enne kui neid teadmisi saab rakendada patsientide heaolu suurendamiseks ja ravitulemuste parandamiseks.

Praktilised soovitused

1. Viljatusravi planeerides peab arvesse võtma nii endometrioosi raskusastet kui eelnevat viljatusravi anamneesi. Antud töö tulemusena saame väita, et endometrioosi kahtluse ja mitmete ebaõnnestunud IVF protseduuridega patsiendid peaksid kaaluma endometrioosi kombineeritud ravi.
2. Viljatusravi läbivaid patsiente on vajalik nõustada elustiili tegurite võimalikust mõjust viljatusravi tulemustele. Naistele tuleks soovitada jätkata nende tavapärast aktiivset elustiili või vähemalt mitte vähendada oma füüsilist aktiivsust.
3. Endomeetriumi molekulaarsetes uuringutes peab arvestama menstruaaltsükli faaside mõjuga endomeetriumi geenide ekspressioonile. Seega, kui kasutada uuringutes varem kogutud endomeetriumi biopsiaid, peaks kõigepealt kindlaks tegema, millises menstruaaltsükli faasis need on kogutud. Meie uuring näitas, et selleks sobib näiteks endomeetriumi retseptiivsuse test.
4. Endometrioosi molekulaarsete mehhanismide väljaselgitamine vajab jätkuvat uurimist. Märklaudgeenide leidmiseks kasutatud endomeetriumi ja endometrioosi kollete geeniekspressiooni võrdluskatsed on ennast ammendanud andmata igapäevases töös praktiliselt kasutatavaid tulemusi. Seega oleks väga vaja uusi uurimissuundi. Ühe võimaliku strateegiana võib pakkuda endomeetriumi ja kolde rakkude mõjutamist erinevate keemiliste ühenditega, mis võimaldaks tuvastada endometrioosi tekkes osalevaid uusi faktoreid ja aitaks paremini mõista selle haiguse molekulaarseid põhjusi.

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I

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Pregnancy Rate in Endometriosis Patients according to the Severity of the Disease after Using a Combined Approach of Laparoscopy, GnRH Agonist Treatment and in vitro Fertilization

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Endometriosis · GnRH agonists · Infertility · In vitro fertilization · Laparoscopy

Abstract
Aim: To evaluate the effects of combined treatment approaches on endometriosis-associated infertility in different stages of endometriosis using laparoscopy, gonadotropin-releasing hormone (GnRH) agonist (GnRHa) therapy and in vitro fertilization (IVF). ***Methods:*** This retrospective study was carried out on 179 women with surgically confirmed endometriosis. Patients were divided into subgroups: group 1 (stage I–II, n = 121) and group 2 (stage III–IV, n = 58). Patients eligible for IVF, who were found to have adenomyosis or moderate to severe endometriosis, were also given postoperative GnRHa. Pregnancy and delivery rates were cumulatively calculated during 5 years according to the severity of the disease. ***Results:*** The overall pregnancy, delivery and miscarriage rates were 66.5, 56.4 and 15.1%, respectively, for all patients following spontaneous and assisted conception. There were no significant differences in reproductive outcomes between the study groups. The pregnancy and delivery rates were also comparable within group 1 between the patients with and without GnRHa treatment. ***Conclusion:*** Pregnancy and delivery rates at different stages of endometriosis were not affected by the different approaches used for infertility treatment, with >60 and >50% of patients having conceived and delivered a baby, respectively, in both groups. The usefulness of GnRHa treatment for endometriosis patients with minimal to mild forms is questionable and deserves further studies.

Introduction

Endometriosis is one of the most frequent benign chronic gynecological disorders, and it influences female health negatively by causing abdominopelvic pain and infertility. It has been estimated that endometriosis affects approximately 10% of women of reproductive age and up to 50% of infertile women [1]. Endometriosis-associated infertility is poorly treatable, and treatment mainly consists of surgical approaches, hormonal medication and in vitro fertilization (IVF) or their combination.

The purpose of surgery is to remove all visible implants of endometriosis and adhesions. Women surgically treated at any stage of endometriosis have an approximately 50% chance of spontaneous conception 1–2 years after surgery [2]. Previously, it has been confirmed that surgical treatment improves fertility and has a positive effect on

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pregnancy rate at all stages of the disease [3]. Large-scale randomized controlled trials and meta-analyses have shown higher pregnancy rates in women with minimal and mild-stage endometriosis-associated infertility who underwent surgical treatment compared with diagnostic laparoscopy [4]. However, the real value of surgical approaches in women with early-stage endometriosis-associated infertility remains unclear [5]. Surgery for moderate to severe endometriosis is mainly aimed at the alleviation of pain classically associated with these lesions [6] and the restoration of distorted pelvic anatomy [7]. The negative aspect of moderate to severe endometriosis surgery is that it could harm the ovaries, especially in women with bilateral endometriomas, and impair the ovarian reserve [8].

Previous studies have shown that gonadotropin-releasing hormone (GnRH) treatment without laparoscopy for women with minimal to mild and moderate to severe endometriosis seems to be ineffective for endometriosis-related infertility and does not improve the pregnancy rates among infertile women. GnRH therapy may even worsen the situation as a result of treatment delay [9]. Long-term pituitary downregulation, using GnRH therapy, after surgery in women with endometriosis-associated infertility has often been advocated to improve the pregnancy rate, but its real value is still uncertain [10]. However, surgically diagnosed endometriosis patients had better pregnancy rates if pretreated with GnRH analogues for 3 months before IVF [11]. Also, a meta-analysis of 165 women has confirmed the benefit of a 3- to 6-month administration of GnRH analogues before initiation of IVF [12].

When surgery and hormonal medication fail, or when spontaneous conception is impossible due to tubal or male factor infertility, the use of IVF is recommended [13]. Thus, controlled ovarian hyperstimulation and IVF are commonly used for the treatment of endometriosis-associated infertility. The effect of surgery for ovarian endometriomas on IVF outcome is still unclear [14]. Nevertheless, in cases of several unsuccessful IVF attempts, surgical treatment could improve the pregnancy rate [15].

The aim of this retrospective study was to evaluate the effects of combined treatment approaches on endometriosis-associated infertility in minimal to mild and advanced stages of endometriosis using laparoscopy, GnRH agonist (GnRHa) therapy and IVF. In addition, we were interested in the usefulness of applying the GnRHa treatment in minimal to mild stages of endometriosis by calculating the cumulative pregnancy and delivery rates for patients with and without GnRHa treatment.

Materials and Methods

This retrospective study was carried out among 179 infertile women (22–42 years of age) with surgically confirmed endometriosis, who underwent curative laparoscopy at the Elite Clinic from 2005 to 2008. In our clinic, diagnostic-curative laparoscopy is the first choice of treatment for all patients with endometriosis-associated infertility.

Laparoscopy was performed using a 10-mm laparoscope in the umbilical position and two 5-mm trocars. After a thorough check of the pelvic and abdominal organs, adnexal adhesions were removed. All visible foci of endometriosis, most commonly on ligaments, peritoneum, cavum Douglasi and less frequently on ovaries, parametrium and fossa ovarica, as well as superficial foci on the bowel and bladder were coagulated by bipolar diathermy. The appendix was removed if endometriotic foci were found on it. Out of 58 patients with moderate to severe endometriosis, 32 (55%) had endometriomas. When endometriotic lesions were found on the uterus, or the presence of adenomyosis had previously been diagnosed by ultrasonography, the whole exterior uterine wall was coagulated. Vascular extensions of the uterus and the fallopian tube angle region were also coagulated. However, in our clinical practice, we do not support the surgical treatment of adenomyosis alone, and this procedure is performed only in case of laparoscopy for endometriosis. We did not perform bowel and bladder resection, and a maximum of 6 months of GnRHa treatment was prescribed in these patients.

The patients were divided into group 1 (stage I–II endometriosis, n = 121) and group 2 (stage III–IV endometriosis, n = 58) according to the American Society for Reproductive Medicine (ASRM) classification system [16]. The patients of group 1 and group 2 were further divided according to the GnRHa treatment status (with and without GnRHa treatment). There were no patients without GnRHa treatment in group 2.

Patients eligible for IVF, or who were found to have adenomyosis or moderate to severe endometriosis, were postoperatively given a GnRHa, either Diphereline (Ipsen Pharma Biotech, France) or Zoladex (AstraZeneca UK, Ltd., UK). The first dose of GnRHa was administered on the first postoperative day, and each following dose was administered after every 28 days for 3–6 months. Some patients received GnRHa treatment, but did not undergo IVF treatment because they spontaneously achieved pregnancy prior to the scheduled IVF treatment. In patients scheduled for IVF, GnRHa treatment and pituitary suppression were continued by IVF without waiting for a menstrual period.

Natural conception was recommended for couples with patent fallopian tubes and normal sperm quality. IVF, intracytoplasmic sperm injection (ICSI) and slow embryo freezing were performed according to standard protocols. Pregnancy was documented by the presence of gestational sac(s) at 6–7 weeks of gestation, with miscarriages occurring between the detection of a pregnancy and the 22nd week of gestation. The cumulative pregnancy rate for all IVF/ICSI/frozen embryo transfer (FET) cycles was calculated for patients participating in the IVF program until December 2009. The study involved only those women for whom we had a complete pregnancy follow-up, including both natural and assisted conceptions and deliveries.

All statistical tests were performed using the R2.14.1 environment (Free Software Foundation, Boston, Mass., USA). The unpaired t test, Wilcoxon's rank sum test and the χ^2 test were applied to compare the groups. Logistic regression analysis adjusted for

Table 1. General characteristics and laparoscopic findings in the study groups

Characteristics	Group 1	Group 2
Patients, n	121	58
Age, years	33.2±4.4	32.5±4.4
BMI	22.4±2.9[a]	21.5±2.6[a]
Regularity of menstruation	105 (86.8)	53 (91.4)
Secondary dysmenorrhea	31 (25.6)[b]	24 (41.4)[b]
Primary infertility	41 (33.9)	25 (43.1)
Secondary infertility	80 (66.1)	33 (56.9)
Duration of infertility, years	6.1±3.3 (n = 111)	6.3±3.6 (n = 55)
Previously laparoscopically diagnosed endometriosis	19 (15.7)[c]	30 (51.7)[c]
Adenomyosis	44 (36.4)[d]	36 (62.1)[d]
Chronic and adhesive pelveoperitonitis	53 (43.8)	34 (58.6)
At least one fallopian tube permeable	91 (75.2)	45 (77.6)
Myoma of uterus	21 (17.4)	7 (12.1)

Values for age, BMI and duration of infertility are expressed as mean ± standard deviation. Figures in parentheses indicate percentages unless otherwise indicated. Groups with common superscript are different: [a] $p = 0.041$; [b] $p = 0.032$; [c] $p < 0.001$; [d] $p = 0.001$.

age, body mass index (BMI), presence of adenomyosis and severity of endometriosis was used to assess pregnancy and delivery outcomes in different study groups.

The study was approved by the Research Ethics Committee of the University of Tartu, Tartu, Estonia.

Results

General characteristics and laparoscopic findings for the two groups are presented in table 1, with no statistically significant differences between the groups regarding age, regularity of menstruation, pelveoperitonitis, fallopian tube permeability or the reproductive characteristics. The mean BMI value was higher in group 1 than in group 2 ($p = 0.041$). Secondary dysmenorrhea was observed more often in the group with moderate to severe endometriosis ($p = 0.032$). Also, the women in group 2 had adenomyosis ($p = 0.001$) more often than those in group 1, while the incidence of myomas (size up to 3 cm) was similar in both groups.

Comparisons and the results of GnRHa treatment and IVF used for the study groups are presented in table 2. The average duration of GnRHa therapy after surgical treatment was longer in group 2 ($p = 0.021$). More than 75% of the women had undergone IVF treatment; the value was the highest (89.7%) in the group with moderate to severe endometriosis who had also received GnRHa therapy. The total number of IVF and FET cycles and the average number of IVF/FET cycles per patient were similar in all groups. Although the number of retrieved oocytes was statistically higher in group 1 (10.1 ± 5.8 vs. 7.4 ± 4.3, $p = 0.008$), two embryos were transferred in IVF/FET cycles in both groups.

During this retrospective study, until 2009, 119 (66.5%) patients became pregnant, 33 (18.4%) conceived spontaneously, and 86 (48.0%) conceived through IVF. Patients with minimal to mild endometriosis conceived similarly to patients with moderate to severe endometriosis. Twenty-four (19.8%) patients from group 1 and 9 (15.5%) patients from group 2 conceived spontaneously; 25 of them (16 patients from group 1 and 9 from group 2) had undergone previous GnRHa treatment. Fifty-seven (47.1%) patients from group 1 and 29 (50.0%) patients from group 2 conceived through IVF; 66 of them (37 patients from group 1 and 29 from group 2) had undergone previous GnRHa treatment. Seventy-seven patients (89.5% of all IVF pregnancies) conceived during the first two IVF attempts.

The overall clinical pregnancy rate for all patients was 66.5%, and altogether 81 (66.9%) patients with minimal to mild endometriosis and 38 (65.5%) patients with moderate to severe endometriosis achieved pregnancy. Seventy-one (58.7%) patients in group 1 and 30 (51.7%) patients in group 2 delivered. There were no significant differences in miscarriage and delivery rates between the groups with milder and more severe forms of the disease.

We were unable to demonstrate any statistical differences in total pregnancy, miscarriage and delivery rates between the patients with and without GnRHa treatment

Table 2. Results of treatment of endometriosis patients

Characteristics	GnRH treatment after laparoscopy			
	group 1			group 2
	GnRH+/–	GnRH+	GnRH–	GnRH+
Patients, n	121	76 (62.8)	45 (37.2)	58
Age, years	33.2±4.4	33.3±4.7	33.0±3.9	32.5±4.4
Duration of postoperative GnRH treatment, months	–	4.3±1.4[a]	–	4.9±1.6[a]
Patients with IVF/FET treatment per all patients	89 (73.6)	57 (75.0)[b]	32 (71.1)	52 (89.7)[b]
Total IVF cycles	142	89	53	71
IVF cycles per IVF patient	1.6	1.6	1.7	1.4
Total FET cycles	15	13	2	8
FET cycles per IVF patient	0.2	0.2	0.1	0.2
Retrieved oocytes	10.1±5.8[c]	9.7±5.5	10.7±6.4	7.4±4.3[c]
IVF/FET cumulative pregnancies per IVF patient	57 (64.0)	37 (64.9)	20 (62.5)	29 (55.8)
Conceived during I–II IVF attempt per all IVF/FET pregnancies	51 (89.5)	33 (89.2)	18 (90.0)	26 (89.7)
Spontaneous pregnancies per all patients	24 (19.8)	16 (21.1)	8 (17.8)	9 (15.5)
Total pregnancy rate per all patients	81 (66.9)	53 (69.7)	28 (62.2)	38 (65.5)
Total miscarriage rate per all pregnancies	10 (12.3)	6 (11.3)	4 (14.3)	8 (21.1)
Total delivery rate per all patients	71 (58.7)	47 (61.8)	24 (53.3)	30 (51.7)

Values for age, duration of postoperative GnRH treatment and retrieved oocytes are expressed as mean ± standard deviation. Figures in parentheses indicate percentages. GnRH+/– denotes patients with and without GnRH treatment. Groups with common superscript are different: [a] p = 0.021; [b] p = 0.030; [c] p = 0.008.

in group 1. This observation was equally true for those women who became pregnant following IVF as well as for those who conceived spontaneously.

Discussion

In the present retrospective study, we evaluated the effects of combined treatment approaches on endometriosis-associated infertility using laparoscopy, GnRHa therapy and IVF. Treatment of endometriosis-associated infertility is complicated and depends largely on patient age, previous treatment, duration of infertility and the severity of the disease. Optimal treatment is often difficult to choose as the course of the disease is unpredictable and sometimes causes no complaints, while on other occasions it can be aggressive and cause severe pelvic pain and infertility, being referred to as 'active endometriosis' [17]. The ASRM classification system, which is widely used, provides the stage of endometriosis but does not always correlate with the activity of the disease and the degree of pelvic pain, nor is it predictive of fertility after treatment [2, 18, 19].

The results of studies focusing on treatment options for different stages of endometriosis are conflicting, even when involving identical treatment approaches [9, 20, 21]. Thus, it is possible that the patients with the same disease stage according to the ASRM classification are actually not comparable when it comes to symptoms and activity of endometriosis [2, 22]. The ideal management approach should involve the consideration of the course of the disease, and for very active forms of endometriosis early combined treatment is recommended. It is common practice that a combination of surgery, hormonal medication and IVF is usually recommended to women with endometriosis-associated infertility. However, there are limited data about the efficiency of the various forms of treatment because the number of randomized controlled trials has so far been too small [23].

