DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS
135

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ANTIBODIES TO FOLLICLE STIMULATING HORMONE

Significance in female infertility

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PRESS

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Dissertation is accepted for commencement of the degree of Doctor of Medical Sciences on April 18, 2007 by the Council of the Faculty of Medicine, University of Tartu, Estonia

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Commencement: May 30, 2007

Publication of this dissertation is granted by University of Tartu

ISSN 1024–395x
ISBN 978–9949–11–585–3 (trükis)
ISBN 978–9949–11–586–0 (PDF)

Tartu Ülikooli Kirjastus
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To my family

CONTENTS

1. ABBREVIATIONS

7AAD	7-aminoactinomycin D
ACA	Anti-cardiolipin autoantibodies
ANA	Antinuclear autoantibodies
ANA-H	Antinuclear autoantibodies detected on human antigen
ANA-R	Antinuclear autoantibodies detected on rodent antigen
AOA	Antiovary autoantibodies
APA	Antiphospholipid autoantibodies
ART	Assisted reproductive technology
Asn	Asparagine
Asp	Aspartate
ASRM	American Society for Reproductive Medicine
B2-GPI	Anti-beta 2-glycoprotein I autoantibodies
CI	Confidential interval
COH	Controlled ovarian hyperstimulation
EDTA	Ethylenedinitrilotetraacetic acid
EIU	Enzyme-immunological units
ELISA	Enzyme-linked immunosorbent assay
ESHRE	European Society for Human Reproduction & Embryology
ET	Embryo transfer
FF	Follicular fluid
FITC	Fluorescein isothiocyanate
FSH	Follicle stimulating hormone
FSHB	Follicle stimulating hormone β-subunit gene
GnRH	Gonadotrophin-releasing hormone
GPH	Glycoprotein hormone
HAP	Haplotype
hCG	Human chorionic gonadotropin
HEp2	Human Epitheliod Cell Line: type 2
HLA	Human leukocyte antigen
HSP	Heat shock proteins
ICSI	Intracytoplasmic sperm injection
Ig	Immunoglobulin
IUI	Intrauterine insemination
IVF	*In vitro* fertilization
LH	Luteinizing hormone
MHC	Major histocompatibility complex
MTF	Women with male or tubal factor infertility
OD	Optical density
OPU	Oocyte pick-up
OR	Odds ratio
PBS	Phosphate buffered saline

PCA	Parietal cell autoantibodies
PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction
PEU	Women with polycystic ovary syndrome, endometriosis, unexplained infertility or infertility due to other reasons
PID	Pelvic inflammatory disease
PN	Pronucleus
POF	Premature ovarian failure
RFLP	Restriction fragment length polymorphism analysis
SCA	Steroid cell autoantibodies
SD	Standard deviation
SLE	Systemic lupus erythematosus
SMA	Smooth muscle autoantibodies
SNP	Single nucleotide polymorphism
SPC	Spontaneous menstrual cycle
STD	Sexually transmitted diseases
$TGF\beta_1$	Transforming growth factor β-1
TMA	Thyroid microsomal autoantibodies
TPO	Thyroid peroxidase
TRIS	2-amino-2-(hydroxymethyl)propane-1,3-diol
TSH	Thyroid stimulating hormone
V14D	Synthetic peptide corresponding to the 78–93 region of the human FSH β-chain
WHO	World Health Organization

2. ORIGINAL PUBLICATIONS

I **Haller K, Mathieu C, Rull K, Matt K, Béné MC, Uibo R.** IgG, IgA and IgM antibodies against FSH: serological markers of pathogenic autoimmunity or of normal immunoregulation? Am J Reprod Immunol 2005, 54:262–269.

II **Haller K, Salumets A, Grigorova M, Talja I, Salur L, Béné MC, Laan M, Uibo R.** Putative predictors of antibodies against follicle-stimulating hormone in female infertility: a study based on in vitro fertilization patients. Am J Reprod Immunol 2007, 57:193–200.

III **Haller K, Sikut A, Karro H, Uibo R, Salumets A.** Levels of anti-follicle-stimulating hormone immunoglobulin A (IgA) correlate with anti-sperm IgA in sera of female infertility patients: a sperm-prone reaction of mucosal tolerance? Manuscript submitted to Am J Reprod Immunol.

IV **Haller K, Sarapik A, Talja I, Salumets A, Uibo R.** Controlled ovarian hyperstimulation changes the prevalence of serum autoantibodies in *in vitro* fertilization patients. Am J Reprod Immunol 2006, 56:364–370.

V **Haller K, Salumets A, Talving E, Karits P, Uibo R.** Effects of antibodies against follicle stimulating hormone on controlled ovarian hyperstimulation (COH) utilizing the GnRH antagonist protocol. Manuscript submitted to Fertil Steril.

3. INTRODUCTION

Reproductive health implies that people are able to have a satisfying and safe sex life and that they have the capability to reproduce and the freedom to decide if, when and how often to do so. Reproductive illhealth accounts for >30% of the overall burden of disease among women and for 12% among men (The ESHRE Capri Workshop, 1996). Infertility contributes a great proportion to overall reproductive illhealth, since there are ~60–80 million infertile couples (~15% of couples) around the world (The ESHRE Capri Workshop, 1996). Accordingly, approximately 15 000 infertile couples would be suspected in Estonia. Although infertility *per se* may not threaten physical health, it may have a serious impact on the mental and social well-being of couples and may result in detrimental social consequences, such as divorce or ostracism (The ESHRE Capri Workshop, 1996). In addition, infertility contributes to low birthrate, which is a major social and national issue in whole Europe as well as in Estonia.

Infertility represents an increasing medical problem. A progressive decrease in fertility rate (number of live births to the total population of women of reproductive age) has been indicated since 1955 and that of within the same age groups (The ESHRE Capri Workshop Group, 2002). The decrease in fertility rate is associated with both medical and non-medical factors. Women's age is the major determinant of the average time required to conceive in all couples and particularly in those who have deferred conception until the woman is in her mid thirties. The highest live birth rates are in the age group of 25–30 years and declines sharply after the age of 35 years (Templeton *et al.,* 1996). Also, the duration of infertility contributes meaningful information to the estimation of future fertility, since each year of infertility diminishes the chance of a birth significantly (Templeton and Morris, 1998). In addition, the longer the duration of infertility, the higher the probability of the presence of genetic or more severe pathological factors. Chromosomal aberrations, monogenic diseases, endocrine dysfunctions, sexually transmitted diseases (STD) and immune system dysfunctions are medical situations, which can contribute both to male and female infertility. Unfortunately, still in about 20% of couples, the infertility cause remains unknown (The ESHRE Capri Workshop, 1996). However, autoimmune mechanisms have been associated with premature ovarian failure (POF), ´subclinical´ ovarian failure and with recurrent pregnancy loss (The ESHRE Capri Workshop Group, 2002).

Merely, every second infertile couple seeks for medical advice (The ESHRE Capri Workshop, 1996). Historically, infertility has been difficult to treat, with only <50% of treated infertile women being successful in achieving a live birth until the 1980's (The ESHRE Capri Workshop, 1996). Nowadays, the utilization of assisted reproduction technology (ART) has improved the prospects of infertility treatment. First child following *in vitro* fertilization

(IVF) was born in 1978 (Steptoe and Edwards, 1978). In Estonia, IVF has been available since 1994. Today, approximately 1.4% of newborns account for IVF treated couples in Estonia, which corresponds to the majority of other European countries, but 1/3 of the same figure in Nordic countries (Nygren and Andersen, 2002). Regardless of constant improvement of pregnancy rate in IVF, the success rates are still around 30% per cycle (Nygren and Andersen, 2002). Autoimmunity and the presence of autoantibodies have been invoked as a possible mechanism of IVF failure. There are contradicting data regarding the importance of certain antibodies to damage directly the preimplantation embryo, interfering with implantation process or formation of placenta (Geva *et al.,* 1994; Gleicher *et al.,* 1994; Van Voorhis and Stovall, 1997; Mecacci *et al.,* 2000; Hornstein *et al.,* 2000; Chilcott *et al.,* 2000). Consequently, the overall activation of the immune system in female infertility has been suggested (Gleicher, 2001).

For the purpose of improving infertility treatment, the mechanisms of immune system associated with healthy reproduction as well as with infertility should be carefully evaluated. This study was carried out in the Immunology Group, Institute of General and Molecular Pathology, University of Tartu, Estonia. Infertility associated immune disorders has been one of the research projects of our laboratory since 1990's. Earlier studies on immune mechanisms of infertility have been carried out in collaboration with andrologists and colleagues from Women's Clinic of Tartu University Hospital, Estonia; but also from the University of Sheffield, UK and from the University of Tampere, Finland. The current study was carried out in collaboration with the Nova Vita Clinic, Centre for Infertility Treatment and Medical Genetics, Viimsi, Estonia; Laboratoire d'Immunologie, Faculté de Médecine, Université Henri Poincaré, Nancy, France; the Institute of Molecular and Cell Biology, University of Tartu, Estonia and the Department of Obstetrics and Gynecology, University of Tartu, Estonia.

4. REVIEW OF THE LITERATURE

4.1. Infertility

Infertility is a condition that affects a couple and is defined as the lack of conception after an arbitrary period of 12 months without using any contraception (The ESHRE Capri Workshop Group, 2002). These couples comprise the infertile population and include the sterile members of the population, for whom is no possibility of natural pregnancy, and the remainder who are subfertile (The ESHRE Capri Workshop, 1996). The latter inadvertently includes normal fertile females who failed to conceive by chance during the 12 or 13 opportunities a woman has per year (The ESHRE Capri Workshop Group, 2002).

Approximately 15% of couples suffer from infertility in all over the world. Half of the infertility in couples has been caused by the female and a half by the male factor infertility. However, the etiology of the infertility remains to be unexplained in more than 20% of the couples. Considering the pattern of diagnoses for both partners of the infertile couples shows, that minor fertility impairment are seen in both partners more frequently than expected (>70% of infertile couples). Such fertility impairments are not necessarily associated with infertility when present only in one partner, but may become important when present in both partners. Only about 3–15% of infertile couples represent a firm cause of sterility of at least one partner (The ESHRE Capri Workshop, 1996). Thus, there is a strong case of investigating and treating both partners except when there is a condition associated with sterility.

4.1.1. Male infertility

Male infertility is assessed based on medical history, physical examination and semen analysis (Table I). If a male infertility factor is present, it is almost defined by the finding of an abnormal semen analysis. However, the prevalence of diminished sperm count among men population can vary in different countries (Jørgensen *et al.,* 2002; Punab *et al*., 2002). The idiopathic abnormal semen quality (Table II) without identification an etiological factor has been noted in about half of the cases where any extent of fertility impairment in men could be detected (The ESHRE Capri Workshop, 1996). In presence of a non-obstructive azoospermia or oligoasthenoteratozoospermia one could suspect the chromosomal aberrations, such as numerical sex chromosomal aberrations (47XXY, 46XY/47XXY, 47XYY), 46XX sex-reversal males, structural Y-chromosomal anomalies, Robertsonian and reciprocal translocations, Y-chromosome microdeletions or monogenic defects such as cystic fibrosis (The

ESHRE Capri Workshop Group, 2002; Shah *et al.,* 2003). Recently, it has been suggested that impaired semen parameters can be associated with poor sperm DNA integrity (The Practice Committee of the American Society for Reproductive Medicine, 2006b). Approximately 25% of sperm cells exhibited DNA denaturation and 28% have DNA fragmentation in infertile men, while these DNA damages were detected only in 10% and 13% of fertile men, respectively (The Practice Committee of the American Society for Reproductive Medicine, 2006b). The causes for poor DNA integrity have been suspected to be protamine deficiency, various reasons for DNA packaging defects, abortive apoptosis in spermatozoa, DNA damaging by reactive oxygen species, but also the external factors such as heat, chemotherapeutic agents, radiation, cigarette smoking, genital tract inflammation, varicocele or hormonal deficiency (The Practice Committee of the American Society for Reproductive Medicine, 2006b).

Table I. Normal values of semen parameters (WHO, 1999)

Standard tests	
Volume	≥2.0 ml
pH	≥7.2
Sperm concentration	$\geq 20 \times 10^6$ spermatozoa/ml
Total sperm count	$\geq 40 \times 10^6$ spermatozoa per ejaculate
Motility	≥50% with motile (grades a + b) or ≥25% with progressive motility (grade a) within 60 min of ejaculation
Morphology	>15% with normal forms
Vitality	≥50% live
White blood cells	$<1 \times 10^6$/ml
Immunobead test	<50% motile spermatozoa with beads bound
Mixed agglutination reaction test	<50% motile spermatozoa with adherent particles

Varicocele is defined as abnormally dilated scrotal veins. Venous reflux and testicular temperature elevation appear to play important roles in varicocele-induced testicular dysfunction, but only larger palpable varicoceles have been clearly associated with infertility (The Practice Committee of the American Society for Reproductive Medicine, 2006c). Varicocele is the most common abnormality found on examination the testis, and is present in 15% of the normal male population and in approximately 40% of men presenting with infertility (The Practice Committee of the American Society for Reproductive

Medicine, 2006c). However, in association with abnormal semen measurements approximately 15% of men in infertile couples have been diagnosed with varicocele (The ESHRE Capri Workshop, 1996).

Infections, autoimmune orchitis, anti-sperm antibodies, endocrine hypogonadism, maldescended testes, ejaculatory or erectile dysfunctions, general diseases, testicular tumors and obstructions are also considerable causes for male infertility (The ESHRE Capri Workshop, 1996).

Table II. Nomenclature for normal and pathological findings in semen analysis (WHO, 1999)

Nomenclature	Description
Normozoospermia	Normal ejaculate (as defined in Table I)
Oligozoospermia	Sperm concentration less than reference value
Asthenozoospermia	Less than reference values for motility
Teratozoospermia	Less than reference values for morphology
Oligoasthenoteratozoospermia	Signifies disturbance of all three variables (combinations of only two may also be used)
Azoospermia	No spermatozoa in the ejaculate
Aspermia	No ejaculate

4.1.2. Female infertility

Female fertility is regulated by a series of highly coordinated and synchronized interactions in the hypothalamic-pituitary-ovarian axis. Therefore, female fertility can be affected by diseases or dysfunctions of reproductive tract, neuroendocrine system, and immune system or by any severe or exhausting general disease. The etiology of female infertility in a diagnostic and treatment point of view is summarized in Table III (based on the guidelines provided by The ESHRE Capri Workshop, 1996 and The ESHRE Capri Workshop Group, 2002).

In Western Europe and North America, where tubal diseases are relatively uncommon, endocrine dysfunctions can be identified in about 10–20% of women presenting with infertility (The ESHRE Capri Workshop Group, 2000). The primary defect may lie in any component of the hypothalamic-pituitary-ovarian axis leading to anovulation. A pituitary adenoma can be identified in almost 50% of women with hyperprolactinaemia. Hyperprolactinaemia may affect fecundity solely by affecting ovulation and implantation through hormonal but also through immunological processes, since prolactin enhances antibody production (Gleicher, 1998). Hypogonadotrophic hypogonadism can

be caused by multiple factors associated with failure of secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH). Weight loss is far most common cause of it in western societies. Anovulation can occur with modest losses (around 10% of body weight) due to decreased secretion of gonadotrophin-releasing hormone (GnRH) by the hypothalamus (The ESHRE Capri Workshop Group, 2002).

Table III. Etiology of female infertility (based on the diagnostic and treatment guidelines provided by The ESHRE Capri Workshop, 1996 and The ESHRE Capri Workshop Group, 2002)

Anovulatory infertility
Hyperprolactinaemia
Pituitary adenoma
Hypogonadotrophic hypogonadism
Kallmann's syndrome
Weight loss
Hypergonadotrophic hypogonadism
Premature ovarian failure (POF) and early menopause
Gonadotropin resistance due to a receptor defect
'Normogonadotrophic' oligo-anovulation
Polycystic ovary syndrome (PCOS)
Adrenal cause of hyperandrogenism
Genetic determinants
Turner syndrome, Swyer syndrome
Androgen insensitivity syndrome
Androgen synthesis disorders
Tuboperitoneal infertility
Tubal factor infertility
Endometriosis
Autoimmunity
POF
Recurrent pregnancy loss
Autoimmunity associated infertility
Uterine abnormalities
Malformations
Submucous myomas
Endometrial adhesions
Unexplained infertility

Most common cause for hypergonadotrophic hypogonadism is POF (The ESHRE Capri Workshop Group, 2002). POF is defined as secondary amenorrhea with elevated gonadotrophin levels observed under the age of 40. However, if it occurs between 41 and 44 years, it is defined as early menopause.

The two forms affect 1–2% and 5% of women of the general population (The ESHRE Capri Workshop Group, 2002). POF is highly heterogeneous condition and can be associated with autoimmune disorders, ovarian surgery, iatrogenic causes such as chemo-radiotherapy, systemic diseases such as galactosaemia, or with genetic factors. Both, X-chromosomal abnormalities (45,X; 45,X/46,XX mosaicism, deletions in X-chromosome, fragile X syndrome) and mutations in autosomal (FSH receptor gene) genes have been associated with the development of POF (The ESHRE Capri Workshop Group, 2002). In more than half of the cases, the development of POF has been associated with autoimmune reactions to ovarian tissue (Monnier-Barbarino *et al.,* 2005).

