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TRIIN LAISK-PODAR

Genetic variation as a modulator of susceptibility to female infertility and a source for potential biomarkers

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To my grandparents,

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II. **Laisk T**, Haller-Kikkatalo K, Laanpere M, Jakovlev U, Peters M, Karro H, Salumets A. Androgen receptor epigenetic variations influence early follicular phase gonadotropin levels. Acta Obstetricia et Gynecologica Scandinavica 2010; 89:1557–1563

III. **Laisk T**, Peters M, Saare M, Haller-Kikkatalo K, Karro H, Salumets A. Association of CCR5, TLR2, TLR4 and MBL genetic variations with genital tract infections and tubal factor infertility. Journal of Reproductive Immunology 2010; 87:74–81

IV. **Laisk T**, Peters M, Salumets A. Mannose-binding lectin genotypes: potential role in tubal damage and adverse IVF outcome. Journal of Reproductive Immunology 2011; 92:62–67

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Paper II: Participation in the study design, performing the experiments and data analysis, writing the paper

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Paper V: Participation in the study design, performing the experiments and data analysis, writing the paper

ABBREVIATIONS

AMH	anti-Müllerian hormone
AR	androgen receptor (gene *AR*)
ART	assisted reproductive technologies
ASRM	American Society for Reproductive Medicine
AUC	area under the curve
BMI	body mass index
CAG-BM	*AR* CAG biallelic mean
CAG-WBM	*AR* CAG weighted biallelic mean
CCL5	chemokine (C-C motif) ligand 5
CCR5	C-C chemokine receptor type 5 (gene *CCR5*)
COS	controlled ovarian stimulation
CT	*Chlamydia trachomatis*
DHEAS	dehydroepiandrosterone sulfate
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ESHRE	European Society of Human Reproduction and Embryology
FET	frozen embryo transfer
FNDC4	fibronectin type III domain containing 4 (gene *FNDC4*)
FSH	follicle stimulating hormone
FSHB	FSH β-subunit gene
FSHR	follicle stimulating hormone receptor (gene *FSHR*)
GnRH	gonadotropin releasing hormone
GSPT1	G1 to S phase transition 1 (gene *GSPT1*)
GWAS	genome-wide association study
hCG	human chorionic gonadotropin
HLA	human leukocyte antigen
HOMA-R	homeostasis model assessment index ratio for insulin resistance
ICSI	intracytoplasmic sperm injection
IGF	insulin-like growth factor
IL	interleukin
INS	insulin gene
IU	international unit
IUI	intra-uterine insemination
IVF	in vitro fertilisation
KPNA3	karyopherin alpha 3 (importin alpha 4) (gene *KPNA3*)
L-CAG	longer *AR* CAG allele

LD	linkage disequilibrium
LH	luteinizing hormone
LHR	luteinizing hormone receptor
MBL	mannose binding lectin
MBL2	MBL gene
METAP1D	methionyl aminopeptidase type 1D (mitochondrial) (gene *METAP1D*)
NLRP11	NLR family, pyrin domain containing 11 (gene *NLRP11*)
OHSS	ovarian hyperstimulation syndrome
OPU	ovarian puncture
PAMP	pathogen-associated molecular pattern
PCOS	polycystic ovary syndrome
PCR	polymerase chain reaction
PID	pelvic inflammatory disease
POI	premature ovarian insufficiency
PRR	pattern recognition receptor
RFLP	restriction fragment length polymorphism
rFSH	recombinant FSH
RHBDL2	rhomboid, veinlet-like 2 (Drosophila) (gene *RHBDL2*)
ROC	receiver operating characteristic
S-CAG	shorter *AR* CAG allele
SNP	single nucleotide polymorphism
STD	sexually transmitted disease
SYCP2L	synaptonemal complex protein 2-like (gene *SYCP2L*)
TDRD3	tudor domain containing 3 (gene *TDRD3*)
TFI	tubal factor infertility
TLR	Toll-like receptor
TMEM150B	transmembrane protein 150B (gene *TMEM150B*)
TNF-α	tumor necrosis factor α
VNTR	variable number of tandem repeats
WBM	weighted biallelic mean
XCI	X-chromosome inactivation

INTRODUCTION

The formation of a new human being in the female body begins with the production of oocytes and ends with the miraculous event of birth. It is a delicately balanced process that requires the immaculate cooperation of different parts of the female reproductive tract – the ovaries, the fallopian tubes and the uterus. Unfortunately, 10% of all couples tackle with various infertility issues and female infertility is the cause in approximately 40% of cases, meaning that in these women, this fine-tuned balance and cooperation has been disturbed. Female infertility can be caused by impaired tubal function, ovulatory disorders, impaired endometrial function or endometriosis, while male infertility is mainly associated with abnormal semen quality. A study conducted among infertility treatment clinic patients in 2004–2005 allows to assume that in Estonia, the leading cause of female infertility is tubal factor infertility (TFI), followed by polycystic ovary syndrome (PCOS). This means that the main causes of female infertility in Estonia are due to disturbed ovarian and fallopian tube functions.

In vitro fertilisation (IVF) treatment, where in vitro fertilised and cultured embryos are transferred directly to the uterus in theory helps to overcome most of the abovementioned problems. Unfortunately, IVF treatment is successful in only 1/3 of the cases, as calculated per treatment cycle. The outcome greatly depends on the effectiveness of controlled ovarian stimulation (COS), which in turn is highly dependent on how the ovaries respond to exogenous stimulation with follicle stimulating hormone (FSH).

Over the years, great effort has been directed towards elucidating the molecular mechanisms behind the conditions causing female and male infertility and it has been concluded that individual genetic variation is definitely one of the factors that contributes to reduced fertility. Indeed, multiple links between genetic polymorphisms and infertility have been established. Individual genetic variation is also a good candidate for predicting natural reproductive function and perhaps even IVF treatment success, and recently a lot of research has focused on finding such genetic markers. Owing to these studies, we know now significantly more about the genetic background that affects fertility in humans; however, a lot still waits to be uncovered. Hence this study was conducted to add our contribution to the ever-growing pool of knowledge on (in)fertility in humans.

The general objective of the current thesis was to assess the importance of various genetic factors in the etiology of female infertility and especially those conditions affecting the function of the ovaries and the fallopian tubes, more specifically PCOS and TFI. In addition, several genetic variants that could be related to ovarian function, COS and IVF treatment parameters were evaluated.

1.1. REVIEW OF LITERATURE

1.1. Human reproduction: the role of the female

Human reproduction is a delicately balanced process that, in addition to interindividual compatibility and collaboration, requires the immaculate cooperation of different parts of the female reproductive tract – the ovaries, the fallopian tubes and the uterus that all have a specific role in the process of procreation (Figure 1). The ovaries are responsible for oocyte maturation and ovulation, but also participate in the production of steroid and peptide hormones. The fallopian tubes connect the ovaries with the uterus and thus facilitate the transport of the sperm and the capture and transport of the oocyte. Since the ampullar region of the fallopian tubes is the preferred site of actual conception shortly after ovulation, the fallopian tubes are also responsible for providing an appropriate environment for early embryo development and transporting the developing embryo into the uterus. The developing embryo that has reached the uterus some four days after fertilisation embeds itself in the endometrium that lines the uterus and thus it is the uterus that has to provide the perfect place for embryo implantation and support the embryo and the placenta throughout the remaining pregnancy (Roy and Matzuk, 2011).

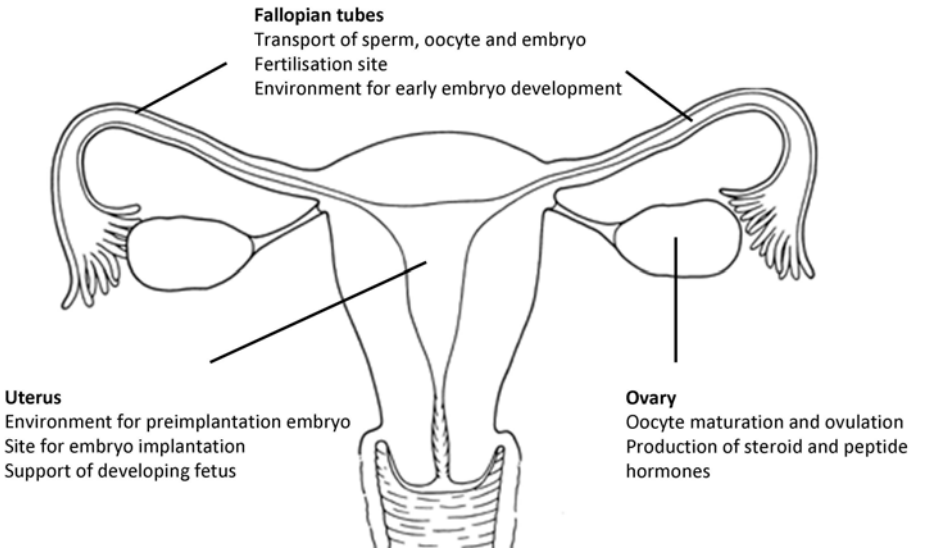

Figure 1. Functions of the female upper reproductive tract. Adapted from Roy and Matzuk (2011).

1.1.1. The ovary, folliculogenesis and the ovarian reserve

The human ovaries are small (3–5 cm in length) paired organs in the abdominal cavity. The ovaries are covered by tunica albuginea, a capsule made of connective tissue, which in turn is covered by a single layer of simple squamous epithelium – the germinal epithelium. The ovarian tissue itself can be divided into two distinct regions – the ovarian cortex that contains the ovarian follicles at various developmental stages and stroma, and the medulla that is the highly vascular innermost zone that usually does not contain developing follicles. The main functions of the human ovary are the production and release of oocytes and the secretion of hormones and other factors necessary for follicular maturation and other stages of the reproductive cycle (such as endometrial receptivity and embryo implantation).

According to the currently accepted concept, the human ovary contains a finite number of follicles established before birth (the ovarian reserve) that steadily decline with age and are virtually depleted by the time of the menopause (Wallace and Kelsey, 2010). While the number of primordial follicles is approximately 1–2 million at birth, only ~450 oocytes are ovulated and the rest (99%) go through atresia and degenerate, leaving some 1000 primordial follicles left by the time of the menopause (reviewed by Broekmans et al., 2009). In addition to the decrease in follicle quantity, the quality of the follicles and oocytes also decreases with age, reflected by the structural damage and increasing frequency of aneuploidies (Broekmans et al., 2009). The process of ovarian follicle maturation, also known as folliculogenesis, is the maturation of the ovarian follicle, which contains a developing oocyte. The process is controlled by endocrine, autocrine, paracrine and juxtacrine factors [inhibins, activins, insulin-like growth factors (IGFs), androgens, estrogens], but the main controllers are the two gonadotropic pituitary hormones – the follicle stimulating hormone (FSH) and the luteinizing hormone (LH), which act through corresponding receptors (FSHR and LHR, respectively). Although the initial recruitment of primordial follicles is mainly controlled by factors of ovarian-origin [IGF-1, epidermal growth factor (EGF), anti-Müllerian hormone (AMH), etc.], the later stages are entirely dependent on the action of FSH and LH (reviewed by Messinis et al., 2010). The gonadotropin-releasing hormone (GnRH) regulates the release of both FSH and LH from the anterior pituitary. In the first half of the menstrual cycle (known as the follicular phase in the ovary and the proliferative phase in the endometrium), FSH stimulates follicle growth, recruitment and selection of the dominant follicle. FSH also stimulates the production of steroid hormones from granulosa cells (cells surrounding the ovum), while LH stimulates the production of androgens from theca cells (cells surrounding the granulosa cells), which are in turn transferred to granulosa cells and aromatised into estrogens under the influence of FSH (reviewed by Messinis et al., 2010). Estrogens consecutively provide negative feedback to the hypothalamus and pituitary to inhibit FSH and LH secretion. FSH levels are the highest in the beginning of the follicular phase, but then begin to drop as a

response to the inhibin B produced by small antral follicles. The follicle with an optimal level of FSH in the follicular fluid and an optimal number of FSHRs in the granulosa cells is "rescued", while others become atretic (Messinis et al., 2010). It is believed that serum FSH measured at the beginning of the follicular phase (most commonly on menstrual cycle day 3) may give some information on how well the hypothalamic-pituitary-ovarian axis is functioning (Barnhart and Osheroff, 1998). With age, the number of follicles recruited in each cycle decreases and consequently FSH secretion is not suppressed as efficiently, which leads to increased FSH levels. Hence elevated early follicular phase FSH levels represent a smaller ovarian reserve (Practice Committee of the American Society for Reproductive Medicine, 2015).

In the second half of the follicular phase, LHRs appear on the granulosa cells and the follicle becomes dependent on LH. Approximately in the middle of the menstrual cycle, the LH levels rise sharply and trigger ovulation. After ovulation, during the luteal phase, LH stimulates the formation of the corpus luteum that produces estrogen and progesterone.

Under hormonal orchestration, the maturing follicles go through distinct stages during folliculogenesis – primordial, primary, secondary, early/late tertiary (also known as antral follicles) and finally preovulatory (Figure 2). The number of antral follicles (measuring <10 mm in size) correlates best with the size of the remaining follicular pool (Practice Committee of the American Society for Reproductive Medicine, 2015). Folliculogenesis comes to an end when the limited number of follicles – the ovarian reserve – is virtually depleted. The process itself is continuous, meaning that at all times the ovary contains follicles in all developmental stages. A cycle ends when a mature oocyte is released during ovulation.

The estrogens (estradiol and estrogen) secreted by the ovarian follicles during the follicular phase (corresponds to the proliferative phase of the endometrium) also stimulate the endometrial cells to proliferate, while in the second half of the menstrual cycle – the luteal phase (corresponds to the endometrial secretory phase) – the corpus luteum in the ovary produces progesterone that is responsible for converting the endometrium to its receptive state.

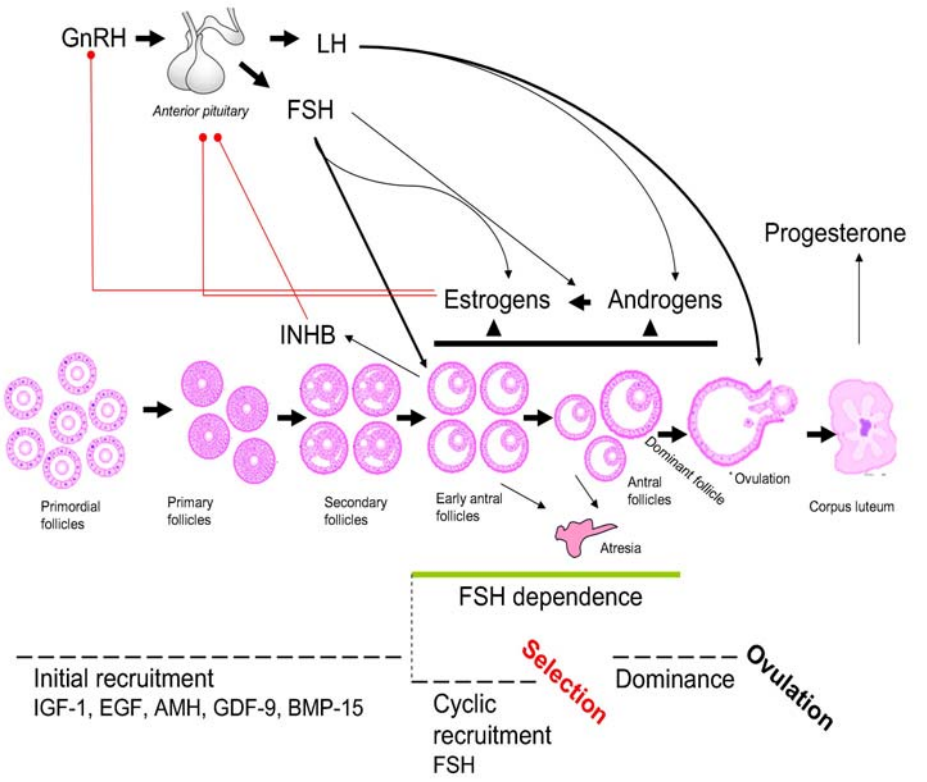

Figure 2. Schematic overview of folliculogenesis in humans. Figure created using data from a review by Messinis and colleagues (Messinis et al., 2010).

1.1.2. Fallopian tubes

From a histological viewpoint, the fallopian tubes consist of four layers: serosa, smooth muscle layer, lamina propria and a mucosal layer that is arranged in complex folds. The muscle layer is responsible for the peristaltic movements of the tubes. The fallopian tubes are lined with ciliated epithelium that consists of ciliated cells and peg cells that produce tubal secretions. Similarly to the endometrium that lines the uterine cavity, the cells of the tubal epithelium also express receptors for estradiol and progesterone and the expression of these receptors is dependent upon the menstrual cycle phase (Shah et al., 1999) that modulates the number and functioning of epithelial cells.

As mentioned above, one of the main functions of the fallopian tubes is tubal transport, which is influenced by tubal contractions, ciliary beating and tubal secretion flow that need to work in a well-coordinated manner to capture the ovulated oocyte, direct the sperm to the oocyte and finally transport the embryo into the uterus, where the developing embryo (hopefully) implants (reviewed by

Lyons et al., 2006). There is also evidence that the fallopian tubes facilitate sperm storage to create a sperm reservoir and thus increase the likelihood of conception. It has been shown that human sperm bind by their heads to the tubal epithelial cells and preserve their viability (Lyons et al., 2006). The tubal secretions produced by peg cells are known to promote sperm capacitation, modify the affinity of human sperm for the oocyte (Munuce et al., 2009) and in addition provide nutrients and a favourable environment for the developing embryo (Buhi et al., 2000). A growing body of evidence suggests that tubal transport is not a passive process, but rather an active process that involves complicated interactions and can modulate fertilisation, embryo growth and differentiation. Considering this information, it is difficult to underestimate the importance of fallopian tubes in natural human conception.

1.1.3. Endometrium

The endometrium is the inner lining of the uterus that provides the optimal environment for embryo implantation once the developing embryo has reached the uterine cavity with the help from the fallopian tubes. The endometrium can roughly be divided into two regions: the basalis adjacent to the myometrium (the muscle layer of the uterus), and the functionalis that is situated between the endometrial surface epithelium and the basalis. The surface epithelium is formed from a single layer of columnar epithelial cells that rest on the endometrial stroma (connective tissue) containing endometrial glands and a rich supply of blood vessels. Throughout the menstrual cycle, the endometrium (especially the functionalis that is highly sensitive to hormonal fluctuations) undergoes the cycle of proliferation, secretion and degeneration as orchestrated by estrogen and progesterone produced by the ovaries. As already mentioned, in the proliferative phase estrogen stimulates cellular proliferation, whereas in the secretory phase progesterone promotes cellular differentiation, the endometrial glands reach their peak secretory activity and the endometrium becomes receptive to the implanting embryo. However, if implantation does not occur, the progesterone levels decrease and the endometrial lining is shed during menstruation.

Stemming from tissue characteristics, the main function of the endometrium (or decidua during pregnancy) is to be receptive at the “right time”, have a successful dialogue with the implanting embryo (Altmäe et al., 2012) and provide a favouring environment and support throughout the pregnancy.

As with all intricate mechanisms, a slightest disturbance can affect the functioning of the entire system and so is the case with the female reproductive system. In many women either the individual parts of the reproductive tract are dysfunctional or the cooperation between different parts is for some reason disrupted, resulting in a serious medical, emotional and socioeconomical problem – infertility.

1.2. Infertility

Infertility is defined as the failure to achieve a successful pregnancy after one year or more of appropriate, timed unprotected intercourse (Practice Committee of American Society for Reproductive Medicine, 2013). Given that one in six couples is faced with some sort of infertility problems and the current prevalence of infertility among reproductive-aged women is approximately 10% [European Society of Human Reproduction and Embryology (ESHRE) 2014 fact sheet, http://www.eshre.eu/Guidelines-and-Legal/ART-fact-sheet.aspx], infertility can be considered a serious and widespread medical and socioeconomical problem. According to the "Women's Health in Estonia" study conducted in 2004 (Part et al., 2007), 18% of women aged 18–44 reported having experienced periods of involuntary infertility (defined as difficulties in conceiving during one year of regular sexual intercourse). Although the epidemiology of infertility in Estonia is poorly studied, it is estimated that approximately 15–20,000 couples are infertile, which is especially worrisome, considering the current demographic trends (aging population and emigration).