Many studies have demonstrated the benefit of curative laparoscopy in endometriosis-associated infertility treatment. The pregnancy rate achieved after operative laparoscopy was shown to be similar at all stages of endometriosis [2], which was also observed in our study. In another study, the removal of visible foci of endometriosis during laparoscopy enhanced fertility in infertile women [4]. Sometimes, endometriotic lesions may be located in places (e.g. the myometrium of the uterus or the pararectal region) where surgical removal is technically impossible or associated with an increased risk of complications; in such cases, a combined treatment approach

could prove valuable. The following treatment options have been used: (a) surgical removal of endometriotic lesions, (b) laparoscopy and hysteroscopy when endometrial changes are expected (e.g. endometrial hyperplasia, uterine polyps, adenomyosis or repeated IVF failure), (c) postoperative GnRHa therapy depending on the severity of the endometriosis, and (d) in cases with indication for IVF, immediate postoperative GnRHa therapy continuing with IVF [3, 11, 12, 24, 25].

GnRH supplements are often used in the treatment of endometriosis. In cases of minimal and mild endometriosis, there is no clear view about the usefulness of GnRH therapy [20, 21]. Furthermore, the information available about the activity of endometriotic lesions in minimal to mild endometriosis is very scarce [9]. However, GnRH therapy alone without laparoscopy might be ineffective to alleviate endometriosis-associated infertility [26].

The results of our study further raised the question about the benefit of using GnRHa treatment in cases of milder forms of endometriosis unless laparoscopic surgery is continued with IVF. According to our results, with similar pregnancy and delivery rates in endometriosis patients with/without GnRHa treatment, the GnRHa treatment seems to be not advocated for patients with less severe forms of endometriosis. However, further studies are required before any final conclusion can be made about the recommended treatment protocols for endometriosis patients with milder forms.

When it comes to moderate and severe endometriosis and associated infertility, GnRH therapy after surgery should be considered the 'first-line' treatment and IVF is only indicated as a second treatment option [27]. Furthermore, several case studies where endometriosis was combined with adenomyosis have shown the efficacy of GnRHa therapy in enhancing pregnancy rate [28–30], although its exact fertility-improving impact is not yet entirely clear [29]. In our study, GnRHa therapy in patients with minimal to mild stages of endometriosis was mainly used when adenomyosis was observed during transvaginal ultrasonography. Thus, in our opinion, the reason for administering GnRHa medication after laparoscopy is the regulation of the menstrual cycle before starting IVF as well as the improvement in implantation in cases when endometrial pathology is detected during hysteroscopy.

Consensus opinion on the benefit of medical treatment after surgery in women with endometriosis-associated infertility has never been reached, and its real value in improving pregnancy rate and in preventing recurrence of endometriosis is uncertain [10]. According to the latest ASRM committee opinion [18] and the Royal College of Obstetricians and Gynaecologists guidelines (2006; http://www.rcog.org.uk), postoperative GnRH treatment is not recommended as no proven value is available in terms of a better pregnancy rate. Furthermore, the prolonged GnRH treatment prior to IVF may decrease the ovarian reserve as patients become older and thereby may have a deleterious effect on the pregnancy outcome. As such, urgent IVF after surgery might have some benefit over the postoperative GnRH treatment. However, other studies have still reported contrasting results and have proposed that women who are postoperatively pretreated with analogues of GnRH prior to IVF show better pregnancy rates [11, 12, 31].

Our results confirm that the use of combined treatment can lead to good pregnancy rate, even in patients with moderate to severe endometriosis. Namely, the percentage of women who conceived after treatment of endometriosis in our study (overall pregnancy rate of 66.5%) is comparable to that reported by other authors [32, 33]. Also, our findings emphasize the importance of using combined treatment for infertility patients affected by endometriosis of different stages and in the case of coexisting adenomyosis. In contrast, there is evidence that adenomyosis has a negative impact on the pregnancy outcome [34]. However, the results of our study suggested that the pregnancy rate was not affected by the higher frequency of adenomyosis in the patients with more severe stages of endometriosis compared to the patients with minimal to mild endometriosis.

Certain limitations of this study must be underlined. Firstly, it is a retrospective follow-up study, with patients undergoing curative laparoscopy during 4 years. Secondly, there were no patients without GnRHa treatment in the group with severe endometriosis. This is because the benefits of the GnRHa treatment in cases of severe endometriosis are clearly proved [12, 27]. Therefore, we were unable to estimate the pregnancy rate in patients with more severe stages of the disease, but without the GnRH treatment.

However, the strong point of this study is that all patients were treated by one gynecologist, eliminating the differences in surgery techniques and biases in diagnosing and interpreting the occurrence and progression of the disease.

In conclusion, we found that the outcome of infertility treatment among patients with different stages of endometriosis was not affected by the different approaches used. Sixty-six percent of the studied endometriosis-associated infertility patients conceived and 56% delivered during the study period. Patients with minimal to mild endometriosis conceived similarly (either spontaneously or through IVF) to those who had moderate to severe endometriosis.

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Disclosure Statement

The authors have no conflicts of interest to disclose.

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II

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ASSISTED REPRODUCTION TECHNOLOGIES

Maternal physical activity and sedentary behaviour before and during in vitro fertilization treatment: a longitudinal study exploring the associations with controlled ovarian stimulation and pregnancy outcomes

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Abstract

Purpose To evaluate the association of objectively measured physical activity (PA) and sedentary behaviour before and during in vitro fertilization (IVF) with controlled ovarian stimulation (COS) and pregnancy outcomes.

Methods This longitudinal study involved 107 infertile women undergoing IVF treatment. PA and sedentary behaviour were measured for 14 consecutive days using accelerometry as follows: (1) before IVF treatment, (2) during IVF at the implantation time, immediately after embryo transfer, and (3) after positive pregnancy test. Total screen time was assessed by questionnaires. COS results were measured as the number of oocytes and embryos obtained, and the study outcomes included positive hCG, clinical pregnancy, and live birth.

Results Compared with baseline activity levels, women significantly reduced their PA and increased sedentary behaviour during IVF ($p \leq 0.001$). Higher average PA, light PA, and ratio between breaks in every ≥ 30-min blocks of sedentary time showed positive associations, while sedentary time, number, and time accumulated in blocks of ≥ 30 min of sedentary time associated negatively with oocyte and embryo counts (all $p < 0.05$). Women with high total screen time during non-work days (≥ 7 h) obtained 4.7 oocytes ($p = 0.005$) and 2.8 embryos ($p = 0.008$) less in COS. PA and sedentary behaviour before and during IVF did not affect the positive hCG, clinical pregnancy, and live birth outcomes.

Key message Higher levels of light physical activity and shorter sedentary behaviour and screen-watching times before entering infertility treatment associate with better ovarian stimulation outcomes under IVF treatment protocols.

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Conclusion Our study results suggest that higher time spent in PA and lower time spent in sedentary behaviour before entering assisted reproduction is associated with better COS outcomes, while activity levels before and during IVF do not affect the implantation, pregnancy, and live birth outcomes.

Keywords Controlled ovarian stimulation · In vitro fertilization · Physical activity · Pregnancy outcome · Sedentary behaviour

Introduction

Infertility is a growing medical and global public health concern [1], with a prevalence of 12–15% of couples in childbearing age [2]. Infertility is experienced by an estimated 48.5 million couples worldwide, and is steadily increasing with the trend to delay the time of first pregnancy in developed countries, and now also in developing countries [3]. Even though the development of assisted reproductive technologies (ARTs) and the constant improvements in the treatment protocols have helped many infertile couples to achieve pregnancy, the rates of pregnancy and live births among all ART-treated couples still remain ~ 30% per treatment cycle [4]. Ways to improve ARTs outcomes have become a critical topic for both clinicians and couples undergoing infertility treatment.

Several of the most influential factors of infertility treatment, such as female age and genetic factors, are non-modifiable, while there is emerging evidence that modifiable factors, like smoking, weight, nutrition, or physical activity (PA) among others, can influence assisted reproduction [5, 6]. In fact, comparative studies indicate that PA intervention may be as effective as other clinical interventions used for improving reproductive health outcomes, while the type, intensity, frequency, and duration of optimal PA remain unclear [7]. The American Congress of Obstetricians and Gynaecologists recommends pregnant women to engage in moderate intensity exercise for at least 30 min most days per week [8], and suggests for overweight women to lose weight and become more physically active prior to pregnancy [9]. There are, however, no specific guidelines of PA for women attempting conception or women undergoing infertility treatment. In fact, no consensus has been reached regarding the effect of PA before/during ART on pregnancy outcome [10]. The few studies of female PA and infertility treatment success have produced mixed results, where higher PA has been associated with decreased embryo implantation and live birth in in vitro fertilization (IVF) [11], no effect [5, 1, 2], or others have reported beneficial effect of PA on IVF outcomes [10, 13–16]. Nevertheless, in all these studies, PA was measured using self-reported questionnaires, which are prone to recall bias and reliance problems [6], and clearly, more studies evaluating objectively PA effects on assisted reproduction are needed.

High levels of sedentary behaviour, any waking behaviour characterized by a very low energy expenditure while in a sitting or reclining posture, on the other hand, have been associated with many detrimental health consequences [17], but its effect on infertility treatment outcomes remains unexplored. Another important unexplored question to clinical practice is what PA levels to recommend for women after embryo transfer, during the 2 weeks of critical time of embryo implantation and early pregnancy establishment. Clarifying the role of PA and sedentary behaviour in infertility treatment, using objective methods for assessing PA and sedentary behaviour, may help to provide the long-sought-after PA recommendations for women undergoing infertility treatment.

The objective of this study was to examine the associations of objectively measured PA and sedentary behaviour before and during assisted reproduction with IVF treatment outcomes.

Materials and methods

This longitudinal study was carried out among infertile women in reproductive age, entering IVF/ICSI (intracytoplasmic sperm injection) treatment cycle and receiving fresh embryo transfer (ET) at Elite Clinic and Tartu University Hospital's Women's Clinic, Estonia from January 2013 to December 2016. IVF treatment cycles with donor oocytes were excluded. After a detailed explanation, in total, 107 women agreed to participate in the study (see Fig. 1 for the study design). Power calculation analyses showed that sample size of 100 participants with an alpha error of 5% and a power of 90% would be enough to detect an association of an effect size of 10% variance explained of the study outcomes. At enrolment, all participants were asked to fill out a questionnaire of general characteristics, and reproductive health. Patient's measurements of weight, height, and waist and hip circumference were obtained by an assistant nurse. The BMI was calculated as weight (kg)/height^2 (m).

IVF treatment protocol was conducted according to the gonadotropin-releasing hormone (GnRH) antagonist or agonist protocols. All patients started recombinant FSH (Gonal F, Merck Serono, Italy; or Bemfola, Finox Biotech AG, Switzerland) injections on day 2–7 of menses, continuing daily for 9 ± 2–3 days until 3 follicles achieved 18 mm of diameter, and human chorionic gonadotrophin (hCG) (Merck Serono, Italy) was administered. The controlled ovarian stimulation (COS) follow-up included 3–4 ultrasound assessments

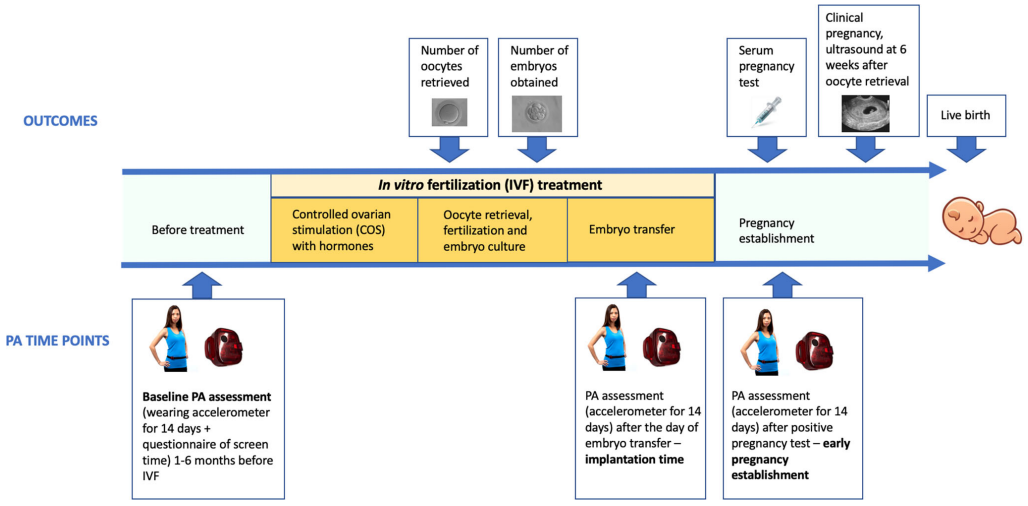

Fig. 1 Study design. Three time points for physical activity (PA)/sedentary behaviour and 5 time points for infertility treatment outcomes were assessed in this longitudinal study. PA and sedentary behaviour were measured for 14 consecutive days using accelerometry at each of the three time points: (1) before IVF treatment, (2) during IVF at the implantation time, immediately after embryo transfer, and (3) after positive pregnancy test. Total screen time was measured at the baseline, before any IVF treatment, using a questionnaire. The study outcomes included the number of oocytes and embryos obtained after COS, and positive hCG test, clinical pregnancy, and live birth

of endometrium and follicular growth. Final follicular maturation was achieved using 250 mcg of hCG followed by oocyte retrieval 36 h later.

Patients who received IVF or ICSI were both included (later indicated in conjunction as 'IVF'). Embryo transfer (ET) was done after COS on days 2 to 5. A serum pregnancy test, referred to as positive hCG, was performed on 14 ± 2 days after ET and considered to be positive if β-hCG > 10 mLU/mL. The ultrasound evaluation for defining clinical pregnancy was performed 6 weeks after oocyte retrieval, and was considered positive in the presence of at least one intrauterine gestational sac and cardiac activity on ultrasound. Live birth was abstracted from medical records.

All women were Caucasians. Patients did not receive any restrictions or recommendations for PA during the infertility treatment. The study was approved by the Research Ethics Committee of the University of Tartu, and written informed consent was obtained from all subjects before their participation.

Assessment of PA and sedentary behaviour

PA and sedentary behaviour were measured for 14 consecutive days using accelerometry at each of the three time points as follows: (1) before IVF treatment, (2) during IVF at the implantation time, immediately after embryo transfer, and (3) after positive pregnancy test (see Fig. 1 for the study design).

Uniaxial accelerometers GT1M (ActiGraph LLC, FL, USA) were placed on participants' waists to objectively assess PA and sedentary activities' time. Detailed information on accelerometer assessment is described elsewhere [18]. Briefly, patients were asked to wear accelerometers for 14 consecutive days and to only remove them for swimming, bathing, and sleeping. Three measurements of PA and sedentary behaviour, up to 14 days each, were performed within the study as follows: (1) one to 6 months before IVF, termed as baseline activity; (2) after ET, starting from the day after the embryo transfer (embryo implantation time); and (3) after positive serum pregnancy test, from the next day after the test (early pregnancy establishment) (Fig. 1).

ActiLife software version 6.10.2 (ActiGraph LLC, FL, USA) was used to process accelerometer data. Accelerometer's output consists in a score calculated in a minute basis intended to measure movement, usually referred to as 'activity counts'. Non-wear time was defined using algorithm proposed by Choi et al. [19, 20], which consists of 90 min of consecutive 0 cpm with an allowance of 2 min of activity when it is placed between two 30-min windows of 0 cpm. This algorithm outperformed other algorithms on the detection of non-wear time according to our recent systematic review on accelerometry's data analyses methods [21]. Only women wearing the accelerometer for at least 10 h/day for 4 or more days were included into the analysis ($N = 98$) [21].

PA is usually classified into different intensity categories, i.e., different levels of energy expenditure required to perform

certain PA, usually measured as metabolic equivalents. Metabolic equivalents are usually measured as oxygen consumption relative to body weight per minute (ml/kg/min). In this regard, all activities occurring below 1.5 metabolic equivalents in a seated or reclined posture are considered sedentary behaviour. PA requiring energy expenditure between 1.5 and 3 metabolic equivalents is considered of light intensity, and between 3 and 6 metabolic equivalents is considered moderate PA. Vigorous PA included the activities requiring hard physical effort and they require more than 6 metabolic equivalents.