Normogonadotrophic anovulation represents about 50% of women with an endocrine cause of infertility, and includes mostly the patients with polycystic ovary syndrome (PCOS). PCOS affects up to 4–10% of all women of reproductive age (Dunaif, 1995; Knochenhauer *et al.,* 1998). PCOS is characterized by polycystic ovaries, oligo-anovulation, insulin resistance, and hyperandrogenism or hyperandrogenaemia (Dunaif, 1997; The Rotterdam ESHRE/ASRM-sponsored PCOS workshop group, 2004). Infertility in PCOS is associated with an alteration in folliculogenesis and in the selection of the dominant follicle leading to anovulation (Harborne *et al.,* 2003). The etiology of this syndrome is unknown, but it has a genetic component, which includes variations in genes involved in metabolic homeostasis (Franks, 1995). Thus, it has become clear that polycystic ovaries are a secondary functional derangement and not a specific primary local defect (Jonard and Dewailly, 2004).

Tubal factor infertility accounts for 10–30% in developed countries and up to 85% in developing countries of reported cases of infertility (The Practice Committee of the American Society for Reproductive Medicine, 2006a, Land and Evers, 2002). Decreased fecundity may be attributed to impaired ovum transport due to fimbrial damage and/or adnexal adhesions. The factors responsible for tubal disease are diverse and include infections, pelvic surgery, and endometriosis. Pelvic inflammatory disease (PID) represents the link between STD and infertility. In majority of cases, acute PID results from acute bacterial endometritis and salpingitis. Most of the long-term consequences of PID, however, stem from the destruction of normal tubal structure, with or without tubal occlusion (The ESHRE Capri Workshop Group, 2002). While in western countries, there has been a decline in the incidence of STD salpingitis and correspondingly in PID by the end of 1980's, a significant rise in the incidence of STD in Estonia, similar to that in Eastern Europe and central Asia, has been documented at the beginning of 1990's (The ESHRE Capri Workshop, 1996; Uusküla *et al.,* 1997; Uusküla *et al.,* 2001). The increased incidence of STD in Estonia has been associated with the elevated incidence of traditional venereal diseases such as gonorrhea and syphilis (Uusküla *et al.,* 1997). Still, every year, 1–2% of all young, sexually active women suffer from PID. The incidence of infertility following the acute PID depends on various factors and

varies from 6 to 60% (The ESHRE Capri Workshop Group, 2002). In addition, there is a silent, relatively asymptomatic PID, which, according to different authors, could be the case of as much as in 30–80% of chlamydial infections (Land and Evers, 2002; Wiesenfeld *et al.,* 2002). Genital infection of *Chlamydia trachomatis* is currently the most common bacterial STD (in 20–40% of cases) and it coexists with the infection of *Neisseria gonorrheae* in 25–50% of cases (The ESHRE Capri Workshop Group, 2002).

Endometriosis is characterized by the growth of endometrial tissue outside the uterine cavity. It is a common disorder, affecting 10–20% of all women of reproductive age (Wheeler, 1989; Goldman and Cramer, 1990). Studies have been suggested that up to 50% of infertile women have endometriosis and likewise, that up to 50% of women with endometriosis are infertile (The Practice Committee of the American Society for Reproductive Medicine, 2006f). The most frequent clinical presentations of endometriosis include dysmenorrhea, pelvic pain, dyspareunia, infertility, and pelvic mass. The diagnosis of this condition is often problematic and direct assessment of endometriotic foci at laparoscopy has been viewed as a 'gold standard' for identifying endometriosis (American Society for Reproductive Medicine, 1997). However, the correlation of laparoscopic observations with histological findings and with clinical symptoms is often low. Distorted pelvic anatomy, altered peritoneal function, altered humoral and cell-mediated immune system reactions, impaired implantation, and endocrine and ovulatory abnormalities, all contribute to the endometriosis-associated female infertility (The Practice Committee of the American Society for Reproductive Medicine, 2006f). It has been proposed; that infertility in patients with lesions of endometriosis stage I–II is predominantly caused by immunological factors, while in more advanced stages of III–IV the infertility has been associated with distorted pelvic anatomy (Ulĉová-Gallová *et al.,* 2002).

Approximately 20–30% of couples who are unable to conceive are determined to have unexplained infertility (The ESHRE Capri Workshop, 1996; The Practice Committee of the American Society for Reproductive Medicine, 2006d). Unexplained infertility is a term applied to an infertile couple whose standard investigations (semen analysis, tubal potency and laboratory assessment of ovulation) yield normal results. A longer period has been suggested to be required for this group of patients to achieve pregnancy without treatment, as 70% of fertility rate is achieved in two years for the group of unexplained infertility, whereas only nine months are required for the fertile group to achieve the same rate (Barnea *et al.,* 1985). However, about 20–30% of these patients remain infertile even after 9 years of attempting to conceive (Templeton and Penney, 1982). Therefore, unexplained infertility appears to represent either the lower extreme of normal distribution of fertility, or it arises from a defect in fecundity that cannot be detected by the routine infertility evaluation (The Practice Committee of the American Society for Reproductive Medicine,

2006d). Although no biological defect is known, older age of female partner or the presence of latent or undetectable defects are believed to be major problems contributing to unexplained infertility (The ESHRE Capri workshop, 1996). Dysregulation in immune system reactions and subclinical endometriosis have been putative etiologic candidates for this group of patients (Dmowski, 1995; Luborsky *et al*., 1999; Jasper *et al.,* 2006).

4.2. Autoimmune reactions in female infertility

4.2.1. Autoimmunity

Active tolerance mechanisms are required to prevent inflammatory responses to the many innocuous air-borne and food antigens that are encountered at mucosal surfaces. However, the most important aspect of tolerance is self tolerance, which prevents the body from mounting an immune attack against its own tissues — prevention from autoimmune reactions. Autoimmunity is associated with a dysbalance of various components of the immune response and with the development of autoantibodies directed against normal host antigens. The susceptibility to autoimmune reactions is regulated at several levels (Nepom and Kwok, 1998). The proliferation of mature T-lymphocytes in response to either self- or foreign antigenic stimuli is affected by the nature and strength of antigenic peptide-MHC (major histocompatibility complex) stimulation (Nepom and Kwok, 1998; Muraro and Douek, 2006). Human leukocyte antigen (HLA)-class II molecules influence the stability of the antigenic-peptide-HLA complex in an allele-specific manner, affecting the induction of central tolerance (Nepom and Kwok, 1998). As revealed by the studies on anti-insulin autoimmunity in the murine models of diabetes, the stimulation provided by antigenic peptide-MHC stimulation could also be modulated by genetic variations of the insulin gene, potentially influencing the gene expression in the thymus (Vafiadis *et al.,* 2001; Jasinski and Eisenbarth, 2005). Tissue-specific autoimmunity appears to be additionally dependent on local factors, including infection-related tissue damage (Nepom and Kwok, 1998), iatrogenic manipulations (Gobert *et al.,* 1992), and the level of autoantigen in periphery (Fénichel *et al.,* 1999; Byersdorfer *et al.,* 2005). Thus, the expansion of cells responding to low-affinity ligands (self-antigen) or anomalies in the deletion of high-affinity autoreactive T-cells can lead to autoimmune reactions (Muraro and Douek, 2006). Once an autoimmune disease has been developed, a wider range of autoimmune reactions may progress, meaning that an individual may have more than one autoimmune disease (Tuohy *et al.,* 2000; Mackay and Rowley, 2004).

4.2.2. Reproductive autoimmune failure

The reproductive autoimmune failure syndrome was originally described by Gleicher *et al.* in women with endometriosis, infertility and increased autoantibodies (Gleicher *et al.,* 1989). Autoimmune mechanisms as well as an increased production of multiple autoantibodies are involved in such infertility disorders as POF, endometriosis, PCOS, unexplained infertility, repeatedly unsuccessful IVF attempts and may be responsible for the pathophysiology of preeclampsia or spontaneous abortions (Geva *et al.,* 1997; Fénichel *et al.,* 1999; Reimand *et al.,* 2001; Matarese *et al.,* 2003; Forges *et al.,* 2004).

Endometriosis has been labeled an 'autoimmune syndrome' because of the three major immune system abnormalities suggested and investigated: (i) the presence of endometriotic lesions has been proposed to trigger a specific B-cell response leading to production of anti-endometrial antibodies (to endometrial transferrin and α_2-Heremans–Schmid glycoprotein), (ii) deficiency in cellular immunity, particular natural killer cells and CD8+ T-lymphocytes, towards endometriotic tissue, (iii) endometriosis has been linked to classical autoimmune diseases, such as systemic lupus erythematosus (SLE) (Gleicher, 1994; Burns and Schenken, 1999; Mathur *et al.,* 1999; Mathur, 2000; Lang and Yeaman, 2001). Classical autoimmune diseases, as well as endometriosis, are characterized by polyclonal B-cell activation and production of multiple different autoantibodies. About 40–60% of patients with endometriosis have elevated autoantibody titers when tested against a panel of autoantigens (Lebovic *et al.,* 2001). Patients with endometriosis often possess antiovary antibodies (AOA), antinuclear autoantibodies (ANA), smooth muscle autoantibodies (SMA), and antiphospholipid antibodies (APA) (Mathur *et al.,* 1982; Geva *et al.,* 1997; Van Voohris and Stovall, 1997; Ulĉová-Gallová *et al.,* 2002).

An autoimmune mechanism has also been suggested in some cases of PCOS, where increased prevalence of AOA and common organ- and non-organspecific autoantibodies has been detected (Fénichel *et al.,* 1999; Reimand *et al.,* 2001; Forges *et al.* 2004; Janssen *et al.,* 2004).

Manifestation of tubal destruction, however, is dependent also from the ability to activate autoimmune inflammation. During chlamydial infection, as that of in most infections, the synthesis of heat shock proteins (HSPs) is strongly up-regulated. HSPs are the major antigens and can induce a strong immune response. Because there is a strong amino acid sequence homology between microbial and human HSPs, the induced immune response against microbial HSPs may incite an autoimmune inflammatory reaction in the host, culminating in tubal damage (Land and Evers, 2002).

Many cases of POF are unexplained, and since POF could be associated with nearly all organ-specific autoimmune diseases, it is hypothesized that some of these arise from ovarian autoimmunity (Forges *et al.,* 2004). An investigation of antiovarian autoimmune reactions and autoantibodies may be severely

hampered by the fact that POF represents an end-stage of disease. By the time a women is diagnosed, she has by definition exhausted her follicular supply and, presumably, also the target antigen for the autoimmune attack on her ovary. Thus, the autoimmunity causal of POF can be difficult to detect retrospectively. Regardless of that, high prevalence of AOA (30–67%) and others organ- and non-organspecific autoantibodies have been observed in patients with POF (Fénichel *et al.,* 1997; Luborsky *et al.,* 1999; Luborsky, 2002; Monnier-Barbarino *et al.,* 2005; Kelkar *et al.,* 2005).

Thyroid autoantibodies have been associated with recurrent pregnancy loss, POF and repeatedly unsuccessful IVF attempts (Gleicher, 1998; Bussen *et al.,* 2000; Mecacci, 2000; Poppe and Velkeniers, 2002). The frequent association of the presence of thyroid autoantibodies and miscarriages is hypothetically explained by the fact that organ specific autoimmune diseases may be secondary to some basic cellular abnormality that directly affects pregnancy outcome (Poppe and Velkeniers, 2002; Putowski *et al.,* 2004). Repeated IVF failure has been associated with increased prevalence of many autoantibodies, including AOA, APA, ANA, SMA, and anti-sperm antibodies (Geva *et al.,* 1999; Luborsky, 2002; Putowski *et al.,* 2004). Therefore, the failure in differentiation of uterine T-cells into T-regulatory cells, as a key determinant of fertility in women has been suggested to be a case in unexplained infertility (Jasper *et al.,* 2006). Since the prevalence of AOA in unexplained infertility and POF has been detected similar, the unexplained infertility was suggested to represent an early stage of autoimmune POF (Luborsky *et al.,* 1999).

The impact of a particular autoantibody on the pathogenesis of infertility is not uniformly understood. ANA could interfere with early implantation of embryo and SMA could alter the fallopian tube function (Geva *et al.,* 1997). It is concluded, that APA may be involved in uterine vascular modifications affecting implantation processes (Battaglia *et al.,* 1998). Except AOA in ovulatory dysfunctions and disease-specific autoantibodies described in case of endometriosis (Mathur *et al.,* 1999; Mathur, 2000; Lang and Yeaman, 2001), autoantibodies detected in infertile patients are usually not specific to infertility or to the gynecological diseases leading to infertility. Furthermore, the number of detectible autoantibodies, in particular, has been proposed to predict the pregnancy rate of IVF treatment (Dmowski *et al.,* 1995). Therefore some studies suggest lesser importance of specific autoantibodies and stress the key role of overall activation of the immune system in reduced fecundity (Dmowski *et al.,* 1995; Kaider *et al.,* 1999; Gleicher, 2001). Consequently, the autoimmune-associated infertility might be a polyclonal event characterized by immunological defects at the T-cell level which, similarly to classical autoimmune diseases, may manifest itself in abnormal antibody production (Gleicher, 1998).

4.2.3. Antiovary autoantibodies

Although the presence of AOA immunoglobulin G (IgG) has been documented in different groups of infertile patients, there are no epidemiological studies of ovarian autoimmunity. Using an estimated prevalence of autoimmune POF, about 1.1 million women potentially have ovarian autoimmunity solely in US, which makes ovarian autoimmunity far more common than Addison's disease, myasthenia gravis or SLE (Luborsky, 2002).

Some antibodies in the pool of AOA are suggested to associate with a direct action on ovarian tissue, whereas others do not have cellular effects, similar to autoantibodies in other autoimmune diseases (Luborsky, 2002). Therefore, it is possible, that several different antigens are involved in ovarian autoimmunity, as both ovarian cellular and zona pellucida/oocyte antibodies have been reported. Anti-oocyte antibodies were identified already in 1966 and this was also one of the first descriptions of antiovarian autoimmunity (Vallotton and Forbes, 1966; Forges *et al.,* 2004). High prevalence of anti-zona pellucida antibodies have been detected in infertile women, but also in healthy fertile women and even in men (Forges *et al.,* 2004). Antibodies to steroid cells (SCA) are more prevalent in POF patients with Addison's disease (73–87%), but rare in those patients with other autoimmune disease (0–8%) or in 0–10% of patients with isolated POF (Forges *et al.,* 2004). Steroidogenic enzymes such as 17α-hydroxylse, desmolase (P450-side chain cleavage), 3β-hydroxysteroid dehydrogenase, 21-hydroxylase have been detected as the molecular targets of SCA (Chen *et al.,* 1996, Arif *et al.,* 1996; Peterson *et al.,* 1997). Gonadotrophin receptors have been also investigated as a potential autoantibody targets. While antibodies against LH receptor were first identified in 30% of IVF patients and in 50% of infertile patients with endometriosis (Moncayo *et al.,* 1989; Wheatcroft *et al.,* 1994), only few cases of POF patients possessing antibodies to FSH receptor was documented (Chiauzzi *et al.,* 1982). A later study on FSH receptor blocking ability of these antibodies has allowed the questioning of pathophysiological role of anti-FSH receptor antibodies in ovarian failure (Reznik *et al.,* 1998).

Although blocking antibodies are usually considered to interact with receptors, the FSH and LH activity-inhibiting antibodies could also directly recognize gonadotrophins themselves. The presence of anti-FSH and anti-LH antibodies in poor responder IVF patients has been associated with immunization against exogenous gonadotrophins (Meyer *et al.,* 1990). To date, anti-gonadotrophin antibodies have been described only in POF patients and that with conflicting results. By using different antibody assays, some authors suggest the importance of only anti-LH antibodies (Luborsky *et al.,* 1990), while others evidence the association of POF with anti-FSH antibodies (Gobert *et al.,* 2001). The latter group presented antibodies against β-subunit of FSH in nearly all of the studied AOA-positive POF patients and no anti-LH activity

was detected in these samples. Moreover, these antibodies recognized epitopes all over the β-subunit molecule, but a region between amino acids 78 and 93 (V14D) was predominantly recognized in all samples, probably representing the immunodominant epitope (Gobert *et al.,* 2001). The antibodies detected could readily explain the ovarian failure in POF patients, since this part of the β-subunit of FSH molecule is directly involved in determining the specificity of receptor binding (Fox *et al.,* 2001). The ability of anti-FSH to inhibit the function of FSH hormone has been detected in men (Westhoff *et al.,* 1996; Yao *et al.,* 2004). However, there is no data available according to the presence of anti-FSH IgG, not to mention of IgA or IgM, neither in different etiologic groups of female infertility nor in healthy women. In addition, the etiologic factors for producing anti-FSH antibodies of all subtypes as well as the putative pathological role of these antibodies in folliculogenesis or effectiveness of infertility treatment are not described. In addition, no data is available about the detection of these antibodies locally in ovarian follicles. Also, nothing can be concluded regarding to the production of these antibodies during pregnancy. Pregnancy itself is accompanied with a suppression of the development of new ovulating follicles. This ovulatory quiescence is due to an inhibition of the pituitary during pregnancy, as seen in the decreased response of FSH and LH to GnRH administration (Rubenstein *et al.,* 1978).