According to the ESHRE 2014 fact sheet, physiological causes in men and women both account for about one-third of infertility cases, whereas a problem in both partners is to be blamed in 25–40% of cases, and in 10–20% of infertility cases the cause remains unclear. Male infertility can mostly be attributed to suboptimal semen quality as seen on semen analysis (Cooper et al., 2010), while the causes for female infertility are more diverse and more difficult to diagnose. The major causes of female infertility are ovulatory disorders [premature ovarian insufficiency (POI), PCOS, age-related infertility], tubo-peritoneal infertility (TFI, endometriosis), and uterine abnormalities (anatomical malformations, fibroids). Unexplained infertility is diagnosed when current standard infertility evaluation methods (Cooper et al., 2010; Practice Committee of American Society for Reproductive Medicine, 2012) in both partners yield normal results. There are also regional differences in the causes of infertility worldwide. While damaged fallopian tubes due to an infection is the leading cause of infertility in developing countries (Audu et al., 2009), infertility caused by advanced maternal age and decreased ovarian function is an increasing problem in the western countries (Schmidt et al., 2012). Although the exact distribution of different causes of infertility in Estonia is unknown, a study conducted among infertility treatment clinic (the Nova Vita clinic in Viimsi, Estonia) patients in 2004–2005 allows assuming that in Estonia, the leading cause of female infertility is TFI, followed by PCOS (Haller et al., 2006).

1.2.1. Genetic component of human fertility and infertility

The female reproductive lifespan is delineated by two events – menarche, the onset of first menstruation and attainment of reproductive capacity, and the menopause, the cessation of menstruations and the end of the normal

reproductive function. Both menarche and menopause are directly linked with the ovarian function and folliculogenesis. Although several environmental and lifestyle factors (nutrition, smoking) are known to modify the onset of both events, a considerable genetic background has also been identified (Stolk et al., 2012; Carty et al., 2013; Fernandez-Rhodes et al., 2013; Perry et al., 2013; Perry et al., 2014), meaning that the female reproductive lifespan and ovarian-part of the fertility are to some extent determined by genetic factors. Furthermore, some candidate genes related to the hypothalamic-pituitary ovarian axis and folliculogenesis (*FSHR*, *FST*, *INHBA*, *CGB/LHB* gene cluster) have been directly associated with female fertility (measured by the number of children) (Kuningas et al., 2011). (Epi)genetically determined molecular processes are also known to influence functioning of the fallopian tubes (reviewed by Shaw et al., 2010) and the endometrium (Garrido-Gomez et al., 2013). The entire spectrum of genetic factors affecting the functioning of different parts of the female reproductive tract is currently unknown.

Infertility as a condition is multifactorial in nature in the majority of cases, and thus multiple factors (lifestyle, environmental, physiological and genetic) can contribute to the condition. Over the years, great effort has been directed towards elucidating the molecular mechanisms behind the conditions causing female and male infertility and it has been concluded that, similarly to many other diseases, individual genetic variation is definitely one of the factors behind reduced fertility. While a few monogenic defects (for example in *CFTR, PTPN11, SOX9)* are known that lead to infertility (reviewed by Shah et al., 2003), majority of infertility cases have a polygenic background and genetic variation (such as single nucleotide polymorphisms (SNPs) or tandem repeat sequences) in several genes contributes to the overall infertile phenotype. Diseases associated with infertility are believed to have a considerable heritability (Treloar et al., 1999; Vink et al., 2006), and as a result of extensive research, some genetic susceptibility factors have been discovered for most infertility-causing conditions, such as male infertility (Kosova et al., 2012), endometriosis (Rahmioglu et al., 2014), POI (Perry et al., 2013), PCOS (Goodarzi, 2007; Shi et al., 2012; Lee et al., 2015) and TFI (Morre et al., 2009b). The genetic component of the latter two conditions will be discussed in more detail in chapters 1.2.3. and 1.2.5. The genetic background of infertility-causing conditions has also been studied among Estonians, both in women (Altmäe et al., 2010; Lamp et al., 2010; Saare et al., 2010; Lamp et al., 2011; Lamp et al., 2012) and in men (Saare et al., 2008; Lend et al., 2010; Peters et al., 2010). Owing to the above-mentioned studies, we know now significantly more about the genetic background that affects fertility in humans; however, a lot still waits to be uncovered.

1.2.2. Tubal factor infertility

TFI is characterised by partial or complete blockage of the fallopian tubes that interferes with the natural functions of the fallopian tubes – gamete transportation and fertilisation – and leads to decreased fertility. Difficulties in gamete transport may be attributed to scar tissue and adhesions in the region, or to functional damage to the ciliated cells in the fallopian tube epithelium. TFI accounts for approximately a third of all reported infertility cases (Miller et al., 1999); however, in developing countries the prevalence of TFI can be as high as 70% (Audu et al., 2009). The exact prevalence of TFI in Estonia is unknown, but according to the Estonian IVF Effectiveness and Cost report (Tonsiver et al., 2013), TFI (diagnose code N97.1) is the main indication (46% of all National Health Insurance Fund covered treatment bills) for IVF treatment in Estonia.

The main cause of TFI is pelvic inflammatory disease (PID), which can result in infertility in up to 60% of cases and the likelihood of developing TFI is greater after several episodes of PID (The ESHRE Capri Workshop Group, 2002). PID is commonly caused by classic sexually transmitted pathogens, such as *Neisseria gonorrhoeae* and *Chlamydia trachomatis,* but recent studies have also linked *Mycoplasma genitalium* and bacterial vaginosis-associated anaerobic microorganisms with PID (reviewed by Mitchell and Prabhu, 2013). *C. trachomatis* is the causing agent of the most common bacterial sexually transmitted disease (STD), urogenital chlamydia (Adams et al., 2004; Torrone et al., 2014). The prevalence of genital chlamydia infection in Estonia is estimated to be approximately 5%, but among young (aged 18–35) females the prevalence is 7%, which is among the highest in Europe (Redmond et al., 2015). *C. trachomatis* is responsible for approximately half of TFI cases (reviewed by Debattista et al., 2003), but the exact prevalence of chlamydia-related TFI in Estonia is unknown.

Tubal patency is usually evaluated using hysterosalpingography or laparoscopic surgery. However, it has been found that tubal blockages are strongly associated with the presence of anti-chlamydial antibodies in serum, and more specifically, anti-chlamydial IgG antibodies have been associated with TFI (Debattista et al., 2003). For this reason, serum *C. trachomatis* antibody testing is widely used to screen for tubal pathology and decide upon the need for subsequent laparoscopy during fertility work-up (den Hartog et al., 2006a). Serum *C. trachomatis* antibody testing is also commonly used in scientific research to determine the chlamydia-status in study subjects to distinguish potential chlamydia-related TFI.

In females, *C. trachomatis* targets mainly the columnar epithelium of the cervix. Symptoms of genitourinary chlamydia infection in women include dysuria and abnormal vaginal discharge. Unfortunately, most *C. trachomatis* infections are asymptomatic or present with only mild symptoms, meaning that serious complications can occur silently before a woman even recognizes a problem. If left untreated on time, the infection can ascend to the upper genital tract (endometrium and fallopian tube epithelium) and cause PID. In addition to

PID and infertility, chlamydia infection in women increases the risk for chronic pelvic pain and ectopic pregnancy (Haggerty et al., 2010), that all result from damage and scarring of the fallopian tube tissue caused by proteases, clotting factors and tissue growth factors released from infected epithelial cells and inflammatory cells (Darville and Hiltke, 2010).

While the consequences of genital chlamydia infection are thoroughly studied and recognized, the exact nature of the pathologic mechanisms leading to those consequences is unclear. Similarly to other infections, the nature of the host immune response and the interactions between the host and the pathogen determine the efficiency of infection clearance and occurrence of post-infection complications and pathological changes. It is hypothesised that the inflammatory damage caused by *C. trachomatis* can result from either too weak immune response (pathology due to chronic infection) or too strong immune response (pathology caused by hyperinflammation) (Debattista et al., 2003). Anti-chlamydial immunity in the female reproductive tract is provided by elements of both the innate and acquired immunity. While the acquired immune system, consisting of humoral (antibody-mediated) and cell-mediated (mainly T-lymphocytes) immunity, provides a more "tailored" immune response, the innate immune system responds first and provides a rapid and more general immune response. Elements of the innate host response include epithelial cells, host vaginal microbiota, antimicrobial peptides and pattern recognition receptors (PRRs), such as the Toll-like receptors (TLRs), components of the complement system and cells of the innate immune system (macrophages, neutrophils, natural killer cells) (den Hartog et al., 2006a; Horne et al., 2008; Hafner et al., 2013). Since *C. trachomatis* is an intracellular pathogen, the epithelial cells targeted by the infection initiate the host response. Infected cells secrete chemokines, such as CCL5 (Sakthivel et al., 2008), that attract inflammatory leukocytes, and cytokines (such as IL-1) that further augment cellular inflammatory response, eventually leading to tissue damage (reviewed by Darville and Hiltke, 2010). The nature of anti-chlamydial immune response is determined by many factors: pathogen strain and viral load, presence of co-infections, but also by hormones that fluctuate throughout the menstrual cycle (Hafner et al., 2013), and by individual genetic variability (Morre et al., 2009a).

1.2.3. Genetics of tubal factor infertility

The studies on the genetic susceptibility to TFI have entirely focused on chlamydia-related tubal damage and infertility. Data from twin studies allows estimating that host genetic factors contribute approximately 40% of the variation in immune response to *C. trachomatis* infection (Bailey et al., 2009). Since the susceptibility to *C. trachomatis* infection, the course and outcome of the infection depend on many factors, it is reasonable to believe that susceptibility to TFI is determined by many genes and the individual contribution of each gene and genetic variant is small. Majority of the studies have compared *C.*

trachomatis positive TFI patients with individuals with a different course (no tubal damage or TFI) of infection. Several studies using candidate gene approach have been carried out and all tested variants are located in genes that associate with host immune response and can roughly be divided into three major groups – cytokine signalling (*IL-1B*, *IL-1RN*, *IL-6*, *IL-10*, *IL-12A*, *IL-12B*, *TGFB*, *TNF-α*, *IFNG*), antigen presentation (*HLA*, *MICA*) and PRRs (*TLR2*, *TLR4*, *TLR9*, *CD14*, *CARD15/NOD2*, *MBL2*) (Cohen et al., 2000; Kinnunen et al., 2002; Cohen et al., 2003; Morre et al., 2003; Murillo et al., 2003; den Hartog et al., 2006b; Sziller et al., 2007b; Karimi et al., 2009; Mei et al., 2009; Ohman et al., 2009; Ouburg et al., 2009; Ohman et al., 2012). Only a few markers have been tested in more than one study, and thus it is difficult to draw any firm conclusions. As far as cytokine signalling and antigen presentation are concerned, an increased risk of developing TFI after genital chlamydia infection has been associated with certain HLA alleles (Kinnunen et al., 2002) and with genetic variation in *IL-10* and *TNF-α* (Ohman et al., 2009), and *IL-12B* (Ohman et al., 2012). Findings related to PRRs are discussed in the following paragraphs.

1.2.3.1. Toll-like receptors

TLRs represent the most studied PRRs in the context of TFI. TLRs are expressed on macrophages, dendritic and epithelial cells, and recognize specific pathogen-associated molecular patterns (also known as PAMPs), such as bacterial cell surface components. Activation of TLRs consecutively results in recruitment of phagocytic cells and the production of pro-inflammatory cytokines and chemokines. Genetic variation in the form of SNPs has been described in several TLRs, but the most studied are the two cosegregating SNPs Asp299Gly (rs4986790) and Thr399Ile (rs4986791) in *TLR4* (9q33.1), and Arg753Gln (rs5743708) in *TLR2* (4q32), which affect PAMP-receptor binding and have been associated with susceptibility to several infections (reviewed by Schroder and Schumann, 2005). It has been shown that TLR2, TLR4 and TLR9 are among the main TLRs that are involved in the immune response toward *C. trachomatis* (reviewed by Joyee and Yang, 2008). Thus, SNPs in these three TLRs have been studied in relation to *C. trachomatis* infection and tubal damage (Morre et al., 2003; den Hartog et al., 2006b; Karimi et al., 2009; Ouburg et al., 2009). Apart from a protective effect regarding development of tubal pathology reported for one specific haplotype in *TLR2* (Karimi et al., 2009), no significant associations have been found.

1.2.3.2. Mannose-binding lectin

Mannose binding lectin (MBL) is a secreted PRR that binds a wide variety of pathogens and is an important part of the complement system, thus providing the first-line defence against invading pathogens, including *C. trachomatis*

(Swanson et al., 1998). In addition to mediating complement system activation, MBL also modulates inflammation and apoptotic cell clearance (Dommett et al., 2006). MBL is encoded by the *MBL2* gene (10q11.2), and more than 400 SNPs have been described in the gene region. Most studied are the six common polymorphisms −550 H/L (rs11003125) and −221 X/Y (rs7096206) in the promoter region, +4 P/Q (rs7095891) in the non-translated region, and three polymorphisms located in exon 1 at codon 52 A/D (rs5030737), codon 54 A/B (rs1800450) and codon 57 A/C (rs1800451) (Madsen et al., 1995; Madsen et al., 1998). However, the latter is very rare in European populations (Dommett et al., 2006). These six SNPs also have an effect on serum MBL levels (Lipscombe et al., 1992; Madsen et al., 1994; Naito et al., 1999). Given the important role of MBL in immune system regulation, genetically determined variation in MBL concentration has been associated with the susceptibility and course of different infections (reviewed by Eisen and Minchinton, 2003) and fertility-related conditions (van de Geijn et al., 2007a; Christiansen et al., 2009). Genetic variation in MBL, particularly codon 54 A/B (rs1800450), has been associated with an increased risk of developing TFI after genital chlamydia infection (Sziller et al., 2007b). The importance of other *MBL2* SNPs in TFI has not been studied.

1.2.3.3. Chemokine receptor CCR5

Chemokine receptor CCR5 is generally not considered as a PRR, but it is a transmembrane receptor expressed on several cells of the innate and adaptive immune system, such as monocytes, dendritic cells, T helper 1 cells and macrophages. CCR5 binds proinflammatory chemokines (i.e. CCL5, MIP-1α and MIP-1β) produced by activated immune cells and thus the primary role of CCR5 is to facilitate immune cell cross-talk. However, CCR5 can also act as a PRR (Floto et al., 2006). *C. trachomatis* infection up-regulates production of the chemokine CCL5, a ligand for CCR5 in epithelial cells (Maxion and Kelly, 2002) and T cells expressing CCR5 dominate in inflammatory disease and mucosal surfaces (Sallusto et al., 1998). The CCR5 is encoded by the *CCR5* gene (3p21.31), and the gene region includes over 400 SNPs. In addition, a functional 32-bp deletion has been described in *CCR5* (*CCR5*delta32), which results in premature termination of the protein (Mueller and Strange, 2004). As a consequence, individuals homozygous for this deletion do not express CCR5 on the cell surface, while heterozygous individuals have a reduced number of CCR5 receptors (Liu et al., 1996). The *CCR5*delta32 is mainly known for its ability to hinder HIV-1 transmission (Paxton and Kang, 1998). So far, only two studies have investigated the role of *CCR5*delta32 in TFI, and while Barr and colleagues (Barr et al., 2005) reported a protective effect regarding development of tubal pathology for *CCR5*delta32, no such effect was seen in a later study (Mania-Pramanik et al., 2011).

1.2.4. Polycystic ovary syndrome

PCOS is a common endocrine disorder among reproductive-aged women, with an estimated prevalence of approximately 10% (but it can be as high as 20%, depending on the diagnostic criteria used) (Sirmans and Pate, 2013). The exact epidemiology of PCOS in Estonia is unknown, but the prevalence is estimated to be relatively similar worldwide. The clinical presentation in patients varies considerably, but symptoms include menstrual cycle disturbances, hyperandrogenism (presenting as hirsutism, acne, and alopecia), polycystic ovarian morphology, obesity and insulin resistance. The syndrome is often accompanied by infertility, which affects approximately 40% of women with PCOS, and moreover, PCOS is the most common cause of anovulatory infertility (Teede et al., 2010).

Due to heterogeneous clinical manifestation, diagnosis of PCOS cannot be made based on a single criterion. Currently, there are three sets of proposed criteria for diagnosing PCOS. The NIH/NICHD criteria include clinical and/or biochemical hyperandrogenism and menstrual dysfunction. Diagnosis according to the so-called Rotterdam criteria is made when two of the following characteristics are present: clinical and/or biochemical hyperandrogenism, oligo- or anovulation, and polycystic ovaries (presence of eight or more follicles <10 mm in diameter and/or increased ovarian stroma) (The Rotterdam ESHRE ASRM-Sponsored PCOS Consensus Workshop Group, 2004). Finally, the criteria of the Androgen Excess Society include clinical and/or biochemical hyperandrogenism, and ovarian dysfunction and/or polycystic ovaries (Azziz et al., 2006). All three also state that other androgen excess or related disorders (thyroid disease, hyperprolactinemia, nonclassic congenital adrenal hyperplasia) have to be excluded. Currently, experts suggest using the Rotterdam criteria for diagnosing PCOS (Legro et al., 2013).

Clinical or biochemical androgen excess is a central feature in all proposed diagnostic criteria, underlining its important role in the disease pathogenesis, although signs of androgen excess are not always present (reviewed by Goodarzi et al., 2011). The three main cornerstones of the disease – hyperandrogenism, ovarian dysfunction and hyperinsulinism resulting from insulin resistance interact in a loop system to produce the clinical picture characteristic to PCOS (Figure 3), with a variable degree of contribution from each component.

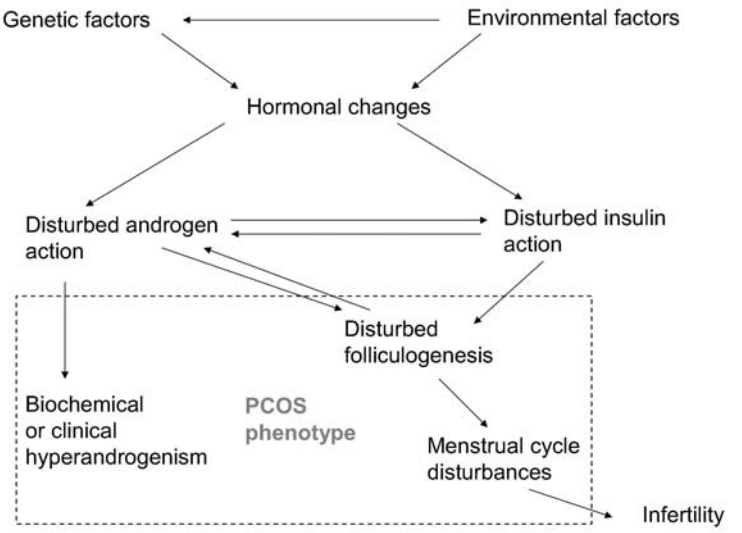

Figure 3. Schematic overview of PCOS pathophysiology. (Adapted from Diamanti-Kandarakis, 2008).