Accelerometers are unable to measure the energy expenditure required to perform PA, but the existing linear relationship between movement/accelerations, expressed as cpm, and metabolic equivalents allow to estimate PA intensity using validated cpm cut-points [22]. In this regard, we calculated time per day spent at different intensities of PA using the Freedson's cut-points [22]. Briefly, sedentary time, light PA, moderate PA, and vigorous PA were considered when cpm accumulation was < 100 cpm, 100–1951 cpm, 1952–5724 cpm and > 5724 cpm, respectively [22]. Moderate-to-vigorous PA (MVPA) was calculated by summing the time spent at moderate and vigorous PA. Average physical activity, expressed as mean cpm, was computed as the sum of counts per all valid days divided by the total wear time in minutes in these days. Beyond, as maintained time in certain behaviours can be important of health outcomes [23], the number and time spent in bouts of at least 10 min of MVPA and 30 min of sedentary time were computed. An interruption up to 2 min below or above the threshold (i.e. < 1952 cpm for MVPA and > 100 cpm for sedentary time, respectively) was allowed to consider bouts of activity. A variable of sedentary breaks was also computed as the total number of 'breaks' during continuous sedentary bouts. Finally, we computed a ratio between the number of breaks divided by the total time accumulated in 30 min (or longer) bouts of sedentary time.

In addition to the objectively measured PA and sedentary time, the women filled in a questionnaire, where different screen times (TV watching, DVD, computer) were assessed by asking women to indicate the total screen time spent during the last 5 work days and total screen time spent during the last non-work days (e.g. weekend). These questions were obtained from previous large-scale surveys and have shown good reliability, validity, and relation with health outcomes [24].

Statistical analyses

Descriptive characteristics of the sample and baseline PA/sedentary behaviour were calculated. The intra-subject changes in PA/sedentary behaviour from baseline (before starting infertility treatment) to the implantation period (2 weeks onwards from embryo transfer) were tested using repeated measured analysis of variance (ANOVA) models. Associations of baseline PA/sedentary variables with oocytes and embryos obtained after hormonal stimulation were examined using linear regression models, after adjustment for a set of potential confounders selected based on existing clinical knowledge: accelerometer registered time, age, body mass index, educational level, smoking, infertility diagnosis, infertility duration, and the amount of follicle stimulation hormone administered. Finally, the associations of PA/sedentary levels at different time points (i.e. baseline, after embryo transfer, and after positive serum hCG pregnancy test) or changes from baseline to implantation period with different pregnancy outcomes (i.e. positive hCG, clinical pregnancy, and live birth) were examined using binary logistic regression models after adjustment for the same set of confounders. Further, we tested whether PA/sedentary levels at the three different time points varied between women who became pregnant and those who did not (positive hCG, positive clinical pregnancy, and live birth) using one-way analysis of covariance (ANCOVA) models, with the PA/sedentary variables as dependent variables (one per model), the pregnancy groups (Yes vs. No) as fixed factor, and the same confounders previously selected. Next, we performed isotemporal substitution modelling (also called reallocation modelling, which is becoming more frequently used in the field of sport sciences [25, 26]. The isotemporal concept is based on the assumption that the sum of time spent in sedentary activities, plus those of light intensity, plus those of moderate-to-vigorous intensity (MVPA) result in the total waking time registered by the accelerometer, so that if a woman increases the time spent in one of the components (e.g. light PA), the time spent in another component (e.g. sedentary time) must be reduced proportionally if the rest of components remain unchanged. Statistically, this is handled with linear regression models entering all the activity/sedentary variables (e.g. total registered time, light PA, and MVPA) except one (e.g. sedentary time), plus all the potential confounders.

All analyses were performed using the IBM-SPSS software, version 20.0. Armonk, NY, USA. The level of significance was set at $p < 0.05$ for all the analyses.

Results

Characteristics

Characteristics of the study sample are presented in Table 1. In total, 107 women agreed to participate in this longitudinal study, but as 6 women cancelled the IVF treatment, the final number of women for the analyses remained 101. Majority of women (70%) underwent infertility treatment because of male factor infertility, unexplained infertility, tubal factor infertility, advanced age, or because of no partner. Seven per cent of women were infertile due to polycystic ovary syndrome (PCOS) and 23% due to endometriosis. The average time of infertility in the study group was 4.6 years. Following IVF/

Table 1 Characteristics of the study population (patients undergoing in vitro fertilization, IVF)

	n	Mean	SD
Age (years)	101	33.5	4.1
Height (cm)	101	167.1	5.5
Weight (kg)	101	66.9	12.8
BMI (kg/m^2)	101	23.9	4.4
Infertility duration (years)	101	4.6	3.5
Total FSH dose (IU)	100	1560.7	575.0
Oocytes retrieved	101	11.6	7.1
Embryos obtained	101	6.6	4.5
Embryos transferred	101	1.5	0.6
		Frequency	%
Weight status (UW/NW/OW/OB)	101	7/61/23/10	6.9/60.4/22.8/9.9
Smoking (0/1/2)	101	57/36/8	56.4/35.6/7.9
Education (university)	101	65	64.4
Diagnosis (0/1/2)	101	71/7/23	70.3/6.9/22.8
Menstrual cycle (regular)	101	87	86.1
IVF protocol (agonist)	101	72	71.3
IVF/ICSI (IVF)	101	39	38.6
ET stage (2-3 day)	101	59	62.8
hCG (positive)	101	44	43.6
Clinical pregnancy (positive)	101	40	39.6
Live birth (yes)	101	29	28.7

BMI body mass index, *ET* embryo transfer, *FSH* follicle stimulating hormone, *ICSI* intracytoplasmic sperm injection, *IVF* in vitro fertilization, *UW* underweight if BMI < 18.5 kg/m^2, *NW* normal-weight if BMI between 18.5 and 24.9 kg/m^2, *OW* overweight if BMI between 25.0 and 29.9 kg/m^2, and *OB* obese if BMI ≥ 30 kg/m^2

ET – embryo transfer

SD standard deviation

Smoking was coded as 0 = never, 1 = formerly, and 2 = currently

Educational level was coded as 0 = under university and 1 = university

Diagnosis group 0—infertility is due to male factor infertility, unexplained infertility, tubal factor, female age, or no partner; Diagnosis group 1—infertility is due to PCOS (including male factor infertility and PCOS; tubal factor and PCOS); Diagnosis group 2—infertility is due to endometriosis (including women with endometriosis and spouses' male factor infertility)

Menstrual cycle was coded as regular vs. irregular

IVF protocol was coded as agonist vs. antagonist

IVF/ICSI was coded as IVF vs. ICSI

ET stage was coded as 2-3 day vs. blastocyst

hCG test and clinical pregnancy were coded as positive vs. negative

Live birth was coded as yes vs. no.

ICSI, 43.6% of women had a positive hCG test, 39.6% of women had clinical pregnancy and finally 28.7% of women gave live birth. As twin pregnancy was only reported for six (15%) out of 40 women with clinical pregnancy, we were unable to perform any analyses to assess the associations between PA/sedentary patterns and twinning rate. Spare embryos from 50 women were frozen for future embryo transfers, but as most of them are still being stored, we were not able to calculate the PA and sedentary behaviour effects on cumulative pregnancy rate.

Baseline PA and sedentary behaviour among women before infertility treatment are presented in Supplementary Table 1. The compliance wearing the accelerometer was high, i.e. although the minimum required to be included in the analyses was 4 days with ≥ 10 wearing hours ($N = 98$), among those included, 90% of them had 10 valid days or more registered ($N = 88$). When comparing the baseline activity levels of the infertile women, i.e. before IVF, with their PA levels during the IVF treatment, specifically at the time of embryo implantation, the women significantly reduced their PA levels and increased sedentary time as follows: average PA reduced 18% ($p < 0.001$), light PA reduced 6% ($p = 0.001$), MVPA reduced 27% ($p < 0.001$), MVPA accumulated blocks of 10 min reduced 39% ($p < 0.001$), vigorous PA reduced 72% ($p = 0.008$), and number of steps per day reduced 14% ($p < 0.001$), while daily total sedentary time increased 4% ($p < 0.001$) and sedentary time accumulated in 30-min blocks increased 9% ($p < 0.001$) (Fig. 2).

Relationships between baseline PA and sedentary behaviour with COS outcomes

Linear regression analysis of the associations of PA/sedentary variables with oocytes and embryos obtained after COS are summarized in Table 2. The average PA ($p = 0.04$), light PA ($p = 0.03$), and ratio between breaks in every 30-min sedentary time ($p = 0.002$) showed positive association with the number of oocytes obtained in COS, while sedentary time ($p = 0.02$), number of 30-min blocks of sedentarism ($p = 0.02$), and time accumulated in ≥ 30-min blocks of sedentarism ($p = 0.01$) associated negatively with the oocyte count in COS (Table 2).

Regarding the obtained embryos in COS and IVF, light PA ($p = 0.03$) and ratio between breaks in every 30-min sedentary time ($p = 0.01$) showed positive association with the number of embryos obtained, while sedentary time ($p = 0.03$), number of 30-min blocks of sedentarism ($p = 0.01$), and time accumulated of 30-min blocks of sedentarism ($p = 0.04$) associated negatively with the number of obtained embryos.

When assessing the self-reported total screen time spent during work days and non-work days, the women with high self-reported total screen time during non-work days (≥ 7 h TV, DVD, computer screen time) obtained 4.7 oocytes ($p = 0.005$) and 2.8 embryos ($p = 0.008$) less in COS than women with low-medium screen time (Fig. 3).

Next, we performed an isotemporal analysis of the time spent in different intensities of baseline PA and sedentarism in association with the number of oocytes and embryos obtained in COS (Fig. 4). This analysis predicted that if a woman increased her daily light PA for 1.5 h, thereby reducing her daily sedentary behaviour for 1.5 h, she would obtain 1.8

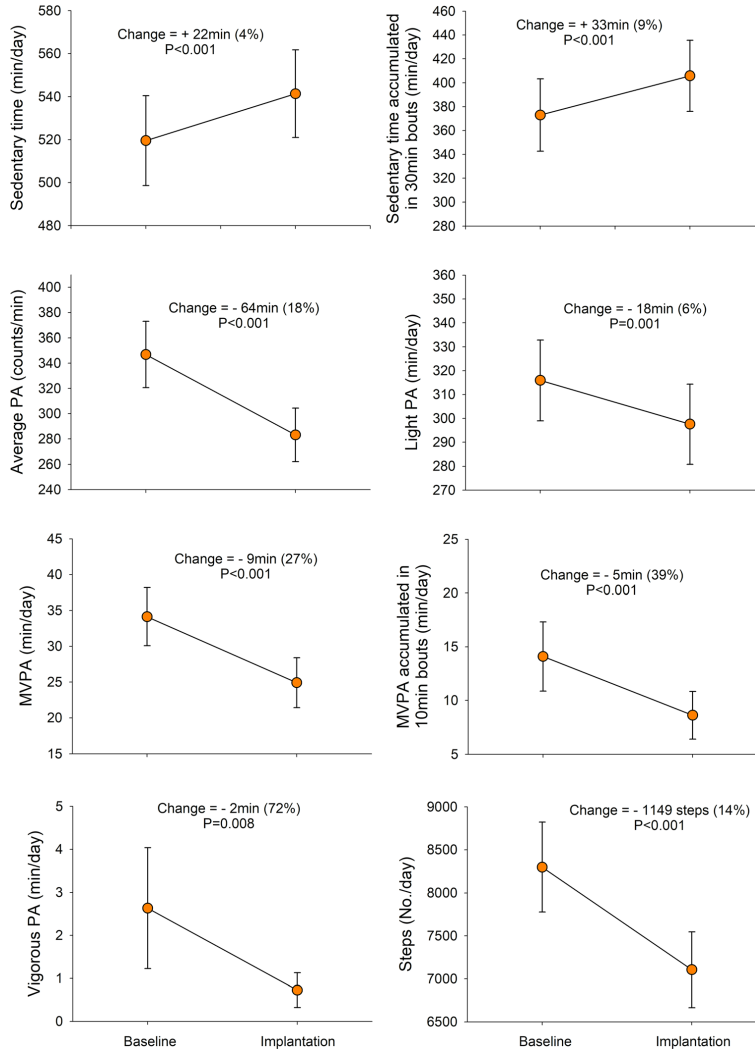

Fig. 2 Changes in physical activity and sedentary behaviour from baseline levels to the infertility treatment procedure, at the period of embryo implantation (14 days registered after embryo transfer) ($N = 79$ women with valid accelerometer data at both time points). PA, physical activity; MVPA, moderate-to-vigorous PA. *P* values report the significance of the intra-subject changes tested by repeated measured analysis of variance (ANOVA). The ranges (min. and max.) of the scale used in the Y axes are based on the percentile 25 and 75 of the present study sample

oocytes and 1.2 embryos more under COS ($p = 0.03$ and $p = 0.03$, respectively).

Relationships between PA and sedentary behaviour with pregnancy outcomes

Logistic regression analysis of possible associations of PA/sedentary behaviour at different time points (at baseline and during IVF) with IVF outcomes such as positive hCG, clinical pregnancy, and live birth are presented in Tables 3 and 4. No statistically significant association of different PA and sedentary indicators with IVF outcomes were detected.

Next, we analysed whether changes in PA/sedentary behaviour from baseline to implantation were associated with pregnancy outcomes. When the analyses of Tables 3 and 4 were conducted using changes in PA and sedentary time from baseline to implantation period, the results were consistent to those shown in the tables, i.e. no significant associations were observed. Likewise, no significant association was found when entering into the model activity/sedentary behaviour of a person in a specific time point plus the change observed over

Table 2 Associations of baseline physical activity and sedentary time with controlled ovarian stimulation (COS) outcome – oocytes retrieved and embryos developed ($N = 97$)

	Oocytes					Embryos				
	UnStnd. B	Lower CI	Upper CI	Stnd. B	*p* value	UnStnd. B	Lower CI	Upper CI	Stnd. B	p value
Average PA (counts/min)	0.01	0.00	0.02	0.21	0.04	0.01	0.00	0.01	0.19	0.07
Sedentary time (hours/day)	− 1.29	− 2.36	− 0.22	− 0.29	0.02	− 0.77	−1.46	−0.08	−0.27	0.03
Light PA (hours/day)	1.25	0.13	2.37	0.23	0.03	0.79	0.07	1.50	0.23	0.03
Moderate PA (min/day)	0.04	− 0.05	0.12	0.09	0.40	0.01	−0.05	0.06	0.03	0.78
Vigorous PA (min/day)	0.06	− 0.19	0.31	0.05	0.64	0.06	−0.10	0.22	0.08	0.44
MVPA (min/day)	0.04	− 0.04	0.12	0.11	0.30	0.01	−0.04	0.06	0.05	0.61
10 min bouts of MVPA (No./day)	0.64	− 1.94	3.22	0.06	0.62	− 0.57	−2.21	1.07	−0.08	0.49
Time accumulated in 10 min bouts of MVPA (min/day)	− 0.01	− 0.11	0.10	− 0.01	0.93	− 0.03	−0.09	0.04	−0.09	0.45
30 min bouts of sedentarism (No./day)	− 0.88	− 1.59	− 0.18	− 0.27	0.02	−0.58	−1.03	−0.13	−0.28	0.01
Time accumulated in 30 min bouts of sedentarism (hours/day)	− 0.87	− 1.55	− 0.19	− 0.28	0.01	− 0.47	−0.91	−0.03	−0.24	0.04
Ratio breaks:sedentary time in 30 min bouts (No./hours each day)	9.79	3.74	15.85	0.34	0.002	5.12	1.18	9.05	0.28	0.01
Steps (1000/day)	0.21	− 0.36	0.77	0.08	0.47	0.06	−0.30	0.42	0.03	0.75

Linear regression models adjusted for accelerometer registered time, age, body mass index, educational level (university vs. below university), smoking (never, before but not now and currently), infertility diagnosis (see Table 1 for coding), infertility duration (years), and follicle stimulation hormone administered. Exploratory additional adjustments for treatment protocol (agonist/antagonist) or infertility centre did not alter the results

PA physical activity, *MVPA* moderate-to-vigorous PA, *CI* confidence intervals, *UnStnd.* unstandardized beta coefficient B, *Stnd. B* standardized beta coefficient

time. Moreover, those women with proven positive hCG pregnancy tests were asked to wear the accelerometer for 14 more days to test whether the PA and sedentary behaviour at that period of pregnancy had any relation with the risk of miscarriage and early pregnancy establishment. We observed no statistically significant associations (data not shown).