4.3. Treatment of infertility

Treatment of primary disease could also improve the fertility in both men and women. However, among all infertility causes, STD as the prerequisite for most common cause of infertility — tubal factor infertility — is the only preventable cause. Treatments for tubal damage include reconstructive surgery (The Practice Committee of the American Society for Reproductive Medicine, 2006a). Varicocele repair is option for the management of couples with male factor infertility associated with varicocele (The Practice Committee of the American Society for Reproductive Medicine, 2006c). Endometriosis can be treated medically with ovulation-suppressing drugs or by surgical ablation of the pelvic implants (The ESHRE Capri Workshop, 1996). In the absence of correctable abnormality, the therapy for unexplained infertility is empiric. Depending on the etiology of infertility, further treatment regimens include intrauterine insemination (IUI), ovulation induction with oral or injectible medications (clomiphene citrate or gonadotrophin therapy), combination of IUI with ovulation induction, and ART (The Practice Committee of the American Society for Reproductive Medicine, 2006d).

The IVF-embryo transfer (ET) has become a promising treatment for infertility of many causes. During IVF procedure, multiple follicles are enabled to grow and mature by stimulating the ovaries with the administration of exo-

genous FSH by either GnRH antagonists' or agonists' protocols. This procedure is routinely known as controlled ovarian hyperstimulation (COH). Cumulus-oocyte complexes are retrieved from the ovaries by vaginal ultrasound-guided needles. In the classical IVF, punctured cumulus-oocyte complexes are inseminated a few hours later with progressively motile spermatozoa. In procedure called intracytoplasmic sperm injection (ICSI) cumulus cells are removed from punctured oocytes and a single sperm is selected and injected directly into the cytoplasm of oocyte (Palermo *et al.,* 1996). ICSI is utilized for the treatment of infertility due to male factor or selected female factors including, but not limited to, morphologic abnormalities of the oocyte, limited quantities of oocytes, and anomalies of the zona pellucida, or when poor fertilization occurred in a prior cycle (The Practice Committee of the American Society for Reproductive Medicine, 2006e). Normal fertilization is assessed after 16–18 h and further embryo cleavage of the normally fertilized oocytes is assessed on the second day of *in vitro* insemination or injection. There are different morphological scoring systems for the cleaved embryos, which are used to select the best embryos to be transferred (Staessen *et al.,* 1994; Salumets *et al.,* 2001; Salumets *et al.,* 2003). An uterine embryo transfer is usually carried out on the second or third day after insemination and, for most patients (aged <40 years) the number of transferred embryos are limited to two to avoid as much as possible the occurrence of multiple pregnancies (Templeton and Morris, 1998; Vilska *et al.,* 1999).

Although the cumulative pregnancy rate per patient can be as high as ~70%, success rates per IVF-ET cycle are still around 30% (Nygren and Andersen, 2002; Kikuchi *et al.,* 2003). The presence of autoantibodies has been invoked as a possible mechanism of IVF failure (Geva *et al.,* 1994; Gleicher *et al.,* 1994; Van Voorhis and Stovall, 1997; Mecacci *et al.,* 2000; Hornstein *et al.,* 2000; Chilcott *et al.,* 2000). On the other hand, repeatedly performed IVF attempts themselves have been proposed to increase the production of autoantibodies (Fisch *et al.,* 1991; Birdsall *et al.,* 1996; Monnier-Barbarino *et al.,* 2005; Delgado Alves *et al.,* 2005). The success of attaining a pregnancy following IVF is primary dependent on the effectiveness of COH. While the COH outcome is unpredictably variable between patients, the identification of factors that influence the response to standard treatment with FSH would be of great clinical advantage.

4.4. Follicle stimulating hormone

4.4.1. Regulation of gonadal function by FSH

FSH is one of the two pituitary gonadotrophins involved in the regulation of the gonadal function. In females, FSH targets the receptor expressed only on

granulose cells, and induces the maturation of ovarian follicle (Themmen and Huhtaniemi, 2000). FSH can influence the development of preantral follicles via paracrine factors (Thomas and Vanderhyden, 2006). However, growth of antral follicles becomes critically dependent on FSH support, making a pre-ovulatory follicle capable of ovulation and forming a corpus luteum in response to the mid-cycle surge of LH (Knight and Glister, 2006). The role of FSH and its signaling system is central in the normal reproductive function since mutations in human FSH and its receptor are associated with altered ovarian responses to the hormone, resulting in various degrees of reduced reproductive function (Conway, 1996; Huhtaniemi and Aittomaki, 1998; Levallet *et al.,* 1999; Touraine *et al.,* 1999; Layman *et al.,* 2002).

In males, FSH acts on testicular Sertoli cells and has an importance of initiation and maintenance of spermatogenesis quantitatively. LH stimulates the testosterone production in Leydig cells, which is essential for the attainment of spermatogenesis and other functions of androgens in the male (Tena-Sempere and Huhtaniemi, 2003). FSH along with LH and sex hormones are present also in semen in both healthy and in infertile men (Vasquez *et al.,* 1986; Bujan *et al*. 1993; Luboshitzky *et al.,* 2002).

4.4.2. Coding genes and molecular structure of FSH

FSH is a member of the family of pituitary glycoprotein hormones (GPH), which also include human chorionic gonadotrophin (hCG), LH and thyroid-stimulating hormone (TSH). GPHs are heterodimers, each consisting of a common α-subunit (92 amino acids) and a unique β-subunit (111 amino acids in FSH). Glycosylation of the GPH has been shown to be important in circulatory persistence and clearance and in bioactivity (Ulloa-Aguirre and Timossi, 1998). In a solvent content, two FSH molecules form an asymmetric unit in clasped hands-like fashion, with all carbohydrate chains extended into the open channel formed in the middle of the unit (Fox *et al.,* 2001). Two FSH dimers in the asymmetric unit are similar, except there is a small difference in the angle between the subunits. The α-subunit carboxy terminus as well as carbohydrate residues linked to the α-subunit have been implicated in receptor binding and activation (Ulloa-Aguirre and Timossi, 1998; Fox *et al.,* 2001). However, there is a cysteine noose, or determinant loop on the β-subunit of FSH molecule (between amino acids 87 and 94), the residues of which (Asp 88, Asp 90 and Asp 93) play a role in determining the specificity of FSH receptor binding (Fox *et al.,* 2001) (Figure 1).

The receptor-binding and hormone specificity determining β-subunit of FSH hormone is coded by *FSHB* gene at the 11p13 (Fox *et al.,* 2001). It is composed of three exons and two introns. The first exon contains the 5' untranslated region while second and third exons contain all the coding

sequence (Ulloa-Aguirre and Timossi, 1998). Haplotype analysis has revealed two most prevalent variants of *FSHB* gene — HAP1 and HAP13, covering together about 90% of Estonians (Grigorova *et al.,* 2007). These two core haplotypes have been suggested to be associated with female's fecundity (Grigorova *et al.,* 2007), but the association with autoimmunity to FSH through gene expression in central tolerance induction towards FSH has not been studied before.

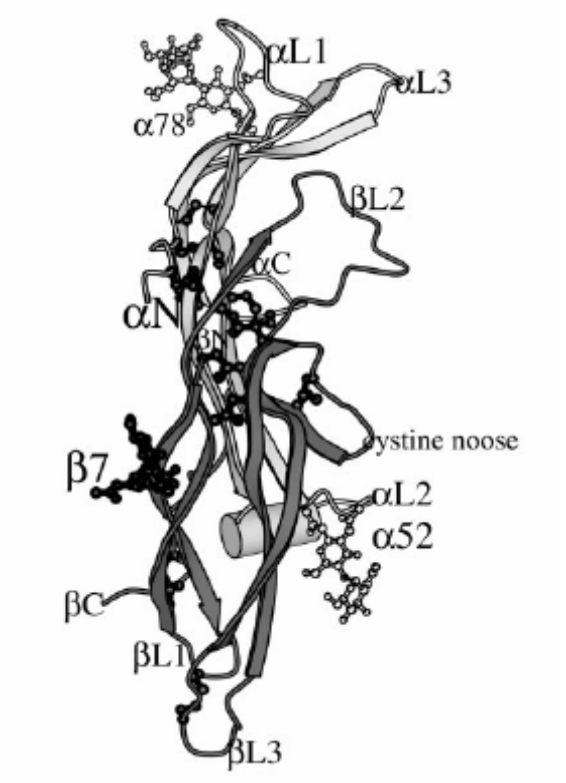

Figure 1. Schematic overview of three-dimensional structure of follicle stimulating hormone with termini and main loops identified (Fox *et al.,* 2001).

5. AIM OF THE PRESENT STUDY

The general objective of the study was to investigate the presence and significance of antibodies to follicle stimulating hormone in female infertility.

Accordingly, the study had the following aims:

1. To compare the levels of serum anti-FSH IgG, IgA and IgM antibodies in women suffering from infertility, from ovarian dysfunction, and in healthy women during pregnancy and in general population.

2. To examine putative factors inducing the production of anti-FSH.

3. To investigate the changes in serum anti-FSH antibody levels during COH in comparison with anti-FSH in the follicular fluid.

4. To evaluate the potentiality of anti-FSH antibodies to interrupt with FSH receptor binding.

5. To assess the effect of serum anti-FSH on folliculogenesis — a study based on the evaluation of COH outcome in IVF treatment.

6. MATERIALS AND METHODS

6.1. Subjects and study design

The study was approved by the Ethics Committee of the University of Tartu and informed consent was obtained from all participants. Study groups are characterized in Table IV.

Non-IVF patients and healthy women. Serum samples were collected from patients with PCOS [n = 75, mean age ± standard deviation (SD) 25.6 ± 4.9 years], endometriosis (n = 103, 31.1 ± 6.7 years), uncomplicated pregnant women at the end of 1st trimester (n = 31, 25.2 ± 6.9 years) and at term (n = 44, 26.8 ± 5.8 years), and from female blood donors participating as controls. PCOS was diagnosed according to the revised diagnostic criteria by The Rotterdam ESHRE/ASRM-sponsored PCOS consensus group (The Rotterdam ESHRE/ASRM-sponsored PCOS workshop group, 2004) (Paper I). Endometriosis was confirmed by diagnostic laparoscopy, as recommended by the American Society for Reproductive Medicine (American Society for Reproductive Medicine, 1997). None of the patients had undergone IVF as treatment for infertility, the group of pregnant women included both nulli- and multiparous women and all controls were healthy according to their medical records. Patients had been suffering from primary or secondary infertility for at least a year before entering the study. No additional clinical data was available for these women.

IVF-patients. A total of 233 consecutive women undergoing IVF treatment were included and were classified into five groups of infertility diagnoses: tubal factor infertility (39.9%, 34.1 ± 4.2 years), male factor infertility (25.3%, 34.0 ± 5.3 years), endometriosis (8.2%, 34.5 ± 4.7 years), PCOS (14.6%, 32.1 ± 4.5 years), unexplained infertility (7.3%, 35.6 ± 5.4 years) and infertility due to the other reasons (4.7%, 36.7 ± 4.5 years). Tubal factor infertility due to occlusion of the fallopian tubes was diagnosed either by hysterosalpingography or diagnostic laparoscopy (Forti and Krausz, 1998). The main cause for tubal occlusion was an episode of PID. A couple was diagnosed to have male factor infertility when the woman was lacking any reason for infertility, while her partner experienced decreased semen quality (WHO, 1999). PCOS and endometriosis were diagnosed according to the abovementioned criteria. Other causes for infertility were endometrial hyperplasia, myoma uteri, and ovulatory dysfunction. Unexplained infertility was assumed if a woman was lacking any of the abovementioned reasons for infertility and her partner was shown to have normal semen quality, but the couple had experienced infertility of more than one year.

The following clinical data were collected from medical records and from the physicians' interviews and examinations for the current thesis. Sixty three percent of patients were about to start their first IVF procedure, while others had

had at least one previous IVF attempt. Both, patients undergoing IVF and ICSI were included in the study. Ovarian hormonal stimulation was conducted accordingly to the GnRH antagonist or agonist protocols with administration of recombinant FSH (Gonal–f®, Serono, Rome, Italy). Patients following GnRH antagonist protocol started COH with injection of recombinant FSH (Gonal-F) on day 1–3 of menses, continuing daily up to a day before hCG (Ovitrelle, Serono, Rome, Italy) administration. The COH follow-up included 3–4 ultrasound assessments of endometrium and follicular growth. Daily GnRH antagonist administration (0.25 mg) (Cetrotide, Serono, Rome, Italy or Orgalutran, N.V. Organon, Oss, The Netherlands) 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 oocyte pick-up (OPU) 36 h later. Patients who followed GnRH agonist protocol for COH underwent pituitary down regulation with GnRH agonist (Diphereline; Ipsen Pharma. Biotech, Paris, France) commenced in the mid-luteal phase of previous menstrual cycle. When suppression was achieved, ovarian stimulation was performed with recombinant FSH and followed similarly to the GnRH antagonist protocol. None of the patients had received non-IVF FSH treatment prior to entering the study. All patients had been suffering from infertility for at least a year.

Transvaginal ultrasound scanning of ovaries was performed prior to initiation of IVF cycle during the first 5 days of patient's spontaneous menstrual cycle. Ovarian volume was estimated according to the following formula: ½(A × B × C), where A was the longitudinal diameter, B the anteroposterior diameter, and C the transverse diameter of the ovary (Sample *et al*., 1977). The number of small antral follicles (4–7 mm) was determined by ultrasound scanning of each ovary in longitudinal cross-section (Robinson *et al*., 1992). Mean follicle number and mean ovarian volume were calculated as the sum of left and right ovaries divided by two. The diameter of the follicle from where the follicular fluid (FF) was aspirated during oocyte retrieval was measured by ultrasound scanning. Serum progesterone, testosterone, and serum and FF estradiol and FSH levels were measured using chemiluminescence immunoassay (Immulite 2000® station, DPC, Los Angeles, CA, USA). Albumin concentration in the FF was measured by bromcresol green dye-binding assay (Cobas Integra® 800, Roche Diagnostics, F. Hoffmann-La Roche Ltd, Basel, Switzerland).

Multiple parameters were utilized to evaluate the efficacy of COH, including the number of follicles punctured at the day of oocyte pick-up (OPU follicles) and the number of cumulus-oocyte complexes obtained by OPU (OPU oocytes). The number of mature oocytes was calculated for both IVF and ICSI. IVF oocytes were assessed for maturity one day after OPU and following insemination by counting fertilized oocytes and Meiosis II (M II) unfertilized oocytes. ICSI oocytes were considered mature if they reached M II stage 4–6 h

after OPU. The total number of embryos gained included the embryos with two pronuclei (2PN). The embryos representing at least four blastomeres and <20% of fragmentation on day 2 after insemination or ICSI were considered good quality embryos. The following parameters were calculated from the total amount of recombinant FSH used for ovarian stimulation: the amount of FSH (IU) needed to mature one follicle punctured at OPU, the amount of FSH needed for one oocyte obtained at OPU, the amount of FSH per mature oocyte, the amount of FSH per 2PN embryo, and the amount of FSH per good quality embryo.

Blood samples from IVF patients were drawn once during the 3–5 days of the patient's spontaneous menstrual cycle, before ovarian stimulation with exogenous FSH, and once again on the day of oocyte retrieval. EDTA-collected peripheral blood samples were obtained for genomic DNA extraction. Aspirates containing fluid from a single follicle were collected during oocyte retrieval.

Table IV. Characteristics of study groups

Study group (n)	Mean age ± SD (years)	Comments	Institution (period of collection)	Study material	Paper
Non-IVF patients (178)	28.9 ± 6.6	Endometriosis and PCOS patients	University of Tartu, Women's Clinic (1997–2004)	Sera from various time points of menstrual cycles	I
Pregnant women (75)	26.6 ± 6.5	1st trimester and term of uncomplicated pregnancy	University of Tartu, Women's Clinic (2002–2004)	Sera	I
Controls (85)	44.9±10.7	Healthy female blood donors	Blood donation centre of Kuressaare Hospital (March – April 2003)	Sera from various time points of menstrual cycles	I, II
IVF patients (233)	34.0 ± 4.9	Male factor infertility, tubal factor infertility, unexplained infertility, endometriosis, PCOS, other reasons	Nova Vita Clinic, Centre for Infertility Treatment and Medical Genetics, Viimsi, Harjumaa (July 2004 – December 2005)	SPC (n = 129…135) OPU (n = 129…182) Follicular fluid (n = 170) Blood for genomic DNA extraction (n = 182)	II, III*, IV*, V** IV, V** V** II

SD — standard deviation
SPC — sera obtained from day 3–5 of patients' spontaneous menstrual cycles
OPU — sera obtained at the day of oocyte retrieval
* Except IVF (*in vitro* fertilization) patients with infertility due to other reasons
** Except IVF patients with polycystic ovary syndrome (PCOS) and/or following GnRH agonist protocol for controlled ovarian hyperstimulation

6.2. Immunological methods

6.2.1. Detection of anti-FSH antibodies

Indirect enzyme-linked immunosorbent assay (ELISA) with purified FSH [Metrodine® HP 75, Serono, Italy (Paper I) or Fostimon® 75, IBSA, Switzerland (Papers II, III, V)] as the antigen was used to detect serum and FF anti-FSH antibodies of IgG, IgA and IgM isotypes according to the protocol provided in Paper II. The results of two ELISA protocols (Metrodine® and Fostimon®) were in good correlation for IgG, IgA and IgM autoantibodies (Pearson's correlation coefficients were 0.37, $p = 0.002$, 0.37, $p = 0.002$ and 0.49, $p<0.0001$, respectively). Therefore, Fostimon® was used when Metrodine® was no longer available for laboratory practice. The intra- and inter-assay coefficients of variation for IgG, IgA and IgM measured in Fostimon®-ELISA were as follows — 7.2%, 8.6%, 3.7% and 10.1%, 7.3%, 3.8%, respectively.