Hyperinsulinism observed in PCOS women is probably caused by both increased basal insulin secretion and decreased insulin clearance (insulin resistance) (Dunaif, 1997). Furthermore, hyperandrogenism contributes to insulin resistance and hyperinsulinemia in turn augments androgen production, resulting in a vicious circle (Dunaif, 1997). The androgen excess characteristic to PCOS is mainly of ovarian origin stemming from a steroidogenic defect in theca cells that is further augmented by increased LH and insulin levels (reviewed by Diamanti-Kandarakis, 2008). The intra-ovarian hyperandrogenism most likely promotes early follicular growth, which leads to a 2–5 mm follicle excess, and together with hyperinsulinism/insulin resistance, results in disturbed follicle selection process, follicular arrest and polycystic ovarian morphology (Jonard and Dewailly, 2004). The disturbances in folliculogenesis are also to blame for the anovulation and resulting subfertility.

The exact mechanisms leading to development of PCOS are in large part unknown, but it is clear that environmental factors, especially lifestyle and weight, have a huge impact on how severely the disease is manifested (Goodarzi et al., 2011). There is also growing evidence that genetic variants and epigenetics contribute to the dysregulation behind PCOS symptoms, providing new clues about PCOS pathogenesis.

1.2.5. Genetics of polycystic ovary syndrome

There is strong evidence to suggest that susceptibility to PCOS is determined by genetic factors. For example, according to a twin-study, the heritability of PCOS can be as high as 70% (Vink et al., 2006). At the same time, it has become evident that complex interactions between genetic and environmental factors are not completely understood (reviewed by Crosignani and Nicolosi, 2001), which together with the broad spectrum of PCOS manifestation hinders the detection of genetic susceptibility loci. However, it is clear that PCOS is a complex genetic trait, meaning that many different genes contribute to the etiopathogenesis of the disease. Similar to other complex traits, majority of studies have used a candidate gene approach and more than 70 candidate genes have been tested for association with PCOS in different populations, unfortunately with inconsistent results (reviewed by Urbanek, 2007; Barber and Franks, 2010). Candidate genes have been selected based on the main characteristics of the PCOS syndrome, such as hyperandrogenism, obesity and insulin resistance, disturbed folliculogenesis, therefore the tested genetic variants have mainly been chosen from pathways related to androgen production and action (*AR*, *BMP15*, the *CYP* family, *GDF9*, *STAR*, etc.), insulin action (*INS*, *INSR*, *IGF1*, *IGF2*, *IGF1R*, *LEPR*, etc.), and gonadotropin secretion (*FSHB*, *FSHR*, *FST*, *GNRHR*, *INHA*, *LHB*, *SHBG*, etc.) (Goodarzi, 2007; Urbanek, 2007; Barber and Franks, 2010). The tandem repeats in the androgen receptor gene (*AR*) and insulin gene (*INS*) are two of the most studied genetic variants and their association with PCOS will also be discussed below.

During the last couple of years, researchers have also performed genome-wide association studies (GWAS) to use a hypotheses-free approach for finding disease associations, and have found susceptibility loci on chromosomes 2, 8, 9, 11, 12, 16, 19 and 20 (Chen et al., 2011; Shi et al., 2012; Lee et al., 2015). Genes within or near these loci include *DENND1A*, *KHDRBS3*, *FSHR*, *HMGA2*, *INSR*, *LHCGR*, *RAB5B*, *SUOX*, *SUMO1P1*, *THADA*, *TOX3*, *YAP1*. Although these loci were first identified in PCOS patients of Asian origin, later meta-analyses observed largely similar results among Europeans (Louwers et al., 2013; Brower et al., 2015), suggesting patients with different ethnic backgrounds share common pathogenic mechanisms. Whether an independent GWAS among Europeans confirms these results or unravels additional novel susceptibility loci remains to be seen in the nearest future.

1.2.5.1. Androgen receptor gene

All androgens manifest their actions through the androgen receptor (AR). The AR is a nuclear transcription factor and thus the binding of androgens to AR regulates the expression of a wide variety of genes, several of which are expressed during folliculogenesis in the developing follicle. The gene encoding the receptor (*AR*) is located at Xq11-12 and contains 8 exons (Lubahn et al.,

1988). The N-terminal transcription activation domain in encoded by exon 1, which also contains a highly polymorphic CAG repeat that results in a polyglutamine stretch on translation and is commonly referred to as the *AR* CAG microsatellite. The number of CAG repeats ranges from ~10 to ~30 in the general population, and the most common alleles among Scandinavians contain approximately 20 repeats (Lund et al., 2000). It has been shown that the length of the CAG repeat correlates inversely with the transactivation function of the AR, at least in vitro (Chamberlain et al., 1994). Thus, several authors have tried to find associations between *AR* CAG microsatellite length and various pathogenic conditions. Very long repeats (>40) have been associated with androgen insensitivity and related conditions, whereas shorter *AR* CAG tracts have been linked with increased androgen sensitivity and concomitant increased risk for prostate cancer (reviewed by Kumar et al., 2011). Since increased androgen action is also characteristic to PCOS, numerous studies have assessed the *AR* CAG microsatellite length in PCOS patients of different ethnic origin, and while some authors have found associations with shorter *AR* CAG repeats (Shah et al., 2008; Van Nieuwerburgh et al., 2008; Xita et al., 2008; Schuring et al., 2012; Xia et al., 2012), others have not (Mifsud et al., 2000; Jaaskelainen et al., 2005; Ferk et al., 2008b; Kim et al., 2008; Echiburu et al., 2012; Skrgatic et al., 2012; Rajender et al., 2013), and one study even found that longer *AR* CAG microsatellites are more prevalent among PCOS patients (Hickey et al., 2002). Recently published systematic reviews and meta-analyses conclude that the *AR* CAG microsatellite is most likely not a susceptibility factor for PCOS (Wang et al., 2012; Rajender et al., 2013; Zhang et al., 2013), but may contribute to the hyperandrogenemic aspect of the disease (Zhang et al., 2013; Peng et al., 2014).

One factor that could help to explain these inconsistent results is the phenomenon of X-chromosome inactivation (XCI). Since women have two copies of the X-chromosome, one of them has to be inactivated in all somatic cells. The XCI is random in roughly half of the women, but skewed inactivation patterns can be seen in the other half, and these women have the same X-chromosome inactivated in >60 % of somatic cells (Naumova et al., 1996). Such skewed inactivation can affect the activity of *AR* (and other X-linked genes), and ideally, the epigenetic effect of XCI should be taken into account in association studies. Indeed, some studies have proposed that alterations in XCI pattern may be associated with PCOS (Hickey et al., 2002; Hickey et al., 2006; Shah et al., 2008; Dasgupta et al., 2010; Schuring et al., 2012), but the results are inconclusive.

1.2.5.2. Insulin gene

Insulin is mostly known for mediating glucose uptake in muscle and adipose tissue; however, insulin can also serve as a para- or autocrine regulator of gene transcription, and cell growth and differentiation, including steroidogenesis in the ovaries (Franks et al., 1999). The insulin gene (*INS*, 11p15.5) includes 3

exons and there are more than 200 SNPs in the gene region. The 5' region of the *INS* gene also includes a minisatellite (VNTR – variable number of tandem repeats), which is in linkage disequilibrium (LD) with other SNPs in the area (Bennett et al., 1995). The *INS* VNTR is polymorphic due to the ranging number of a certain 14–15 bp tandem repeat and the VNTR alleles are divided to 3 classes, based on the number of tandem repeats: class I alleles (30–60 repeats), class II alleles (60–120 repeats), class III alleles (120–170 repeats); however, class II alleles are very rare among Europeans. The length of the *INS* VNTR affects insulin transcription, such that longer (class III) VNTR alleles correlate with greater transcriptional activity in vitro (Kennedy et al., 1995); however, in the pancreas, the class I (shorter) alleles are associated with greater transcriptional activity (reviewed by Pugliese and Miceli, 2002). Moreover, there is evidence the *INS* VNTR may also affect the expression of the neighbouring insulin-like growth factor II gene (*IGF2*) (Paquette et al., 1998).

Because of the central role of insulin action in PCOS, the *INS* VNTR has also been studied in the context of PCOS, but similar to *AR* CAG, with inconsistent results (Waterworth et al., 1997; Vankova et al., 2002; Ferk et al., 2008a; Xu et al., 2009; Yun et al., 2012; Skrgatic et al., 2013). Even meta-analyses conducted on this matter or studies including multiple data sets have yielded different results both in favour (Yan et al., 2014) and against (Powell et al., 2005; Song et al., 2014) an association.

1.3. Treatment of infertility

Due to the diverse nature of conditions behind infertility, a variety of treatment options are used to tackle the problem – hormonal medications, surgical treatment, intrauterine insemination, and in more difficult cases assisted reproductive technologies.

Hormonal treatment is the first choice in cases where female infertility due to ovarian dysfunction is diagnosed. Ovarian stimulation drugs (such as Clomiphene, Tamoxifen) can be prescribed to women with ovulatory problems, while some women with PCOS may benefit from treatment with drugs (Metformin) that alleviate insulin resistance (Legro et al., 2013). Surgical treatment is used in cases where removal of adhesions is indicated – such as TFI or endometriosis. Surgery is also used to remove endometriosis-associated endometrial lesions from the abdominal cavity (Practice Committee of the American Society for Reproductive Medicine, 2012) and remove ovarian cysts typical to PCOS. Quite often, hormonal treatment is used together with surgical intervention (especially in endometriosis and PCOS).

Intrauterine insemination (IUI), whereby sperm is introduced directly to the uterus using a special catheter, is a relatively non-invasive, safe and cost-effective treatment option, but unblocked fallopian tubes and normal ovulation are a prerequisite for the procedure.

However, if the aforementioned treatments fail or in more complex cases of infertility, assisted reproductive technologies (ART) are used.

1.3.1. Assisted reproductive technologies and controlled ovarian stimulation

ART include all treatments or procedures that include the in vitro handling of human oocytes and sperm, or embryos, for the purpose of establishing a pregnancy (Zegers-Hochschild et al., 2009). This also includes IVF and intracytoplasmic sperm injection (ICSI). IVF alone or in combination with ICSI is the most used procedure for infertility treatment and can help to overcome both female and male factor infertility. In 2011, nearly 600,000 ART treatment cycles were reported from 33 European countries and more than 5 million IVF babies have been born worldwide since the birth of the first IVF baby in 1978 (ESHRE 2014 ART fact sheet). According to the National Institute for Health Development health statistics, today approximately 2–3% of all babies born in Estonia are IVF babies.

IVF treatment includes three stages: a) controlled ovarian stimulation (COS), b) in vitro fertilisation of the obtained oocytes and incubation of the embryos, and c) embryo transfer into the uterus (Figure 4). Any good quality embryos not transferred can be frozen and stored for future use in frozen embryo transfer (FET).

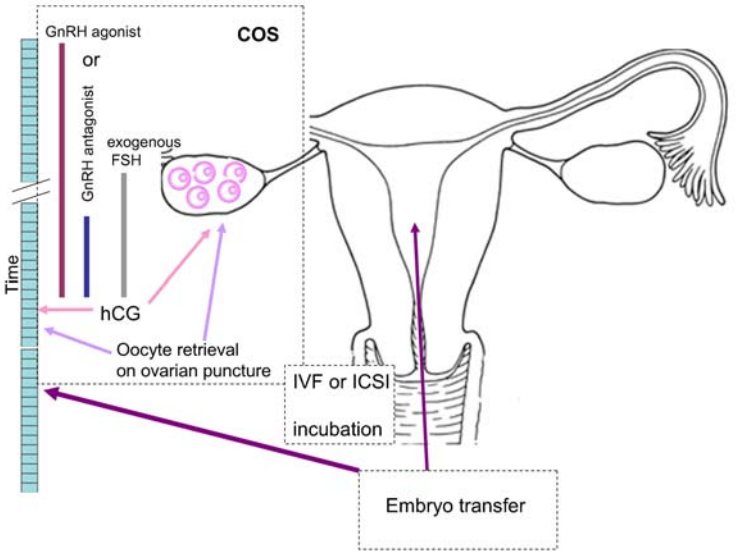

Figure 4. Schematic overview of the IVF treatment process. Exogenous FSH together with the GnRH agonist or antagonist protocol is used to stimulate the growth and maturation of multiple follicles. Oocytes are retrieved on ovarian puncture and then fertilised in vitro. After incubation, good quality embryos are transferred to the uterus.

One of the main goals of IVF treatment is to obtain multiple oocytes with good quality that could then be fertilised, making COS a key step in IVF treatment. During COS, multiple ovarian follicles are induced to grow and mature by stimulating the ovaries with exogenous FSH together with either the GnRH agonist or antagonist protocol. Final follicular maturation is achieved using exogenous human chorionic gonadotropin (hCG), which is followed by oocyte retrieval on vaginal ultrasound-guided ovarian puncture (OPU). The ovarian response to exogenous hormonal stimulation varies greatly among individuals, and unfortunately, in some women may result in suboptimal numbers of retrieved oocytes (poor responders), while in others the standard amount of hormones administered is too much and they develop a serious complication – the ovarian hyperstimulation syndrome (OHSS). In 2011, 0.6% of all treatment cycles in Europe resulted in OHSS (ESHRE ART 2014 fact sheet). Over the years, COS protocols have undergone various changes and doctors and scientists worldwide are constantly looking for ways to improve the protocols and make them safer and more effective. Today, the optimal clinical management of IVF-COS includes a) the prospective identification of the ovarian reserve, b) the individualisation of the COS treatment protocol, and c) the prevention of potential complications (Arslan et al., 2005).

The oocytes obtained during COS are then fertilised in vitro with motile spermatozoa (traditional IVF), or a single selected sperm is injected directly into the oocyte (ICSI). ICSI is the recommended choice of treatment in couples with male factor infertility or with unexplained treatment failure in previous IVF cycle, but also in certain cases of female infertility. According to the ESHRE 2014 ART fact sheet, ICSI is the most common treatment technique that accounts for two-thirds of all treatments worldwide, and both IVF and ICSI yield a pregnancy rate of ~30% per embryo transfer. The proportion of used treatment techniques (IVF or ICSI) is similar in Estonia (Tonsiver et al., 2013).

Fertilised oocytes are then incubated and routinely assessed 16-18h after insemination, and the day after to evaluate the embryo cleavage of normally fertilised oocytes. Morphology of cleavage stage embryos is one of the markers used to assess the quality of the embryos, and different scoring systems have been developed (reviewed by Machtinger and Racowsky, 2013). If the embryos are developing normally, generally 1–2 embryos are transferred to the uterus 2-3 days after fertilisation (4–8 cell stage) or 5 days after fertilisation (blastocyst stage).

Despite advancements in IVF technologies, the success rate per IVF treatment cycle remains around 30% even if embryos with a seemingly excellent quality are transferred. Currently, systemic oxidative stress (Ahelik et al., 2015) and overweight (Lapp, 2013), but also female age, duration of subfertility, basal FSH levels and the number of oocytes obtained on COS (all indicative of the ovarian function) are considered somewhat predictive of pregnancy following treatment with IVF/ICSI (van Loendersloot et al., 2010). Markers prognostic of treatment success are extensively sought for and novel methods have been proposed for

timing embryo transfer to match endometrial receptivity (Ruiz-Alonso et al., 2013) and for assessing embryo quality (Rodrigo et al., 2014). Although the importance of endometrial receptivity and embryo-endometrial cross-talk has recently been underlined, the success of the first stage of IVF treatment – the ovarian stimulation stage – remains the main modifier of treatment efficiency and reliable markers targeting this treatment stage could substantially improve the overall treatment outcome.

1.3.2. Markers of ovarian function in natural and assisted conception

Because the ovarian reserve and the ovarian function are the key limiting factors in achieving pregnancy both in natural and assisted conception, various ovarian reserve tests are used to a) predict the ovarian reserve and reproductive potential, and b) predict the ovarian response and outcome of IVF-COS (reviewed by Jirge, 2011). In short, the most used ovarian reserve tests consider woman's age, hormone levels (basal FSH, AMH, etc.) and ultrasound parameters (antral follicle count, ovarian volume and blood flow) either separately or in various combinations. As with other markers in clinical practice, an ideal marker for ovarian function should be easily measurable and inexpensive, minimally invasive and maximally informative, and most importantly, minimally affected by other factors. All currently used ovarian reserve markers have their pros and cons, but together with the personalised medicine revolution and because the ovarian function is directly related to reproductive aging, which has a considerable heritable component (as high as 90%) (de Bruin et al., 2001), a lot of research has been directed on finding the genetic predictors of reproductive aging and ovarian function. By definition, a genomic/genetic biomarker is a DNA or RNA characteristic indicative of normal biologic, or on the contrary pathogenic, processes, and/or response to therapeutic or other intervention (Novelli et al., 2008). DNA characteristics that could be considered as biomarkers include SNPs, tandem repeat sequences, DNA modifications (such as methylation), insertions, deletions and copy number variations (Novelli et al., 2008). The diagnostic value of genomic biomarkers lies in their robustness and in theory they could be used for screening, diagnosing and monitoring the activity of a disease, but also for personalising treatment regimens and assessing therapeutic responses (Novelli et al., 2008).

These markers are believed to also complement the existing methods used for monitoring the ovarian reserve and function (ultrasound, hormone measurements), mainly because genetic markers are stable, reliable, and could also provide some information about the reproductive potential of the ovarian reserve. Therefore, such markers could be of use in clinical ovarian reserve and function assessment in natural and assisted conception, where reliable genetic markers are extensively sought for in order to develop a pharmacogenetic approach for IVF-COS and thereby increase treatment effectiveness and reduce

harmful side effects (reviewed by Altmäe et al., 2011; Lledo et al., 2014). Although several markers with a clinical potential (*FMR1*, *FSHR*) have been proposed (Gleicher et al., 2009a; Gleicher et al., 2009b; Yan et al., 2013), genetic markers of ovarian reserve/IVF-COS are currently not used in clinical practice.

Until recently, the search for genetic predictors of ovarian reserve/IVF-COS focused on obvious candidates (FSHR) and certain pathways (estrogen synthesis/metabolism, AMH signalling), meaning that the overall number of tested variants was small. Advancements in genotyping technologies and the formation of large international consortiums have enabled scientists to use a genome-wide hypotheses-free approach in the search for genetic markers of reproductive aging and the ovarian reserve (He et al., 2009; Schuh-Huerta et al., 2012a; Stolk et al., 2012; Carty et al., 2013). These studies have identified several loci that show strong associations with studied traits (*UIMC1*, *SYCP2L*, *MCM8* and age at menopause; *MYADML* and FSH levels); however, these variants explain only a small proportion of the phenotypic variation, and in most part their functional significance is unclear. Unfortunately the GWAS-approach has been unsuccessful for identifying genetic predictors for IVF success or IVF-COS (van Disseldorp et al., 2011), probably because a genome-wide approach needs very large and rather homogenous study cohorts that are difficult to achieve in IVF setting and the stringent analysis criteria dismiss the variants with smaller effect sizes. However, the reproductive aging and the ovarian function most likely share some of the genetic background (Rosen et al., 2010), as also shown by a study that successfully associated menopause-related variants with ovarian reserve markers (Schuh-Huerta et al., 2012b). In this study, rs16991615 (*MCM8*) which has been associated with menopausal age in Caucasian women (He et al., 2009; Stolk et al., 2012) was associated with the number of antral follicles (Schuh-Huerta et al., 2012b). This demonstrates that the genetic variants associated with reproductive aging are good candidates for markers of ovarian function and IVF-COS.

2. AIMS OF THE STUDY

The general objective of the current study was to assess the importance of various genetic factors in the etiology of female infertility and especially in those conditions affecting the function of the ovary and the fallopian tubes. In addition, several genetic variants that could be related to the ovarian reserve, controlled ovarian stimulation and in vitro fertilisation treatment outcome parameters were evaluated.

Accordingly, the specific aims were:

1. To assess whether genetic variation in insulin and androgen receptor genes is associated with PCOS.
2. Determine the prevalence of chlamydia-induced TFI among Estonian TFI patients.
3. To analyse polymorphisms in genes modulating immune response to assess whether they affect susceptibility to TFI.
4. To evaluate if genetic variants related to ovarian aging are associated with ovarian function, COS and IVF treatment parameters.
5. To consider the suitability of these genetic variants for use as markers of ovarian function, COS and IVF treatment.