In further exploratory analyses, we tested whether PA and sedentary behaviour at the different time points (baseline, after ET, after positive hCG test) differed between women who became pregnant and those who did not using ANCOVA models and adjusting for the same set of confounders as in the logistic regression models indicated in Tables 2, 3, and 4. The results showed no significant differences in PA and sedentary behaviour between women who had a successful pregnancy and those who did not (in all cases $P > 0.1$, data not shown). Further, no associations between IVF outcomes and total screen time spent during work days and non-work days were detected (data not shown).

Discussion

To the best of our knowledge, this is the first study where PA and sedentary behaviour is objectively measured for 14 days before any infertility treatment and for 4 weeks during IVF procedure, 14 days after ET and 14 days after positive pregnancy test, in order to closely observe how the activity levels relate to IVF outcomes. In our cohort of infertile women, higher baseline light PA and lower baseline sedentary behaviour were associated with higher number of oocytes and embryos obtained in IVF, while PA and sedentary behaviour before and during IVF did not affect the implantation, pregnancy, and live birth outcomes in fresh embryo transfers. These associations suggest that lifestyle changes such as increase in PA and reduction of sedentary time may positively influence ovarian hyperstimulation under ART protocols. Indeed, a recent systematic review and meta-analysis concluded that PA may be an affordable and feasible alternative or complementary therapy to fertility treatments [7]. In our study, increased PA and reduced sedentary time support obtaining higher number of oocytes and embryos per COS at ovarian puncture, leading to more embryos available for spare embryo freezing. In fact, a recent study shows that higher oocyte yield is independently associated with more embryos obtained and higher cumulative live birth rates [27]. Nevertheless, due to the limited number of patients enrolled in our study, we cannot directly prove that higher PA and decreased sedentary behaviour are related to higher cumulative pregnancy rate combining the pregnancies from fresh and all frozen-thawed embryo transfers. This seems highly likely and should be demonstrated in larger patient cohorts. Our study findings also suggest that PA (at the levels observed in our study) during the most vulnerable period of establishing a

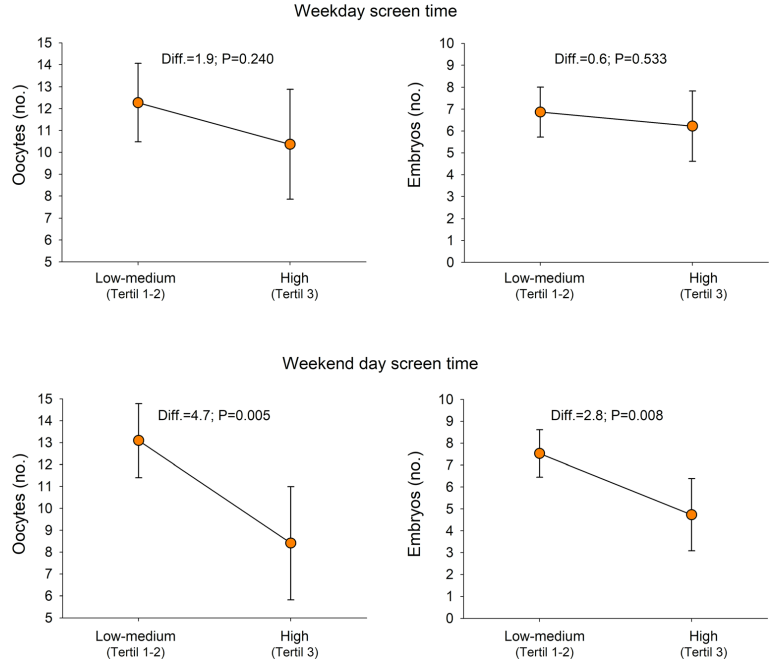

Fig. 3 Differences in the number of oocytes and embryos obtained after controlled ovarian stimulation according to different levels of self-reported screen time in work days and during non-work days ($N = 98$). Analysis of covariance (ANCOVA) models with oocytes or embryos as dependent variables (in separate models), groups of screen time as fixed factors, and a set of potential confounders as covariates: age, body mass index, educational level (university vs. below university), smoking (never, before but not now and currently), infertility diagnosis (see Table 1 for coding), infertility duration (years), and follicle stimulation hormone administered. High screen time was defined as belonging to the 3rd tertile, while low-middle screen time was defined as belonging to 1st or 2nd tertiles. High screen time in the non-work days (i.e. 3rd tertile) was equivalent to watch screens (TV, DVD, computers) for 7 h or more during the non-work days, i.e. about 3–4 h per day. When high screen time was defined using the 4th quartile instead of 3rd tertile, the results were consistent

pregnancy, e.g., after embryo transfer and until confirmation of a clinical pregnancy, is not harmful for the IVF procedure to succeed.

The mechanisms through which PA affect fecundability could be as follows: (1) PA may influence ovarian function by altering production of oestrogens and other steroid

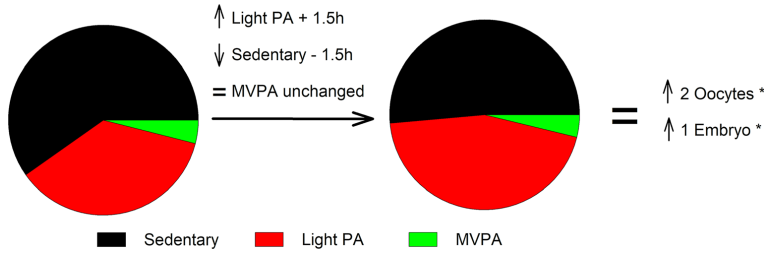

Fig. 4 Graphical illustration of the accelerometer isotemporal analyses showing the association of the time spent in different intensities of physical and sedentarism activities with oocytes and embryos obtained after stimulation in our study group ($N = 98$). The interpretation of this model is that by a given value of MVPA and registered time, an increase in, for example, 1.5 h in light PA accumulated during the whole day (slow walking, slow biking, some houseworks, etc.) should be mirrored by a decrease in sedentary time (the only variable left out of the model), which in turn would result in the change in the dependent variable indicated by the unstandardized regression coefficient. In our study, the regression coefficient for oocytes and embryos of the described models were 1.8 ($P = 0.03$) and 1.2 ($P = 0.03$), respectively (these values were rounded to the closest entire number in the figure for simplicity)

Table 3 Associations of baseline physical activity and sedentary time with in vitro *fertilization* (IVF) outcomes (N = 97)

Baseline physical and sedentary activities	Positive hCG			Clinical pregnancy			Live birth		
(14 days registered)	OR	Lower CI	Upper CI	OR	Lower CI	Upper CI	OR	Lower CI	Upper CI
Average PA (counts/min)	1.00	0.99	1.00	1.00	0.99	1.00	1.00	0.99	1.00
Sedentary time (hours/day)	1.09	0.79	1.52	1.06	0.76	1.48	1.08	0.75	1.57
Light PA (hours/day)	0.95	0.68	1.34	1.01	0.71	1.43	0.96	0.65	1.41
Moderate PA (min/day)	0.99	0.97	1.02	0.99	0.96	1.01	0.99	0.96	1.02
Vigorous PA (min/day)	0.93	0.82	1.04	0.93	0.83	1.05	0.95	0.83	1.09
MVPA (min/day)	0.99	0.96	1.01	0.98	0.95	1.01	0.99	0.96	1.02
10-min bouts of MVPA (No./day)	1.07	0.51	2.26	0.80	0.36	1.77	0.86	0.35	2.11
Time accumulated in 10 min bouts of MVPA (min/day)	0.99	0.96	1.02	0.99	0.95	1.02	0.99	0.95	1.03
30-min bouts of sedentarism (No./day)	1.06	0.85	1.31	1.05	0.84	1.31	1.17	0.90	1.52
Time accumulated in 30-min bouts of sedentarism (hours/day)	1.05	0.85	1.30	1.03	0.83	1.28	1.06	0.83	1.34
Ratio breaks:sedentary time in 30-min bouts (No./hours each day)	0.72	0.11	4.55	0.84	0.13	5.54	2.26	0.28	18.58
Steps (1000/day)	0.93	0.79	1.11	0.91	0.77	1.09	0.95	0.78	1.16

Binary logistic regression models adjusted for accelerometer registered time, age, body mass index, educational level (university vs. below university), smoking (never, before but not now and currently), infertility diagnosis (see Table 1 for coding), infertility duration (years), and follicle stimulation hormone administered. Additional adjustment for the treatment protocol (agonist/antagonist), infertility centre and transferred embryo stage did not change the results

PA physical activity, *MVPA* moderate-to-vigorous PA, *OR* odds ratio, *CI* confidence intervals

hormones via the hypothalamic-pituitary-ovarian-axis [28]; (2) PA can impact reproductive function through its ability to regulate energy balance and affect BMI, which, in turn, are correlated with the reproductive system [29]; (3) PA may influence lipid profiles and inflammation [30]; (4) moderate PA levels have been shown to increase the expression of antioxidant enzymes throughout the body [31]; (5) PA may improve the ART outcome through insulin sensitization, which has been shown to have an effect on ovarian response to clomiphene citrate during ovulation induction [32]; and (6) PA can help to relieve stress and anxiety, which have been shown to affect negatively ARTs [33, 34]. On the other hand, extreme levels of exercise and being underweight may result in disruption of menstrual cyclicity and increase risk for amenorrhea and subfertility, mostly via disruption of hypothalamic-pituitary endocrine axis [35]. However, we lack the information on thresholds for the amount and intensity of activity to achieve optimal fertility.

Our study results demonstrate that women who were engaged in light PA before entering infertility treatment obtained more oocytes and embryos after the stimulation than women who were less active. Also a recent study has associated light PA prior to pregnancy with improved fecundity among overweight/obese women with previous pregnancy loss [36]. Different types of PA (e.g. household, recreational, work, transportation) or exercise (e.g. aerobic, strength) may have different impacts on fertility. Vigorous PA for instance has been believed to be detrimental, while moderate PA could have beneficial effects on fertility [6]. To our knowledge, only two previous studies have assessed the influence of PA on COS, and no association between PA and the number of oocytes and embryos obtained was found [12, 15], although the number of oocytes and embryos tended to be lower among inactive women [12]. In both of these studies, PA was measured using self-reported questionnaires, which are more biased than objectively measured PA [13, 37] which could explain these differences.

Based on the current study, we believe that the public health message is to encourage light PA among infertile women by reducing the time spent for sedentary behaviours. Indeed, according to the recently published 2018 Physical Activity Guidelines Advisory Committee Scientific Report, the current evidence supports that, among individuals with low levels of activity, replacing sedentary behaviour with light-intensity PA is associated with a better systemic health [23]. Our study results demonstrate that women with high sedentary behaviour (> 8 h per day) and 30-min period blocks of sedentarism (> 7) before treatment associate with a lower number of oocytes and embryos in IVF. Furthermore, women who broke more frequently their sedentary behaviour obtained better COS outcomes than women who did less breaks. Sedentary behaviour for prolonged periods of time has been identified as an important public health concern, associated with a range of health issues [38–40], and is another potential

Table 4 Associations of physical activity and sedentary time during IVF (during 14 days after embryo transfer, ET) with treatment outcomes ($N = 79$)

Physical and sedentary activities after ET	Positive hCG			Clinical pregnancy			Live birth		
	OR	Lower CI	Upper CI	OR	Lower CI	Upper CI	OR	Lower CI	Upper CI
Average PA (counts/min)	1.00	0.99	1.00	1.00	0.99	1.00	1.00	0.99	1.00
Sedentary time (hours/day)	1.11	0.75	1.64	1.09	0.73	1.63	1.22	0.79	1.88
Light PA (hours/day)	0.94	0.63	1.41	0.99	0.65	1.50	0.87	0.56	1.35
Moderate PA (min/day)	0.98	0.94	1.01	0.96	0.92	1.00	0.95	0.90	1.01
Vigorous PA (min/day)	1.14	0.85	1.51	1.16	0.87	1.57	1.33	0.97	1.82
MVPA (min/day)	0.98	0.94	1.01	0.96	0.92	1.00	0.95	0.90	1.01
10-min bouts of MVPA (No./day)	1.17	0.40	3.44	0.83	0.27	2.55	0.73	0.20	2.61
Time accumulated in 10-min bouts of MVPA (min/day)	0.99	0.94	1.05	0.98	0.93	1.04	0.98	0.92	1.05
30-min bouts of sedentarism (No./day)	1.12	0.86	1.46	1.14	0.86	1.50	1.31	0.96	1.81
Time accumulated in 30-min bouts of sedentarism (hours/day)	1.11	0.87	1.41	1.10	0.85	1.41	1.18	0.90	1.55
Ratio breaks:sedentary time in 30-min bouts (No./hours each day)	1.60	0.31	8.37	1.63	0.28	9.34	2.92	0.38	22.50
Steps (1000/day)	1.03	0.96	1.10	0.83	0.61	1.12	0.73	0.51	1.04

Binary logistic regression models adjusted for accelerometer registered time, age, body mass index, educational level (university vs. below university), smoking (never, before but not now and currently), infertility diagnosis (see Table 1 for coding), infertility duration (years), and follicle stimulation hormone administered. Additional adjustment for the treatment protocol (agonist/antagonist), infertility centre, and transferred embryo stage did not change the results

PA physical activity, *MVPA* moderate-to-vigorous PA, *OR* odds ratio, *CI* confidence intervals

modifiable risk factor for infertility. Indeed, in a recent study, sedentary behaviour was strongly associated with female infertility [41]. Even further, our isotemporal analysis predicted that if a woman decreased her daily sedentary behaviour for 1.5 h a day and thereby increased 1.5 h light PA, she would obtain 2 oocytes and 1 embryo more in COS. In our cohort of 101 women, we obtained in total ~ 1200 oocytes, while by increasing PA and reducing sedentary behaviour, this number could grow up to ~ 1400. A previous extensive study analysing the reproductive potential of 207,000 oocytes in fresh and frozen embryo transfers yielded 4.5% live birth per oocyte [42], indicating that by exploiting these extra 200 oocytes in IVF, the birth of nearly 10 babies is expected.

Another intriguing result in our study is the link observed between TV/screen watching and assisted reproduction outcomes, which has not been studied before. We found that total screen time at non-work days was strongly associated with the number of oocytes and embryos obtained in COS. The effect is even stronger than sedentary behaviour measured by accelerometry, which could be explained by the fact that not all sedentary behaviours seem to be equally harmful for health, and TV watching seems to be the most harmful [17]. In fact, screen time during work days (which tends to be more work-related) was not associated with COS outcomes among our cohort of women, while the screen time during non-work days should be more volunteer behaviour and more likely to be TV based. TV watching has been associated with detrimental health consequences [43, 44], including lower sperm concentration and lower total sperm count in young men [45].

In our study, women while undergoing IVF significantly reduced PA and increased sedentary behaviour, which means that few women engaged in vigorous activity during IVF and therefore we cannot adequately assess whether high-intensity PA during treatment affects pregnancy outcomes. In fact, we did not detect any associations of objectively measured PA and sedentary behaviour before and during IVF treatment with IVF outcomes such as positive hCG, clinical pregnancy, and live birth. Our study results are in line with the only previous study that has objectively assessed PA, where women significantly reduced their PA and increased sedentary behaviour levels during IVF and no association of PA on IVF outcomes was detected [13]. Although they measured objectively PA during IVF, for 1 week, and the activity levels 1 year before entering IVF were assessed with self-reported questionnaires [13]. In line with our objectively measured data are a number of previous studies demonstrating that bed rest after ET in IVF programs does not influence pregnancy outcomes and is unnecessary [46–50]. Our results contribute to the existing knowledge suggesting that, at least within the levels of PA observed in our participants, higher levels of PA during this critical period of implantation of the embryo had no negative impact on the changes to become pregnant, supporting the notion that there is no evidence to suggest to avoid PA during this period. Nevertheless, we did not find very high/extreme levels of PA within our sample; therefore, we cannot know how these extreme doses of PA could affect successful implantation.

Limitations and strengths

First, the sample may not be generalizable and should be confirmed in other populations. Next, the accelerometer used in the study required removal while in the water, meaning that water-related activities could not be recorded. Our observational study design does not allow to draw conclusions on causality. Likewise, despite the inclusion of a broad set of potential confounders in this study, there is always the possibility that other confounding factors not included in the analyses could influence the results. There are, however, several strengths in our study that should be highlighted. Firstly, that PA and sedentary behaviour were measured objectively using accelerometry and that we measured activities for 14 consecutive days during three critical periods. Also, our unique study design should be acknowledged, where we included only fresh embryo transfers, and measured PA before any infertility treatment and 4 weeks during the treatment, which enabled us to closely observe the periods of embryo implantation and early pregnancy establishment. Further, the confirmation of the study findings with self-reported method, screen time measure, strengthens our conclusions.