In brief, serum samples were diluted to 1:100, corresponding to albumin levels of 0.35–0.50 g/L and FF samples were diluted to an albumin concentration of 0.40 g/L. Horseradish peroxidase-conjugated rabbit *anti*-human IgG, IgA and IgM (DAKO, Glostrup, Denmark) were used as secondary antibodies. The optical density (OD) at 690 nm was subtracted from OD at 450 nm, and the difference was recorded as signal. Antibody levels were expressed as:

Corrected OD value =

$$(\text{Sample mean OD}_{\text{IgG, IgA or IgM}}) - (\text{Blank mean OD}_{\text{IgG, IgA or IgM}})$$

(Papers I and III)

or

Sample OD ratio to pool OD =

$$\frac{(\text{Sample mean OD}_{\text{IgG, IgA or IgM}}) - (\text{Blank mean OD}_{\text{IgG, IgA or IgM}})}{(\text{Pool median OD}_{\text{IgG, IgA and IgM}}) - (\text{Blank mean OD}_{\text{IgG, IgA or IgM}})}$$

(Papers II and V)

A calibrator (pool of sera from 200 healthy fertile women) was included in each plate in later studies, in order to facilitate between-plates comparison of antibody levels. Corrected OD value of anti-FSH antibodies was reused in Paper III in order to be comparable to the anti-sperm antibody test approach. Blank wells contained all the assay components but serum or FF samples.

Serum anti-FSH IgG antibody concentration was predicted by creating a concentration curve. Instead of sera, FSH-coated (Fostimon®) plates were incubated with series of dilutions of mouse monoclonal IgG antibody [INN-fFSH-60] to human FSH beta (Abcam®, Cambridge, UK). Peroxidase conjugated goat *anti*-mouse IgG (Sigma Chemical Co., St. Louis, MO) diluted

to 1:2000 was used as secondary antibody. Otherwise, the protocol provided for anti-FSH ELISA was followed (Paper II). OD values of detected signals were calculated for each monoclonal antibody dilution (Figure 2).

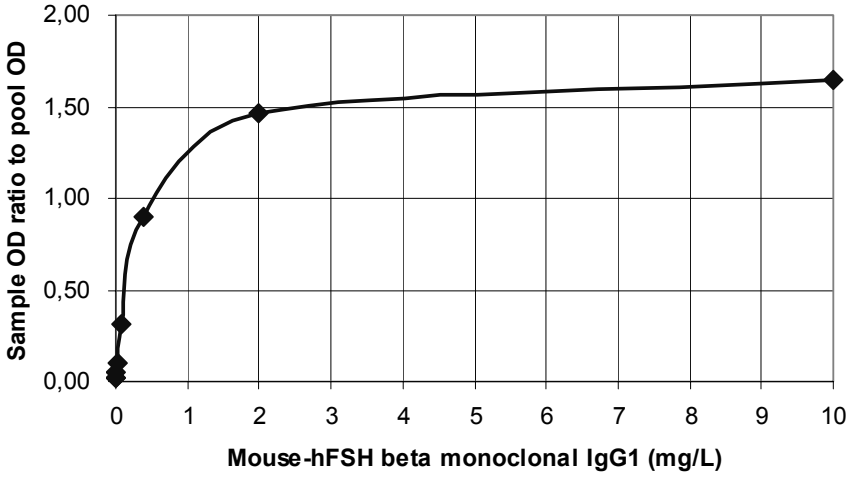

Figure 2. Mouse monoclonal IgG1 to human FSH beta concentration curve to optical density (OD) ratio. Prediction of serum anti-FSH IgG antibody concentration based on OD ratio.

6.2.2. Detection of anti-V14D antibodies

Serum IgG, IgA and IgM type of anti-V14D antibodies were detected with a synthetic peptide corresponding to the sequence of the 78–93 region (V14D) of human FSH β-chain (Laboratoire d'Immunoessais, Biomérieux, Marcy L'Etoile, France). ELISA protocol used for anti-V14D was the same as for anti-FSH, except the plates were coated with 10 μg/ml peptide V14D solution in carbonate-bicarbonate buffer and serum samples were diluted to 1:25. Antibody values were expressed as corrected OD values (Paper I) or sample OD ratio to pool OD (paragraph 7.4. in the section of Results).

6.2.3. Detection of common autoantibodies

Indirect immunoflourescence method was used to assess common autoantibodies: nuclear [ANA-H and ANA-R on human HEp2 cell line (ImmunoConcepts®, Sacramento, CA) and rodent antigen, respectively], SMA, thyroid microsomal autoantibodies (TMA) and parietal cell (PCA) autoantibodies (Reimand *et al.,* 2001). The 5 μm cryostat sections of rat liver and kidney, mouse stomach, human thyroid tissue or commercial HEp2 cells were used as antigenic substrates. Sera were diluted 1:10 (lower titer) and 1:40 (higher titer for ANA-H) or 1:100 (higher titer for other autoantibodies) in phosphate buffered saline (PBS: 8 g/l NaCl, 0.2 g/l KCl, 1.44 g/l $Na_2HPO_4 \cdot 7H_20$, 0.24 g/l KH_2PO_4, pH 7.2). Diluted sera were added to substrates and incubated for 30 min followed by two 10-min washes with PBS. As a secondary antibody, Fc-specific fluorescein isothiocyanate (FITC) conjugated rabbit *anti*-human IgG (DAKO, Glostrup, Denmark) in a 1:40 dilution was incubated for 30 min. After two 10-min washes, test glasses were covered with cover slip and the fluorescence signal was explored using a UV microscope (Olympus BX50F4, Tokyo, Japan). The antibody levels were expressed as negative or as positive at lower or higher titers.

Indirect in-house ELISA was used to detect antibodies against β2-glycoprotein I (B2-GPI) (Reimand *et al.,* 2001) and cardiolipin (ACA) (Khamashta *et al.,* 1993). A polystyrene 96 well plate (Nunc Maxi Sorp, Roskilde, Denmark) was coated with antigenic solutions of 50 μl per well. Antigenic solutions were 5 μg/mL β2-glycoprotein I (Crystal Chem, Chicago, IL) in borate buffered saline (BBS, 200 mM H_3PO_3, 75 mM NaCl, pH 8.4) or 50 μg/mL cardiolipin (Sigma, Glostrup, Denmark) in 95% ethanol, respectively. Plates were incubated over night at +4°C, washed and blocked by 0.5% (B2-GPI) or 10% (ACA) bovine serum albumin and 0.4% Tween-20 in BBS for 1.5 h at room temperature. The wash buffer consisted of 0.1% Tween-20 in BBS. Plates were washed four times after each incubation step. Sera dilutions of 1:100 (B2-GPI) or 1:50 (ACA) in the blocking buffer were reacted for 2 h (B2-GPI) or 3 h (ACA) at room temperature. Alkaline phosphatase-conjugated *anti*-human IgG (DAKO, Glostrup, Denmark) was diluted 1:1000 in the blocking buffer, followed by incubation for 1 h (B2-GPI) or over night (ACA) at room temperature. Color was developed by adding 100 μl (B2-GPI) or 50 μl (ACA) substrate, *p*-nitrophenyl phosphate 1 g/l in 1 M diethanoleamine buffer (9.7% $C_4H_{11}NO_2$, 0.01% $MgCl_2 \cdot 6H_2O$, 0.02% NaN_3, pH 9.8). Absorbancies were read at 405 nm with 492 nm subtraction. Antibody levels were expressed as:

Enzyme-immunological units (EIU) =

$$\frac{(OD_{\text{sample}} - OD_{\text{negative control}})}{(OD_{\text{positive control}} - OD_{\text{negative control}})} \times 100$$

Patients' sera with known test results were chosen for negative and positive controls. The cut-off values for weak and strong positive result were 10 and 30 EIU for B2-GPI, and 30 and 60 EIU for ACA.

Immunological tests for ANA-H, ANA-R, SMA, PCA, B2-GPI and ACA are clinically available and have been periodically subjected to external quality assessment by UK NEQAS (Sheffield, UK).

Antibodies against thyroid peroxidase (anti-TPO IgG) were detected using a fluoroenzyme immunoassay ImmunoCAP technology according to the manufacturer's instructions (UniCAP, Phadia OY, Finland). Results were expressed as negative or positive (>100 IU/mg of anti-TPO IgG).

6.2.4. Detection of anti-sperm antibodies

Anti-sperm antibodies were detected as previously reported (Nikolaeva *et al.,* 1997) with some modifications. Anti-sperm antibody negative donor spermatozoa were prepared using discontinous Percoll (Medicult, Denmark) density gradient procedure (McClure*et al.,* 1989) adjusted with Sperm Preparation Medium (SPM) (Medicult, Denmark) to a final concentration of 10 – 12 x $l0^6$/mL of motile spermatozoa, were used as antigens. The serum samples were heat inactivated at +56°C for 30 min, diluted to 1:3 in SPM and incubated at +4°C for 15 min followed by the incubation with 20 μL of motile sperm suspension at +4°C for 40 min. Negative controls consisting of 30 μL SPM and 20 μL of sperm suspension were included in all experiments.

After the incubations with sera, all samples were washed twice in phosphate-buffered saline (PBS, pH 7.4) and the cell pellet was suspended in 25 μL of PBS. Fc-specific FITC-labelled rabbit $F(ab')_2$ fragmented anti-human IgG, IgA and IgM (DAKO, Denmark) were diluted to 1:10 (IgG and IgM) or 1:20 (IgA) in SPM and added to the spermatozoa suspension (final volume 50 μL), followed by incubation at +4°C for 20 min in dark. Cells were washed three times with PBS, suspended in 300 μL of PBS, and stained with 10 μg/mL 7AAD (7-aminoactinomycin D, Invitrogen, USA) to distinguish living spermatozoa. After incubation at +4°C in dark for 20 min, samples were analysed using FACScalibur flowcytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA).

Twenty thousand events were recorded and analysed using the Cell Quest software (Becton Dickinson, Immunocytometry Systems, Mountain View, CA).

The sperm population was gated on the basis of the forward — (FSC) and side — scatter (SSC) properties. According to the labelling pattern in the bivariate FITC/7AAD analysis, 3 cell populations were identified (Figure 3): (i) viable spermatozoa with no surface bound anti-sperm antibodies (FITC- and 7AAD-negative pool), (ii) viable spermatozoa with surface bound anti-sperm antibodies (FITC-positive but 7AAD-negative pool), and (iii) non-viable spermatozoa (7AAD-positive and FITC-positive or negative pool). The percentage of antibody-positive sperm was defined as the ratio of the FITC-positive and 7AAD-negative sperm population to the total 7AAD-negative living sperm. The levels of anti-sperm antibodies were expressed as:

Corrected amount (%) of antibody-positive spermatozoa =

(Sample mean % $_{\text{IgG, IgA or IgM}}$) − (Negative control median % $_{\text{IgG, IgA and IgM}}$)

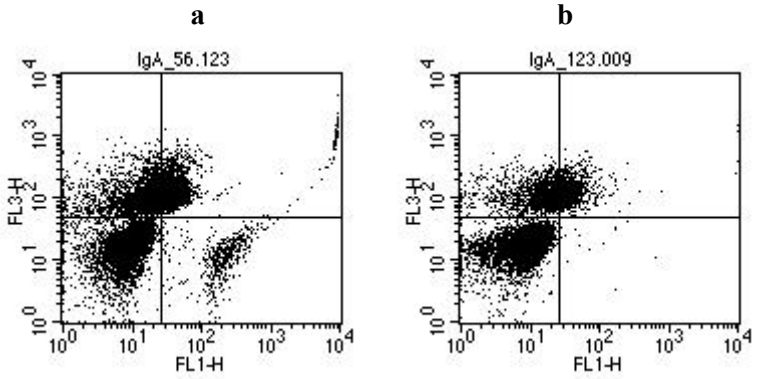

Figure 3. The sperm pools of anti-sperm IgA positive (**a**) and anti-sperm IgA negative (**b**) IVF patient. Lower left quadrant represents FITC-negative/7AAD-negative viable sperm with no antibodies bound to them. Lower right quadrant shows FITC-positive/7AAD-negative viable sperm with bound anti-sperm antibodies. Both upper left and right quadrants represent the pool of non-viable sperm.

6.3. Molecular-genetic methods

6.3.1. Genomic DNA extraction

Genomic DNA was extracted from peripheral EDTA-blood (2 ml) using modified salting-out method (Miller *et al.,* 1988). Erythrocytes were eliminated by adding erythrocyte lysis buffer twice the amount of blood starting volume (16 mM saccharose, 0.05% Triton-X-100, 0.25 mM $MgCl_2$, 0.6 mM Tris-HCl) and by removing the supernatant after centrifugation at 2500 rpm 10 min. Leucocytes in the precipitate were washed 3–4 times with 1 mL MQ and centri-

fuged at 13000 rpm 2 min and supernatant was removed. Leucocytes were incubated with 800 µl of protein lysis buffer [0.2x protein-K buffer (1x buffer: 375 mM NaCl, 120 mM EDTA), 87 µg/ml protein-K, 1% SDS] at +55°C for 1 h. Approximately 300 µl of 5 M NaCl were added and shacked for 15 s. Proteins were precipitated by centrifugation at 13000 rpm for 5 min. Supernatant was poured into 1 ml cooled (–20°C) 99.5% ethanol and DNA was collected with pipette-tip. Pipette-tips with DNA were washed with 70% ethanol and were allowed to dry at room temperature. DNA was dissolved in 0.5 ml 10/1 TE buffer (9.97 mM TRIS, 1.27 mM EDTA) at +55°C overnight. DNA concentration was measured by spectrophotometer (Ultraspec 2000, Amersham Pharmacia Biotech, Piscataway, NJ) and stored at –20 or –70°C.

6.3.2. Genotyping of *FSHB* gene

Haplotypes of the *FSHB* gene were detected by the haplotype tagging single nucleotide polymorphisms (SNPs) of *FSHB*: +1234 C/A (rs594982) in intron 2 and +1736 C/T (rs6169) in exon 3 (Grigorova *et al.,* 2007). The polymerase chain reaction (PCR) primers and restriction enzymes are listed in Table V.

Table V. Primer sequences and restriction enzymes used for *FSHB* genotyping

SNP rs #	Primer sets 5'-3'	Restriction enzymes	Allele size (bp)
rs594982	CAGTTGCTAGTCTGTGTTTGCAG CCTTGAAGGTACATGTTTTCTGG	*Xap*I	C: 108+234+312+319 A: 108+234+312+143+176
rs6169	GCCATAGGAAGTAAGAAAAGA TACCTCAAACATCGTCTTCCAGT	*Bst 1107*I	T: 326+670 C: 50+276+670

Amplification of the genomic DNA (~100 ng) was performed in a total volume of 25 µl containing 250 µM dNTP-s (MBI Fermentas, Vilnius, Lithuania), 2.5 mM $MgCl_2$, 1x PCR buffer (Naxo Ltd., Tartu, Estonia), 0.01 µM of primers (Metabion, Martinsried, Germany) and 0.04 U/µl Hot Smart-Taq DNA poly-

merase (Naxo Ltd., Tartu, Estonia). Amplifications were performed using thermal cycler (Eppendorf, Hamburg, Germany). The reactions were initiated with a denaturation at +95°C for 15 min, followed by 10 cycles of denaturation at +95°C for 20 s, annealing at +68°C (-1°C per cycle) for 30 s, and elongation at +68°C for 2 min. Next 10 cycles were performed with annealing at +56°C for 30 s, following by 10 cycles with annealing at +54°C for 30 s. Final 10 cycles were performed with annealing at +51°C for 30 s followed by final extension step at +68°C for 10 min. PCR products were visualized under the UV light on 2% agarose gel with ethidium bromide staining. For SNP determination, restriction fragment length polymorphism (RFLP) method was used. The PCR products were digested with 1 U/μl of respective restriction enzymes (MBI Fermentas, Vilnius, Lithuania) (Table V) at +37°C overnight. The DNA fragments were visualized under the UV light on 3% agarose gel with ethidium bromide staining. Haplotype no. 1 (HAP1) was determined by the presence of +1234 C and +1736 T, and haplotype no. 13 (HAP13) consisted of +1234 A and +1736 C alleles (Grigorova *et al.,* 2007).