3. MATERIALS AND METHODS

All the studies included in this thesis have been approved by the Research Ethics Committee of the University of Tartu, and an informed consent has been obtained from all participants. Studies are summarised in Table 1.

3.1. Study subjects, clinical data and IVF

IVF patients

All patients undergoing IVF or ICSI at the Nova Vita Clinic (Estonia) from 2004 to 2007 were recruited in the study. All included women had been suffering from primary or secondary infertility for at least one year. The reason for infertility was diagnosed as follows:

- *PCOS*

PCOS was diagnosed based on the criteria established by the Rotterdam PCOS consensus group (The Rotterdam ESHRE ASRM-Sponsored PCOS Consensus Workshop Group, 2004): oligo- or anovulation; clinical or biochemical hyperandrogenism and/or polycystic ovaries. The presence of two out of the three aforementioned criteria was necessary for diagnosing PCOS.

- *TFI*

Either hysterosalpingography or diagnostic laparoscopy was used for diagnosing tubal occlusions. The main findings on laparoscopy were unspecified blockage and/or surgery of fallopian tubes, sacto- or hydrosalpinx, adhesions, and abscess, all characteristic to a previous episode of PID, which was therefore considered to be the main cause for TFI.

- *Endometriosis*

The presence of endometriotic lesions characteristic to the disease was confirmed by diagnostic laparoscopy, as suggested by the American Society for Reproductive Medicine (Practice Committee of the American Society for Reproductive Medicine, 2012).

- *Male factor infertility*

Male factor infertility was diagnosed when the female counterpart was lacking any obvious reason for infertility, while decreased semen quality as diagnosed by World Health Organization criteria was present in the male.

- *Other*

Other causes for infertility included endometrial hyperplasia, uterine myoma, ovulatory dysfunction, and unexplained infertility, which was considered to be present when the woman was lacking all of the abovementioned reasons for infertility and the male semen quality was normal, but the couple had nevertheless experienced infertility for more than a year.

From this dataset including 439 women, smaller subsets were selected for individual studies depending on study objectives as shown in Table 1 and on Figure 5. The schematic distribution of diagnoses for infertility is shown on Figure 6.

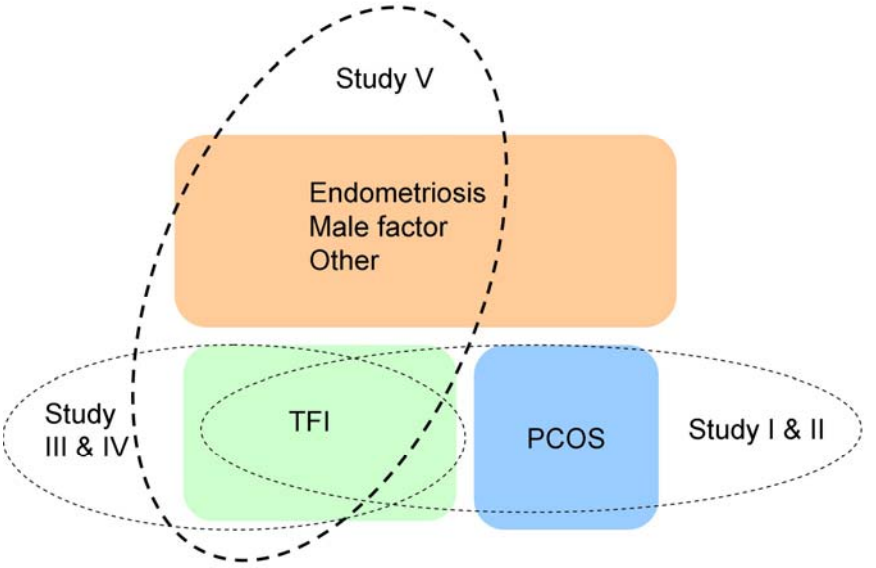

Figure 5. Schematic representation of patient overlap between different studies.

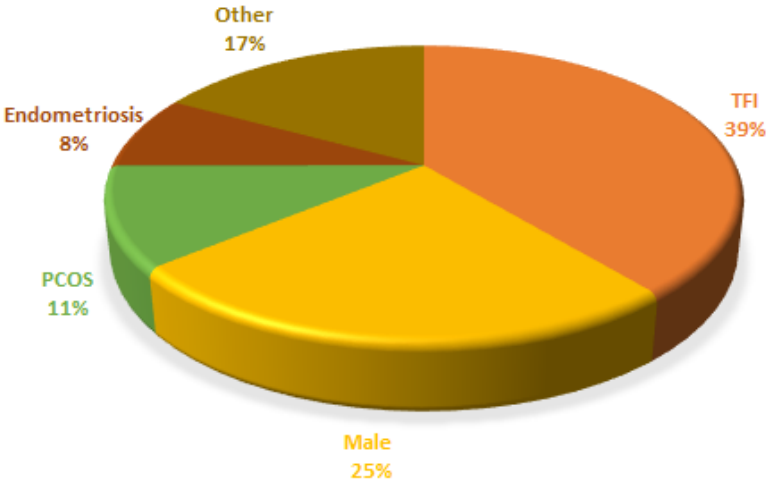

Figure 6. Distribution of diagnoses for infertility in study cohort.

Women with ectopic pregnancy

Study IV also included women with previous ectopic pregnancy (n=178, age 39.3±5.2 years), who represented women with tubal damage but without infertility. DNA and serum samples, as well as medical history for this group were obtained from the Estonian Genome Center of University of Tartu.

Controls

Two hundred women (age 39.8±5.3 years) with proven fertility (i.e. at least 2 children) and 200 women (age 45.7±18.1 years) representing the general population were enrolled in Studies III and IV as controls. Control group DNA and serum samples together with medical history were also obtained from the Estonian Genome Center of University of Tartu.

Clinical data

Metabolic assays (Studies I & II)

Serum samples for measuring metabolic parameters were collected during the first five days of a spontaneous menstrual cycle. Serum levels of fasting insulin as well as glucose were measured by chemiluminescence immunoassay (Immulite 2000; Siemens Healthcare Diagnostics, Deerfield, IL, USA) and glucose oxidase method (Cobas Integra 800®, Mannheim, Germany), respectively. The homeostasis model assessment index ratio for insulin resistance (HOMA-R index) was also calculated (Vrbikova et al., 2002).

History of sexually transmitted diseases and Chlamydia trachomatis-specific IgG antibodies (Studies III & IV)

Any information regarding previous episodes of genital infections associated with *Chlamydia trachomatis* was collected from patient medical records. In addition, patient serum samples were tested for presence of *C. trachomatis*-specific IgG antibodies by ELISA (EUROIMMUN, Medizinische Labordiagnostika AG, Germany). Patients with a positive antibody analysis or a past infection in anamnesis were considered positive for *C. trachomatis*. The serum samples of women with ectopic pregnancy and control group women used in Study III and IV were also tested for presence of *C. trachomatis*-specific IgG antibodies by ELISA (EUROIMMUN).

Early follicular phase parameters (Studies I, II & V)

Transvaginal ultrasound to assess ovarian volume and early follicle count were performed during a spontaneous menstrual cycle. Ovarian volume, measured by ultrasound, was estimated according to the following formula: $1/2(A \times B \times C)$, where A is the longitudinal diameter, B is the anteroposterior diameter and C is the transverse diameter of the ovary. The number of small antral follicles was estimated by transvaginal ultrasound scanning of each ovary in longitudinal cross-section. Mean follicle number and mean ovarian volume were calculated as the sum of the left and right ovaries divided by two.

Serum samples for measuring hormonal levels were collected during the first five days of a spontaneous menstrual cycle. Levels of FSH, LH, estradiol,

testosterone and dehydroepiandrosterone sulphate (DHEAS) were measured using chemiluminescence immunoassay (Immulite 2000; Siemens Healthcare Diagnostics, Deerfield, IL, USA).

COS parameters (Study V)

Patients undergoing IVF as well as ICSI were included in the study. COS was conducted according to the GnRH antagonist (Cetrotide, Merck Serono, Geneva, Switzerland) or agonist (Cetrotide) protocol with the administration of recombinant FSH (rFSH) (Gonal-F, Merck Serono, or Puregon, Schering-Plough, Kenilworth, USA) for 9–10 days until 1 day before hCG (Ovitrelle, Merck Serono) administration. The COS follow-up included 3–4 ultrasound assessments of endometrial and follicular growth. Daily GnRH antagonist administration (0.25mg, Cetrotide) was initiated if at least one follicle reached the size of ≥14 mm. The GnRH antagonists were given for 4–5 days up to and including the day of hCG administration. Final follicular maturation was achieved using 250 μg of hCG, followed by ovarian puncture (OPU) 36 h later. The ovarian stimulation parameters analysed in Study V included: a) total amount of rFSH used (IU); b) amount of FSH administered per retrieved oocyte (IU/oocyte); c) number of ovarian follicles punctured (OPU follicles) and d) number of oocytes retrieved at puncture.

IVF treatment parameters (Studies IV & V)

Patients undergoing frozen embryo transfer were excluded from the study. A maximum of three 2-day embryos were transferred into the uterus, with vaginal progesterone (Lugesteron, Leiras, Turku, Finland) used for luteal support. Biochemical pregnancy was confirmed with a positive serum hCG test performed 14 days after embryo transfer, whereas clinical pregnancy was defined as the presence of gestational sac(s) with fetal heartbeat on transvaginal sonography 6–7 weeks of gestation. The IVF treatment parameters analysed in Study V included: a) biochemical pregnancy and b) clinical pregnancy.

3.2. Genotyping of polymorphisms

Different genotyping methods were used to analyse 47 polymorphic loci (tandem repeats, deletions and SNPs) in 24 different genomic regions (Table 1). In addition, for the *AR* gene located on the X-chromosome, the XCI pattern was also determined.

Genomic DNA was extracted from peripheral EDTA blood using the salting-out method (Aljanabi and Martinez, 1997). Before genotyping, polymerase chain reaction (PCR) was performed. Loci of interest were amplified individually, except when SNaPshot Multiplex primer extension (duplex PCR) or Sequenom MassArray iPLEX assay (multiplex PCR) were used.

3.2.1. Tandem repeat analysis (Studies I & II)

Tandem repeats are stretches of DNA where a pattern of one or more nucleotides is repeated and the repetitions are directly adjacent to each other. Tandem repeats are further classified as microsatellites (<10 repeating nucleotides, also known as short tandem repeats) or minisatellites (10–60 repeating nucleotides). When the number of repeating nucleotides in a minisatellite is variable, the term variable number of tandem repeat (VNTR) can also be used. In our study, two tandem repeats were analysed: *INS* VNTR and *AR* CAG microsatellite together with the XCI pattern.

The INS VNTR was genotyped using the surrogate marker –23 *Hph*I A/T (Lucassen et al., 1993), which was genotyped using PCR combined with restriction fragment length polymorphism (PCR-RFLP) analysis.

The number of CAG repeats in the *AR* CAG microsatellite was determined by electrophoresis of fluorescently labelled PCR products. The $(CAG)_n$ repeat region was also used to determine XCI pattern using comparative quantitative detection of fluorescently labelled PCR products using intact and methylation sensitive restriction enzyme *Hpa*II digested DNA templates. Amplicon size and inactivation ratios were determined by electrophoresis on an ABI Prism 377 DNA Sequencer (PE Applied Biosystems, Foster City, CA, USA) under standard conditions and analysed by Genescan 2.1 software (PE Applied Biosystems). Rox 500® (PE Applied Biosystems) was used as an internal size standard. The shorter (S-CAG) and longer (L-CAG) allele of each individual, biallelic mean (CAG-BM, the arithmetic mean of allele lengths), weighted biallelic mean (CAG-WBM, mean allele length adjusted for relative activity of shorter and longer allele) and XCI difference (XCI-Dif, represents the gross difference from the 50:50 inactivation ratio) were used in statistical analyses. CAG-WBM was calculated using the following formula:
CAG-WBM = [(S-Act/100) × S-CAG] + [(L-Act/100) × L-CAG], where S-Act and L-Act correspond to the percentage of cells with active shorter or longer CAG alleles, respectively. The area under the curve obtained by visualisation of PCR products was used for XCI pattern calculations using the following formula:

$$\text{XCI-Dif} = |\text{S-Act} - \text{L-Act}|.$$

For example, if S-Act and L-Act are both 50% inactive, the XCI-Dif is 0%, but if S-Act and L-Act are 90 and 10% inactive, respectively, the XCI-Dif is 80%.

3.2.2. *CCR5*delta32 deletion genotyping (Study III)

The *CCR5*delta32 deletion was detected using PCR, followed by visualisation of PCR products by electrophoresis on an agarose gel, which identified the following fragments: 189 bp (wild-type allele) and 157 bp (variant allele).

3.2.3. SNP genotyping (Studies III, IV & V)

A total of 44 SNPs in 21 genomic regions were analysed.

SNPs in *TLR4* and *MBL2* genes were detected using SNaPshot Multiplex primer extension (PE Applied Biosystems, Foster City, CA, USA). SNaPshot products were run on an ABI 3130xl Genetic Analyzer (PE Applied Biosystems), analysed using GeneScan 4.0 software (PE Applied Biosystems) and validated by direct sequencing.

The *MBL2* codon 54 polymorphism, as well as *TLR2* Arg753Gln polymorphism were genotyped by RFLP analysis. The corresponding PCR products were digested overnight at 37°C using restriction enzymes *Bsh*NI and *Pst*I, respectively. All restriction fragments were separated according to their length by gel electrophoresis and visualised under UV transillumination.

Since the selected polymorphisms in the *MBL2* gene are in strong LD, the genotyped SNPs were further combined to also include *MBL2* haplotypes in the analysis. The first two positions in the haplotype are determined by polymorphisms in the promoter area and the third position in the haplotype, A or B/D, is a combination of the two polymorphisms in exon 1, with A indicating a wildtype allele in both positions and B/D representing a variant allele in the corresponding locus. Promoter haplotypes HY, LY and LX correlate with high, intermediate and low serum levels of MBL, respectively (Naito et al., 1999), while polymorphic structural variants (B/D) are associated with low levels or an absence of functional MBL (Sumiya et al., 1991; Lipscombe et al., 1992; Madsen et al., 1994). The two *MBL2* variant alleles (B/D) in exon 1 were grouped together as allele O, and the two wild-type alleles were grouped as allele A (Garred et al., 2006). Overall 7 *MBL2* haplotypes were present in groups analysed (HYPA, LYPA, LYQA, LXPA, HYPD, LYPB and LYPD), which formed 22 *MBL2* combined genotypes. To facilitate interpretation of the data, simplified *MBL2* haplotypes HYA (HYPA), LYA (LYPA, LYQA), LXA (LXPA) and O (HYPD, LYPB and LYPD) were used. Since *MBL2* genotypes correlate with serum MBL levels, the latter were not measured and genotypes were divided into groups based on their estimated expression potential as follows: high-producing (HYA/HYA, HYA/LYA, LYA/LYA, HYA/LXA and LYA/LXA), low-producing (LXA/LXA, HYA/O and LYA/O) and MBL-deficient (LXA/O and O/O) (Steffensen et al., 2000).

All other SNPs were genotyped by Sequenom MassArray iPLEX Assay (Sequenom, San Diego, CA, USA). Genotypes were called automatically and confirmed manually using the MassARRAY Typer 4.0.22 software (Sequenom).

3.3. Statistical analysis

Data for continuous variables are presented as mean ± SD, unless stated otherwise. Allele frequencies were tested for deviations from the Hardy-Weinberg equilibrium. For all analyses, p value <0.05 was considered statistically significant. Statistical tendency or trend was considered to be present when $0.05 < p < 0.1$.

The Student's t-test, Wilcoxon rank sum test with continuity correction, χ^2 test or Fisher's exact test were used to compare the distribution of studied characteristics in study groups. Linear and logistic regression analyses were used when adjustment for different confounding factors was necessary (details given in the Results and Discussion section). To consider the suitability of genetic variants associated with ovarian aging for use as markers of ovarian function, COS and IVF treatment, the leave-one-out cross-validation algorithm was applied and squared correlation between observed and predicted values (R^2) or area under the ROC-curve (AUC) were calculated, respectively for logarithm transformed (continuous) variables or binary variables.

For statistical analyses, the following software were used in different studies – The R2.0.0 and R 2.3.1 A Language and Environment (Free Software Foundation, Boston, MA, USA), Statistical Package for Social Sciences 17.0 (SPSS Inc., Chicago, IL, USA) and Statistical Analysis System 9.1 (SAS Institute Inc.).

Table 1. Overview of the studies

Study	Groups analysed (n)	Mean age ± SD	Exclusion criteria	Genetic variants analysed, SNP rs-number (alternative name; location in gene) Used methods
I	IVF patients: PCOS (30) TFI (75)	 32.0±4.4 34.1±4.4	All other diagnoses	*INS* VNTR RFLP
II	IVF patients: PCOS (32) TFI (79)	 32.3±4.7 33.4±6.2	All other diagnoses	*AR* CAG; XCI methylation-sensitive restriction; PCR microsatellite analysis
III	IVF patients: TFI (163) Controls: General population (200) Fertile women (200)	 33.7±4.0 45.7±18.1 39.8±5.3	Endometriosis	*CCR5*delta32; *TLR2*: rs5743708 (Arg753Gln; EM); *TLR4*: rs4986790 (Asp299Gly; EM), rs4986791(Thr399Ile; EM); *MBL2*: rs11003125 (-550 H/L; P), rs7096206 (-221 X/Y; P), rs7095891 (+4 P/Q; U), rs5030737 (codon 52; EM), rs1800450 (codon 54; EM) PCR; PCR-RFLP; SNaPshot Multiplex primer extension
IV	IVF patients: TFI (155) Ectopic pregnancy (178) Controls: General population (198) Fertile women (190)	 37.1±4.2 39.3±5.2 45.8±18.1 39.9±5.4	Endometriosis	*MBL2*: rs11003125 (–550 H/L; P), rs7096206 (-221 X/Y; P), rs5030737 (codon 52; EM), rs1800450 (codon 54; EM) SNaPshot Multiplex primer extension
V	IVF patients: Male factor (91) Tubal factor (130) Endometriosis (31) Unexplained (21) Other (32) Unknown (1)	34.2±4.4	1. PCOS 2. Only one ovary 3. FET cycles	*FSHB*: rs10835638 (–211G>T; P), rs11031010 (U), rs34951229 (U), rs34224414 (U), rs611246 (I), rs550312 (I), rs609896 (I), rs6169 (ES), rs613058 (I), rs7951733 (U); *FSHR*: rs11125215 (I), rs4971884 (I), rs7606570 (I), rs1277460 (I), rs2268360 (I), rs2268363 (I), rs4971637 (I), rs6165 (c.919G>A; p.Ala307Thr; EM), rs6166 (c.2039G>A; p.Asn680Ser; EM), rs971831 (I); TLK1: rs10183486 (I); *TMEM150B*: rs11668344 (I); *NLRP11*: rs12461110 (EM); *MCM8:* rs16991615 (EM); *SYCP2L*: rs2153157 (I); *PRIM1*: rs2277339 (EM); *FNDC4*: rs2303369 (I); *ASH2L*: rs2517388 (I); *UIMC1*: rs365132 (ES); *KPNA3*: rs3736830 (I); *RHBDL2*: rs4246511 (I); *TDRD3*: rs4886238 (I); *METAP1D*: rs7606918 (I); *RNF44*: rs890835 (I); intergenic SNPs: rs10852344, rs12294104 Sequenom MassArray iPLEX assay

EM – exon missense, EN – exon non-synonymous, ES – exon synonymous, FET – frozen embryo transfer, I – intron, IVF – in vitro fertilisation; P – promoter, PCOS – polycystic ovary syndrome; PCR – polymerase chain reaction; RFLP – restriction fragment length polymorphism; SNP – single nucleotide polymorphism, TFI – tubal factor infertility, U – untranslated region

4. RESULTS AND DISCUSSION

4.1. Genetic susceptibility factors for PCOS (Studies I & II)

4.1.1. Study design

In this part of the study, we evaluated the *INS* VNTR and the *AR* CAG microsatellite for their association with PCOS. Patients with TFI but with regular menstrual cycles were used as controls in these studies. Therefore, the studies presented in this section represent classic genetic association studies that test whether particular allele(s) occur at a significantly different frequency among the patient group of interest compared to the control group. PCOS is a so-called complex disease, which means that it is caused by a combination of genetic and environmental factors and the individual contribution of each gene and genetic variant is small and individually insufficient to cause the disease. Both the *AR* CAG and *INS* VNTR were selected among genes likely involved in the disease pathogenesis based on the current knowledge about PCOS. On one hand, this approach lowers the number of potential comparisons and thus increases study power, but on the other hand it is hampered by our limited knowledge on the pathogenesis of complex diseases and by the heterogeneous phenotype typical for PCOS. One of the major pitfalls in PCOS candidate gene studies is the varying criteria used to diagnose PCOS (Goodarzi, 2007), therefore all the patients included in our studies were diagnosed according to the Rotterdam criteria (The Rotterdam ESHRE ASRM-Sponsored PCOS Consensus Workshop Group, 2004) which are currently the preferred criteria for diagnosis (Legro et al., 2013).