Conclusions

Infertility treatment is costly in time, money, and emotional stress; therefore, it is of utmost importance to identify factors which dictate the success of infertility treatment, especially the factors that could be modifiable (e.g. lifestyle factors). This is the first study to objectively measure PA before and during infertility treatment and we show that PA and sedentary behaviour can influence ovarian stimulation outcomes in IVF protocol. Our results suggest that increase in light PA and reduction of sedentary behaviour and screen watching before IVF may help infertile women to improve their results in COS. On the other hand, no positive or negative association of PA with pregnancy outcomes in fresh embryo transfers, including during the critical period of embryo implantation, was observed. Thus, we conclude that PA does not seem to affect the quality of the oocytes (as measured by the achieved pregnancies), but rather the quantity of the oocytes obtained (as measured by COS parameters). Whether the increased number of oocytes collected in more active women is transformed into improved pregnancy rate from cumulative fresh and frozen embryo transfers should be proven by the forthcoming studies.

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Author's contributions All authors contributed to the study design, execution, and interpretation of the study. DS, EM, MN, JM, AE, AS, AS, and AS were responsible for the data collection; FBO and JHM were responsible for the data analysis; and DS and SA drafted the first version of the manuscript. All authors revised critically the manuscript and approved the final version of the manuscript.

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Compliance with ethical standards

Ethical approval The study was approved by the Research Ethics Committee of the University of Tartu.

Statement of informed consent Written informed consent was obtained from all subjects before their participation.

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III

Saare M, Laisk T, Teder H, Paluoja P, Palta P, Koel M, Kirss F, Karro H, **Sõritsa D**, Salumets A, Krjutškov K, Peters M. A molecular tool for menstrual cycle phase dating of endometrial samples in endometriosis transcriptome studies. Biol Reprod. 2019;101(1):1–3.

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Letter to the Editor

A molecular tool for menstrual cycle phase dating of endometrial samples in endometriosis transcriptome studies†

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Summary Sentence

Transcriptome profiling of 57 endometrial receptivity genes specifies the menstrual cycle phase of endometrial samples.

Key words: endometriosis, endometrium, menstrual cycle.

Dear Editor,

Here we report the usability of a panel of transcriptomic markers to determine the menstrual cycle phase of undated endometrial tissue samples for gene expression studies. Endometrial tissue transcriptomic studies are an important approach to find molecular characteristics and biomarkers of endometriosis and other endometrium-related diseases. However, endometrial gene expression is under strict hormonal control and the menstrual cycle phase-specific signature has to be considered in molecular studies of reproductive age women to avoid false-positive or -negative findings that may occur if studied individuals are from different menstrual cycle phases. The endometrial specimens' collection is generally well tolerated by patients: however, unnecessary procedures can be avoided if archival well-preserved RNA samples are available for research. Still, the use of archival samples may be complicated if there is no accompanying menstrual cycle information or only patients' self-reported menstrual cycle day is available, and no tissue has been left for histological evaluation and classification of samples. Although the self-reported menstrual cycle history has been extensively used in molecular studies, the length of the normal menstrual cycle varies between 24 and 35 days and thus self-reported menstrual history or calendar-based counting methods are insufficient to accurately determine menstrual cycle phase, or discriminate ovulatory cycles from anovulatory cycles [1]. Ponnampalam et al. [2] utilized high-throughput microarray technology and demonstrated that classification of the endometrial samples according to the global transcriptional profile is concordant with the histological evaluation. However, as global expression profiling is rather costly, we aimed to use a new cost-effective Targeted Allele Counting by sequencing (TAC-seq) methodology [3] to explore the capability of a panel of 57 well-described endometrial receptivity genes [4] to determine the exact molecular menstrual cycle phases of endometrial samples.

For that purpose, RNA was extracted from endometrial tissue samples collected from 45 women with and 33 women without endometriosis (suffering from pelvic pain or infertility) in menstrual (M, cycle days 1–5, n = 4), proliferative (P, cycle days 6–14, n = 17),

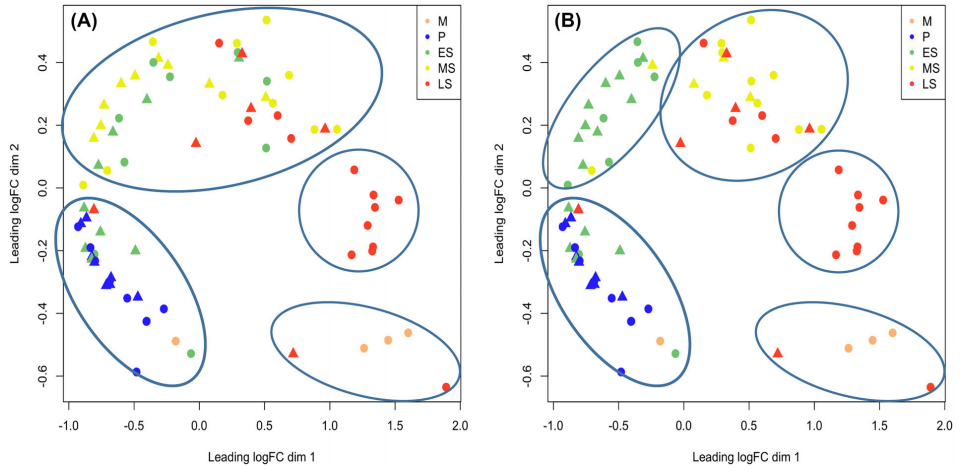

Figure 1. Multidimensional scaling plot of normalized RNA sequencing data of 57 endometrial receptivity genes in women with and without endometriosis. (A) Clustering analysis of RNA sequencing data. (B) Clustering after applying support vector machine classifier to ES and MS phase samples. P—proliferative, ES—early secretory, MS—mid-secretory, LS—late secretory, M—menstrual phase endometrial samples. Triangles represent women without endometriosis and circles mark women with endometriosis.

early-secretory (ES, cycle days 15–18, n = 19), mid-secretory (MS, cycle days 19–23, n = 19), and late-secretory (LS, cycle days 24–28, n = 19) phases according to the self-reported menstrual cycle days (Supplementary Table S1 and Supplementary Materials and Methods). The average age of women with and without endometriosis was 31.0 ± 4.7 and 32.0 ± 5.1 years, respectively, and they had not received any hormonal treatments for at least 3 months prior to the laparoscopy in Tartu University Hospital (Tartu, Estonia). The TAC-seq libraries were sequenced with NextSeq 500/550 v2.5 Kit (Illumina). Sequencing data analysis was performed as described previously [3], and each sample was normalized using geometric mean of gene expression levels of four housekeeper genes. The same sequencing protocol was applied to 54 paired endometrial samples from 27 healthy parous women, collected at the histologically and biochemically [predicted from the luteinizing hormone (LH) peak in urine] confirmed ES and MS cycle phases (described in [5]). The resulting data were used to create a machine learning support vector machine (SVM) model for discrimination of ES and MS phase samples.

Multidimensional scaling plot of normalized RNA sequencing data showed that expression pattern of the 57 endometrial receptivity genes divided the samples roughly into four distinct groups (Figure 1A). Also, no clear segregation was seen between women with and without endometriosis, which is concordant with a recent study by Garcia-Velasco et al. [6]. All endometrial samples from P phase clustered together and a subset of LS phase samples (n = 8) formed a distinct cluster; however, several samples from LS phase (n = 8) were more similar to MS samples and two LS samples grouped together with M phase samples. A similar phenomenon was described by Ponnampalam et al. [2], who suggested that the menstrual cycle is a continuum and the samples from the boarders of cycle phases may cluster to the adjacent phases. Interestingly, one LS sample showed similar gene expression pattern to P samples. We hypothesized that expression of the receptivity-related genes in anovulatory cycles remains similar to P phase throughout the cycle. Although the data about the endometrial receptivity-specific gene expression signature in women with anovulatory menstrual cycles is scarce, the level of glycodelin, which normally increases considerably and stays elevated during the secretory phase, has been shown to remain low throughout the anovulatory cycle [7]. The *PAEP* gene encoding glycodelin was also among the 57 genes analyzed in the current study and its low level in this LS sample was comparable to P samples, supporting our assumption about anovulatory cycle.

Furthermore, ES and MS samples formed one diffuse cluster (Figure 1A), indicating that self-reported menstrual cycle day does not allow reliable distinction between samples from these adjacent phases. Thereafter, the SVM model was successfully applied to segregate the studied self-reported ES and MS samples (Figure 1B) according to the receptivity gene expression pattern in endometrial tissues from women in biochemically confirmed ES and MS phases. After adjustment, 4 out of 19 ES phase samples were re-classified as MS samples and 9 MS samples were re-classified as ES, showing that molecular profiling helped to assign the endometrial samples from adjacent phases correctly even without precise chronological dating. The most widely used method to assign the endometrial samples collected at the second half of the cycle to ES or MS phase is determination of the LH peak from urine, which correlates significantly better with the histological dating than the calculations based on the onset of the next menstrual period [8]. However, as collection of tissue samples for research is for ethical reasons usually combined with clinical procedures that are scheduled long in advance, it is difficult if not impossible to obtain specimens at the particular LH day. Furthermore, the value of histological dating has been questioned as there are too many confounding factors influencing the interpretation of the results [9]. Therefore, new molecular tools, such as described in the current report, are useful to help specify the precise menstrual cycle phase of not only archived endometrial RNA samples but also of endometrial samples from uncertain cycle

phases in transcriptomic studies to facilitate the discovery of true disease-related markers.

Supplementary data

Supplementary data are available at *BIOLRE* online.
Supplementary Table S1. General characteristics of the study participants.

Conflict of interest

The authors have declared that no conflict of interest exists.

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IV

Lavogina D, Samuel K, Lavrits A, Meltsov A, **Sõritsa D**, Kadastik Ü, Peters M, Rinken A, Salumets A. Chemosensitivity and chemoresistance in endometriosis – differences for ectopic versus eutopic cells. Reprod Biomed Online. 2019;39(4):556–568.

RBMO

ELSEVIER

ARTICLE

Chemosensitivity and chemoresistance in endometriosis – differences for ectopic versus eutopic cells

BIOGRAPHY

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KEY MESSAGE

Akt/PKB inhibitor GSK690693, CK2 inhibitor ARC-775, MAPK pathway inhibitor sorafenib, proteasome inhibitor bortezomib, and microtubule-depolymerizing toxin MMAE showed higher cytotoxicity in eutopic cells. In contrast, 10 μmol/l of the anthracycline toxin doxorubicin caused cellular death in ectopic cells more effectively than in eutopic cells, underlining the potential of doxorubicin in endometriosis research.

ABSTRACT

Research question: Endometriosis is a common gynaecological disease defined by the presence of endometrium-like tissue outside the uterus. This complex disease, often accompanied by severe pain and infertility, causes a significant medical and socioeconomic burden; hence, novel strategies are being sought for the treatment of endometriosis. Here, we set out to explore the cytotoxic effects of a panel of compounds to find toxins with different efficiency in eutopic versus ectopic cells, thus highlighting alterations in the corresponding molecular pathways.

Design: The effect on cellular viability of 14 compounds was established in a cohort of paired eutopic and ectopic endometrial stromal cell samples from 11 patients. The biological targets covered by the panel included pro-survival enzymes, cytoskeleton proteins, the proteasome and the cell repair machinery.

Results: Protein kinase inhibitors GSK690693, ARC-775 and sorafenib, proteasome inhibitor bortezomib, and microtubule-depolymerizing toxin monomethyl auristatin E were more effective in eutopic cells. In contrast, 10 μmol/l of the anthracycline toxin doxorubicin caused cellular death in ectopic cells more effectively than in eutopic cells. The large-scale sequencing of mRNA isolated from doxorubicin-treated and control cells indicated different survival strategies in eutopic versus ectopic endometrium.

Conclusions: Overall, the results confirm evidence of large-scale metabolic reprogramming in endometriotic cells, which underlies the observed differences in sensitivity towards toxins. The enhanced efficiency of doxorubicin interfering with redox equilibria and/or DNA repair mechanisms pinpoints key players that can be potentially used to selectively target ectopic lesions in endometriosis.

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KEYWORDS

Cell viability
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Endometriosis
Eutopic/ectopic endometrial stromal cell
Protein kinase inhibitor
Toxin

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INTRODUCTION

Endometriosis is an inflammatory gynaecological disease that manifests itself as a growth of endometrial stromal cells (ESC) and epithelial cells in extrauterine sites. Endometriosis is estimated to affect 2–10% of women in their reproductive years, and as there are still no effective non-surgical treatments, it has a considerable impact on the quality of life of women affected (*Nnoaham et al., 2011*). Endometriosis-associated symptoms such as severe pelvic pain, infertility and impaired psychological and social functioning cause a socioeconomic burden from loss of productivity; furthermore, the risk of developing ovarian cancer is moderately increased in women suffering from endometriosis, being about 1.9% compared with 1.4% in the general population (*Vercellini et al., 2018*). Therefore, the new possibilities in the treatment of endometriosis are being actively explored.

To find potent strategies for treating endometriosis, the mechanisms behind disease initiation need to be understood. The formation of endometriotic lesions presupposes an ability of endometrial cells to attach to peritoneal surfaces, establish neo-angiogenesis and resist apoptosis (*Nasu et al., 2009*). Characteristics such as a high degree of inflammation, an excess of iron and an increase in reactive oxygen species (ROS) have also been described in endometriotic lesions (*Defrere et al., 2008; Lousse et al., 2012; Scutiero et al., 2017*). Furthermore, a comprehensive proteomic study by the current group has shown that extensive metabolic reprogramming (associated with down-regulation of oxidative respiration) and an up-regulation of proteins involved in adhesiveness and motility occur in endometriotic stromal cells (*Kasvandik et al., 2016*), emphasizing the similarities between endometriotic and cancer cells. Therefore toxins affecting various molecular pathways in cancer chemotherapy could find an alternate application in research into – and potentially therapy of – endometriosis. Some such compounds have been briefly explored in the context of endometriosis (*Celik et al., 2008*) yet we are not aware of studies with a focused panel of toxins that would systematically compare the effect of compounds in eutopic and ectopic cells taken from women with endometriosis.

This paper reports on quantification of the cytotoxic effect of 14 compounds (TABLE 1) in a cohort of paired eutopic and ectopic ESC (euESC and ecESC) samples from 11 patients. The biological targets covered by this panel included pro-survival enzymes, cytoskeleton proteins, the proteasome and the cell repair machinery. The rationale behind the choice of compounds took into consideration the high affinity and well-defined selectivity profile of inhibitors in biochemical studies and their applicability in cellular assays. The goal was to find compounds demonstrating different efficiency in eutopic versus ectopic cells from peritoneal lesions, thus highlighting alterations in the corresponding molecular pathways, and to pinpoint compounds that preferentially affect ectopic cells, thus paving the way for future possible therapeutic strategies.