6.3.3. Genotyping of *HLA-DQB1* gene

The specific region in exon 2 of the *HLA-DQB1* gene was amplified using a pair of biotinylated primers [5′- GCATGTGCTACTTCACCAACG, 3′- Bio-CCTTCTGGCTGTTCCAGTACT, provided by *HLA-DQB1* hybridization assay DELFIA® Diabetes reagents (Wallac, PerkinElmer Life Sciences, Boston, MA)]. Amplification of the genomic DNA (~100 ng) was performed in a total volume of 80 μl containing: 1.5 M betaine, 200 μM dNTP-s (MBI Fermentas, Vilnius, Lithuania), 3 mM $MgCl_2$, 1 x PCR buffer (670 mM TRIS-HCl pH 8.8, 0.1% Tween-20), 0.2 μM of primers (CyberGeneAB, Huddinge, Sweden) and 0.02 U/μl Taq DNA polymerase (Naxo Ltd., Tartu, Estonia). Amplifications were performed using PTC-100^{TM} thermal cycler (MJ Research, Inc., Waltham, MA). The reactions were initiated with denaturation at +95°C for 6 min, annealing at +59.5°C (-1°C per cycle) for 4 min, elongation at +73°C for 2 min and followed by 36 cycles with denaturation at +95°C for 50 s, annealing at +59.5°C for 1 min and elongation at +73°C for 1 min (-0.5°C per cycle). *HLA-DQB1* typing was performed by utilizing *HLA-DQB1* hybridization assay DELFIA® Diabetes reagents. Briefly, PCR products were transferred to the streptavidine-coated microtitration plate for streptavidine-biotine binding, denatured and hybridized with five allele-specific oligonucleotide probes, labeled with lanthanides: europium, terbium or samarium. The *DQB1* probes specific for *DQB1*03* class (**0301* and/or **0302*) or *DQB1*06* class (**0602* and/or **0603*) alleles were applied in two mixtures. After hybridization, time resolved fluorescence of lanthanide labels was measured using a Wallac

Victor™ 1420 Multilaber Counter (Perkin-Elmer, Boston, MA) to detect the specifically bound PCR product.

6.4. Statistical analysis

The R2.0.0–2.3.1 A Language and Environment (Free Software Foundation, Boston, MA) was used for statistical analysis. Pearson's correlation, *t*-test, paired *t*-test, proportion test, bivariate and multivariate linear and logistic regression models were used for statistical methods. The methods and adjusted parameters are further explained at the results. $P<0.05$ was considered statistically significant in all cases.

7. RESULTS

7.1. Serum anti-FSH antibodies in different pathological and physiological conditions in women (Papers I and II)

Since study groups represented women from various age groups (see Methods) and infertility problems as well as the propensity to produce antibodies could be age-sensitive; IgG, IgA and IgM type of anti-FSH antibodies were analyzed utilizing age adjusted regression models. Age adjusted model gives the statistical results in the conditions where age is constant.

7.1.1. Anti-FSH antibodies in patients with endometriosis and PCOS, and in IVF patients

Serum anti-FSH IgG, IgA and IgM antibodies were detected in patients with endometriosis (n = 103) and PCOS (n = 75), and compared to the antibody levels in healthy female blood donors (n = 85). Serum samples were obtained from various time points of menstrual cycles. Antibody values were expressed as corrected OD (Paper I).

The age adjusted linear regression model showed significantly higher values of IgA antibodies in infertile patients [regression coefficient (r) = 0.07, $p<0.001$], while the levels of IgG and IgM type of anti-FSH antibodies were similar in patients and controls (Figure 4).

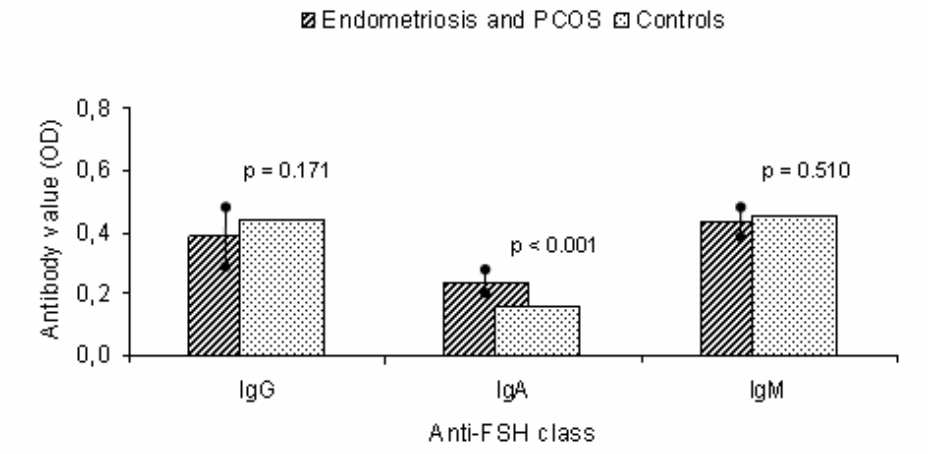

Figure 4. Estimated values of anti-FSH antibodies [optical density (OD)] and 95% confidential intervals for 34 years old endometriosis and PCOS patients compared to controls, from age adjusted linear regression model.

The age adjusted logistic regression model suggested higher levels of serum anti-FSH IgA was risk factor for both endometriosis and PCOS [adjusted odds ratios (ORs) 2.00, p = 0.002, and 2.15, p = 0.015, respectively].

Anti-FSH antibodies were measured in IVF patients with different causes of infertility. The infertility diagnoses included: tubal factor infertility (n = 56), male factor infertility (n = 30), PCOS (n = 21), endometriosis (n = 12), unexplained infertility (n = 11) and infertility due to the other reasons (n = 5). Serum samples obtained between day 3 and 5 of the spontaneous menstrual cycles before starting the ovarian stimulation with exogenous FSH were used in antibody tests. Antibody values were expressed as sample OD ratio to pool OD (Paper II).

Linear regression model adjusted by age of woman revealed that IVF patients displayed significantly increased levels of all subtypes of anti-FSH antibodies (IgG r = 0.26, p = 0.006, IgA r = 0.20, p<0.001, IgM r = 0.63, p<0.001) compared to the controls (Figure 5 and Paper II, Table I). Eighty nine patients (65.9%) were about to start their first IVF procedure, while the rest (34.1%) had had at least one previous IVF procedure. Additional analysis was performed between controls and IVF patients with no previous IVF procedures, using age adjusted linear regression models. The levels of anti-FSH antibodies were significantly increased in IVF patients with no previous IVF procedures (IgG r = 0.26, p = 0.011, IgA r = 0.18, p = 0.001, IgM r = 0.52, p = 0.006) compared to controls.

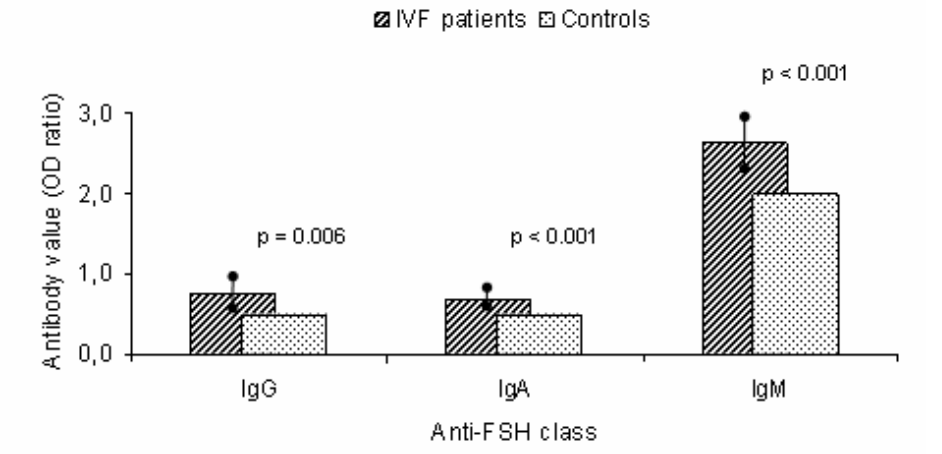

Figure 5. Estimated values of anti-FSH antibodies [optical density (OD) ratio to pool OD] and 95% confidential intervals for 34 years old IVF patients compared to controls, from age adjusted linear regression model.

IVF patients with PCOS, endometriosis, unexplained infertility or infertility due to other reasons (PEU) were studied separately from women with male or tubal factor infertility (MTF). The age adjusted linear regression analysis suggested both patient groups presented increased levels of anti-FSH IgG (PEU r = 0.19, p = 0.021, MTF r = 0.29, p = 0.006), IgA (PEU r = 0.13, p = 0.006, MTF r = 0.23, p<0.001) and IgM (PEU r = 0.49, p = 0.019, MTF r = 0.66, p<0.001) than controls (Figure 6 and Paper II, Table I).

Utilizing Metrodine® as an antigen for measuring anti-FSH, a significant correlation was revealed between the production of IgA and IgM, but not IgG antibodies and the age of woman in control population. The levels of anti-FSH IgA increased with age, while anti-FSH IgM decreased (Pearson's correlation r = 0.28, p = 0.008 and r = –0.35, p = 0.001, respectively) (Paper I). However, the correlation between anti-FSH antibodies and the age of controls were not established with ELISA testing on Fostimon® (Paper II).

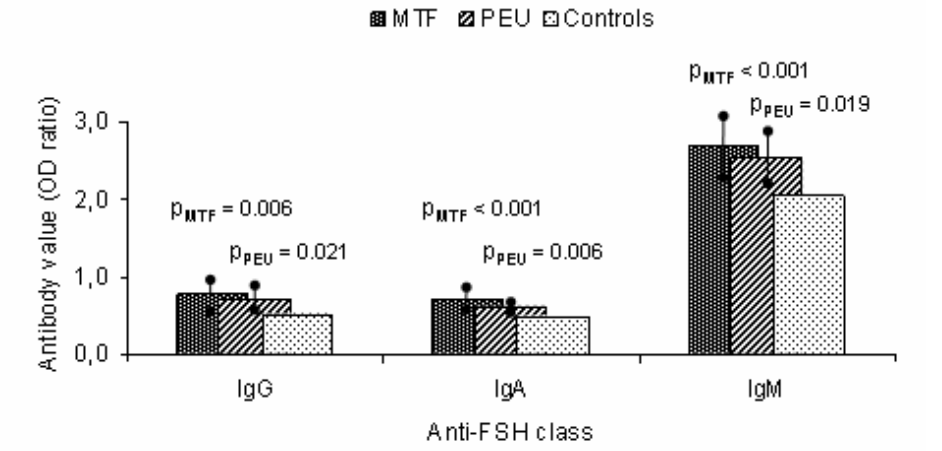

Figure 6. Estimated values of anti-FSH antibodies [sample optical density (OD) ratio to pool OD] and 95% confidential intervals for 34 years old IVF patients compared to controls, from age adjusted linear regression model. MTF — patients with male or tubal factor infertility, PEU — patients with PCOS, endometriosis, unexplained infertility or infertility due to other reasons.

7.1.2. Anti-FSH antibodies during pregnancy

Possible effect of pregnancy on the production of anti-FSH antibodies was assessed by age adjusted linear regression models. Levels of serum anti-FSH antibodies (expressed as corrected OD) in women during pregnancy (at 1st trimester, n = 31 and at term, n = 44) were compared with antibody levels in controls (Paper I). A significantly lower production of anti-FSH IgG (r = –0.15, p = 0.003) and IgM (r = –0.10, p = 0.005), but not IgA, was revealed in pregnant women compared to the controls (Figure 7).

Additionally, a significant decrease in the levels of anti-FSH IgG and IgM was revealed already at the end of 1st trimester of pregnancy (r = -0.15, p = 0.048 and r = –0.41, p<0.001) compared to the controls.

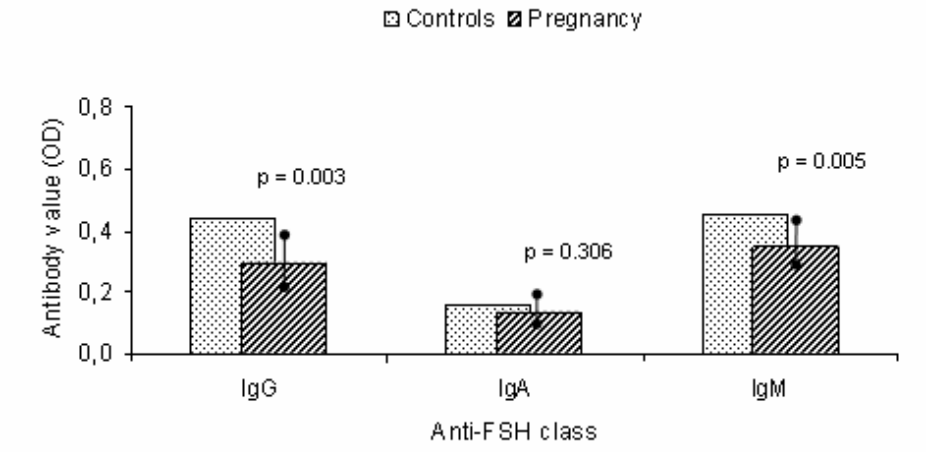

Figure 7. Estimated values of anti-FSH antibodies [optical density (OD)] and 95% confidential intervals for 34 years old pregnant women compared to controls, from age adjusted linear regression model.

7.2. Putative factors inducing anti-FSH antibody production in IVF patients (Papers II and III)

Anti-FSH antibodies detected in sera of infertile women could be autoantibodies to females' endogenous FSH or alloantibodies produced either against seminal plasma FSH (Vasquez *et al.,* 1986; Luboshitzky *et al.,* 2002) introduced to women genital tract mucosa or exogenous FSH used in ovarian stimulation in IVF procedures.

If anti-FSH were autoantibodies, four major etiologic factors were suspected (Paper II): i) the excessive amount of autoantigen (Fénichel *et al.,* 1999;

Byersdorfer *et al.,* 2005) — this was studied by the level of peripheral FSH, ii) impaired expression of autoantigen in thymus to disturb the central tolerance induction (Vafiadis *et al.,* 2001; Jasinski and Eisenbarth, 2005) — studied by genetic variations of *FSHB* gene probably influencing gene expression, iii) autoimmunity related dysregulation of immune reactions (Tuohy *et al.,* 2000; Mackay and Rowley, 2004) — studied by the general propensity to autoimmune reactions assessed by the presence of common autoantibodies with auto-immunity-prone HLA class II gene alleles, and iv) development of auto-immunity via iatrogenic or inflammatory destruction of target tissues (Nepom and Kwok, 1998; Gobert *et al.,* 1992) — studied in relation with repeatedly performed IVF procedures. The associations were studied within all IVF patients and separately in IVF patients with PEU and MTF.

The hypothesis that anti-FSH antibodies could represent alloantibodies developed in response to seminal FSH was assessed by correlation with anti-bodies produced against sperm surface antigens (Paper III).

Sera obtained during day 3–5 of patients' spontaneous menstrual cycle and before starting ovarian stimulation with exogenous FSH were used in antibody tests.

7.2.1. Association between anti-FSH antibodies and peripheral FSH levels

Mean (± SD) peripheral level of FSH at the early follicular phase of the menstrual cycle was 8.73 ± 4.69 IU/L. The linear regression model adjusted by age could not detect any significant association between the levels of anti-FSH antibodies (expressed as sample OD ratio to pool OD) and that of FSH hormone within the group of all IVF patients and separately in the group of PEU (IgG r = 0.36, p = 0.814, IgA r = -0.17, p = 0.961 and IgM r = –1.01, p = 0.248). However, in the group of MTF, the linear regression adjusted by the age and the previous IVF attempts showed positive correlation between the level of peripheral FSH and anti-FSH IgM (r = 0.71, p = 0.043) and also tended to be associated with anti-FSH IgG (r = 1.04, p = 0.058), but not with anti-FSH IgA (r = –0.64, p = 0.532). At the same time, the level of peripheral FSH was slightly but insignificantly lower in the patients of MTF compared to the women with PEU (8.31 ± 4.51 and 9.55 ± 4.92 IU/L, respectively, *t*-test p = 0.064).

7.2.2. Relationship between anti-FSH antibodies and *FSHB* gene haplotypes

The distribution of *FSHB* core haplotypes among IVF patients was as follows: 23.4% were homozygous for HAP1, 45.3% were HAP1/HAP13 and 31.3%

were homozygous for HAP13. The logistic regression model adjusted by the age was unable to detect any significant association between *FSHB* haplotypes and different anti-FSH antibodies (expressed as sample OD ratio to pool OD) within the group of all IVF patients (adjusted ORs for IgG 0.88, p = 0.669, IgA 1.55, p = 0.497 and IgM 0.92 p = 0.647) or if PEU and MTF patients were studied separately.