4.1.2. *INS* VNTR, *AR* CAG and XCI

Both the *INS* VNTR and *AR* CAG microsatellite together with XCI pattern were evaluated for association with PCOS. The distribution of *INS* VNTR genotypes was comparable in patients and in controls (Table 2), meaning that the *INS* VNTR is probably not associated with an increased PCOS risk. This is in concordance with large-scale studies analysing several data sets (Powell et al., 2005; Song et al., 2014), but in disagreement with a meta-analysis claiming that the *INS* VNTR class III alleles increases PCOS risk (Yan et al., 2014). The different results of the meta-analyses may be explained by different i) study designs, ii) inclusion criteria that may result in a certain bias, and iii) analysis methodology. While Powell et al. (2005) conducted case-control, family-based association and quantitative trait analyses, Song et al. (2014) and Yan et al. (2014) conducted meta-analyses of 13 and 9 previously published datasets, respectively. It is also noteworthy that patient groups included in these meta-analyses were diagnosed using various criteria (NIH criteria, ultrasound

symptoms, Rotterdam criteria) which could mean that due to patient heterogeneity these studies are actually not comparable.

The studied *AR* CAG allelic characteristics (Table 2) did not differ between patients and controls, for example, both the AR CAG biallelic mean and weighted biallelic mean were on average approximately 21.5 repeats in PCOS patients and in controls. These results support the findings that the *AR* CAG is not a genetic susceptibility factor for PCOS (Wang et al., 2012; Rajender et al., 2013; Zhang et al., 2013). The patients in this study had similar testosterone levels compared to the control group (Table 1 in Study II) and therefore represent the PCOS subphenotype that does not exhibit profound hyperandrogenism but rather presents with polycystic ovaries and menstrual cycle irregularities. Since there is evidence the *AR* CAG may contribute to the hyperandrogenemic aspect of the disease (Zhang et al., 2013; Peng et al., 2014), the *AR* CAG may modify the phenotype in patients with more pronounced hyperandrogenemic traits.

Table 2. Genetic characteristics evaluated for association with PCOS.

Characteristic	**PCOS**	**Control**
***INS* VNTR genotypes**	**n=30**	**n=75**
III/III	2 (6.7%)	7 (9.3%)
III/I	10 (33.3%)	24 (32.0%)
I/I	18 (60.0%)	44 (58.7%)
***AR* CAG characteristics (mean no of repeats ± SD)**	**n=32**	**n=79**
S-CAG	20.2±2.0	20.0±2.0
L-CAG	22.8±1.9	23.1±2.4
CAG-BM	21.5±1.6	21.6±1.8
CAG-WBM	21.4±1.8	21.6±2.0
XCI characteristics (mean percentage ± SD)	**n=23**	**n=63**
XCI-Dif	21.9±22.8	28.8±20.1

SD – standard deviation, *AR* CAG – androgen receptor CAG microsatellite, CAG-BM – *AR*-CAG biallelic mean, CAG-WBM – *AR* CAG weighted biallelic mean, S-CAG – shorter *AR* CAG allele, L-CAG – longer *AR* CAG allele, XCI-Dif – X-chromosome inactivation difference represented by the absolute difference between the percentage of cells with an active longer or shorter CAG allele.

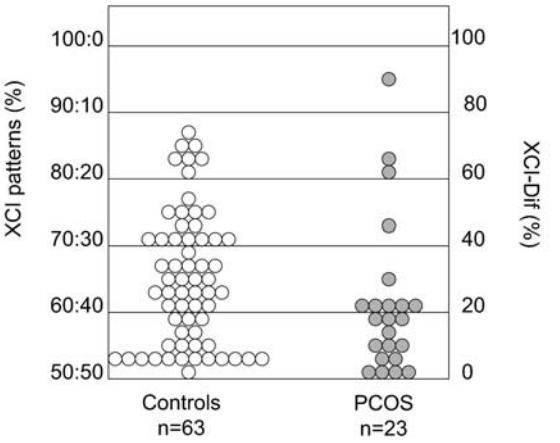

Figure 7. Distribution of XCI patterns and XCI-Dif values in study groups. The XCI-Dif reflects the XCI pattern. For example, if both X-chromosomes are 50% inactive, the XCI-Dif is 0%, but if one is 90% and the other 10% inactive, the XCI-Dif is 80%.

Although there is evidence that the XCI may be altered in PCOS women (Hickey et al., 2002; Dasgupta et al., 2010), the distribution of XCI pattern (represented by the absolute difference between the percentage of cells with an active longer or shorter CAG allele) in our study was similar in both groups (Table 2, Figure 7). Extreme XCI skewing (95:5) was seen in only one PCOS patient, while in the majority of patients and controls the XCI pattern was approximately 60:40 (Figure 7). Together with previous findings (Shah et al., 2008; Schuring et al., 2012) it can be concluded that the XCI is not altered in PCOS women. However, since we studied the XCI phenomenon only in peripheral blood samples, and the XCI pattern can vary between tissues (Gale et al., 1994), the possibility that the XCI pattern is skewed in tissues of importance to PCOS pathogenesis (pituitary, ovarian tissues, etc) cannot be excluded.

The major limitation for the abovementioned studies was the modest sample size, which can result in false-negative findings because, as already mentioned, the effect of each genetic variant in the pathogenesis of PCOS is small and large study groups are needed to reach the necessary statistical power (Diamanti-Kandarakis and Piperi, 2005). Furthermore, our patient group represents only those PCOS patients who have infertility issues. Overall, it seems that the *INS* VNTR and *AR* CAG together with the XCI pattern are not major susceptibility factors for PCOS, which has also been shown by several other studies (Powell et al., 2005; Wang et al., 2012; Zhang et al., 2013; Song et al., 2014). However, like with many other candidate gene association studies, the results of the current analysis must be interpreted bearing in mind that PCOS is a syndrome with a great phenotypic heterogeneity, and although we did not find any associations with the studied variants, in a different subset of patients the associations may be present.

4.2. Prevalence of *C. trachomatis*-related TFI (Study III)

Since chlamydia-related infertility is one of the few forms of infertility that could be avoided, a better estimation of the prevalence of chlamydia-related TFI in Estonia could give valuable directions for future healthcare strategies. A nation-wide multicentre study would be the best study design for this purpose, but given the overall lack of data on TFI prevalence in Estonia and the fact that infertile women are treated in five major hospitals/clinics (Tonsiver et al., 2013), data from a single centre study could also adequately reflect the current situation in Estonia.

In order to determine *C. trachomatis* infection status in the TFI group, if possible, data from antibody analysis was combined with data extracted from medical records. We found that the prevalence of *C. trachomatis*-specific IgG antibodies was lower in the control group (17.8%), which is similar to that previously reported for fertile aged Estonian women (20.2%) (Kibur et al., 2000). On the contrary, approximately half (48.4%) of the patients with TFI had corresponding antibodies (Figure 8).

If additional data from medical records was taken into account, 58.7% of women with TFI were considered positive for *C. trachomatis* (CT+). A high prevalence of *C. trachomatis* seropositivity among women with TFI was to be expected, as *C. trachomatis* infection is one of the main causative factors of TFI (Paavonen and Eggert-Kruse, 1999). In addition to causing TFI, *C. trachomatis* infection can have detrimental effects on pregnancy outcome (Linhares and Witkin, 2010) and cumulative non-IVF pregnancy rate (Keltz et al., 2013), meaning that genital chlamydia infection can have a negative impact on human fertility that involves more than straightforward tissue damage.

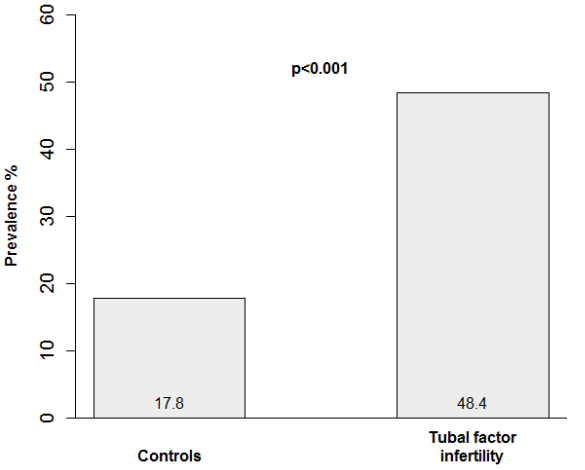

Figure 8. Prevalence of *C. trachomatis* IgG seropositivity among studied groups.

According to these results, approximately half of all TFI cases in Estonia can be categorised as having *C. trachomatis*-related TFI, which is in good correlation with published estimates (Price et al., 2012). Since acquired infertility due to infections is practically the only form of infertility that could be avoided and the prevalence of genital chlamydia among young females is among the highest in Europe (Redmond et al., 2015), preventive measures (sexual health training, screening strategies for target groups) and timely interventions could help to significantly reduce the number of women suffering from chlamydia-related infertility in Estonia.

4.3. Genetic susceptibility factors for TFI (Studies III & IV)

4.3.1. Study design

Identifying the genetic susceptibility factors of *C. trachomatis* infection and tubal damage could give further knowledge on the immunopathogenesis of genital infections and host-pathogen interactions, which in turn can help to identify those individuals at risk for developing serious complications. Similarly to PCOS, the genetic susceptibility factors for TFI have been thus far sought using the candidate gene approach (Morre et al., 2009b). The most common study designs include a) comparing CT+ (either DNA or antibody positive) individuals with CT− individuals, and b) comparing CT+ patients with CT+ individuals with a different course of infection (Morre et al., 2009b). In the studies presented in this section, women with TFI were compared to controls (fertile women and women representing the general population). Additionally, women with tubal damage (ectopic pregnancy) but without infertility complaints were enrolled in Study IV to help establish whether the selected genetic variants are associated merely with tubal damage, or also have a certain impact on fertility as such. In both studies, the patient and control groups were also divided according to their *C. trachomatis* status, as the mechanisms of pathogenesis may differ in chlamydia-related TFI and TFI not involving a chlamydia infection.

According to Morré et al, the most important variables to consider in the analysis stage include clear ethnic background of study population (due to varying SNP allele frequencies in different populations), clinical definition of *C. trachomatis* status and correct definition of tubal pathology (Morre et al., 2009b). All studied TFI patients were Estonians (Caucasian) and all control group DNAs were obtained from the Estonian Genome Centre of the University of Tartu, which is a population-based biobank where the vast majority of participants are also Caucasians (http://www.geenivaramu.ee/en/about-us). This means that all individuals in studies III and IV come from the same region, share a similar racial (and very likely a similar ethnic) background, therefore this factor is unlikely to influence the results. In the patient group, TFI was

diagnosed either by hysterosalpingography or by diagnostic laparoscopy which are both generally accepted and recommended methods for assessing tubal pathology (Practice Committee of American Society for Reproductive Medicine, 2012). For the control groups, the medical history in the biobank is recorded according to the ICD-10 classification, and all individuals with diagnosis code N97.1 (female infertility of tubal origin) were excluded from the analysis. Since the medical history relies on self-reported data, the possibility that some women with TFI were also accidentally included in the control group representing the general population cannot be excluded. The control group also included 200 fertile controls, who had at least 2 children and were therefore unlikely to suffer from infertility. In study IV, we studied an additional group of women from the Estonian Genome Center who had tubal damage (ectopic pregnancy) but no self-reported infertility complaints. This group was selected because similarly to TFI, the main risk factors for ectopic pregnancy include sexually transmitted infections, tubal infection or pelvic adhesions, and the majority (~90%) of ectopic pregnancies are located in the fallopian tubes (Practice Committee of the American Society for Reproductive Medicine, 2013). In these women, the presence of tubal damage was not confirmed by hysterosalpingography or laparoscopy, and the diagnosis of ectopic pregnancy (ICD-10 O00 for ectopic pregnancy or O00.1 for tubal pregnancy) was considered as sufficient evidence for the presence of tubal damage or dysfunction.

*C. trachomati*s status in all studied groups was determined by testing the serum samples for the presence of *C. trachomatis*-specific IgG antibodies, which generally denote previous and resolved infections (Debattista et al., 2003). When possible, *C. trachomatis* infection status was determined by combining both medical history and IgG antibody analysis, since *C. trachomatis*-specific IgG antibodies are detectable in serum for up to 8 years (Debattista et al., 2003). Of course, the self-reported data on chlamydia infection may not be most accurate due to recall bias or because in some patients the infection can clear spontaneously (Geisler, 2010). Using this combined approach we most likely identified the vast majority of individuals who have had a genital chlamydia infection, but it cannot be excluded that some women were mistakenly classified as CT−.

Polymorphic variants in the *CCR5*, *TLR2*, *TLR4* and *MBL2* genes were tested and evaluated for their association with TFI.

4.3.2. *CCR5*, *TLR2* and *TLR4*

The distribution of *CCR5*delta32 deletion, *TLR2* Arg753Gln, *TLR4* Asp299Gly and Thr399Ile genotypes did not differ between TFI patients and controls, or between CT+ and CT− groups (Table 3).

A role for CCR5 in modulating the likelihood of tubal pathology has been proposed (Barr et al., 2005); however, our results do not support this notion. The frequency of variant allele carriers among controls and infertile women was

similar in both studies (~20%), but Barr and colleagues reported a remarkable difference in *CCR5*delta32 carriership when comparing subfertile CT+ women with and without tubal pathology (7% vs. 31%). Our study did not include a subfertile CT+ control group and this could be one of the reasons for contrasting results, stressing the importance of group selection when studying the genetic background of TFI. Although a recent study with a design similar to ours also found no differences in *CCR5*delta32 distribution (Mania-Pramanik et al., 2011), these results must be interpreted with care as the frequency of *CCR5*delta32 variant allele carriers shows great geographic variation and was very low in this study population (2%).

Several TLRs have been studied in the context of tubal pathology development (Darville et al., 2003; den Hartog et al., 2006b), but the results have been inconclusive. In our study, the frequency of studied polymorphisms in *TLR2* and *TLR4* did not differ between studied groups, which is in line with results from similar studies (Morre et al., 2003; den Hartog et al., 2006b; Karimi et al., 2009). Therefore, common genetic variants in *TLR2* and *TLR4* are probably not a major susceptibility factor for TFI.

Table 3. Distribution of CCR5, TLR2 and TLR4 genotypes in studied groups.

Polymorphism Genotype		**Genotype frequency %**					
		TFI	**control**	**TFI CT+**	**control CT+**	**TFI CT−**	**control CT−**
***CCR5*delta32**	wt/wt	79.6	78.6	77.2	80.0	83.3	78.1
	wt/del	18.5	18.4	21.7	17.1	13.6	18.8
	del/del	1.9	3.0	1.1	2.9	3.0	3.1
***TLR2* Arg753Gln**	GG	89.5	88.6	90.0	85.5	88.9	89.2
	AG	10.5	11.1	10.0	14.5	11.1	10.5
	AA	0	0.3	0	0.3	0	0.3
***TLR4* Asp299Gly**	AA	90.4	88.1	90.9	87.1	88.1	88.9
	AG	8.9	11.6	8.0	12.9	11.9	10.8
	GG	0.7	0.3	1.1	0	0	0.3
***TLR4* Thr399Ile**	CC	88.4	88.1	88.6	87.1	86.4	88.9
	CT	11.0	11.6	10.2	12.9	13.6	10.8
	TT	0.7	0.3	1.1	0	0	0.3

CT – *Chlamydia trachomatis*; TFI – tubal factor infertility

As a conclusion, our results show that genetic variants in *CCR5*, *TLR2* and *TLR4* are not major susceptibility loci for TFI. However, in the future it could be worthwhile to consider SNP interactions and conduct a burden analysis, as there is evidence that carriage of two or more SNPs in genes related to TLR signalling can increase the risk of developing tubal pathology following chlamydia infection (den Hartog et al., 2006b).

4.3.3. *MBL2*

When tested separately, no associations were observed for *MBL2* −221 X/Y, +4 P/Q, codon 52 and codon 54 polymorphisms, whereas the distribution of *MBL2* −550 H/L genotypes was significantly different between TFI patients and controls ($p < 0.05$), which correlates with the differential distribution of *MBL2* combined genotypes. The first two positions in the combined genotype are determined by polymorphisms in the promoter area and the third position, A or B/D, is a combination of the two polymorphisms in exon 1, with A indicating a wildtype allele in both positions and B/D representing a variant allele in the corresponding locus. When *MBL2* SNPs were evaluated in the context of combined genotypes, both Study III and IV showed the high-producing HYA/HYA genotype was more frequent in TFI patients than in controls ($p<0.05$ in both studies) (Figure 9). However, study IV also showed the HYA/HYA genotype was more frequent in women with TFI compared to women with tubal damage but no infertility ($p<0.05$) (Figure 9).

According to our results, the high-producing HYA/HYA genotype is a risk factor for TFI. The HYA/HYA genotype has been associated with MBL hyper-production (Madsen et al., 1995; Matsushita et al., 2001) and susceptibility to primary biliary cirrhosis (Matsushita et al., 2001), which supports the 'MBL paradox' hypothesis (Dommett et al., 2006). This hypothesis states that very high MBL levels may be disadvantageous for two reasons: a) certain intra-cellular parasites may benefit from increased complement activation associated with very high MBL levels; and b) excessive complement activation may lead to immunopathological host damage (Dommett et al., 2006). Therefore, the preliminary conclusion was that excessive complement activation and subsequent tissue damage may very well be the mechanism linking the high-producing HYA/HYA genotype with TFI.

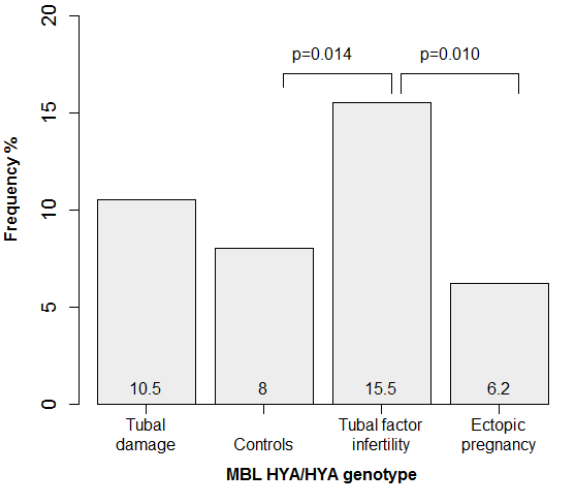

Figure 9. Frequency of MBL HYA/HYA genotype in studied groups. Data from Study IV.