MATERIALS AND METHODS

Chemicals and equipment

Protein kinase inhibitors were obtained from the following sources: SGI-1776 – Axon Medchem (Groningen, Netherlands); H-89 – Biaffin (Kassel, Germany); sorafenib, Y-27632, HA-1077 – Cayman Chemical (Ann Arbor, MI, USA); staurosporine – Cell Guidance Systems (Cambridge, UK); VX-689, CYC116 – Selleckchem (Houston, TX, USA); bortezomib, monomethyl auristatin E (MMAE), doxorubicin – TBD-Biodiscovery (Tartu, Estonia); and GSK690693 – Tocris (Bristol, UK). ARC-775 and ARC-1859 were kindly gifted by Dr Asko Uri (University of Tartu, Tartu, Estonia). The stock solutions of compounds (5–10 mmol/l in dimethylsulphoxide [DMSO]) were stored at –20°C. SYTOX Blue Nucleic Acid

TABLE 1 COMPOUNDS USED IN THE STUDY

Name	Concentrations used (μmol/l)	Major biological target	References
GSK690693	0.4, 2, 10	Akt/PKB 1, 2, 3	(*Levy et al., 2009; Rhodes et al., 2008*)
VX-689 (MK5108)	0.2, 1, 5	Aurora A	(*Chinn et al., 2014; Shimomura et al., 2010*)
CYC116	0.4, 2, 10	Aurora A, B	(*Jayanthan et al., 2014; Wang et al., 2010*)
ARC-775	0.4, 2, 10	CK2	(*Rahnel et al., 2017*)
ARC-1859	0.4, 2, 10	CK2	(*Viht et al., 2015*)
SGI-1776	0.4, 2, 10	PIM 1, 3	(*Chen et al., 2011, 2009*)
H-89	0.4, 2, 10	PKA, PKG1	(*Dabizzi et al., 2003; Yoshino et al., 2003*)
Y-27632	0.4, 2, 10	ROCK 1, 2	(*Grewal et al., 2010; Yotova et al., 2011; Yuge et al., 2007*)
HA-1077 (fasudil)	0.4, 2, 10	ROCK 2	(*Tsuno et al., 2011*)
Sorafenib (BAY 43-9006)	0.4, 2, 10	RAF1, BRAF, KDR (VEGFR2), FLT4 (VEGFR3)	(*Llobet et al., 2010; Moggio et al., 2012*)
Staurosporine	0.2, 1, 5	PKCα, γ, η	(*Izawa et al., 2006; Watanabe et al., 2009*)
Bortezomib (PS-341, Velcade)	0.4, 2, 10	20S proteasome	(*Kao et al., 2014*)
Doxorubicin (adriamycin)	0.4, 2, 10	DNA, topoisomerase-II	(*Byron et al., 2012; Chitcholtan et al., 2012*)
Monomethyl auristatin E (MMAE)	0.04, 0.2, 1	Tubulin	(*Abdollahpour-Alitappeh et al., 2017; Chen et al., 2017*)

Abbreviations: Akt/PKB, protein kinase B; BRAF, V-Raf murine sarcoma viral oncogene homolog B; CK2, casein kinase 2; PIM, proto-oncogene Ser/Thr-protein kinase; PKA, protein kinase A; PKC, protein kinase C; RAF1, V-Raf-1 murine leukemia viral oncogene homolog 1; ROCK, Rho-dependent protein kinase; VEGFR, vascular endothelial growth factor receptor.

Stain and NP40 lysis buffer were from Thermo Fischer Scientific (Rockford, IL, USA); cell culture grade DMSO was from AppliChem (Darmstadt, Germany); resazurin, bovine serum albumin (BSA) and phosphate-buffered saline (PBS) (supplemented with Ca^{2+} and Mg^{2+}; used for the biochemical assays and western blotting) were from Sigma-Aldrich (St Louis, MO, USA). Other solutions, reagents and materials for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were from Thermo Fischer Scientific (Carlsbad, CA, USA).

For the necrosis/late apoptosis and viability assays, the initial number of cells was counted using a TC10 cell counter (Bio-Rad, Hercules, CA, USA), and the cells were seeded onto transparent 96-well, clear, flat-bottom cell culture plates (BioLite 130188; Thermo Fischer Scientific, Rochester, NY, USA). Fluorescence intensity and absorbance measurements were made using Synergy NEO, Cytation 5 (both from Biotek, Winooski, VT, USA) and PHERAstar (BMG Labtech, Ortenberg, Germany) multimode readers.

Patient characteristics and sample collection

The study was approved by the Research Ethics Committee of the University of Tartu (approval 276/M-13) on 18 December 2017 and informed written consent was obtained from the participants. Endometrial tissue samples and peritoneal endometriotic lesions were collected from 11 women endometriosis (TABLE 2) undergoing laparoscopy at the Tartu University Hospital Women's Clinic. Tissue samples were immediately placed into the cryopreservation medium and processed as previously described (*Rekker et al., 2017*). At least one endometriotic lesion sample from each patient was placed into formalin and the diagnosis was confirmed by histopathological examination of the specimens. Disease severity was determined according to the American Society for Reproductive Medicine revised classification system (*American Society for Reproductive Medicine, 1997*). Only women who had not received any hormonal medications for at least 3 months before surgery were enrolled in this study.

Isolation and culturing of cells

Endometriotic and endometrial tissues were treated according to the previously published protocol (*Kasvandik et al., 2016*). Briefly, the tissue was washed twice in 7 ml of fresh medium (a 1:1 mixture of Dulbecco's modified Eagle's medium [DMEM] and Ham's F-12; Sigma-Aldrich, Steinheim, Germany) to remove any debris or excess blood cells. The biopsies were dissociated in 5 ml of DMEM (without phenol red) containing 0.5% collagenase (Sigma-Aldrich) in a shaking incubator rotating at 110 rpm at 37°C until the biopsies had been digested (but not for longer than 1 h). The dispersed cells were filtered through a 50 µm nylon mesh to remove undigested tissue pieces. Next, the cells were resuspended in 10 ml of culture medium in a 15 ml tube; the sealed tubes were placed in an upright position for 10 min to sediment the epithelial glands. The top 8 ml of medium containing stromal cells was then collected and the tube was refilled to 10 ml with fresh medium; the sedimentation process was repeated three times and the collected fractions were pooled. The final purification of stromal cells was achieved by selective adherence of stromal cells to culture dishes for 20–30 min at 37°C in 5% CO_2 in an incubator. Non-adhering epithelial cells were removed by washing the cell layer twice with 5 ml of culture medium.

The isolated ESC were further cultured for 5–6 passages in DMEM/Ham's F-12 medium supplemented with 10% fetal bovine serum (FBS; Capricorn, Ebsdorfergrund, Germany) and a mixture of penicillin, streptomycin and amphotericin B (Capricorn, Ebsdorfergrund, Germany) at 37°C in 5% CO_2 in an incubator.

Necrosis/late apoptosis assay

euESC and ecESC (passage number 5–6) were seeded onto 96-well plates at a density of 4000–6000 cells per well in DMEM/Ham's F-12 medium supplemented with FBS; euESC and ecESC from the same patient were thawed on the same day, and two plates were prepared for both eutopic and ectopic stromal cells. After incubating the cells for 24 h at 37°C in 5% CO_2 in a humidified incubator, the medium was exchanged and dilution series of compounds in PBS were added (see TABLE 1); the final volume per well was 110 µl, and the concentration of DMSO in the treated wells was ≤0.1% by volume. For each plate, each concentration of each compound to be tested was represented in duplicate; the controls (10% DMSO and 0.1% DMSO) were represented in sextuplicate. The cells were incubated with the compounds for 22 h at 37°C in 5% CO_2 in a humidified incubator; next, the medium was removed

TABLE 2 CHARACTERISTICS OF THE STUDY PARTICIPANTS

Patient ID	Age (years)	BMI (kg/m^2)	Endometriosis stage	Location of lesion	Study
E048	29	19.8	III	Lig. sacrouterina SUP	N, V
E044	32	23.7	III	Excavatio vesicouterina SUP	N, V
E041	39	25.6	I	Fossa ovarica SUP	N, V
E205	36	22.2	I	Lig. latum SUP	N, V
E242	30	20.1	I	Lig. sacrouterina SUP	N, V
E262	40	29.8	II–III	Lig. latum SUP	N, V, V2, WB, seq
E267	25	22.1	I	Pouch of Douglas SUP	N, V, V2, WB
E270	33	21.6	III	Lig sacrouterina SUP	N, V
E278	32	20.8	I	Excavatio vesicouterina SUP	N, V, V2, WB, seq
E279	22	21.4	I	Excavatio vesicouterina SUP	N, V, V2, WB, seq
E310	24	23.5	I	Lig. sacrouterina SUP	N, V

Lig., ligamentum; N, necrosis/late apoptosis assay; seq, mRNA sequencing; SUP, superficial; V, viability assay with large cohort; V2, viability assay with small cohort; WB, western blot.

and 1 µmol/l Sytox Blue solution in PBS (containing Ca^{2+} and Mg^{2+}) was added. The plates were placed into a multimode reader and incubated for 10 min at 37°C, and the fluorescence intensity was measured (excitation 430 nm, emission 480 nm, monochromator, top optics, gain 90; area scan mode 5 × 5, read height 2.5 mm, with lid).

Viability assay

The viability assay was performed directly after the necrosis/late apoptosis assay using the same plates. The solution of Sytox Blue was replaced by 50 µmol/l resazurin solution in PBS (containing Ca^{2+} and Mg^{2+}). The plates were placed into the multimode reader, and measurement of absorbance was performed (570 nm and 600 nm, monochromator; kinetic mode with a reading taken every 15 min for 2 h, read height 8.5 mm, with lid). Next, resazurin solution was replaced by fresh sterile DMEM/Ham's F-12 medium supplemented with FBS, and the cells were incubated for 24 h at 37°C in 5% CO_2 in a humidified incubator. Finally, the viability assay was performed again (without the preceding necrosis/late apoptosis assay). In a pilot experiment, it was confirmed that the first application of resazurin for 2 h in PBS did not cause severe cytotoxicity (data not shown).

Western blotting

For the western blot assay, one 6-well plate was prepared for euESC and one plate for ecESC (passage number 5–6). When the confluency of cells was 50% or higher, dilutions of doxorubicin in PBS or DMSO in PBS (control) were added. The final volume per well was 2 ml; the final concentration of doxorubicin was 10 µmol/l, and the final concentration of DMSO was 0.1%. On each plate, both doxorubicin and control incubations were represented in duplicate. The cells were incubated for 48 h at 37°C in 5% CO_2 in a humidified incubator.

After collection and lysis of the cells on ice, the samples for SDS-PAGE were prepared by adding NuPAGE sample loading buffer (ThermoFisher, Carlsbad, CA, USA) to supernatants and heating at 70°C for 15 min. SDS-PAGE was performed on 10% Bis-Tris gels or 4–12% Bis-Tris gradient gel (ThermoFisher, Carlsbad, CA, USA) in MES buffer (ThermoFisher, Carlsbad, CA, USA); samples of treated and untreated euESC and ecESC from the same patients were applied on different lanes of the same gel. Semi-dry transfer followed at 15 V for 60 min using methanol-activated polyvinylidene difluoride (PVDF) membrane and NuPAGE transfer buffer. The membrane was then stained with primary antibody (1,000 × dilution of rabbit anti-procaspase-3; catalogue number 9662 Cell Signaling, RRID: AB_331439) and secondary antibody (5,000 × dilution of goat anti-rabbit conjugated to alkaline phosphatase; T2191 Thermo Fischer Scientific, RRID: AB_11180336) according to the manufacturers' instructions. The same procedure was used for the subsequent staining of the same membrane with mouse antibeta-actin (4,000 × dilution; A1978 Sigma-Aldrich, RRID: AB_476692) and goat anti-mouse conjugated to alkaline phosphatase (5,000 × dilution; T2192 Thermo Fischer Scientific, RRID: AB_11180852).

mRNA isolation and large-scale sequencing

euESC (n = 3) and ecESC (n = 3) were isolated and grown as described in the sections on the isolation and culturing of cells and western blotting, respectively; the cells were isolated from the paired eutopic and ectopic samples that were included in western blot studies. After 24 h incubating the cells with a final concentration of 2 µmol/l doxorubicin or 0.1% DMSO (as a negative control) in growth medium, the medium was removed, the cells were rinsed with PBS and RNA was extracted using RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNase I treatment was performed using the DNA-free DNA removal kit (Invitrogen, Carlsbad, CA, USA). A 2200 TapeStation system in conjunction with RNA ScreenTape (Agilent Technologies, Palo Alto, CA, USA) was used to determine the quality and quantity of purified RNA. For sequencing library construction, RNA from two technical replicates was pooled together. cDNA was synthesized as previously described (*Teder et al., 2018*), converted to the next-generation sequencing library using a Nextera XT Library Prep kit (Illumina, San Diego, CA, USA) and sequenced with NextSeq 500 high output 75 cycles kit (Illumina, San Diego, CA, USA).

Quantitative real-time PCR

The expression levels of selected genes (*HSPA2*, *PTGS2* and *PTN*) were validated by quantitative real-time PCR (qRT-PCR) using RNA from two technical replicates. cDNA was synthesized using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA), and real-time PCR was performed using 1 × HOT FIREPol EvaGreen qPCR Mix Plus (ROX) (Solis BioDyne, Tartu, Estonia). The primer sequences used were as follows: *HSPA2* (F: CTCCACTCGTATCCCCAAGA, R: GTCACGTCGAGTAGCAGCAG), *PTGS2* (F: CCACTTCAAGGGATTTTGGA, R: GAGAAGGCTTCCCAGCTTTT) and *PTN* (F: CAATGCCGAATGCCA GAAGACTGT, R: TCCACAGGTGACA TCTTTTAATCC). *ACTB* (F: TCAAG ATCATTGCTCCTCC and R: ACATCTG CTGGAAGGTGGA) was used as a reference gene.

Statistical analysis

Data are available on request from the authors.

For the necrosis/late apoptosis assay, the mean Sytox Blue fluorescence intensity per well was calculated; the data corresponding to the same concentration of the same compound were pooled and normalized for each plate. For normalization, the signal obtained for incubation with 5 µmol/l staurosporine was considered to be 100% necrosis, and the signal obtained for incubation with 0.1% DMSO as 0% necrosis.

For the viability assay, the ratio of the absorbances at 570 nm and 600 nm was calculated for each well. The data obtained from one plate for the control incubations with 0.1% DMSO or 10% DMSO were pooled and plotted against time, and the linear range of the assay was established. The data corresponding to the same concentration of the same compound were pooled and normalized for each plate. For normalization, data obtained for incubation with 10% DMSO were considered as 0% viability, and data obtained for incubation with 0.1% DMSO as 100% viability.

For western blot data analysis, the membrane was dried and scanned in. The area of bands detected with anti-procaspase-3 and anti-beta–actin was assessed using ImageJ 1.51j8 software (Bethesda, MD, USA), and the ratio of the two values was calculated for each lane; the data were pooled for the lanes where the identically treated samples of the same cells had been applied. Next, data for lanes with samples from euESC and ecESC were normalized separately. For normalization, ratio obtained for incubation with 0.1%

DMSO was considered as 100% to obtain results for one patient; the bottom plateau was fixed at 0%.

In the case of qRT-PCR, the average values of technical replicates were used. The fold change was calculated according to the $2^{-\Delta\Delta CT}$ method (*Livak and Schmittgen, 2001*).

For the final comparison, the results of all the patients were pooled. For the necrosis/late apoptosis and viability assays, the statistical significance for the difference between the inhibitor/toxin-treated cells and the cells treated with 0.1% DMSO was established by ordinary one-way analysis of variance using Dunnett's correction for multiple comparisons ($P \leq 0.05$ was considered significant). For the necrosis/late apoptosis and viability assays as well as the western blotting, the statistical significance of the difference between euESC and ecESC was established by an unpaired t-test with Welch's correction ($P \leq 0.05$ was considered significant). For the qRT-PCR data, the statistical significance of the difference between control and doxorubicin treatment was established using a paired t-test ($P \leq 0.05$ was considered significant), and the statistical significance of the difference between euESC and ecESC was established using an unpaired t-test (again, $P \leq 0.05$ was considered significant). The aforementioned statistical analysis was carried out using GraphPad Prism 6 (San Diego, CA, USA).

The large-scale mRNA sequencing data were acquired using Illumina BaseSpace (San Diego, CA, USA). The reads were quantified with Salmon 0.9.1 (New York, USA / Pittsburgh, PA, USA) in quasi-mapping mode using indexed Ensemble v95 annotation. The quality control of raw sequencing data and statistics on aligned counts was performed with FastQC 0.11.5 (Babraham, UK) and MultiQC 1.7 (Stockholm/Uppsala, Sweden). Based on the quality control, further data transformation was performed by trimming the adapter size with Trimmomatic 0.38 (Jülich, Germany). Quantified transcript read counts were summarized to genes using Bioconductor packages tximport 1.10.1 (Boston, MA, USA / Zurich, Switzerland) and BioMart 2.38.0 (Berkeley, CA, USA / Cambridge, UK). Overall, 175,775 transcripts were identified from all the samples, out of which 28,796 genes with non-zero total counts were summarized. Differential RNA sequencing analysis and ranking was performed with DESeq2 1.22.2 (Heidelberg, Germany). edgeR 3.24.3 (Parkville, Australia) was used in parallel for comparison.

The shortlist of genes with significantly different (*P*-value adjusted for false discovery rate, $P_{adj} < 0.05$) expression in pairwise compared cell types and treatment conditions (control euESC versus control ecESC; control euESC versus toxin-treated euESC; control ecESC versus toxin-treated ecESC; and toxin-treated euESC versus toxin-treated ecESC) was generated as follows. The data for expression of each gene obtained in the same cell type and condition were averaged for three patients, and the binary logarithm of the fold change of averages ($\log_2$FC) was determined. For each pairwise comparison, the latter values were ranked and cut-off values of $\log_2$FC less than or equal to 4 or ≥+4 were applied. The genes showing high variance in expression (for the same cell type and condition between different patients) and the genes for which the number of counts was below 10 in all conditions were eliminated. Finally, after the individual check of the remaining candidates using the GeneCards human gene database (*Weizmann Institute of Science, 2019*) and g:Profiler source (*Reimand et al., 2016*), the pseudogenes and the genes encoding poorly characterized proteins were excluded from the list.