7.2.3. Production of anti-FSH in association with the general propensity to autoimmune reactions

Potential susceptibility of patients to autoimmunity was assessed by the presence of common autoantibodies in low titers in relation to the *HLA-DQB1* alleles. Among the IVF patients, 43.3% carried at least one allele of the *HLA-DQB1*03* class *(0301* and/or *0302)* and 37.0% had at least one allele of the *HLA-DQB1*06* class *(0602* and/or *0603)*. The presence of common autoantibodies was defined when a patient presented reactivity to at least 1 out of the 7 autoantigens investigated (ANA-H, ANA-R, anti-TPO, SMA, B2-GPI, ACA and PCA). The prevalence of common autoantibodies among IVF patients were 40.0% (95% CI 31.8 – 48.9) (Paper II, Table II), representing separate autoantibodies as follows: ANA-H 13.2%, ANA-R 12.4%, anti-TPO 11.0%, SMA 8.8%, B2-GPI 8.1%, ACA 7.4%, and PCA 3.6%. Patients with PEU represented more common autoantibodies (49.0%, 95% CI 34.6 – 63.5, proportion test, $p<0.05$) than patients with MTF (34.9%, 95% CI 25.1 – 46.0).

In order to study the associations between the level of anti-FSH antibodies (expressed as sample OD ratio to pool OD) and the presence of common autoantibodies, the linear regression analysis adjusted by the age and *HLA-DQB1*03* or **06* allele was used. Anti-FSH IgA levels were not associated with the production of common autoantibodies, but were positively associated with the presence of the *HLA-DQB1*03* allele among all IVF patients (r = 0.15, p = 0.020) and separately in PEU patients (r = 0.17, p = 0.022), but not in MTF patients (r = 0.13, p = 0.152). The levels of anti-FSH IgM were in strong positive correlation with the production of other autoantibodies among all IVF patients and separately in MTF (r = 0.70, p = 0.011) but not in patients with PEU (r = 0.14, p = 0.678). The model could not reveal significant associations between anti-FSH IgG antibodies and the presence of common autoantibodies or *HLA-DQB1*03/*06* alleles among all IVF patients and neither separately in PEU nor in MTF.

7.2.4. Effect of previous IVF attempts on anti-FSH antibodies

Among the IVF patients, 65.9% (n = 89) women were starting their first IVF procedure, while 19.3% (n = 26), 8.1% (n = 11) and 6.7% (n = 9) of patients had already undergone 1, 2 and 3 or more IVF attempts, respectively.

The age-adjusted logistic regression model was used to evaluate the association between anti-FSH autoantibodies (expressed as sample OD ratio to pool OD) and the previous IVF attempts. This model showed no significant relationship between the previously performed multiple IVF procedures and anti-FSH antibodies among all IVF patients or separately in the group of MTF (adjusted ORs for IgG 1.04, p = 0.902, IgA 0.75, p = 0.677 and IgM 0.91, p = 0.669). However, the history of previous IVF treatments was positively associated with the level of anti-FSH IgM (adjusted OR 2.96, p = 0.017) but not with anti-FSH IgA or IgG (adjusted ORs 0.61, p = 0.763 and 0.29, p = 0.403, respectively) in the group of PEU. On the average, patients with MTF had undergone more of previous IVF procedures than patients with PEU (mean number ± SD of IVF attempts were 0.7 ± 1.0 and 0.4 ± 0.7, *t*-test p = 0.026).

7.2.5. Correlation between antibodies against spermatozoa and anti-FSH

The levels of anti-sperm and anti-FSH antibodies in IVF patients with different etiologies were detected (Paper III, Table I) and expressed as corrected percentage of antibody-positive sperms or as corrected OD, respectively. Correlations were studied among patients with regard to their similarities in immunotolerating conditions in the genital tract: (i) tubal factor infertility group — women with tubal factor infertility and normal semen quality observed in their partners, (ii) male factor infertility group — healthy women and impaired sperm quality observed in their partners, and (iii) combined group of patients — women with endometriosis, PCOS or unexplained infertility and normal semen quality observed in their partners.

The mean age of study groups was similar. Although patients with PCOS, endometriosis and unexplained infertility seemed to have increased levels of anti-sperm IgG, IgA and IgM antibodies than patients with tubal or male factor infertility, the difference did not reach to statistically significant level (*t*-test, Paper III, Table I). Similarly, the levels of anti-FSH IgG, IgA and IgM did not differ significantly between study groups. The only significant correlation between production of anti-sperm and anti-FSH antibodies was seen with class IgA in the combined group of patients (Pearson's correlation 0.34, p = 0.023). Patients with tubal or male factor infertility did not show any correlation between anti-sperm and anti-FSH IgA. The production of IgG or IgM type of anti-FSH was not correlated to the anti-sperm antibodies among any patients' groups.

7.3. Changes in serum anti-FSH antibody levels during COH in comparison with anti-FSH in FF (Papers IV and V)

Significant increase in serum sex hormone levels were achieved in COH (Table VI). Anti-FSH antibodies (expressed as sample OD ratio to pool OD) detected in sera of IVF patients before and after COH were compared to the anti-FSH in FF relative to follicle size and endocrine markers. Blood samples were drawn once during the 3–5 days of the patient's spontaneous menstrual cycle, before ovarian stimulation with exogenous FSH and administration of GnRH antagonists (SPC, n = 105), and once again on the day of oocyte retrieval (OPU, n = 182). Aspirates containing fluid from a single follicle (n = 170) were collected during oocyte retrieval and the diameter of the follicle was measured by ultrasound scanning (Paper V). Changes in serum common autoantibodies caused by COH was also detected (n = 129) (Paper IV).

Table VI. Patients' hormonal profiles (mean ± SD)

	SPC (n = 129)	**OPU (n = 129)**
Estradiol (pmol/L)	161.1 ± 117.0	5768.0 ± 7867.0 *
Progesterone (nmol/L)	1.6 ± 1.4	32.3 ± 19.1 *
Testosterone (nmol/L)	1.5 ± 0.7	3.3 ± 1.9 *

SPC — day 3–5 of the patients' spontaneous menstrual cycle. OPU — day of oocyte pick-up. * Statistically significant increase (paired *t*-test, $p<0.0001$)

7.3.1. Levels of serum anti-FSH antibodies before and after COH

Paired *t*-tests were used to compare anti-FSH IgG, IgA and IgM antibody levels from the sera before and after COH conducted by GnRH antagonist protocol (Paper V, Table II). Our data suggested there was a significant decrease in serum anti-FSH IgG antibodies following administration of recombinant FSH (mean change of antibody values was –0.17, $p = 0.021$). Additionally, there was a small, but statistically significant decrease in anti-FSH IgA levels (mean difference –0.07, $p = 0.036$), while anti-FSH IgM levels remained unchanged (mean difference –0.01, $p = 0.930$) following COH.

7.3.2. Anti-FSH antibodies in FF compared to anti-FSH in sera

Anti-FSH IgG, IgA and IgM values in FF were 0.48 ± 0.30, 0.34 ± 0.30, and 0.15 ± 0.22, respectively. Multivariate linear regression model data suggested FF levels of anti-FSH IgG increased with follicle diameter ($r = 0.02$, $p = 0.022$), and were also associated with serum anti-FSH IgG levels ($r = 0.34$, $p<0.001$). Anti-FSH IgA levels in FF were not associated with follicle diameter, but were associated with serum anti-FSH IgA levels (r for diameter = –0.01, $p = 0.239$, r for antibodies in sera = 0.23, $p = 0.005$). IgM type anti-FSH in the FF was not associated with either follicle diameter or the level of serum anti-FSH IgM ($r = –0.004$, $p = 0.471$ and $r = 0.013$, $p = 0.447$, respectively).

7.3.3. Association between anti-FSH antibodies in FF and exogenous FSH

There was a positive correlation between levels of anti-FSH IgG in the FF and the total amount of recombinant FSH (IU) used for stimulation ($r = 276.5$, $p = 0.044$), if adjusted by the parameters that might also influence the amount of administered FSH (the value of peripheral FSH of early follicular phase and the mean volume of ovaries). Similarly, multivariate linear regression model adjusted by the value of peripheral FSH and the ovarian volume revealed a significant association between FF anti-FSH IgA levels and the amount of FSH administered ($r = 335.6$, $p = 0.035$). No association was noted between of anti-FSH IgM in the FF and the amount of FSH used for ovarian stimulation ($r = 149.1$, $p = 0.381$).

FSH levels in the FF were correlated positively with the amount of recombinant FSH administered during the ovarian stimulation (Pearson's correlation = 0.37, $p<0.001$). Our data also demonstrated an association between anti-FSH IgG and FSH levels in the FF ($r = 0.21$, $p = 0.012$) when anti-FSH IgG levels in the peripheral blood were included in the multivariate analysis. Similarly, FF anti-FSH IgA levels were associated with FF FSH levels ($r = 0.05$, $p = 0.022$) after adjustment by anti-FSH IgA levels in peripheral blood. Serum anti-FSH IgG and IgA were used in adjustments since the levels of these antibodies affected anti-FSH IgG and IgA values in the FF. Anti-FSH IgM in the FF did not correlate with the FF FSH ($r < –0.01$, $p = 0.773$).

7.3.4. Changes in serum common autoantibodies caused by COH

The presence of IgG type of common autoantibodies: ANA-H, ANA-R, SMA, PCA, TMA, B2-GPI and ACA were measured from SPC and OPU sera.

After the COH, patients falling into the following groups were counted: (i) patients with more autoantibodies detected at OPU than at SPC either at lower

or higher titer, representing the phenomenon of antibody-increase after the COH (ii) patients with unchanged number of detectible autoantibodies after the COH either at lower or higher titer, or (iii) patients with fewer autoantibodies detected at OPU than at SPC either at lower or higher titer, representing the phenomenon of antibody-decrease after the COH. The number of patients with increased antibody titer was compared with the number of patients with decreased antibody titer using the proportion test. Our results showed an overall reduction in autoantibodies during the COH, as there were significantly more patients (18.8% and 9.8% for antibody tests at lower or higher titer, respectively) who showed a decrease in detectible autoantibodies after the COH compared to the patients (9.0% and 3.8% for antibody tests at lower or higher titer, respectively, proportion test, $p<0.05$) with increased number of autoantibodies (Paper IV, Table II). Only 7 (5.4%) patients followed the GnRH agonist protocol none of whom represented the autoantibodies. Thus, these results are restricted to GnRH antagonist protocol only.

7.4. Evaluation of the potentiality of anti-FSH antibodies to interrupt with FSH receptor binding (Paper I)

The reactivity of antibodies directed to the entire FSH molecule were compared to antibodies directed against the immunodominant epitope of the β-chain of the human FSH molecule, the 78–93 amino acid region called V14D (Gobert *et al.,* 2001).

Levels of anti-FSH and anti-V14D antibodies were detected from the sera obtained from various time points of menstrual cycle from non-IVF patients with endometriosis, PCOS and control women. A Pearson's correlation test was performed between OD values of all immunoglobulin isotypes of anti-FSH (detected by Metrodine® antigen) and anti-V14D. There was a good correlation between the immunoglobulin isotypes of anti-FSH and anti-V14D, but the correlation was the weakest for IgG (IgM $r = 0.56$, $p<0.001$, IgA $r = 0.57$, $p<0.001$ and IgG $r = 0.36$, $p<0.001$, Paper I, Figure 3). The correlation was strongest in patients compared to controls and pregnant women for all antibody isotypes (Paper I, Table I). Additionally, similar correlation (IgM $r = 0.41$, $p<0.001$, IgA $r = 0.38$, $p<0.001$, and IgG $r = 0.20$, $p = 0.065$) was revealed also between anti-FSH antibodies detected by Fostimon® antigen and anti-V14D in controls, and when both antibodies were expressed as sample OD ratio to pool OD (Figure 8).

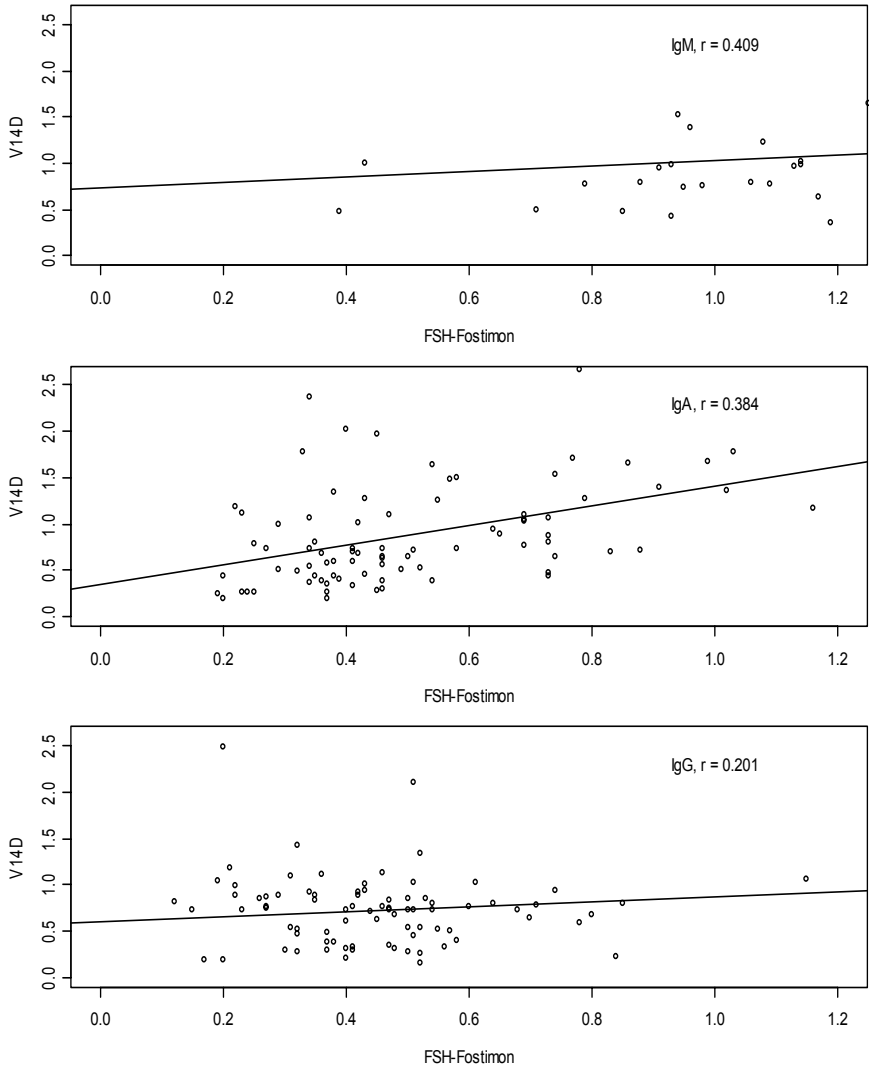

Figure 8. The Pearson's correlation (r) between optical density (OD) ratio to pool OD of anti-V14D and anti-FSH antibodies (detected by Fostimon® antigen) among controls.

7.5. Assessment of the effect of serum anti-FSH on folliculogenesis — a study based on COH outcome in IVF treatment (Paper V)

The roles of serum anti-FSH antibodies to the outcome of COH was assessed with regards to clinical parameters characterizing ovarian reserve e.g. age,

ovarian volume, and number of day 3–5 follicles. Since PCOS is associated with alteration in folliculogenesis and in the selection of the dominant follicle (Harborne *et al.,* 2003), patients with PCOS were excluded from the analysis of the role of anti-FSH antibodies to the folliculogenesis. In addition, only IVF patients who followed the GnRH antagonist protocol for COH were included in the study.

7.5.1. Basic clinical characteristics and COH parameters

The mean (± SD) ovarian volume was 5.0 ± 2.1 cm^3 and the mean number of follicles at the early follicle phase of patient's spontaneous menstrual cycle was 4.6 ± 1.8. Serum FSH levels at day 3–5 of patient's natural menstrual cycle was 9.0 ± 4.9 IU/L. While the majority of patients (81.9%, 149/182) had a regular menstrual cycle of 26–35 days (eumenorrhea), 9.3% (17/182) had polymenorrhea of <26 days, 8.2% (15/182) had oligomenorrhea of >35 days, and 1 patient (0.5%) had an irregular menstrual cycle.

The average of 1918.0 ± 503.6 IU of recombinant FSH was administered subcutaneously during 9.6 ± 0.7 days of ovarian stimulation. The average level of serum estradiol at the day of OPU was 4121.0 ± 4829.2 pmol/L. While the average number of OPU follicles was 13.2 ± 6.7, the 11.3 ± 6.7 of OPU oocytes were obtained. The average number of mature oocytes was 9.4 ± 5.5 and 6.4 ± 4.1 2PN embryos and 3.0 ± 2.7 good quality embryos were gained per patient. The mean amount of recombinant FSH administered was 228.8 ± 263.4 IU per OPU follicle, 290.0 ± 309.2 IU per OPU oocyte, 345.0 ± 359.3 IU per mature oocyte, 443.8 ± 401.2 IU per 2PN embryo and 905.7 ± 732.5 IU per good quality embryo.