Interestingly, study IV showed the HYA/HYA genotype frequency in the ectopic pregnancy group (women with tubal damage but no infertility) was similar to the control group (Figure 9). If the HYA/HYA genotype was associated only with tubal damage, we would expect to see similar frequencies in both patient subgroups, so this finding is contradictory to our initial conclusion. One differentiating characteristic of the two patient groups is that unlike the TFI group, the ectopic pregnancy patients had no infertility complaints in their anamnesis and additional analysis of their reproductive history (data not shown) revealed that they had had an average of 4 pregnancies per woman. Unfortunately, it is not known whether they had had any pregnancies after being diagnosed with ectopic pregnancy; however, spontaneous conception is very likely after treatment of ectopic pregnancy, regardless of the treatment option (medical or surgical) (Fernandez et al., 2013). Although increase in MBL levels and MBL complement pathway activity is a necessary part of maintaining pregnancy (van de Geijn et al., 2007b), high-producing genotypes or higher MBL levels have also been linked with conditions associated with excessive complement action and inflammatory reactions at the maternal-fetal interface, such as pre-eclampsia (Sziller et al., 2007a). Considering the role of MBL in normal pregnancy and adverse pregnancy outcomes, it is possible that MBL genotypes and levels also play a part in embryo implantation. Indeed, higher MBL levels have been associated with both unexplained infertility (Oger et al., 2009) and low implantation rates (Chaouat et al., 2009), demonstrating the role of MBL in embryo implantation and implantation failure. Furthermore, deposits of one particular isoform of MBL at the implantation sites are linked with pregnancy failure in mice (Chaouat et al., 2009), indicating that higher MBL levels and complement deposits both in mice and humans may lead to adverse pregnancy outcome (Chaouat et al., 2009). Even though our TFI group is primarily diagnosed with tubal infertility, the additional component of implantation failure or incomplete implantation cannot be ruled out. Unfortunately *MBL2* genotype frequencies were not determined in previous studies (Chaouat et al., 2009; Oger et al., 2009).

To further analyse the associations between genetic variants and tubal damage and because we were first and foremost interested in genetic susceptibility factors for chlamydia-related TFI, the studied groups (controls and women with tubal damage and with/without infertility) were divided according to their CT-status. We found the high-producing HYA/LYA genotype was more prevalent in the CT+ control group ($p<0.05$ in both studies) (Figure 10A), suggesting a protective effect associated with this genotype, which can mean that a certain genotype or MBL level can provide the optimal immune response during *C. trachomatis* infection. The heterozygous HYA/LYA is associated with a somewhat lower level of MBL expression compared to HYA/HYA homozygotes and similar levels compared to LYA/LYA homozygotes (Madsen et al., 1995).

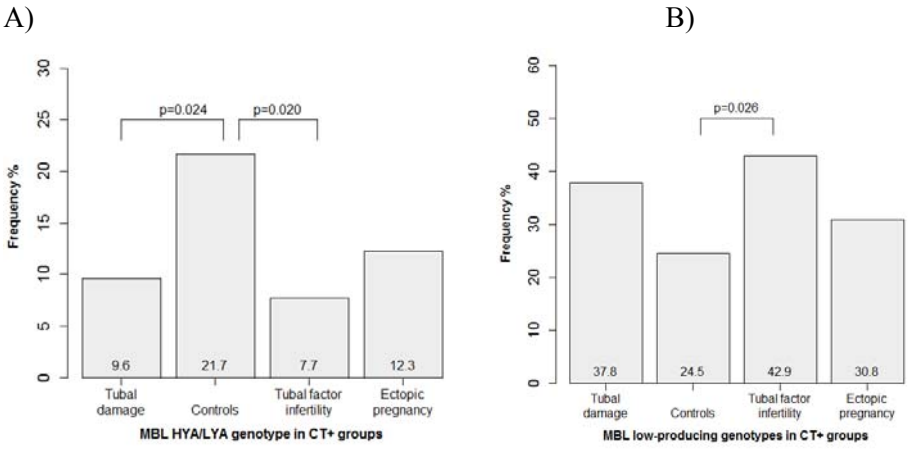

Figure 10. A) Frequency of MBL HYA/LYA genotype, and B) frequency of MBL low-producing genotypes in studied *C. trachomatis*+ groups. Data from Study IV.

In addition, we found that the low-producing *MBL2* genotypes as a cluster are more common among CT+ TFI patients ($p<0.05$ in both studies) (Figure 10B), indicating increased susceptibility to tubal damage after genital chlamydia infection. Contrary to previously reported results (Sziller et al., 2007c), our analysis showed no difference in codon 54 or MBL-deficient genotype distribution among *C. trachomatis*-positive patients and controls. Previously, the *MBL2* codon 54 heterozygous A/B genotype has been associated with decreased IVF success rate in women with TFI (Spandorfer et al., 2003), however, the possible association with other *MBL2* genotypes was not evaluated. The codon 54 A/B genotype is also a low-producing genotype and taken together, these data indicate that *MBL2* genotypes with intermediate protein expression pattern may be associated with decreased IVF success rates.

We identified several *MBL2* genotypes that may increase, or on the contrary, decrease the susceptibility to TFI. However, further studies are needed to decide whether these different genotypes have any functional consequences during *C. trachomatis* infection or facilitate tissue damage in the fallopian tubes, and also whether similar results are observed in independent studies. In addition, a study designed specifically for evaluating associations between pregnancy outcome and *MBL2* genotypes should be conducted in order to further elucidate how *MBL2* genotypes modify female fertility. TFI genetic susceptibility studies in general may benefit from larger study cohorts and international collaborations to identify variants with smaller effects and perhaps use a pathway approach in addition to candidate gene approach.

4.4. Genetic variants related to ovarian aging – association with ovarian function, COS and IVF treatment parameters (Study V)

4.4.1. Study design and studied parameters

In study V, the genetic variants previously associated with age at natural menopause (Stolk et al., 2012) and common polymorphisms in the *FSHB* and *FSHR* genes (in total 36 SNPs) were evaluated for their association with early follicular phase characteristics and ovarian stimulation and IVF outcome parameters in a cohort of women undergoing IVF treatment. The most popular study design for evaluating genotype effects on ovarian stimulation outcome includes the use of data from women undergoing IVF-COS for infertility treatment. The use of assisted reproduction treatment data presents numeral statistical challenges, as the process involves several interdependent steps and often women undergo more than one treatment cycle. For data analysis, usually all but one cycle are discarded, resulting in decreased statistical power, especially in typical single-site IVF studies (Missmer et al., 2011). The major shortcomings in IVF data analysis include quantifying statistical significance without evaluating the magnitude of effect, ignoring confounding factors, and using only a subset of the data at hand. Thus, several sophisticated statistical models have been proposed to make full use of the collected multicycle data (Missmer et al., 2011). In order to fully use the data available and increase study power, we included all treatment cycles for women who underwent two or more treatment cycles during the study period. For data analysis, generalized linear mixed models with logit or logarithm link function were used and all tested models were adjusted for appropriate confounding factors. The effect of repeated measurements from the same woman was considered as a random effect in statistical models.

The distribution of different studied parameters is shown in Table 4. The study included data for a total of 471 treatment cycles from 306 women [age 33.8±4.4 years in first cycle, 34.2±4.4 years across all treatment cycles; body mass index (BMI) 22.6±3.2]. 123 women underwent two or more treatment cycles during the study period, and on average, the participants had undergone 0.9±1.2 treatment cycles before entering this study. The distribution of diagnoses for infertility was as follows: TFI (42.5%), male factor (29.7%), endometriosis (10.1%), unexplained (6.9%), and other (10.5%). For one participant, the diagnosis was unknown. Ultrasound data for assessing ovarian parameters (ovarian volume and antral follicle count) was available for 135 participants, while a blood sample for measuring early follicular phase FSH levels was obtained from 128 women.

Table 4. Main characteristics of study participants and analysed parameters in Study V.

Characteristic		Mean±SD or n (%)
Ovarian surgery	Yes	19 (14.1%)
	No	115 (85.2%)
	Unknown	1 (0.7%)
Early follicular phase parameters	Mean ovarian volume (mL)	4.7±1.9
	Mean follicle count	4.8±1.9
	FSH (IU/L)	8.1±3.0
Ovarian stimulation	Agonist	19 (4.0%)
	Antagonist	384 (81.5%)
	Unknown	68 (14.4%)
Ovarian stimulation outcome	rFSH total (IU)	1980.7±585.3
	OPU follicles	13.5±6.7
	Oocytes retrieved	11.8±7.0
	rFSH per oocyte (IU)	271.6±298.9
IVF procedure	IVF	197 (41.8%)
	ICSI	274 (58.2%)
IVF outcome	No pregnancy	277 (58.8%)
	Biochemical pregnancy	163 (34.6%)
	Unknown	31 (6.6%)
	Clinical pregnancy	119 (25.3%)

FSH – follicle stimulation hormone, ICSI – intracytoplasmic sperm injection, IVF – in vitro fertilisation, OPU – ovarian puncture, rFSH – recombinant FSH, and SD – standard deviation

4.4.2. Genetic factors associated with early follicular phase parameters

In this section, associations between selected genetic variants and early follicular phase FSH levels, antral follicle count and ovarian volume were evaluated.

Early follicular phase FSH level analyses were adjusted for age, BMI, cycle day, mean follicle count and for any ovarian surgery. We found the early follicular phase FSH levels were significantly lower in rs2303369 (*FNDC4*) CC homozygotes ($p<0.05$, Figure 11A). This finding deserves special attention, especially when planning future studies aimed at revealing the genetic regulation of FSH action. rs2303369 is in LD with other SNPs in the adjacent *GCKR* (glucokinase regulator) gene, and the *FNDC-GCKR* locus has been associated with levels of albumin (Franceschini et al., 2012) and C-reactive protein (Ridker et al., 2008), indicating that the locus may have a more general effect on the levels of different serum components, including FSH. This means that when studying the genetic factors affecting FSH levels in circulation, variants other than those directly in *FSHB* region should also be considered.

In addition, early follicular phase FSH levels were significantly lower in rs611246 (*FSHB*) AA homozygotes ($p<0.05$, Figure 11B). Our study included the four SNPs representing the *FSHB* core haplotypes, but as these SNPs were

in complete LD, data is shown only for rs611246. As far as we know, the effect of *FSHB* core haplotypes on hormone levels is unknown. In our study, women with the T-allele had slightly higher FSH values (8.5 IU/L), which still remained in the normal range for follicular phase (3.5–12.5 IU/L). The polymorphisms forming the haplotype, rs611246 among them, are most likely regulatory SNPs (Grigorova et al., 2007), which may affect transcription levels or stability and thereby also protein levels, but their exact functional significance is yet to be elucidated.

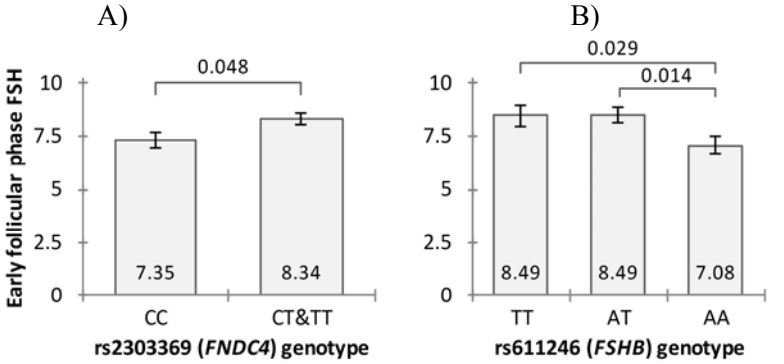

Figure 11. Early follicular phase FSH levels according to A) rs2303369 (*FNDC4*), and B) rs611246 (*FSHB*) genotypes. Data presented as least square means (± standard error) of the studied trait; from linear regression adjusted for age, BMI, cycle day, mean follicle count, previous ovarian surgery.

None of the tested genetic variants was associated with ovarian volume. However, antral follicle count was significantly higher in individuals carrying the G-allele of rs1277460 (*FSHR*) and also in rs6166 (*FSHR*) GG homozygotes ($p<0.05$, Figure 12) (antral follicle count analyses were adjusted for age, any ovarian surgery, mean ovarian volume, early follicular phase FSH and estradiol values).

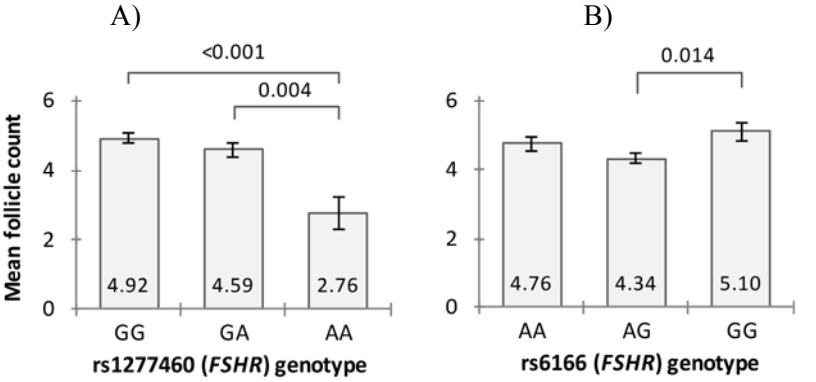

Figure 12. Mean antral follicle count according to A) rs1277460 (*FSHR*), and B) rs6166 (*FSHR*) genotypes. Data presented as least square means (± standard error) of the studied trait; from linear regression adjusted for age, previous ovarian surgery, mean ovarian volume, early follicular phase FSH and estradiol values.

Polymorphisms in the *FSHR* gene, especially rs6165 and rs6166, have thoroughly been studied in the context of ovarian reserve but the results are somewhat conflicting probably due to heterogeneity of study groups (reviewed in Simoni and Casarini, 2013). In our study, the two SNPs showed complete LD, therefore data are presented only for rs6166, which was associated with mean antral follicle count; however, the individual genotype effects were unclear. Unfortunately the genotyping of *FSHR* promoter polymorphism rs1394205 (–29G>A) failed (call-rate <90%), thus it was not possible to use the analysis methodology recommended by Simoni et al (Simoni and Casarini, 2013), that is the combined analysis of rs6166 and rs1394205 in *FSHR*, and rs10835638 in *FSHB* promoter. The grouping of these three SNPs results in 27 combinations that associate with different FSH levels and FSHR activity, and failure to consider the interactions of these SNPs may cause incorrect results (Simoni and Casarini, 2013). rs1277460 represents one of the less-studied *FSHR* polymorphisms, but it has been associated with female fertility (Kuningas et al., 2011).

In conclusion, several of the analysed SNPs in various genes (*FSHB*, *FSHR*, *FNDC4*) were associated with early follicular phase parameters (early follicular phase FSH levels and mean antral follicle count). However, our study included women of different age and with various reasons for infertility, and therefore may not represent the ideal cohort for studying such parameters. Thus, further studies are needed to clarify if the effects seen in this study also apply to healthy women.

4.4.3. Genetic factors associated with COS outcome

To test the statistical significance of effects of preselected genotypes on repeatedly measured ovarian stimulation parameters (total amount of rFSH administered, number of follicles and oocytes obtained on ovarian puncture, the amount of rFSH administered per retrieved oocyte), generalized linear mixed models with logarithm link function were fitted. All tested models were adjusted for appropriate confounding factors (age, diagnosis and stimulation protocol used, and also for the amount of rFSH used in OPU follicle/retrieved oocyte analysis) and the effect of repeated measurements from the same woman was considered as a random effect. The distribution of analysed characteristics is shown in Table 4.

Three SNPs from our selection [rs10852344 (intergenic), rs12461110 (*NLRP11*), and rs4246511 (*RHBDL2*)] apparently modulate the amount of rFSH necessary for ovarian stimulation (Figure 13A-C). Individuals with the minor homozygotic genotype at rs10852344 and rs12461110 needed significantly larger amounts of rFSH during stimulation, whereas minor homozygotic genotype at rs4246511 seems to be associated with smaller doses of rFSH (all $p<0.05$). The same SNP also has an impact on the amount of rFSH necessary

for retrieving one oocyte during stimulation, as do rs11668344 in *TMEM150B* and rs2153157 in *SYCP2L* ($p<0.05$) (Figure 13D-F).

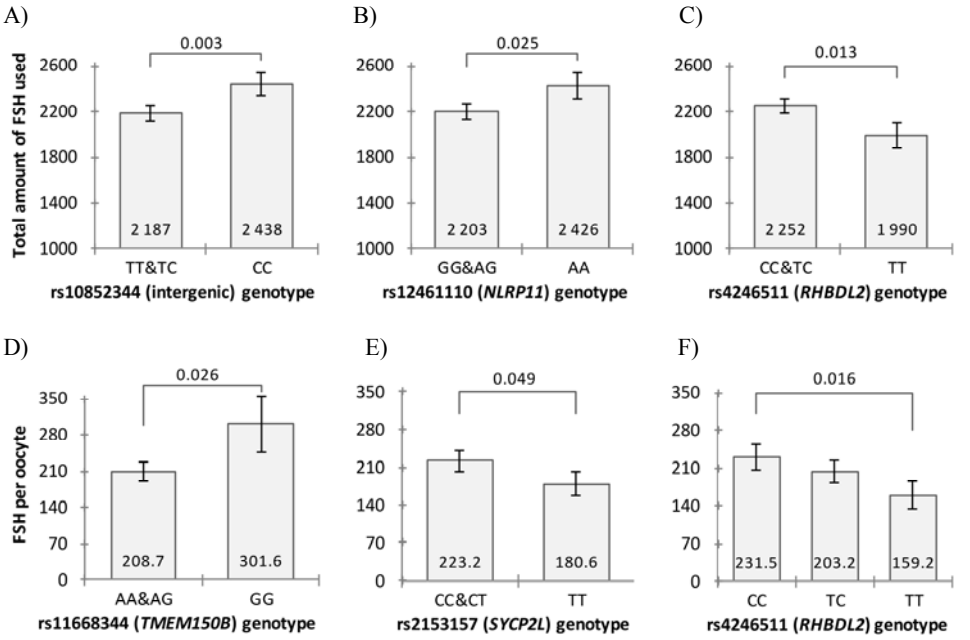

Figure 13. Total amount of rFSH used according to A) rs10852344 (intergenic), B) rs12461110 (*NLRP11*) and C) rs4246511 (*RHBDL2*) genotypes, and the amount of rFSH administered per retrieved oocyte according to D) rs11668344 (*TMEM150B*), E) rs2153157 (*SYCP2L*) and F) rs4246511 (*RHBDL2*) genotypes. Data presented as least square means (± standard error) of the studied trait

RHBDL2 [rhomboid, veinlet-like 2 (Drosophila)] is an intramembrane serine protease from the rhomboid family that releases soluble growth factors by proteolytic cleavage of membrane-bound substrates, including EGF, ephrins and thrombomodulin (reviewed by Etheridge et al., 2013). The EGF network is involved in many aspects of human reproduction, including ovarian steroidogenesis and oocyte maturation (Jamnongjit et al., 2005). TMEM150B is a member of the damage-regulated autophagy modulator family of membrane-spanning proteins, while NLRP11 is a cytoplasmic protein implicated in the activation of proinflammatory caspases. Both of these genes have repeatedly come up in studies of menopause timing (Stolk et al., 2009; Chen et al., 2012; Stolk et al., 2012; Perry et al., 2013; Shen et al., 2013; Chen et al., 2014), indicating their influence on the trait is similar and conserved among women of different ethnicities; however, the exact mechanism of effect remains unclear. The major alleles of these SNPs were associated with decreased amount of total rFSH/rFSH per oocyte, and also with increased age at menopause (Stolk et al.,

2012), suggesting the proteins might somehow mediate the action of FSH on follicular maturation, which is supported by the fact that the NLRP proteins are important for oogenesis (McDaniel and Wu, 2009). rs2153157 (*SYCP2L*; synaptonemal complex protein 2-like) has repeatedly been associated with age at natural menopause (He et al., 2009; Stolk et al., 2012; Carty et al., 2013) and studies from mice show that SYCP2 is necessary for synaptonemal complex assembly and chromosomal synapsis during male meiosis, and furthermore, female SYCP2 knockout mice show signs of subfertility and reduced litter size (Yang et al., 2006). rs2153157 minor homozygotes also need significantly smaller amounts of rFSH/oocyte, which combined with the fact that the minor allele increases age at menopause (Stolk et al., 2012), indicates that the minor allele carriers have a better ovarian reserve. rs10852344 is located in an intergenic region and is surrounded by three genes (*GSPT1*, *SNX29* and *TNFRSF17*) that harbour many SNPs that are in LD with rs10852344. The nearest gene is *GSPT1* (G1 to S phase transition 1) that encodes the eRF3a protein involved in chromosomal segregation and cytokinesis during mitosis.