RESULTS

Viability assay

To establish the effect of the compounds (shown in TABLE 1) on the viability of euESC and ecESC, an assay was used that measures the change in absorbance spectrum of the cell membrane-penetrating dye resazurin upon its biochemical reduction in metabolically active cells. TABLE 3 summarizes the results of the viability assay in which a statistically significant reduction of viability ($P \leq 0.01$) was observed after 22 h incubation of cells with the studied compounds and after an additional 24 h incubation in growth medium; the full versions of the data are presented in the Supplemental Tables S1 and S2.

As expectedly, the lowest viability after 22 h of treatment was observed for both euESC and ecESC treated with the well-known apoptosis inducer staurosporine. SGI-1776, a pan-inhibitor of proto-oncogene Ser/Thr-protein kinase Pim family, caused a significant fall in viability at 10 µmol/l concentration in both euESC and ecESC ($P \leq 0.001$); it was also the only compound in the panel demonstrating a large patient-dependent effect: out of 11 patients' samples, low viability of the cells was evident in the samples from three patients, whereas those from four patients were practically insensitive (Supplemental Figure S1A). Other inhibitors of protein kinases did not cause an extended amount of cell death in either euESC or ecESC (the viability of cells remained at 75% or more relative to the 0.1% DMSO control). Interestingly, after 22 h incubation of cells with the Rho-dependent protein kinase inhibitor HA-1077, an apparent increase in viability was observed in both in euESC and ecESC (i.e. cells treated with 10 µmol/l inhibitor had higher values for resazurin reduction than cells treated with 0.1% DMSO). A similar phenomenon was evident in both in euESC and ecESC on treatment with different concentrations of VX-689, and in ecESC on treatment with 10 µmol/l or 2 µmol/l ARC-1859 (see Supplemental Table S1). Chemotherapeutic drugs bortezomib and MMAE were more efficient in eutopic cells, although a significant fall in viability was observed in both euESC and ecESC ($P \leq 0.001$). Conversely, treatment with 10 µmol/l and 2 µmol/l doxorubicin was more efficient in ecESC than euESC, showing a similar effect across all patients (see Supplemental Figure S1A).

The measurement of cell viability after the subsequent 24 h incubation in growth medium demonstrated that the viability of most toxin-treated euESC and ecESC had decreased further, and differences in results between euESC and ecESC had become smaller (TABLE 3). In addition, a significant decrease of viability was now observed for cells treated with the mitogen-activated protein kinase (MAPK) pathway inhibitor sorafenib ($P \leq 0.05$), protein kinase A (PKA) inhibitor H-89 ($P \leq 0.01$) and Aurora A inhibitor VX-689 ($P \leq 0.01$; a fuller version of 22 h + 24 h results from TABLE 3 is presented as Supplemental Table S2). Although sorafenib and H-89 were slightly more active in euESC, the effect of VX-689 was more pronounced in ectopic cells. Notably, after prolonged

TABLE 3 COMPOUNDS INDUCING A SIGNIFICANT DECREASE IN VIABILITY OF EUESC AND/OR ECESC AFTER 22 H AND PROLONGED TREATMENT

Compound	Concentration	Incubation time[a]	% of viability in euESC[b]		% of viability in ecESC[b]		Difference euESC versus ecESC[c]
GSK690693	10 µmol/l	22 h	86 ± 2	***	94 ± 2	ns	* (euESC)
		22 h + 24 h	78 ± 2	***	87 ± 2	***	** (euESC)
	2 µmol/l	22 h	89 ± 2	**	94 ± 2	ns	ns
		22 h + 24 h	85 ± 2	***	89 ± 2	***	ns
CYC116	10 µmol/l	22 h	89 ± 2	***	103 ± 2	ns	*** (euESC)
		22 h + 24 h	87 ± 2	***	93 ± 2	**	* (euESC)
ARC-775	10 µmol/l	22 h	77 ± 2	***	90 ± 2	***	*** (euESC)
		22 h + 24 h	67 ± 2	***	70 ± 2	**	ns
	2 µmol/l	22 h	90 ± 2	***	102 ± 2	ns	*** (euESC)
		22 h + 24 h	92 ± 2	***	91 ± 2	***	ns
SGI-1776	10 µmol/l	22 h	56 ± 5	***	62 ± 5	***	ns
		22 h + 24 h	48 ± 4	***	57 ± 4	***	ns
Staurosporine	5 µmol/l	22 h	15 ± 1	***	6 ± 1	***	*** (ecESC)
		22 h + 24 h	4 ± 1	***	3 ± 1	***	ns
	1 µmol/l	22 h	27 ± 3	***	24 ± 1	***	ns
		22 h + 24 h	16 ± 2	***	15 ± 2	***	ns
	0.2 µmol/l	22 h	41 ± 3	***	50 ± 2	***	** (euESC)
		22 h + 24 h	30 ± 2	***	41 ± 2	***	*** (euESC)
Bortezomib	10 µmol/l	22 h	26 ± 2	***	40 ± 2	***	*** (euESC)
		22 h + 24 h	5 ± 1	***	16 ± 2	***	*** (euESC)
	2 µmol/l	22 h	33 ± 1	***	42 ± 2	***	*** (euESC)
		22 h + 24 h	12 ± 1	***	23 ± 2	***	*** (euESC)
	0.4 µmol/l	22 h	39 ± 2	***	53 ± 2	***	*** (euESC)
		22 h + 24 h	18 ± 1	***	39 ± 2	***	*** (euESC)
Doxorubicin	10 µmol/l	22 h	78 ± 2	***	59 ± 2	***	*** (ecESC)
		22 h + 24 h	38 ± 2	***	22 ± 2	***	*** (ecESC)
	2 µmol/l	22 h	78 ± 2	***	64 ± 2	***	*** (ecESC)
		22 h + 24 h	39 ± 2	***	37 ± 2	***	ns
	0.4 µmol/l	22 h	85 ± 2	***	83 ± 2	***	ns
		22 h + 24 h	67 ± 3	***	68 ± 2	***	ns
Monomethyl auristatin E (MMAE)	1 µmol/l	22 h	60 ± 2	***	65 ± 1	***	* (euESC)
		22 h + 24 h	47 ± 2	***	53 ± 2	***	* (euESC)
	0.2 µmol/l	22 h	60 ± 2	***	66 ± 2	***	* (euESC)
		22 h + 24 h	49 ± 2	***	57 ± 2	***	** (euESC)
	0.04 µmol/l	22 h	61 ± 2	***	64 ± 1	***	ns
		22 h + 24 h	49 ± 2	***	59 ± 2	***	*** (euESC)

Data are mean normalized viability ± SEM.

[a] Incubation with inhibitors was performed for 22 h, followed by the addition of growth medium for 24 h.

[b] $n = 11$ for the 22 h measurement and $n = 10$ for the 22 h + 24 h measurement. Data obtained for incubation with 10% dimethylsulphoxide (DMSO) were considered to show 0% viability, and data obtained for incubation with 0.1% DMSO were considered to show 100% viability. Significance of effect difference relative to the negative control (treated with 0.1% DMSO): ***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$; ns, $P > 0.05$.

[c] Significance of effect difference between euESC and ecESC; the cell type with the lowest viability is shown in brackets. ***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$; ns, $P > 0.05$. ecESC, ectopic endometrial stromal cell; euESC, eutopic endometrial stromal cell.

incubation, 10 µmol/l doxorubicin still affected ecESC more than euESC. The increased sensitivity of ecESC towards high concentrations of doxorubicin was confirmed in a repeated assay with samples representing four patients from the same cohort (Supplemental Figure S2).

All in all, based on the results of the viability assay, characteristic differences in the viability fingerprint between euESC and ecESC could be formulated (FIGURE 1).

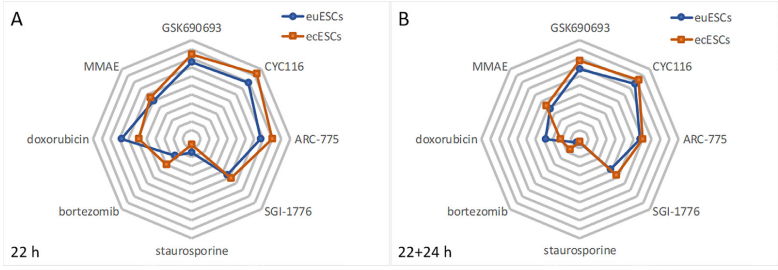

FIGURE 1 Viability fingerprint of euESC versus ecESC (blue and orange lines, respectively) after 22 h (A) or 22 h + 24 h (B) of treatment with various compounds. The compounds were chosen based on *Table 3*. Mean data corresponding to treatment with the highest concentrations of compounds was plotted. The axis scale ranges from 0% (centre of the plot) to 110% (outer line) with a grid interval of 10%. ecESC, ectopic endometrial stromal cell; euESC, eutopic endometrial stromal cell.

Necrosis/late apoptosis assay

To confirm the trends observed in viability studies, we applied an additional assay by using cell membrane-impermeable Sytox Blue dye after 22 h incubation of euESC and ecESC with the different compounds. The increase in fluorescence of Sytox Blue resulting from intercalation of dye into the DNA is only possible in cells with compromised membrane structure, indicating an elevated extent of necrosis/late apoptosis.

The results of the assay are presented in the Supplemental Table S3. The highest effect in euESC as well as in ecESC was observed for 5 µmol/l staurosporine, a generic protein kinase inhibitor, which was therefore chosen as the standardizing condition setting the maximal threshold for normalization of the data. ecESC seemed overall less prone to necrosis/late apoptosis than euESC; however, high levels of cell death in both euESC and ecESC were also observed upon treatment with 10 µmol/l SGI-1776 (which targets PIM family protein kinases) and 10 µmol/l ARC-775 (which targets casein kinase 2 [CK2]). The Akt/protein kinase B (PKB) inhibitor GSK690693 in the 10 µmol/l concentration induced more necrosis/late apoptosis in eutopic cells; furthermore, the toxins bortezomib and MMAE were more effective in euESC than ecESC at all concentrations. Other compounds showed no effect even at the highest concentrations used (5–10 µmol/l).

The data for doxorubicin were not included as here we observed a characteristic fall in Sytox Blue signal below the value observed for the negative control (cells treated with 0.1% DMSO), which occurred in both euESC and ecESC from all patients. We propose that such behaviour is related to the mode of action of doxorubicin, which intercalates into DNA; in this way, doxorubicin competes with Sytox Blue for the binding sites, and necrosis or apoptosis assays based on dyes that gain fluorescence upon binding to DNA are incompatible with doxorubicin studies.

Western blotting

To gain further independent evidence related to the elevated efficiency of doxorubicin in ecESC versus euESC, we proceeded with an alternative assay. Due to the strong autofluorescence of doxorubicin (*Wang et al., 2016*), most of the 'classical' techniques such as imaging or fluorescence-activated cell sorter (FACS) using immunostaining or BrdU detection can be highly prone to artefacts; therefore, we chose western blotting to quantify the fall in procaspase-3 concentrations in doxorubicin-treated samples of euESC and ecESC from four patients (the same samples used for the repeated viability assay; see above). The ratio of signals corresponding to procaspase-3 and beta-actin was quantified for each treatment condition, and the data were normalized separately for euESC and ecESC from each patient according to the corresponding negative control (0.1% DMSO; FIGURE 2A).

The results confirmed that 48 h of treatment with 10 µmol/l doxorubicin caused a statistically significant ($P \leq 0.05$) difference in apoptosis in ectopic versus eutopic cells, with mean normalized procaspase-3 content reduced to 39% (±8% SEM) in ecESC and 60% (±4% SEM) in euESC relative to the corresponding negative controls (0.1% DMSO) (FIGURE 2B).

mRNA sequencing

Finally, to obtain detailed insight into the signalling pathways affected by doxorubicin in euESC and ecESC, large-scale mRNA sequencing was performed after 24 h incubation of cells from three patients with 2 µmol/l doxorubicin or 0.1% DMSO control. The concentration of doxorubicin was chosen based on the results of the viability assay, in order to see a significant difference between euESC and ecESC while still yielding a sufficient population of surviving cells for mRNA isolation.

The comparison of treated versus control cells yielded 4009 significantly differentially expressed genes in the case of euESC, yet only 249 significantly differentially expressed genes for ecESC (using a base mean cut-off value of >10 and a P_{adj} cut-off value of <0.05). To shortlist genes showing a significantly different expression in different cells and treatment conditions (TABLE 4), we sorted the sequencing data as described in the section on statistical analysis, above. Overall, several genes that had a higher expression in control euESC relative to control ecESC (*MMP1/3/10*, *PENK*, *PTN* and *GRP*) or in control ecESC relative to control euESC (*ESM1*, *IL33* and *PTX3*) also showed greater expression in the same cell type following treatment with doxorubicin. Furthermore, treatment with doxorubicin resulted in a reduced expression of several genes in euESC (e.g. *DUSP1/10* and *BARD1*) as well as in ecESC (e.g. *DKK1*, *HAS2*) relative to the control cells of the same type. On the other hand, although the expression of some genes (such as histone cluster 1 and 2 family members *HIST1H2AE*, *HIST1H2BK* and *HIST2H2AA4*) in euESC increased upon treatment with

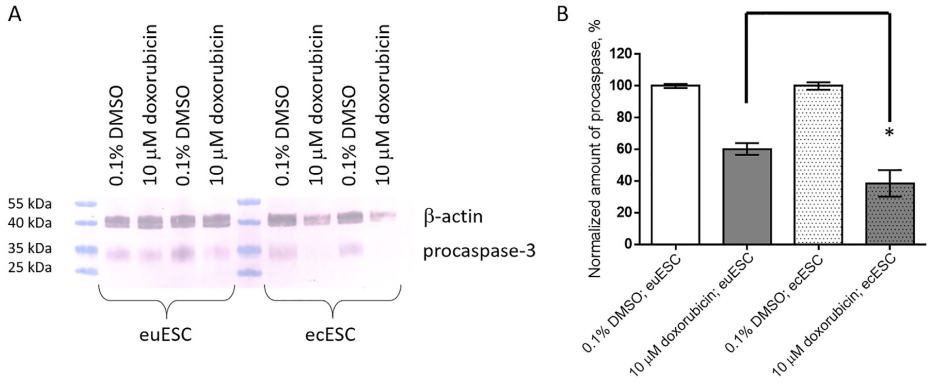

FIGURE 2 Effect of doxorubicin on procaspase-3 concentrations in euESC and ecESC. (A) Representative example of western blot membrane with euESC and ecESC from one patient; different lanes represent independent incubations. (B) Pooled normalized western blot data of euESC and ecESC from four patients (mean ± SEM). Significance of effect difference between euESC and ecESCs: *$P \leq 0.05$. DMSO, dimethylsulphoxide; ecESC, ectopic endometrial stromal cell; euESC, eutopic endometrial stromal cell.

doxorubicin relative to control cells, there was no significant increase in gene expression in toxin-treated ecESC relative to the control treatment.

For technical validation of the results of large-scale mRNA sequencing, qRT-PCR analysis of *PTN* and *HSPA2* was carried out, as examples of genes considerably highly expressed in eutopic cells, with *PTN* expression elevated in both control and toxin-treated euESC relative to the correspondingly treated ecESC (TABLE 4). Also validated was the expression of *PTGS2*, which, according to large-scale mRNA sequencing data, possessed higher expression in ectopic relative to eutopic cells after doxorubicin treatment; however, the statistical significance of this difference was slightly higher than the classical cut-off P_{adj} value of 0.05 (Supplemental Table S4). qRT-PCR confirmed the general trends observed in large-scale transcriptomic analysis, indicating significantly higher expression of *PTN* in both control and doxorubicin-treated euESC versus the corresponding ecESC (both $P < 0.05$), and significantly higher expression of *HSPA2* in control euESC versus ecESC ($P = 0.05$). In addition, doxorubicin treatment elevated the level of *PTN* and *HSPA2* in eutopic and ectopic stromal cells, respectively (both $P < 0.05$). Furthermore, qRT-PCR showed significantly higher expression of *PTGS2* in control ecESC versus euESC as well as doxorubicin-treated ecESC versus euESC (both $P < 0.05$), confirming that *PTGS2* can indeed serve as an important target in endometriosis.