The association between basic clinical parameters and characteristics of COH (Paper V, Table I) were studied in order to select clinical parameters that best predicted the outcome of COH, and therefore necessary to consider in assessments of the role of anti-FSH antibodies. Bivariate linear regression analysis revealed that younger women, larger ovarian volume, and greater numbers of follicles counted at early follicular phase of spontaneous menstrual cycles were linearly associated with the better COH outcomes. The presence of both ovaries compared to just one, lower level of serum FSH at early follicular phase of menstrual cycle, and normal menstrual cycle compared to oligo- or polymenorrhea were not accurate predictors of COH efficacy.

7.5.2. Association between serum anti-FSH antibodies and COH outcome

The association between anti-FSH antibodies and parameters indicative of COH outcome were assessed utilizing multivariate linear regression models adjusted by patient age, number of follicles (counted during the early follicular phase of menstrual cycle), or level of FSH measured during the follicular phase. Adjusted regression models were used in order to investigate the role of anti-FSH antibodies independently from the influence of clinical (adjusted) parameters (Paper V, Table I) on COH outcomes.

Higher levels of serum anti-FSH IgG at OPU associated with longer ovarian stimulation ($r = 0.28$, $p = 0.028$) and a greater amount of FSH needed to obtain one 2PN embryo ($r = 220.6$, $p = 0.002$). Linear regression analysis, adjusted by peripheral FSH levels at the early follicular phase of menstrual cycle, showed an association between anti-FSH IgG and the need for more recombinant FSH to get one OPU oocyte ($r = 92.4$, $p = 0.046$). Linear regression model adjusted by patient age and follicle number, suggested that anti-FSH IgA was positively associated with greater total amount of exogenous FSH used in ovarian stimulation ($r = 158.7$, $p = 0.014$), and increased amount of FSH needed to mature one OPU follicle ($r = 131.7$, $p<0.001$), to obtain one oocyte ($r = 107.2$, $p = 0.014$) and one mature oocyte ($r = 113.7$, $p = 0.029$), and tended to be associated with fewer OPU follicles matured after COH ($r = –1.9$, $p = 0.055$). Serum anti-FSH IgM on the OPU day was not associated with any of parameters characterizing the outcome of COH.

In addition to a linear association between anti-FSH antibodies and COH outcome, the cut-off value for anti-FSH IgG and IgA that was indicative of a poor ovarian response (≤3 oocytes in COH) was provided (Paper V, Table III). Patients with anti-FSH IgG and IgA coefficients >1.0 revealed increased risk for poor ovarian response (IgG crude OR = 4.52, $p = 0.013$, IgA crude OR = 3.87, $p = 0.017$) compared to patients with lower levels of these antibodies. The risk of anti-FSH IgG and IgA was independent of the influence of age and mean follicular number at early follicular phase, as measured by adjusted logistic regression models (IgG adjusted OR = 6.95, $p = 0.005$, IgA adjusted OR = 3.60, $p = 0.039$) (Paper V, Table III).

Series of dilutions of mouse anti-human-FSH monoclonal IgG antibody were used in ELISA test to create a concentration curve. According to the curve, the levels of anti-FSH IgG >1.0 was presumed to correspond to the antibody levels higher than 0.5–0.6 mg/L and could therefore represent 0.004% of expected amount of total IgG (8–17 g/L).

8. DISCUSSION

8.1. Anti-FSH antibodies being primarily natural antibodies

We observed the physiological presence of antibodies directed to FSH in a control group of healthy non-pregnant women, significantly lower values of IgG and IgM anti-FSH antibodies during uncomplicated pregnancy, and increased levels of these antibodies in infertile women.

In our study on IVF patients, the production of anti-FSH IgM antibodies was associated with peripheral FSH hormone levels (i.e. with the amount of hormone produced), only among patients with tubal and male factor infertility. Several conditions have been reported where the production of autoantibodies is associated with the elevated level of autoantigen, such as elevated FSH levels and AOA in premature menopause (Fénichel *et al.,* 1999). Similarly, autoantibodies and insulin levels in pancreatic β cells are correlated in murine autoimmune diabetes models (Byersdorfer *et al.,* 2005). In the current study, the level of FSH remained between the reference values for the majority of patients, with only 5 patients having FSH level above the reference value indicative for ovarian failure (>21.7 IU/L). Moreover, the anti-FSH IgM correlated with the level of peripheral FSH in the patients with tubal and male factor infertility. Those patients' hormonal level was rather lower than in other patients and their infertility was predominantly caused by other than immune system dysregulation. These results suggest anti-FSH antibodies being primarily the naturally-occurring antibodies rather than markers for autoimmunity against FSH hormone. This hypothesis is further supported by the discussion provided by Thomas (2001) who concluded that physiological hormone levels remain below a critical threshold for the stimulation of relevant autoimmune reactions (Thomas, 2001). The reason for the correlation between anti-FSH IgM and the level of peripheral hormone is still unknown, but could be associated with regulation of FSH bioactivity or with cyclic changes in ovary. The ovulatory process has been compared to a classical local inflammatory reaction and leukocytes have been suggested to participate actively in the cyclic events in the ovary (Murdoch *et al.,* 1988; Espey, 1994; Richards *et al.,* 2002; Hernandez-Gonzales *et al.,* 2006). Recently, cumulus and granulosa cells were shown to express cell surface signaling molecules known as pattern recognition receptors acting as sensors of the external environment important for the innate immune system to discriminate self from non-self or altered self (Shimada *et al.,* 2006). Moreover, a distinct compartment of mature B-lineage cells, termed B-1 cells are believed to produce IgM natural antibodies, which interact with variety of self determinants and may also cross-react with bacterial antigens (Binder and Silverman, 2005). The natural IgMs have been suggested to represent a primitive innate-like layer of adaptive immune system to provide a primary line

of defense against systemic infection from viral and bacterial pathogens. There is also evidence that the natural antibodies may contribute to the elimination of autoantigens exposed during tissue damage, for instance (Binder and Silverman, 2005).

In addition to the presence in female serum and in ovarian tissue, FSH is also introduced to the genital tract mucosa as a constituent of semen (Vasquez *et al.,* 1986; Bujan *et al*. 1993; Luboshitzky *et al.,* 2002). Female immune system recognizes and reacts to the constituents of semen during insemination, a phenomenon called seminal 'priming'. Its appropriate activation to induce sperm-prone mucosal tolerance facilitates subsequent pregnancy by sustaining 'semi-allograft' embryo development (Robertson *et al.,* 2003; Hegde *et al.,* 2001). During the process of partner-specific tolerance, cell-mediated and humoral immune reactions are initiated along with the production of antibodies against semen-specific and shared maternal antigens (Hegde *et al.,* 2001), such as FSH (Vasquez *et al.,*1986; Luboshitzky *et al.,* 2002). Therefore, the anti-FSH IgA antibodies detected in the female circulation could be alloantibodies derived from semen. According to this hypothesis, levels of anti-FSH IgA would be, depending on how closely tolerance is induced, correlated with IgA antibodies produced against sperm surface antigens. Anti-sperm antibodies in the sera of IVF patients were compared with the values of anti-FSH antibodies detected in the same individuals. Among all subtypes of antibodies, anti-FSH IgA correlated with anti-sperm IgA. These results suggest that both detected antibodies are of a shared antigenic origin and we propose anti-FSH IgA represent a natural activation of female immune system in induction the mucosal tolerance to partner antigens. This idea is supported by the previous study, where anti-FSH-β-chain antibodies were shown to be absent in the sera of children (Gobert *et al.,* 2001).

Somewhat surprisingly, this correlation was only seen in IVF patients with PCOS, endometriosis and unexplained infertility, not in patients with male factor or tubal factor infertility. The common feature for the former three infertility groups is disturbed regulation of the immune system (Fénichel *et al.,* 1999; Mathur, 2000; Reimand *et al.,* 2001; Matarese *et al.,* 2003; Jasper *et al.,* 2006). Similarly in the current study, patients with endometriosis and PCOS possessed higher prevalence of common autoantibodies. Disruptions of the immune system perturb the female's immune response to semen that is necessary for partner-specific tolerance and thereafter elimination of activated clones to prevent autoimmunity during pregnancy (Hegde *et al.,* 2001). Semen exerts its 'tolerance inducing' effect due to immunomodulating factors, most importantly transforming growth factor β_1 (TGFβ_1) (Roberston *et al.,* 2002; Ochsenkühn *et al.,* 2006). Seminal levels of TGFβ_1 correlate with sperm concentration in ejaculate (Ochsenkühn *et al.,* 2006), the most decisive criterion for diagnosing male infertility (WHO, 1999). However, there is some evidence that male factor infertility is not associated with altered TGFβ_1 levels (Loras *et al.,*

1999). Although we did not distinguish subgroups of patients with male infertility by sperm parameters, generally their levels of anti-sperm and anti-FSH antibodies, or correlations between the two, were similar to other patients. Unlike other IVF patients participating in this study, patients with infertility caused by tubal factor do not have disturbances in female immune system regulation or seminal environment. Thus, the diagnosis-restricted correlation of anti-sperm and anti-FSH IgA cannot be easily explained. However, higher levels of anti-FSH IgA showed an association with the presence of the *HLA-DQB1*03* allele. In this context, it is interesting to refer to the published associations between the *HLA-DQB1*03* allele, and the presence of the sperm-immobilizing antibodies in cervical secretions (Tsuji *et al.,* 2000). Higher production of anti-sperm antibodies has been detected in patients with increased intestinal permeability in bowel inflammatory disease, as a result of immunization against intestinal microflora, which seems to share common antigenic epitopes with spermatozoa (Dimitrova *et al.,* 2005). Consequently, the elevated levels of anti-FSH IgA antibodies in IVF patients could be explained by an up-regulation of the normal mucosal immune response. Another possible explanation of the increased anti-FSH IgA in IVF patients could be a deficits in producing antibodies that neutralize anti-FSH immunoglobulins, which has been noted in case of anti-sperm antibodies in these patients (Naz *et al.,* 1993; Naz and Menge, 1994). These results together suggest the elevated values of anti-FSH IgA in IVF patients could represent a failure in mucosal tolerance in the genital tract, which could be genetically determined.

Correlation analysis of anti-FSH antibody values among healthy controls showed that the levels of both anti-FSH IgM and IgA correlated with the values of anti-FSH IgG.

Compared to the non-pregnant healthy women, significantly lower values of IgG and IgM anti-FSH antibodies were observed during pregnancy. This decreased production of anti-FSH antibodies cannot be easily explained by the general view of a shift towards Th2 cytokines favoring humoral immunity during pregnancy (Saito *et al.,* 1999; Gleicher, 1992; Ito *et al.,* 2001). However, in fact, actual elevations of autoantibodies have been detected in patients with pregnancy loss or recurrent abortion rather than in healthy non-complicated pregnancy (Gleicher, 1992; Eroglu and Scopelitis, 1994). Therefore, we believe that the development of the FSH-antibodies could reflect some other pregnancy-associated mechanism and that anti-FSH antibodies could be the natural antibodies also in this occasion.

Our findings are summarized with Figure 9 and suggest that antibodies detected against FSH could be natural antibodies also subjected to pregnancy-associated immune system regulations. Anti-FSH IgA detected in female circulation could be a part of the mucosal response involved in inducing immune tolerance to seminal constituents. Anti-FSH IgM associates with the peripheral level of FSH hormone and possibly contributes along with the

mucosal-associated induction of IgA to the production of circulating anti-FSH IgG. Although the humoral immune memory associated with natural antibody-producing B-cells has been postulated to contribute to the homeostasis of the internal milieu, these cells are also believed to be responsible for autoantigen-mediated clonal selection in the process of initiating autoimmune reactions (Binder and Silverman, 2005).

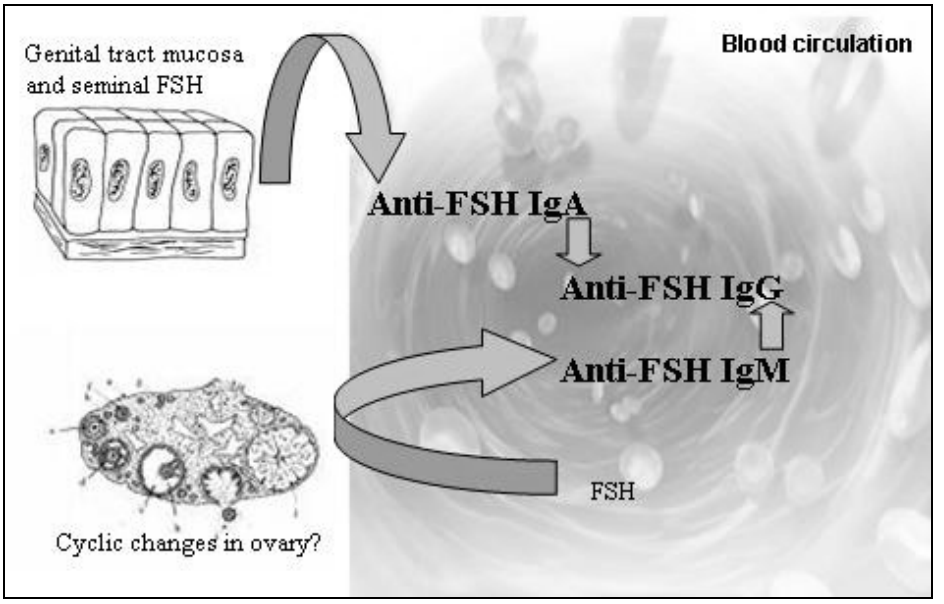

Figure 9. Schematic overview of anti-FSH antibodies in healthy female.

8.2. Increased production of anti-FSH antibodies contributes to female infertility

8.2.1. Higher values of anti-FSH in infertile women

We observed that anti-FSH antibodies were predominantly produced in infertile patients compared to healthy female blood donors. Only IgA type of anti-FSH was detected in higher levels in non-IVF patients with endometriosis or PCOS, while the increase of all isotypes of anti-FSH antibodies were seen in IVF patients with different etiologies. The results of these two studies support the idea of increased production of anti-FSH antibodies in female infertility, despite of (1) the use of a different antigen source in the detection of anti-FSH antibodies and (2) different selection of patients.

Two different urinary purified FSH antigens were used in ELISA — Fostimon® and Metrodine®. Due to developed purification technique Fostimon® represents only FSH without the presence of LH and has improved pureness than formerly used Metrodine®. Latter study group consisted of infertile women who were indicated for IVF, but with serum samples obtained before the administration of exogenous FSH. Thirty-four percent of patients had had at least one previous IVF procedure, but at least three months had past since the last FSH ovarian stimulation. Furthermore, using stratification by previous IVF procedures, anti-FSH antibody levels were also increased in IVF patients who had never undergone IVF procedures before. The further analysis demonstrated no significant differences in anti-FSH antibody levels between the combined groups of patients with tubal and male factor infertility compared to the women with PCOS, endometriosis, unexplained infertility, and female infertility due to the other causes. These data together suggest infertility itself, rather than the cause of infertility, could be a predictive factor for the emergence of anti-FSH antibodies, as previously concluded in case of AOA (Geva *et al.,* 1996). The intriguing question of what associates the production of anti-FSH antibodies and female infertility stemmed directly from this context.

Female infertility has been shown to be associated with a higher occurrence of autoantibodies (Geva *et al.,* 1997; Gobert *et al.,* 1992; Fénichel *et al.,* 1999; Reimand *et al.,* 2001; Matarese *et al.,* 2003). Except disease-specific autoantibodies described in case of endometriosis and POF (Mathur *et al.,* 1999; Lang and Yeaman, 2001; Forges *et al.,* 2004), autoantibodies detected in infertile patients (Geva *et al.,* 1997; Gobert *et al.,* 1992; Fénichel *et al.,* 1999; Reimand *et al.,* 2001; Matarese *et al.,* 2003) are usually not specific to infertility or to the gynecological diseases leading to infertility. Thus, a general immune dysbalance and activation of autoimmune processes are expected to be characteristic for female infertility (Gleicher, 2001). Here we assessed a potential susceptibility of a patient to autoimmunity by the presence of at least 1 out of 7 common autoantibodies in relation to the autoimmunity-prone *HLA-DQB1* alleles. We demonstrated that anti-FSH IgM were associated with the production of common autoantibodies and this association was not confounded by the presence of *HLA-DQB1* alleles. Our results along the ones from the literature discussed above indicate, that the increased production of anti-FSH IgM could be related to a general propensity to autoimmunity in infertile women.

The female infertility has often been studied in the context of IVF. The follicular puncture performed in IVF, in particular, can induce the production of AOA (Gobert *et al.,* 1992). Patients with AOA have often antibodies against FSH, as one of the major autoantigens for the pool of autoantibodies forming AOA (Gobert *et al.,* 2001). In concordance with these data, we showed that the level of anti-FSH IgM was higher in the patients who had undergone previous IVF procedures. The association was revealed among IVF patients who were suffering from PCOS, endometriosis, unexplained infertility, and infertility due

to the other causes, but not among the women with tubal or male factor infertility. These results encourage us to speculate, that repeatedly performed ovarian punctures do not enhance anti-ovarian autoimmunity unless a patient's infertility is caused by the diseases associated with disturbances in immune regulation (Geva *et al.,* 1997; Gobert *et al.,* 1992; Fénichel *et al.,* 1999; Reimand *et al.,* 2001; Matarese *et al.,* 2003). However, simply based on the association study performed here, we cannot substantiate whether the antibodies themselves may cause the need for multiple IVF procedures, or alternatively, the use of IVF procedure *per se* may enhance the production of anti-FSH.