Both the numbers of OPU follicles and oocytes retrieved were associated with two SNPs – rs4886238 (*TDRD3*) and rs7606918 (*METAP1D*) (Figure 14). The minor homozygotic genotype at rs4886238 and the major homozygotic genotype at rs7606918 result in smaller numbers of OPU follicles ($p<0.05$) and retrieved oocytes ($p<0.05$). In addition, rs3736830 (*KPNA3*) and rs2268363 (*FSHR*) were associated with the number of OPU follicles (Figure 14), but not with the number of retrieved oocytes.

Of these three SNPs, rs4886238 (*TDRD3*; tudor domain containing 3) is probably the most interesting in this framework because TDRD3 interacts with the FMR1 protein (Linder et al., 2008) implicated in the development of POI (Sullivan et al., 2011). In our study the ovarian stimulation in rs4886238 G-allele carriers resulted in more follicles and oocytes, whereas previously the same allele was associated with earlier age at menopause (Stolk et al., 2012). These two results indicate that the ovarian pool is depleted quicker in women carrying the G-allele. Not much is known about rs3736830 in *KPNA3* (karyopherin alpha 3, also known as importin α4), which belongs to importin alpha family and mediates nuclear protein import. Importin α4 mRNA is expressed in rodent testis (Hogarth et al., 2006) and importin α4 protects against oxidative stress in murine spermatids (Young et al., 2013), further connecting the protein with germ cell development. However, until now the association with menopause timing was the only link with female reproductive biology, as is the case with rs7606918 in *METAP1D* (methionyl aminopeptidase type 1D), a methionyl aminopeptidase found in mitochondria and often overexpressed in different cancers (Randhawa et al., 2013).

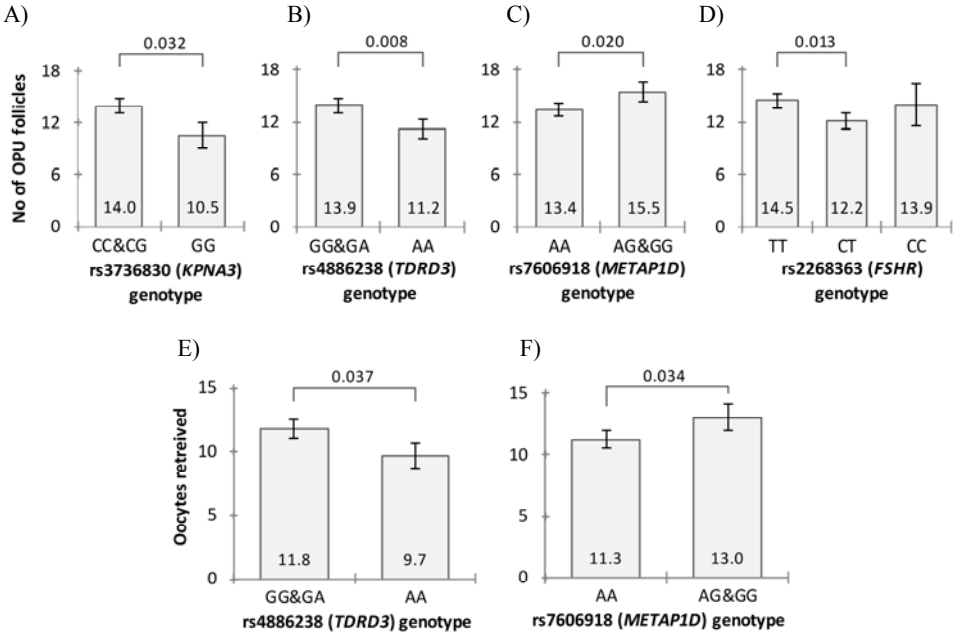

Figure 14. Number of OPU follicles according to A) rs3736830 (*KPNA3*), B) rs4886238 (*TDRD3*), C) rs7606918 (*METAP1D*) and D) rs2268363 (*FSHR*) genotypes and the number of obtained oocytes according to E) rs4886238 (*TDRD3*) and F) rs7606918 (*METAP1D*) genotypes. Data presented as least square means (± standard error) of the studied trait.

The *FSHR* gene is currently the most studied genetic factor with regard to IVF-COS (Altmäe et al., 2011; Simoni and Casarini, 2013) and the rs6165 and rs6166 SNPs have been associated with reduced IVF-COS outcome, elevated FSH administration and lower clinical pregnancy rate (reviewed by Altmäe et al., 2011). However, the clinical relevance of the *FSHR* polymorphisms alone is limited (Simoni and Casarini, 2013) and recent studies have proposed that rs6165 and rs6166 polymorphisms may be useful for distinguishing women undergoing IVF-COS who are at a risk of extreme ovarian response one way or the other (poor responders vs. ovarian hyperstimulation syndrome) (Yan et al., 2013; Pabalan et al., 2014), rather than predicting the entire spectrum of ovarian response. Our study did not find any associations between IVF-COS parameters and rs6165 and rs6166 in the *FSHR*, but instead, the rs2268363 in *FSHR* was linked with the number of OPU follicles. This SNP has previously come up in a GWA study of erectile dysfunction (Kerns et al., 2010). These results suggests that the range of SNPs having an effect on FSH functioning goes beyond the most studied rs6165 and rs6166 and future studies should include other SNPs from the *FSHR* region and perhaps even consider SNP interactions both in the *FSHR* region and with SNPs in the *FSHB* gene (Simoni and Casarini, 2013).

While a genome-wide hypotheses-free approach would be the best for discovering genetic associations, it cannot always be applied. To the best of our knowledge, the study by van Disseldorp et al (van Disseldorp et al., 2011) is so far the only IVF-COS GWAS conducted. Their study involved 102 women undergoing IVF treatment but unfortunately no genetic variant significantly correlated with parameters of IVF-COS and the authors concluded that it is possible that the GWAS methodology may dismiss variants with smaller effects and due to GWAS stringent analysis criteria, the study would need to be repeated in a much larger study cohort (van Disseldorp et al., 2011). However, since the very large and rather homogenous study cohorts suitable for GWAS are difficult to achieve in a single-centre IVF setting, an (international) multi-centre effort is needed to achieve the necessary sample size for conducting a successful GWAS. Until then, other approaches have to be used for detecting potential biomarkers for IVF-COS traits. A pre-selection (based on the association with the timing of age at natural menopause) used in Study V helped to narrow down the number of tested markers and thus to bypass the analysis bottleneck associated with GWAS. To conclude, these results shed some light on how the genes associated with female reproductive aging exert their effect, providing new directions for future research. Furthermore, the results show that genetic variants associated with reproductive aging are good candidates for searching IVF-COS markers.

4.4.4. Genetic factors associated with IVF outcome

To evaluate the effect of genetic variants on IVF outcome parameters (biochemical and clinical pregnancies), generalized linear mixed models with logit link function were fitted and analyses were adjusted for age, diagnosis, endometrial thickness, number of previous IVF treatments, number of good quality embryos, number of transferred embryos and type of IVF procedure. In addition, the effect of repeated measurements from the same woman was considered as a random effect. The values for IVF outcome parameters are shown in Table 4.

rs10852344 (intergenic) and rs2153157 (*SYCP2L*) were associated with both biochemical and clinical pregnancies (Figure 15). rs10852344 minor homozygotes were significantly less likely to achieve biochemical or clinical pregnancy ($p<0.05$), whereas in the case of rs2153157, individuals carrying the minor allele were more likely to get a positive treatment outcome (Figure 15). The positive effect of the T-allele of rs2153157 (*SYCP2L*) on clinical pregnancy rates can stem directly from improved ovarian reserve or be associated with the role the synaptonemal complex plays in preventing chromosome segregation errors and embryo aneuploidy – a major cause of implantation failure and early miscarriage. A similar mechanism of action can be suspected for rs10852344, as the T-allele carriers have increased chances for both biochemical and clinical pregnancy and also need less rFSH during stimulation. As mentioned above,

rs10852344 is near the *GSPT1* that encodes the eRF3a protein, which is involved in chromosomal segregation and cytokinesis during mitosis, and highly expressed in blastocysts (Adjaye et al., 2007), suggesting the protein might be important for early embryonic development, which supports the present results.

While the C-allele of rs4246511 in *RHBDL2* increased the amount of rFSH used during stimulation and necessary for retrieving one oocyte, the T-allele was associated with a decreased chance of clinical pregnancy (Figure 15). RHBDL2 substrates include thrombomodulin, which has been associated with embryo implantation (Dassen et al., 2007; Singh et al., 2011). The fact that the effects of rs4246511 detected in this study are somewhat conflicting – women with the T-allele respond better to ovarian stimulation, and enter menopause later (Stolk et al., 2012), yet have reduced chances of pregnancy – suggests the observed results are manifested via different pathways in the ovary and in the endometrium. However, since the rs4246511 has been scarcely studied in humans, further studies are needed to clarify the role of rs4246511 and *RHBDL2* in human reproduction.

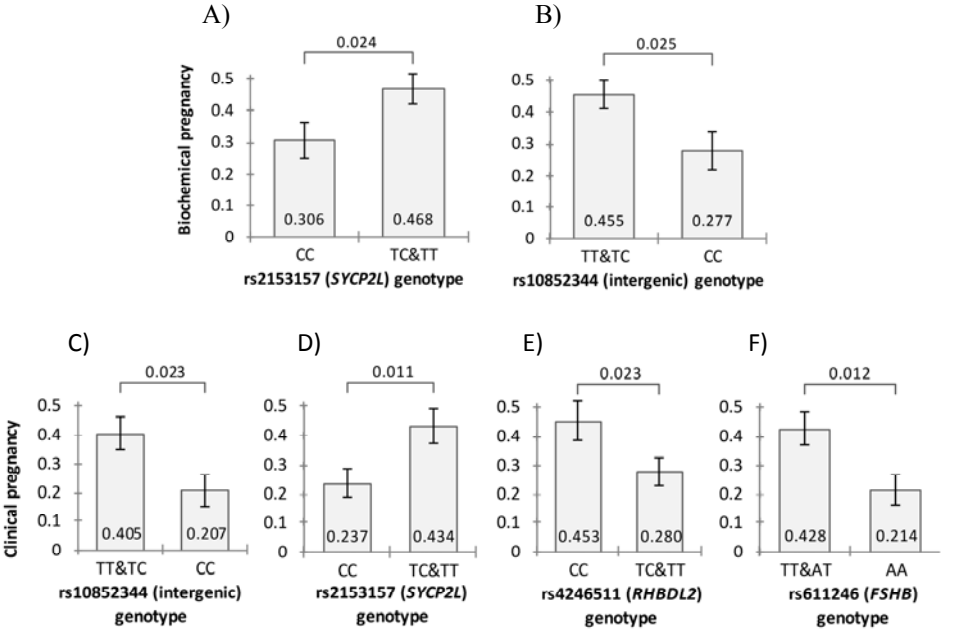

Figure 15. Probability of biochemical pregnancy according to A) rs2153157 (*SYCP2L*) and B) rs10852344 (intergenic) genotypes, and probability of clinical pregnancy according to C) rs10852344 (intergenic), D) rs2153157 (*SYCP2L*), E) rs4246511 (*RHBDL2*) and F) rs611246 (*FSHB*) genotypes. Data presented as estimated probabilities (± standard error) of studied parameters.

In our study, the individuals carrying the *FSHB* rs611246 T-allele were more likely to achieve clinical pregnancy (Figure 15). The core haplotypes identified in the *FSHB* gene region have been associated with conception success in healthy females (Grigorova et al., 2007). Interestingly, in this study the SNPs forming the haplotypes had an effect on the probability of successful (IVF) clinical pregnancy. Grigorova et al. (2007) speculated that *FSHB* gene variants may influence female conception efficiency, and our results seem to support this notion, although contrary to Grigorova et al, our study included only infertile couples. In our study, women with the T-allele had slightly higher FSH values (8.5 IU/L), which still remained in the normal range for follicular phase (3.5–12.5 IU/L), and at the same time had an increased chance of clinical pregnancy compared to AA homozygotes. Whether the positive influence on clinical pregnancy rates is achieved through relatively higher yet normal FSH levels or some other mechanism is currently unclear.

The results of this section highlight the fact that ovarian reserve/IVF-COS effectiveness and chances of a successful pregnancy are closely related, as several of the markers that associated with IVF outcome parameters were also associated with IVF-COS/early follicular phase parameters. Although the most apparent explanation for these associations would be the underlying effect of woman's age, which has a profound effect on both traits (van Loendersloot et al., 2010), this most likely is not the case, as the woman's age was considered as a cofactor in all analyses. For two variants that were linked with several parameters – rs2153157 (*SYCPL2*) and rs4246511 (*RHBDL2*) – two alternative modes of action were proposed that could explain both the associations with IVF-COS and IVF treatment outcome parameters, but for the rest, not enough data is currently available to say anything conclusive about their role in female fertility and further studies are definitely needed.

4.5. Genetic variants related to ovarian aging – potential markers for ovarian function, controlled ovarian stimulation and IVF treatment? (Study V)

Although numerous genetic variants have been associated with traits reflecting the ovarian function and concomitantly proposed as suitable markers, their actual predictive ability is rarely addressed and therefore unknown. Statistical associations obtained in retrospective studies can give valuable hints about the function of the studied variant, yet they are insufficient for assessing the actual clinical validity of this variant. A prospective clinical study would be the preferred method for assessing the true predictive power for any potential biomarker, but at the same time it is also a very time-consuming method, and in certain cases where differential treatment would be applied according to the genotype (such as rFSH dosing in IVF-COS), could also pose serious ethical concerns. As an alternative, retrospective association studies are widely used for

initial selection of biomarker candidates (Gosho et al., 2012) and statistical modelling can be used to initially assess the predictive power of the selected genotypes and then select promising candidates for further clinical validation. Since there is insufficient evidence to currently support the use of any genetic biomarker of ovarian function/IVF-COS in a prospective study, we chose a retrospective study design to test whether genetic variants associated with reproductive aging could be potential candidates that could be considered as markers of ovarian function and IVF-COS. Therefore, in order to evaluate the predictive ability of selected genetic variants and used statistical models, we applied leave-one-out cross-validation modelling and compared the calculated R^2 and AUC values to those obtained for original models. This approach was used both for models including cofactors and for models considering genetic factors without cofactors (clinical data).

A model combining cofactors and all statistically significant genotypes explains 19% and 10% of the variation seen in mean antral follicle count (R^2=0.194) and early follicular phase FSH levels (R^2=0.108), respectively (Table 5). If cross-validation modelling is applied, the numbers decrease to 9% and 2%, respectively for mean follicle count (cross-validation R^2=0.089) and early follicular phase FSH levels (cross-validation R^2=0.019). However, without additional clinical data, the predictive power of statistically significant genotypes decreases remarkably for mean antral follicle count, but remains at ~2–5% for early follicular phase FSH (R^2=0.050, cross-validation R^2=0.017). The relatively poor predictive ability for early follicular phase parameters can be explained by the limited number of participants with early follicular phase data.

The prediction ability of significant genotypes together with selected clinical data for ovarian stimulation parameters is approximately 22–27% (~20% for cross-validation modelling), and without clinical data 4–13% (2–5% for cross-validation modelling) for all studied characteristics. This indicates that the detected markers should have a predictive effect also in prospective studies, if combined with clinical data. The predictive power of selected genotypes without additional clinical data is similar to that reported for other complex traits, such as age at menopause (Stolk et al., 2012) and sex hormone-binding globulin levels (Coviello et al., 2012).

In case of IVF treatment outcome parameters, a cross-validation model combining cofactors and selected (significant) genotypes yielded AUC<0.7 (cross-validation AUC=0.63) for both biochemical and clinical pregnancies (Table 5). If clinical data was excluded from the model, the predictive value of only significant genotypes was modest (AUC<0.6, cross-validation AUC approximately 0.5). This relatively poor predictive ability for IVF treatment outcome parameters is most likely caused by the fact that a successful pregnancy requires a complex dialogue between the embryo and the mother, and the used models are probably insufficient to cover the intricate network of interactions.

Table 5. Results of cross-validation (CV) studies for selected genotypes with a statistically significant effect on studied traits in Study V.

Analysed characteristic	Selected genotypes with cofactors		Selected genotypes without cofactors	
	R^2 / AUC	CV R^2 / AUC	R^2 / AUC	CV R^2 / AUC
Early follicular phase parameters[a]				
Mean ovarian volume[b]	–	–	–	–
Mean antral follicle count	0.194	0.089	0.011	0.000
Early follicular phase FSH	0.108	0.019	0.050	0.017
Ovarian stimulation and outcome parameters[a]				
Total FSH	0.274	0.208	0.132	0.050
Number of OPU follicles	0.248	0.194	0.071	0.028
Number of retrieved oocytes	0.220	0.167	0.050	0.016
Amount of FSH necessary for retrieving one oocyte	0.242	0.199	0.043	0.015
IVF treatment outcome[c]				
Biochemical pregnancies	0.652	0.626	0.574	0.486
Clinical pregnancies	0.694	0.633	0.598	0.523

FSH – follicle stimulating hormone, IVF – in vitro fertilisation, OPU – ovarian puncture
[a] The squared correlation (R^2) between observed and predicted values is reported
[b] No genotypes with statistically significant effect
[c] The area under the ROC-curve (AUC) is reported

The study group for evaluating potential genetic biomarkers was quite diverse and included patients with various diagnoses and of different age. Nevertheless, patients with apparent ovarian dysfunction (PCOS and patients with only one ovary) were excluded from the study. Also, the potential markers of IVF-COS should be usable in everyday practice, which deals with very heterogeneous patients, thus searching for markers in a very homogeneous population of patients might not be very practical in the long run. In addition, all statistical analyses were adjusted for potential confounding factors, which means the reported results are valid regardless of, for example, the diagnosis for infertility, the age of the patient or the stimulation protocol used. Second, since this is a retrospective study, we were not able to assess whether the identified markers hold any actual predictive power in the clinical setting, therefore further studies are warranted for replicating and confirming the current results. Only if results from future studies are consistent with these presented here, a prospective clinical study can be conducted to determine the clinical validity of these marker candidates. However, results of statistical modelling demonstrated that

the detected markers most likely have an actual predictive effect that is more pronounced when combined with relevant clinical data.

Taken together, these results highlight the fact that if in the future a genetic test is used for assessing ovarian function, it is most likely a panel test including tens of SNPs, because the variability seen in complex traits (such as those included in this study) is determined by a large number of genetic variants with a relatively small individual contribution, meaning that even variants that show very strong associations actually explain a very small part of the phenotypic variation, and the specificity and sensitivity of a single marker is insufficient for predictive clinical use (Moron et al., 2007). Furthermore, the statistical modelling performed in our study indicates that for optimal results, genotype data needs to be combined with clinical data and ideally, as proposed before (Wood and Rajkovic, 2013), evaluation of the ovarian function should incorporate genetic testing with relevant medical and family history, and clinical data, including serum markers and ultrasound imaging. Therefore great potential lies in integrating genetic and clinical data and creating predictive algorithms that could be then tested in the clinical setting.

CONCLUSIONS

The main causes for female infertility in Estonia are TFI and PCOS. Both of these conditions are complex diseases, which means that they are caused by a combination of genetic and environmental factors and the individual contribution of each gene and genetic variant is small and individually insufficient to cause the disease. Numerous candidate gene association studies have been carried out for these diseases, but the results have been inconclusive. In the present thesis, the associations between two tandem repeats and PCOS were evaluated. The tandem repeats were selected based on the current knowledge about the pathogenesis of PCOS and were located in the genes for androgen receptor and insulin. In addition, nine genetic variants from four genes involved in immune response regulation were tested for association with TFI.