TABLE 4 GENES SHOWING SIGNIFICANTLY DIFFERENT EXPRESSION IN CONTROL AND TOXIN-TREATED EUESC AND ECESC

Comparison[a]		Gene names and log_2FC values[b,c]
euESC control versus ecESC control	Higher expression in euESC	*MMP12* (8.4), ***MMP10*** (8.0), ***MMP3*** (8.0), *TFAP2C* (7.4), ***RGCC*** (6.8), ***HTR2B*** (6.4), ***GRP*** (6.4), ***DIO2*** (5.7), ***MMP1*** (5.5), *RBP1* (4.9), ***CARD16*** (4.8), *LEPR* (4.8), *PRDM1* (4.7), *CTSK* (4.6), *HSPA2* (4.6), *NID1* (4.6), *GCNT4* (4.5), *PLAU* (4.5), ***PENK*** (4.5), *PTN* (4.4), *IFI6* (4.2), *SEMA5A* (4.1), *AREG* (4.0), *NPY1R* (4.0)
	Higher expression in ecESC	*GIPC2* (–9.7), ***PTX3*** (–9.0), *EFEMP1* (–6.1), ***IL33*** (–6.0), *SFRP4* (–4.5), *PPP1R3C* (–4.3), ***ESM1*** (–4.0)
euESC control versus euESC + toxin	Higher expression in control treatment	***HTR2B*** (8.0), *CCDC107* (7.0), *ING3* (6.4), ***BARD1*** (6.2), *CARNMT1* (5.9), *KRT19* (5.8), *TUBA1A* (5.3), ***DIO2*** (5.2), *PAN3* (5.1), *DUSP1* (4.9), *PKIG* (4.9), *PBK* (4.9), *UTP18* (4.8), *CEMIP* (4.7), *SLC5A3* (4.5), *CITED2* (4.5), *CTGF*(4.4), *SASS6* (4.1), *DUSP10* (4.1), *NOP10* (4.1)
	Higher expression in toxin treatment	*HIST1H2AE* (–7.0), *INSYN2* (–6.7), *TMEFF2* (–6.0), *HIST1H2BPS2* (–5.2), *HIST1H2BK* (–5.0), *HIST2H2AA4* (–4.8), *CXCL3* (–4.7)
ecESC control versus ecESC + toxin	Higher expression in control treatment	*HAS2* (6.9), *MRPL14* (5.0), ***CARD16*** (4.5), *DKK1* (4.0)
euESC + toxin versus ecESC + toxin	Higher expression in euESC	***GRP*** (7.3), ***MMP3*** (7.1), ***MMP10*** (6.1), *PTN* (5.1), ***RGCC*** (4.7), *IFITM1* (4.5), *SOX11* (4.3), ***MMP1*** (4.2), *PENK* (4.1)
	Higher expression in ecESC	***ESM1*** (–6.2), *TFPI2* (–5.3), ***PTX3*** (–4.9), *IL33* (–4.4), ***BARD1*** (–4.1)

[a] Control treatment: 24 h incubation in growth medium containing 0.1% dimethylsulphoxide; toxin treatment: 24 h incubation in growth medium containing 2 µmol/l doxorubicin.

[b] The binary logarithm of the fold change of averages is shown in brackets; $n = 3$. Negative values indicate higher expression in ectopic cells (for euESC versus ecESC comparisons) or in doxorubicin-treated cells (for treatment comparisons).

[c] Genes that are listed under more than one comparison in the table are shown in bold.

ecESC, ectopic endometrial stromal cell; euESC, eutopic endometrial stromal cell; log_2FC, binary logarithm of fold change of averages.

DISCUSSION

Although the molecular players behind the onset and progression of endometriosis are still unclear, several pathways have been closely inspected, with a special focus on inflammation processes, cell migration and adhesion, abnormal proliferation and resistance to apoptosis. The current study explored the differences in cell viability of euESC and ecESC on treatment with selective compounds inhibiting a focused number of molecular players, as well as compounds with a wide profile of biological targets. Methodologically, this study has two major limitations: first, the focus was only on stromal cells, yet the physiological milieu contains epithelial cells that may be involved in unique patterns of signalling and cellular interactions. Second, as only ESCs isolated from superficial peritoneal lesions were investigated, the observed results may not necessarily reflect the effects of toxins in other types of lesions.

Phosphorylation of proteins serves an example of a signalling mechanism that on one hand is ubiquitous, yet on the other can be dissected with a high degree of precision by selective targeting of the catalysing machinery – protein kinases. The human kinome includes 538 protein kinases, most of which have been termed as potentially druggable by virtue of the incorporation of a narrow solvent-hidden pocket (ATP-binding site) that can be selectively targeted by low molecular weight inhibitors. The panel that was used here for screening included 11 inhibitors of protein kinases, 10 of which possessed focused selectivity profiles, while staurosporine was selected as a widely used apoptosis inducer (see TABLE 1 and Supplemental Figure S3). Among the protein kinases targeted by the selective inhibitors were enzymes for which up-regulation in endometriotic cells has been reported: MAPKs (*Ngô et al., 2010; Yotova et al., 2011*), Akt/PKB (*Cinar et al., 2009; Shoji et al., 2009*), PIM1 (*Hu et al., 2006; Jiménez-García et al., 2017*) and CK2 (*Feng et al., 2012; Llobet et al., 2008*). In this study, inhibitors of MAPK (sorafenib), Akt/PKB (GSK690693) and CK2 (ARC-775) were more effective in euESC than ecESC, whereas the PIM inhibitor (SGI-1776) showed a cell type-independent effect: in patients whose euESC were affected, ecESC were also affected (see Supplemental Figure S1B and C). Overall, although the overexpression of certain pro-survival protein kinases in cancer cells can lead to the degeneration of other anti-apoptotic pathways and the establishment of the so-called oncogene addiction (*Ruzzene and Pinna, 2010; Sharma and Settleman, 2007*), this does not seem to be the case for ectopic endometriotic cells.

Surprisingly, CK2 inhibitor ARC-1859, despite featuring a structural design highly similar to that of ARC-775, did not reduce cell viability. Whereas in biochemical assays with recombinant CK2, the affinity of the unmasked counterpart of ARC-775 was indeed higher than that of the unmasked counterpart of ARC-1859 (*Rahnel et al., 2017; Viht et al., 2015*), this is hardly likely to be the only reason underlying the lack of potency of ARC-1859 in assays with ESC. Instead, it is likely that a more hydrophobic ATP-site targeting a fragment of ARC-1859 (the tetrabromobenzimidazole moiety) contributes to the accumulation of inhibitor in membranes, where it is not accessible by either esterases or the cytosolic CK2.

The effect of some compounds included in the panel in this study has previously been explored in the context of endometriosis. The generic protein kinase inhibitor staurosporine has been reported to demonstrate a greater apoptotic effect in euESC from patients without endometriosis than in ecESC from patients with endometriosis (*Watanabe et al., 2009*). In the current study, the sensitivity of eutopic versus ectopic cells to staurosporine depended on its concentration: whereas 5 µmol/l staurosporine caused greater cellular death in ecESC, 0.2 µmol/l staurosporine was more effective in euESC (TABLE 3). The proteasome-targeting compound bortezomib had been shown to reduce the size of endometriotic implants in rats (*Celik et al., 2008*), yet no studies of bortezomib in euESC from women with endometriosis have been reported; in this study study, treatment with bortezomib was significantly more efficient in euESC than in ecESC even after prolonged incubation ($P \leq 0.001$).

The ROCK-targeting inhibitors Y-27632 and HA-1077 have been used to reduce the contractility of ecESC; whereas Y-27632 had demonstrated no cytotoxicity, 0.1–10 µmol/l HA-1077 had caused significant apoptosis of ecESC – albeit after 48 h incubation (*Yotova et al., 2011; Yuge et al., 2007*). In the current study, no reduction in viability was observed even after prolonged incubation of euESC and ecESC with either Y-27632 or HA-1077. In principle, it is possible that the effect of ROCK-targeting inhibitors is only evident in cell motility assays, although it had been hoped that the altered dynamics of cytoskeleton might manifest itself as a retardation of proliferation. The latter was true for the microtubule-depolymerizing compound MMAE; this showed a characteristic concentration-independent profile of effect on cell viability connected to the mode of action of this compound, which serves as an antimitotic agent than an apoptosis inducer (*Abdollahpour-Alitappeh et al., 2017; Chen et al., 2017*).

Furthermore, 22 h treatment of cells with some of the chosen compounds (including inhibitors targeting ROCK, Aurora family kinases or PKA) caused an apparent increase in viability (see Supplemental Table S1), which was alleviated after a subsequent 24 h incubation in medium. This abnormal temporary phenomenon might be triggered by several factors. On one hand, ROCK inhibitors can interfere with the apoptotic caspase 3-ROCK signalling pathway (*Song and Gao, 2011*), and consequently increase the number of viable cells. However, a more likely explanation is that, as a response to treatment with toxins within a certain time window, cell metabolism tends to increase, which manifests itself as enhanced reduction of resazurin.

Overall, the compounds that significantly affected the viability of cells after 22 h of treatment also caused a significant amount of cellular death according to the necrosis/late apoptosis assay (as illustrated by GSK690693, ARC-775, SGI-1776, staurosporine, bortezomib and MMAE; $P \leq 0.05$). The only exception was CYC116, which did not trigger necrosis/late apoptosis yet remarkably reduced viability in euESC at the 10 µmol/l concentration. It is possible that AURORA B-targeting CYC116 acts as an antimitotic substance and hence slows the proliferation of cells rather than triggering cellular death, yet it is not as efficient or as quick as the toxin MMAE, which has a similar mode of action.

Differently from other compounds used in the panel, doxorubicin demonstrated

an enhanced effect on viability in ectopic versus eutopic cells after 22 h as well as 22 h + 24 h of incubation at the 10 µmol/l concentration in the resazurin assay (FIGURE 1), and after 48 h of incubation in the western blot assay (FIGURE 2). Several mechanisms of action have been reported for doxorubicin. It accumulates in cell nuclei, intercalating into DNA and preventing its repair by topoisomerase II (*Thorn et al., 2011*). In addition, doxorubicin can be reversibly oxidized into an unstable semiquinone metabolite, which releases ROS upon spontaneous re-formation of doxorubicin (*Finn et al., 2011*); the liberated ROS attack cellular components, triggering cellular death. In the context of altered redox equilibria in ectopic versus eutopic endometrial cells (*Kasvandik et al., 2016; Scutiero et al., 2017*), enhanced efficiency of doxorubicin in ecESC might be explained by its redox properties.

In this way, although doxorubicin has been used in the treatment of endometrial cancer (*Byron et al., 2012; Chitcholtan et al., 2012*), this compound might also be of remarkable interest for endometriosis studies. Unfortunately, the application of anthracyclines in chemotherapy has revealed high cardiotoxicity for this class of compounds, which complicates their use in model organisms. However, several pharmacokinetic and pharmacodynamic strategies have been actively suggested to prevent anthracycline-induced cardiotoxicity (*Menna and Salvatorelli, 2017*). Furthermore, specifically in the context of doxorubicin, the development of novel derivatives with reduced side effects (*Shaul et al., 2013*) and methods for targeted delivery (*Tran et al., 2017*) have been intensely pursued.

The large-scale transcriptome analysis revealed sets of genes that featured significantly higher expression in eutopic relative to ectopic ESC or in ectopic relative to eutopic ESC, irrespective of the treatment conditions ($P_{adj} < 0.05$, log_2FC of –4 or less or ≥+4; TABLE 4). It was postulated that these sets might reflect variations in survival strategies in eutopic and ectopic endometrium, because it is likely that, after 24 h of treatment of cells with 2 µmol/l doxorubicin, the isolated mRNA profile was characteristic of the population of survivors.

Interestingly, the comparison of treated versus control cells yielded in excess of 10 times more significantly differentially expressed genes in the case of euESC than ecESC ($P_{adj} < 0.05$). Given the fact that the majority of candidate genes in the comparison of control versus doxorubicin-treated ecESC were eliminated on the basis of the P_{adj} cut-off, this difference originates primarily from the large interpatient variation of gene expression in the ecESC group. The latter can in turn be explained by the characteristic heterogeneity of lesions, especially taking into consideration differences in location of the lesions in the three patients whose samples were used for mRNA sequencing (see TABLE 2).

In euESC, among other genes, this set included genes encoding several members of the matrix metalloproteinase (*MMP*) family, and a precursor for the endogenous opioid peptides, preproenkephalin (*PENK*). Another gene with a significantly higher expression in both control and doxorubicin-treated euESC versus ecESC encodes a growth factor, pleiotrophin (*PTN*; $P_{adj} < 0.05$, $log_2FC > +4$); interestingly, doxorubicin treatment further elevated *PTN* expression in drug-treated eutopic but not ectopic cells. Importantly, *MMP*, *PENK* and *PTN* have previously been linked to endometriosis, showing significantly higher expression in eutopic endometrium from women with endometriosis relative to healthy controls, or lower expression in ectopic than eutopic tissue (*Burney et al., 2007; Chung et al., 2002; Kobayashi et al., 2012*), thus pointing to their possible role in initiating peritoneal invasion. Furthermore, PTN has been reported to promote chemoresistance to doxorubicin in several cancers, including osteosarcoma and breast cancer (*Huang et al., 2018; Wu et al., 2017*). Therefore, it can be suggested that the lower expression of *PTN* in untreated ectopic cells is one of the factors responsible for the higher chemosensitivity of this cell type to doxorubicin – although it should be considered that the viability of euESC was still significantly affected by doxorubicin treatment ($P \leq 0.001$; TABLE 3).

A similar effect on cell viability, may be mediated by *HSPA2*, which was, according to sequencing data, more highly expressed in eutopic than ectopic cells. Heat shock-related 70 kDa protein 2 (HSPA2) protects cells from the cytotoxic and growth-inhibiting effects of doxorubicin by several mechanisms, including binding misfolded or damaged proteins and enabling these proteins to acquire proper folding, and controlling the duration of cell cycle arrest (*Karlseder et al., 1996*). According to the qRT-PCR data, the drug treatment enhanced the expression of *HSPA2* in ecESC (average fold change 4.5), suggesting a response to the toxic effect; however, as the initial expression of *HSPA2* in untreated cells was much lower in ectopic then eutopic cells (average fold change –11.8), the expression was still less than that of the eutopic cells.

In ecESC, the set of interest defined by the large-scale transcriptome analysis and qRT-PCR data included genes tightly connected with immune system functioning: these genes encoding interleukin-33 (*IL33*), cyclooxygenase 2 (*PTGS2*) and genes whose expression is regulated by cytokines – pentraxin 3 (*PTX3*) and endothelial cell-specific molecule 1 (*ESM1*). The proteins encoded by all of the aforementioned genes have been reported to be connected with endometriosis (*Cobellis et al., 2004; Fagotti et al., 2004; Kobayashi et al., 2012; Miller et al., 2017; Pelch et al., 2010*), showing a correlation with endometriosis-associated inflammation and angiogenesis; inhibitors of PTGS2 have also been explored in the context of management of endometriosis-related pain (*Cobellis et al., 2004*). Furthermore, IL33 and PTGS2 have been shown to protect cells against doxorubicin-induced apoptosis, albeit in the context of tissues other than endometrium (*Puhlmann et al., 2005; Singh et al., 2008; Yao et al., 2017*). The latter observation indirectly confirms the hypothesis that the mRNA profile identified for doxorubicin-treated euESC and ecESC reflects the corresponding cellular survival strategies. The fact that the viability of ecESC was severely affected by doxorubicin treatment indicates that the major chemoresistance-ensuring players that contribute to the survival of ectopic cells under DNA damage and ROS-triggered conditions of stress might be less efficient than those in eutopic tissue.

The mRNA sequencing results thus underline the interplay of factors that contributing to development and sustainment of endometriosis, and necessitate the application of more complex models, for example enabling the presence of epithelial cells and/or involving immune system components.

Overall, the results of this study seem to have pinpointed a set of clues for future research into endometriosis, both from the aspect of showing a resistance of endometriotic lesions to possible therapeutic candidates, and in terms of providing candidate biomarkers and targets for the succeeding exploration.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.rbmo.2019.05.019.

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DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS

1. **Heidi-Ingrid Maaroos**. The natural course of gastric ulcer in connection with chronic gastritis and *Helicobacter pylori*. Tartu, 1991.
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3. **Eero Vasar**. Role of cholecystokinin receptors in the regulation of behaviour and in the action of haloperidol and diazepam. Tartu, 1992.
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