The second main focus of this thesis revolved around potential genetic biomarkers that could be used for predicting ovarian function in natural and assisted reproduction. For this purpose, the genetic variants identified in studies of ovarian aging and in *FSHB* and *FSHR* genes were evaluated for association with ovarian function, controlled ovarian stimulation and IVF treatment parameters. The potential predictive ability of these markers was assessed using statistical modelling.

Based on the results presented in this thesis, we can make the following conclusions:

I. Genetic variation in insulin (*INS* VNTR) and androgen receptor (*AR* CAG) genes is not a susceptibility factor for PCOS among Estonian women. Furthermore, data from recent studies allow concluding that these two loci are not major susceptibility factors for the disease; however, they may modify certain aspects of the disease phenotype.
II. According to the results presented in this thesis, approximately half of all TFI cases (48–59%) in Estonia can be categorised as having *C. trachomatis*-related TFI. Since infertility caused by infections is practically the only form of infertility that could be avoided, preventive measures and timely interventions could help to significantly reduce the number of women suffering from chlamydia-related infertility in Estonia.
III. Genetic variants in *CCR5* (*CCR5*delta32 deletion), *TLR2* (Arg753Gln) and *TLR4* (Asp299Gly, Thr399Ile) are not associated with TFI. However, several *MBL2* genotypes were identified that may increase (HYA/HYA and *MBL2* low-producing genotypes), or on the contrary, decrease (HYA/LYA) the susceptibility to TFI. Genetic variants in genes regulating immune response are good candidates for finding susceptibility loci for TFI.
IV. Genetic variants related to ovarian aging (e. g. in *RHBDL2* and *SYCP2L*) and in *FSHB* and *FSHR* genes are associated with ovarian function, COS and IVF treatment parameters.

V. Genetic variants related to ovarian aging are potential new biomarker candidates that could be considered as markers of ovarian function, COS and IVF treatment. However, the relatively poor predictive value of only genetic variants highlights the fact that various clinical and genetic biomarkers have to be combined to personalise COS.

In conclusion, the studies in this thesis determined the prevalence of *C. trachomatis*-related TFI in Estonia and identified genetic variants that are associated with susceptibility to TFI in Estonian women. We also showed that genetic variants related to ovarian aging are associated with ovarian function, COS and IVF treatment parameters and could be considered as biomarker candidates if combined with clinical data.

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SUMMARY IN ESTONIAN

Geneetiline varieeruvus kui naisepoolse viljatuse eelsoodumuse mõjutaja ja võimalike uute biomarkerite allikas

Edukaks raseduseks on tarvis naise reproduktiivtrakti erinevate osade – munasarjade, munajuhade ning emaka laitmatut ning hästiajastatud koostööd. Kahjuks on umbes 10%-l paaridest raskusi lapse saamisega ning naisepoolne viljatus on põhjuseks umbes 40%-l juhtudest, mis tähendab et neil naistel on see organite-vaheline koostöö häiritud. Kui mehepoolne viljatus on peamiselt tingitud sperma kvaliteedi langusest, siis naisepoolse viljatuse põhjused on väga mitmekesised ja selle taga võivad olla kahjustunud munajuhad, ovulatsioonihäired, endometrioos või emaka limaskesta probleemid. Eestis on naisepoolse viljatuse peamisteks põhjusteks tubaarne infertiilsus (TFI) ehk munajuhaviljatus ja polütsüstiliste munasarjade sündroom (PCOS), mis tähendab, et neil naistel on häirunud munasarjade ja munajuhade normaalne funktsioon.

TFI korral on koekahjustuse tõttu tekkinud osaline või täielik munajuhade sulgus, mis takistab sugurakkude liikumist ja viljastumist munajuhades ja seega põhjustab probleeme järglaste saamisel. TFI peamiseks põhjuseks on väikevaagna põletikulised haigused, mis on eelkõige tingitud sugulisel teel levivatest patogeenidest – neist levinuim on *Chlamydia trachomatis*, mis põhjustab urogenitaalset klamüüdiainfektsiooni. Lisaks TFI-le võib klamüüdiainfektsiooni tagajärjel suureneda emakavälise raseduse risk. Eestis on TFI kehavälise viljastamise (IVF) ravi peamiseks näidustuseks, samas on urogenitaalse klamüüdiainfektsiooniga seotud TFI levimus Eesti viljatusravi patsientide seas teadmata. On leitud, et geneetiline varieeruvus mõjutab umbes 40% ulatuses seda, milline on immuunvastus klamüüdiainfektsioonile. Kuna inimeste vastuvõtlikkus klamüüdiainfektsioonile, haiguse kulg ja lõpptulem sõltuvad paljudest erinevatest faktoritest, võib eeldada, et ka TFI riski mõjutavad mitmed geenid ja iga geeni ning geenivariandi individuaalne panus on üsna väike. TFI geneetiliste riskifaktorite uuringud on keskendunud kolmele põhilisele grupile – tsütokiinide signaalradadega seotud geenid (*IL-1B*, *IL-1RN*, *IL-6*, *IL-10*, *IL-12A*, *IL-12B*, *TGFB*, *IFNG*), antigeenide esitlemisega seotud geenid (*HLA*, *MICA*) ja mustriretseptorid (*TLR2*, *TLR4*, *TLR9*, *CD14*, *CARD15/NOD2*, *MBL2*), kuid väga väheseid seoseid on näidatud rohkem kui ühes uuringus ja seetõttu on TFI geneetiline taust veel küllaltki ebaselge.

PCOS on hormonaalne häire, mille sagedus reproduktiivses eas naiste hulgas on umbes 10%. Haigusele on iseloomulik väga varieeruv kliiniline pilt, kuid sagedasemate sümptomite seas on menstruaaltsükli häired, kliiniline või biokeemiline hüperandrogenism, polütsüstiline munasarjade morfoloogia, ülekaalulisus ja insuliinresistentsus. Tihti käib haigusega kaasas ka viljatus, mis esineb umbes 40%-l PCOS patsientidest, lisaks on PCOS anovulatoorse viljatuse peamine põhjus. Sündroomi kolm nn. nurgakivi – hüperandrogenism, ovulatsioonihäired ja insuliinresistentsusest tingitud hüperinsulinism on kõik

omavahel tugevas põhjus-tagajärg seoses ja koostoimel tekitavad haigusele iseloomuliku kliinilise pildi, kusjuures iga komponendi individuaalne panus võib olla väga erinev. PCOS-i riski mõjutavaid geene on otsitud peamiselt radadest, mis on seotud haiguse põhitunnustega, ja nii on kaheks enimuuritud variandiks androgeeni retseptori (*AR*) ja insuliini (*INS*) geenides paiknevad tandemjärjestused. Nagu paljudele geneetilistele uuringutele omane, on ka need tööd andnud vastukäivaid tulemusi.

Eelpoolmainitud viljatusprobleemide peamiseks lahenduseks on IVF, mille käigus in vitro viljastatud embrüod siiratakse otse emakasse. Kahjuks on IVF ravi tulemuslikkus umbes 30% ühe ravitsükli kohta ja ravi edukuse määrab eelkõige munasarjade hormonaalse stimulatsiooni (COS) efektiivsus. See aga omakorda sõltub sellest, kuidas reageerivad munasarjad folliikuleid stimuleeriva hormooni (FSH) manustamisele.

Aastate jooksul on palju uuritud nii mehe- kui naisepoolset viljatuse molekulaarset tausta ja on leitud mitmeid seoseid geneetiliste polümorfismide ja viljatust põhjustavate haiguste vahel. Lisaks on jõutud järeldusele, et individuaalne geneetiline varieeruvus mõjutab ka üleüldist viljakust. Kuna individuaalne geneetiline varieeruvus võiks olla ka hea allikas leidmaks reproduktiivfunktsiooni ja ehk isegi IVF ravi tulemuslikkust ennustavaid biomarkereid, on viimasel ajal paljud uuringud keskendunud selliste markerite leidmisele. Tänu nendele uuringutele teame me nüüd oluliselt rohkem viljakust mõjutavate geenide kohta, samas on veel palju ebaselget. Seetõttu soovisime ka oma uuringuga panustada viljakuse ja viljatuse geneetilise tausta selgitamisele. Nii PCOS kui TFI riski mõjutavate geneetiliste faktorite kindlaks tegemine võimaldaks oluliselt paremini mõista nende haiguste tekkemehhanisme ja –põhjuseid. Info reproduktiivfunktsiooni geneetilise tausta kohta võiks olla abiks võimalike geneetiliste biomarkerite valikul ning seeläbi aidata muuta viljatuse ravi ohutumaks ning edukamaks.

Uurimistöö eesmärgid

Käesoleva uurimistöö eesmärgiks oli hinnata seoseid erinevate geneetiliste variatsioonide ja PCOS ning TFI vahel. Lisaks uurisime mitmeid menopausi algusajaga või munasarja funktsiooniga seotud geneetilisi variatsioone, mis võiksid olla seotud ovariaalse reservi, munasarja stimulatsiooni ja IVF ravi tulemuslikkuse parameetritega.

Töö alaeesmärgid olid järgnevad:

I. Analüüsida, kas *INS* ja *AR* geenide varieeruvus on seotud PCOS geneetilise eelsoodumusega.

II. Teha kindlaks urogenitaalse klamüüdiainfektsiooniga seotud TFI levimus Eesti viljatusravi patsientide seas.

III. Uurida, kas immuunvastusega seotud geenide polümorfismid võiksid olla seotud TFI geneetilise eelsoodumusega.

IV. Analüüsida menopausi algusajaga seotud polümorfismide mõju munasarja funktsiooni, munasarja stimulatsiooni ning IVF ravi tulemuslikkust kajastavatele parameetritele.

V. Hinnata, kas menopausi algusajaga seotud polümorfismid võiksid sobida munasarja funktsiooni, munasarja stimulatsiooni tulemuslikkuse ja IVF ravi tulemuslikkuse geneetilisteks biomarkeriteks.

Materjal ja metoodika

Uuringusse kaasati 439 erinevate viljatusdiagnoosidega naist, kellest moodustati uuringu eesmärkidest lähtuvalt väiksemaid uuringugruppe. Uuringus osalevatelt naistelt võeti vereproov, kust eraldati DNA ning seerum. Lisaks olid nende kohta olemas kliinilised andmed üldise tervisliku seisundi, viljatusanamneesi, munasarja stimulatsiooni ja IVF ravi tulemuslikkuse kohta. TFI uuringutesse kaasati lisaks TÜ Eesti Geenivaramu kaasabil 400 naist üldpopulatsioonist (neist 200 tõestatud viljakusega) ja 178 naist, kellel oli diagnoositud emakaväline rasedus, kuid kes teadaolevalt ei olnud viljatud. Nende naiste DNA-d, seerumid ja kliinilised andmed saadi TÜ Eesti Geenivaramust.

Uuringute käigus genotüpiseeriti uuringus osalejatel kokku 47 geenivariatsiooni (tandemjärjestust, deletsiooni ja ühenukleotiidset polümorfismi ehk SNP-d) 22 erinevas geenis (*INS*, *AR*, *CCR5*, *TLR2*, *TLR4*, *MBL2*, *FSHB*, *FSHR*, *TLK1*, *TMEM150B*, *NLRP11*, *MCM8*, *SYCP2L*, *PRIM1*, *FNDC4*, *ASH2L*, *UIMC1*, *KPNA3*, *RHBDL2*, *TDRD3*, *METAP1D*, *RNF44*) ja geenivahelises alas. *AR* geeni varieeruvuse uuringus osalejatel määrati lisaks ka X-kromosoomi inaktivatsiooni muster.

TFI uuringutes osalejatel määrati lisaks vereseerumist *C. trachomatis*-vastased IgG tüüpi antikehad, et teha kindlaks urogenitaalse klamüüdiinfektsiooniga seotud TFI levimus Eesti viljatusravi patsientide seas.

Uurimistöö peamised tulemused ja järeldused

I. Insuliini (*INS* VNTR) ja androgeeni retseptori (*AR* CAG) geenide varieeruvus ei mõjuta oluliselt PCOS haigestumise riski Eesti naiste seas. Lisaks lubavad hiljutiste uuringute tulemused kokkuvõtvalt järeldada, et need kaks variatsiooni ilmselt ei ole seotud PCOS geneetilise eelsoodumusega, küll aga ei saa välistada, et need variatsioonid võivad mõjutada juba avaldunud haiguse fenotüübi erinevaid aspekte.

II. Vastavalt meie uuringu tulemustele on umbes pooled (48–59%) TFI juhud Eestis seotud urogenitaalse klamüüdiainfektsiooniga. Kuna infektsioonidest tingitud viljatus on pea ainuke välditav viljatuse põhjus, võiksid asjakohased ennetusmeetmed ja õigeaegne ravi aidata oluliselt vähendada

urogenitaalsest klamüüdiainfektsioonist tingitud naisepoolse viljatuse esinemist Eestis.

III. Varieeruvus *CCR5* (*CCR5*delta32 deletsioon), *TLR2* (Arg753Gln) and *TLR4* (Asp299Gly, Thr399Ile) geenides ei ole seotud TFI geneetilise eelsoodumusega. Samas leidsime mitmed immuunvastust mõjutavad *MBL2* genotüübid, mis võivad suurendada (kombineeritud genotüüp HYA/HYA ja *MBL2* nn. madala tootlikkusega genotüübid), või vastupidi, vähendada (kombineeritud genotüüp HYA/LYA) TFI riski. Sellest tulenevalt võib järeldada, et immuunvastuse kujunemises osalevate valkude geenid on hea allikas infektsioonide/koekahjustusega seotud TFI geneetilise eelsoodumuse komponentide leidmiseks.

IV. Meie uuring kinnitas, et menopausi algusajaga seotud geenides ja folliikuleid stimuleeriva hormooni β-subühiku (*FSHB*) ja selle retseptori (*FSHR*) geenides olevad SNPd on seotud munasarja funktsiooni, COS ja IVF ravi tulemuslikkuse parameetritega.

V. Menopausi algusajaga seotud geneetilised variatsioonid on võimalikud uued biomarkeri kandidaadid, mida võiks kaaluda munasarja funktsiooni, COS ja IVF ravi tulemuslikkuse markeritena. Samas näitab ainult geneetiliste variantide kesine ennustusvõime seda, et ennustusvõime suurendamiseks tuleb geneetilisi variante kombineerida erinevate kliiniliste andmetega.

Kokkuvõttes näitavad käesoleva töö raames läbi viidud uuringud, et urogenitaalse klamüüdiainfektsiooniga seotud munajuhaviljatus on Eestis suhteliselt sage ja immuunvastust mõjutavate geenide varieeruvus võib mõjutada munajuhaviljatuse riski. Lisaks leidsime, et reproduktiivse vananemisega seotud geneetilised variatsioonid on seotud munasarja funktsiooni, COS ja IVF tulemuslikkuse parameetritega ning tulevikus võiks neid kaaluda võimalike biomarkeritena. Seega võivad uuringu tulemused mõjutada lastetuse ravi edasist arengut, kuna üha täienev infokogum reproduktiivfunktsiooni geneetilise tausta kohta on abiks võimalike geneetiliste biomarkerite valikul ning aitab muuta viljatuse ravi ohutumaks ning edukamaks.

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Employment

2015–…	Competence Centre on Health Technologies, researcher
2009–…	University of Tartu, Women's Clinic, specialist
2010–2013	Competence Centre on Health Technologies (formerly Competence Centre on Reproductive Medicine), researcher
2007–2008	University of Tartu, Institute of Molecular and Cell Biology, department of Biotechnology, specialist

Scientific work

Main research area involves the genetic susceptibility factors for female infertility. Eleven publications have been published in international peer-reviewed journals (excluding abstracts).

Teaching

Lectures on reproductive biology and medicine.

Supervision:
Merlin Pajuva, master's degree in biomedicine, University of Tartu, 2012, (sup) Triin Laisk-Podar, Andres Salumets, "Dopamine receptor expression in endometrium and endometriotic lesions"

Honours & awards

2011	Prof. Marika Mikelsaar's scholarship for student research
2010	Annual Conference of the Estonian Society of Human Genetics, II award for poster presentation
2008	Ministry of Education and Science, The national students' research contest, II award in the field of health research
2006	Ministry of Education and Science, The national students' research contest, II award in the field of health research

ELULOOKIRJELDUS

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Hariduskäik

2008–2015	Tartu Ülikool, doktoriõpingud arstiteaduses
2006–2008	Tartu Ülikool, biomeditsiin
2003–2006	Tartu Ülikool, bioloogia
1997–2003	Tallinna 21. Kool

Ametikäik

2015–…	Tervisetehnoloogiate Arenduskeskus, teadur
2009–…	Tartu Ülikool, naistekliinik, spetsialist
2010–2013	Tervisetehnoloogiate Arenduskeskus (eelnevalt Reproduktiivmeditsiini TAK AS), teadur
2007–2008	Tartu Ülikool, Molekulaar- ja rakubioloogia instituut, biotehnoloogia õppetool, spetsialist

Teaduslik tegevus

Peamiseks uurimisvaldkonnaks on naisepoolse viljatuse geneetilise eelsoodumuse uurimine. Ilmunud on 11 teadusartiklit rahvusvahelistes eelretsenseeritavates ajakirjades.

Õppetöö

Loengud reproduktiivbioloogiast ja -meditsiinist.

Juhendamine:
Merlin Pajuva, magistritöö biomeditsiini erialal, Tartu Ülikool, 2012, (juh) Triin Laisk-Podar, Andres Salumets, “Dopamiini retseptorite ekspressioon endomeetriumis ja endometrioosikolletes”

Teaduspreemiad ja tunnustused

2011	Prof. Marika Mikelsaare üliõpilasteaduse toetamise stipendium
2010	Eesti Inimesegeneetika Ühingu aastakonverents 2010, II preemia posterettekande eest
2008	Haridus- ja Teadusministeerium, II preemia üliõpilaste teadustööde konkursil magistriastmes terviseuuringute valdkonnas
2006	Haridus- ja Teadusministeerium, II preemia üliõpilaste teadustööde konkursil bakalaureuseastmes terviseuuringute valdkonnas

DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS

1. **Heidi-Ingrid Maaroos.** The natural course of gastric ulcer in connection with chronic gastritis and *Helicobacter pylori*. Tartu, 1991.
2. **Mihkel Zilmer.** Na-pump in normal and tumorous brain tissues: Structural, functional and tumorigenesis aspects. Tartu, 1991.
3. **Eero Vasar.** Role of cholecystokinin receptors in the regulation of behaviour and in the action of haloperidol and diazepam. Tartu, 1992.
4. **Tiina Talvik.** Hypoxic-ischaemic brain damage in neonates (clinical, biochemical and brain computed tomographical investigation). Tartu, 1992.
5. **Ants Peetsalu.** Vagotomy in duodenal ulcer disease: A study of gastric acidity, serum pepsinogen I, gastric mucosal histology and *Helicobacter pylori*. Tartu, 1992.
6. **Marika Mikelsaar.** Evaluation of the gastrointestinal microbial ecosystem in health and disease. Tartu, 1992.
7. **Hele Everaus.** Immuno-hormonal interactions in chronic lymphocytic leukaemia and multiple myeloma. Tartu, 1993.
8. **Ruth Mikelsaar.** Etiological factors of diseases in genetically consulted children and newborn screening: dissertation for the commencement of the degree of doctor of medical sciences. Tartu, 1993.
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10. **Katrin Gross.** Multiple sclerosis in South-Estonia (epidemiological and computed tomographical investigations). Tartu, 1993.
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17. **Irja Lutsar.** Infections of the central nervous system in children (epidemiologic, diagnostic and therapeutic aspects, long term outcome). Tartu, 1995.
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