The receptor-binding and hormone specificity determining β-subunit of FSH hormone is coded by *FSHB* gene at the 11p13 (Fox *et al.,* 2001). Haplotype analysis has revealed two most prevalent variants of *FSHB* gene — HAP1 and HAP13, covering together about 90% of Estonians (Grigorova *et al.,* 2007). Similarly to insulin gene polymorphisms affecting central tolerance through the level of gene expression in thymus (Vafiadis *et al.,* 2001), we were looking for an association between the two *FSHB* core haplotypes (Grigorova *et al.,* 2007) and autoimmunity against FSH. As we could not detect such relationship, we suggest that either these SNPs do not affect gene expression in the thymus during central tolerance induction, or that *FSHB*-associated autoimmunity to FSH depends on *HLA-DQB1* allelic variants other than those evaluated in the current study.

The production of anti-FSH IgA is probably related to different factors than those involved in the production of anti-FSH IgM. Anti-FSH IgA were associated with the presence of the *HLA-DQB1*03* allele but not with the cause of infertility, the history of previous IVF attempts or the presence of other autoantibodies. Therefore, it would be tempting to speculate that anti-FSH IgA could not be autoantibodies but alloantibodies triggered by seminal FSH (Vasquez *et al.,* 1986; Luboshitzky *et al.,* 2002) and originating from mucosal response, as discussed above. The reasons for an increased production of this particular IgA isotype of antibodies in IVF patients, however, remain unclear.

Correlation analysis of anti-FSH antibody values among healthy controls showed that the levels of anti-FSH IgM and IgA correlated both with the values of anti-FSH IgG. There is some indirect evidence that anti-FSH IgG antibodies may, however, further worsen female fecundity by reducing the FSH functionality (Meyer *et al.,* 1990; Reznik *et al.,* 1998). These data lead us to investigate the effect of anti-FSH antibodies on folliculogenesis and developing infertility in women.

8.2.2. Effect of serum anti-FSH on folliculogenesis

IVF has become a promising treatment for various causes of infertility. However, the success of attaining pregnancy following IVF depends on the

effectiveness of COH. Our study demonstrated that serum levels of anti-FSH IgG and IgA, but not IgM antibodies at the day of OPU, were linearly associated with the poorer outcome of COH. The outcome of COH was assessed by the duration of FSH stimulation or the total FSH required attaining an adequate response, the number of follicles punctured or oocytes obtained after COH, the number of mature oocytes or embryos, and the amount of FSH required per all of these parameters. The role of anti-FSH antibodies revealed in our study was quite remarkable. For example, our data suggested that a unit (expressed as sample OD ratio to pool OD) difference in anti-FSH IgG was associated with a 220.6 IU increase in FSH needed for one 2PN embryo, while the mean amount of FSH per 2PN embryo was only 443.8 ± 401.2 IU. Furthermore, the cut-off value of >1.0 for anti-FSH IgA and IgG was calculated to be implicated to poor ovarian response (≤3 oocytes). Anti-FSH IgG coefficient >1.0 was corresponding to the estimated antibody concentration of 0.5–0.6 mg/L, which would be expected to form 0.004% of total IgG in peripheral blood. The same or even slightly lower levels of blocking and stimulating serum TSH-receptor autoantibodies has been demonstrated previously in patients with Graves' disease and in autoimmune hypothyroidism (Morgenthaler *et al.,* 2006). As anti-FSH antibodies are often detected in patients with AOA (Gobert *et al*., 2001; Shatavi *et al*., 2006), our results may simply reflect an impaired ovarian function due to ovarian autoimmunity. The association between anti-gonadotrophin (Meyer *et al*., 1990) or AOA (Luborsky *et al*., 2002) IgG in the sera at OPU and poor ovarian response to the FSH stimulation has been shown previously.

In addition to reflecting ovarian autoimmunity, anti-FSH antibodies may impair the function of exogenous or endogenous FSH. For example, anti-FSH could form immune complexes with FSH and induce its clearance, as recently shown for creatine kinase in patients with corresponding antibodies (Warren *et al*., 2006). Also, anti-FSH could interrupt the binding of FSH to its receptor. This hypothesis is supported by our data suggesting anti-FSH antibodies in sera correlated with antibodies directed against the 78–93 amino acid region of the β-chain of the human FSH (Gobert *et al*., 2001), the domain that determines FSH receptor binding specificity (Fox *et al*., 2001). On the other hand, an investigation of the FSH-blocking ability of anti-FSH IgG *in vitro* showing that these antibodies were also present in the women with good response in IVF (Reznik *et al*., 1998) suggested that anti-FSH antibodies may be non-pathogenic. However, this study did not specify which FSH epitopes were bound by the pool of anti-FSH antibodies.

Although the pathophysiology of anti-FSH in association with poor ovarian response is still unclear, the importance of these antibodies pays the attention. Our results confirmed that a woman’s age, as well as her ovarian volume and the number of follicles counted at the early follicular phase of her spontaneous menstrual cycle were significant clinical parameters predicting the outcome of

COH, as also demonstrated previously (Kligman and Rosenwaks, 2001). The association between the levels of anti-FSH antibodies and poor COH outcomes, however, was demonstrated in various associations from multivariate adjusted linear regression models. By adjusting the statistical analysis for the clinical parameters, we were able to assess the role of anti-FSH to the ovarian response as if the clinical parameters were constant in the study population. Though, our data suggest that anti-FSH antibodies could represent an additional importance to the clinical parameters like age, follicle number or ovarian volume, in predicting the outcome of COH. Furthermore, if the influence of anti-FSH on the ovarian response was revealed in the IVF patients (where supraphysiological amounts of FSH were administered to stimulate folliculogenesis) the importance of those antibodies in unstimulated spontaneous folliculogenesis might be substantial.

8.2.3. Changes in serum levels of anti-FSH during COH in relation to FF

We observed that serum levels of anti-FSH IgG and IgA, but not IgM antibodies, were decreased following COH, conducted with GnRH antagonist protocol. Although interpretation of these results is not straightforward, we believe the decrease in anti-FSH antibody levels could partly be explained by the supraphysiological levels of immunosuppressive progesterone and testosterone (Tanriverdi *et al*., 2003; Cutolo *et al*., 2004) produced in COH. This hypothesis is supported by our data suggesting an overall decrease in the number of common IgG autoantibodies during COH. Additionally, anti-FSH antibodies could form immune complexes with administered recombinant FSH or with endogenous FSH (produced in pituitary prior to administration of GnRH antagonists), resulting in the decrease in antibody levels. However, in the current study, levels of anti-FSH IgM remained unchanged after COH. As IgM antibodies also form immune complexes, reactivation of the immune system towards novel epitopes on the FSH molecule and production of anti-FSH IgM during COH might be speculated. As well, immunization against exogenous gonadotrophins has also been previously suggested (Meyer *et al.,* 1990). This hypothesis is further supported by our findings and that found from the literature, that an increase in IgM type of anti-FSH and AOA (Gobert *et al*., 1992; Narayanan *et al.,* 1995; Luborsky *et al.,* 1999) were associated with repeated IVF procedures. However, it was also reported that AOA were initiated by ovarian puncture rather than administered FSH (Gobert *et al*., 1992). Additionally, circulating anti-FSH could pass into the FF during follicle maturation; however this decrease would hardly be detectible in sera by current laboratory tests.

The charge- and size-selective ovarian blood-follicle barrier is open for IgG to pass into the FF (Hess *et al*., 1998). Our data is in agreement with previous

studies suggesting that IgG and IgA concentrations in the FF and the plasma were similar (Clarke *et al*., 1984). In addition, we showed that anti-FSH IgG in the FF were positively associated with the diameter of follicle, reflecting the maturity of a follicle. The increase in FF anti-FSH IgG with the growth of the follicle was not a simple reflection of anti-FSH IgG serum levels, as serum anti-FSH IgG levels were significantly decreased during COH. The positive correlations between FF anti-FSH IgG levels and the amounts of recombinant FSH used for ovarian stimulation and FSH levels measured in the FF were also noted. Previous studies have shown an increase in FSH in the FF during follicular growth (Lambert-Messerlian *et al*., 1997; Glister *et al*., 2006). In the current study, FSH in the FF was also correlated with the amount of FSH administered, in agreement with that previously reported (Luborsky *et al*. 2002). Thus, our results suggest that anti-FSH IgG diffused with the antigen to the FF during the COH. In contrary, research in pigs demonstrated the concentration of total IgG in the FF increased as follicles enlarged (Hussein and Bourne, 1984), suggesting the accumulation of anti-FSH IgG into the FF was not specifically caused by FSH taxis. Although anti-FSH IgA and IgM were detected in the FF, levels of these antibodies were not associated with follicle diameter, which is in agreement with other authors (Hussein and Bourne, 1984). In addition, anti-FSH IgM levels in the FF were very low compared to serum antibody levels, in concordance with that reported by Clarke and coworkers (1984) where total IgM in the FF represented approximately 10% of its plasma concentration (Clarke *et al*., 1984).

In conclusion, our results (Figure 10) suggest that naturally-occurring anti-FSH antibodies were produced in higher levels in infertile women. The production of anti-FSH IgM and IgG antibodies was not associated with *FSHB* gene haplotypes, but could be related to a general propensity to autoimmunity or to previous IVF treatments. The elevated values of anti-FSH IgA in IVF patients could be explained by somewhat different mechanisms including a genetically determined failure in mucosal tolerance in the genital tract. Our data also demonstrated anti-FSH IgG, IgA and traces of IgM antibodies were detectable also in FF. Anti-FSH IgG antibodies, present in sera, accumulated into the preovulatory follicle. Our data further suggested that serum IgG and IgA anti-FSH antibodies, measured at the day of oocyte retrieval, were predictive of COH outcome, additionally to that observed with age and other clinical parameters characterizing the ovarian reserve. These results emphasize the need for further research to elucidate the clinical relevance of anti-FSH antibodies in the spontaneous menstrual cycles.

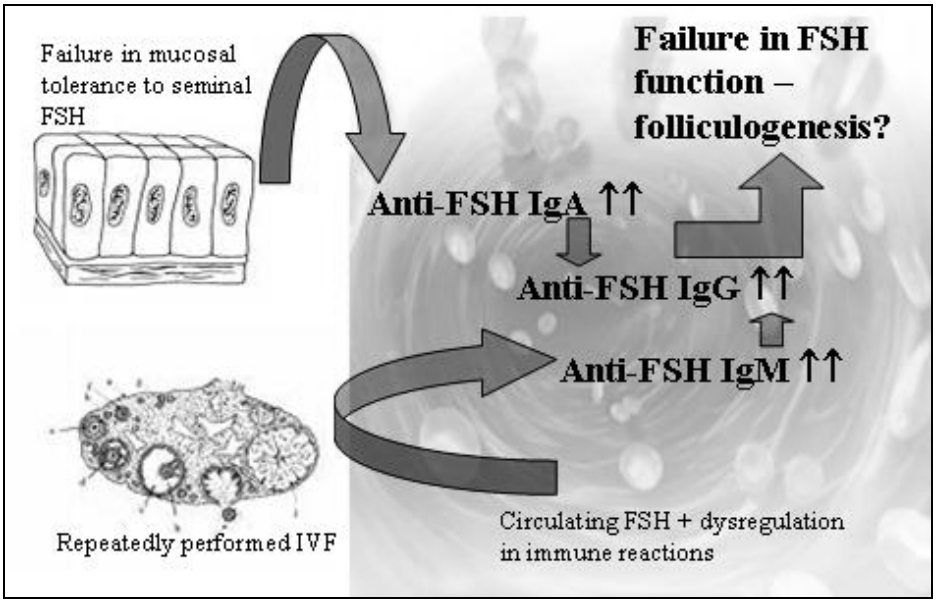

Figure 10. Increased production of anti-FSH antibodies in case of female infertility.

9. SUMMARY AND CONCLUDING REMARKS

1. The presence of antibodies directed to FSH was observed in a group of healthy non-pregnant women. The production of IgG and IgM anti-FSH antibodies were significantly decreased in women with uncomplicated pregnancy and that of already by the end of 1st trimester. Increased levels of these antibodies were detected in infertile women. IgA type of anti-FSH was detected in higher levels in non-IVF patients with endometriosis or PCOS, while the increase of anti-FSH IgG, IgA and IgM antibodies were seen in IVF patients with different etiologies.
2. Higher levels of anti-FSH antibodies in IVF patients were not associated with *FSHB* haplotypes. The increased levels of anti-FSH IgM associated with (i) the levels of FSH in women with male and tubal factor infertility, (ii) the history of IVF in patients with PCOS, endometriosis, and un-explained infertility, and (iii) the production of common autoantibodies among all IVF patients. The levels of anti-FSH IgA were increased in the patients carrying *HLA-DQB1*03* allele. Values of anti-FSH IgA correlated with the levels of serum anti-sperm IgA in patients with PCOS, endometriosis and unexplained infertility. The anti-FSH IgG correlated with the values of anti-FSH IgA and IgM.
3. A significant decrease in serum anti-FSH IgG antibody levels following administration of recombinant FSH was detected. Additionally, there was a small, but statistically significant decrease in anti-FSH IgA levels, while anti-FSH IgM levels remained unchanged following COH. Similarly, there was an overall reduction in IgG type of common autoantibodies during the COH.

   Levels of FF anti-FSH IgG increased along with the growth of a follicle and were also associated with serum anti-FSH IgG levels. Anti-FSH IgA levels in FF were not associated with follicle diameter, but with serum anti-FSH IgA levels, while IgM type anti-FSH in the FF was not associated with either follicle diameter or the level of serum anti-FSH IgM. Anti-FSH IgG and IgA levels in FF were in positive correlation with the total amount of recombinant FSH used for stimulation and the level of FSH in FF.
4. Serum anti-FSH IgG, IgA and IgM antibodies correlated with antibodies directed against the 78–93 amino acid region of the β-chain of the human FSH (V14D) the domain that determines FSH receptor binding specificity. The correlation was the weakest for IgG and strongest in patients compared to controls and pregnant women for all antibody isotypes.
5. Serum levels of anti-FSH IgG and IgA, but not IgM antibodies at the day of OPU were in linear association with the poorer outcome of COH. The cut-off value of >1.0 for anti-FSH IgA and IgG was calculated to be implicated to poor ovarian response (≤3 oocytes).

Summing up, our results suggest that antibodies directed against FSH could be natural antibodies possibly subjected to pregnancy-associated immune system regulations. Anti-FSH IgA detected in female circulation could be a part of the mucosal response involved in inducing immunotolerance to seminal constituents. Anti-FSH IgM associates with the peripheral level of FSH hormone and contributes along with the mucosal-associated induction of IgA to the production of circulating anti-FSH IgG.

Additionally, our data suggest the higher production of anti-FSH antibodies could contribute to female infertility. The induced production of anti-FSH IgM and IgG antibodies could be related to a general propensity to autoimmunity or to previous IVF treatments. The elevated values of anti-FSH IgA in IVF patients could be explained by somewhat different mechanisms including a genetically determined failure in mucosal tolerance in the genital tract. Serum IgG and IgA anti-FSH antibodies, measured at the day of oocyte retrieval, were predictive of COH outcome, additionally to that observed with age and other clinical parameters characterizing the ovarian reserve. A population of anti-FSH antibodies which are produced against 78–93 epitope on the β-chain might modulate the recognition and binding of FSH to its receptor and might therefore have a pathological influence on ovarian function. Our data also demonstrated that anti-FSH IgG, IgA and traces of IgM antibodies were detectable in the FF and that anti-FSH IgG antibodies accumulated into the preovulatory follicle.

These results emphasize the need for further research to evaluate the clinical relevance of anti-FSH antibodies in the spontaneous menstrual cycles. The research investigating IVF patients with their oocytes retrieved from spontaneous menstrual cycles, rather than after COH, could further elucidate this issue. In addition, it would be of interest to investigate the role of anti-FSH antibodies on IVF treatment in patients following the GnRH agonist protocol, where folliculogenesis is stimulated only by exogenous FSH. Additionally, a novel approach would be to study autoantibodies to FSH in men with unspecified impaired sperm count.

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11. SUMMARY IN ESTONIAN

ANTIKEHAD FOLLIIKULEID STIMULEERVA HORMOONI VASTU

Tähendus naisepoolses viljatuses

Paari viljatuse all mõistetakse olukorda, kus naisel ei ole õnnestunud rasestuda kuni 1 aasta kestnud regulaarse suguelu jooksul, ilma rasedusest hoidumata. Viljatus on sage probleem, haarates ~60–80 miljonit (15% paaridest) paari terves maailmas, mis teeb hinnanguliselt umbes 15 000 lastetut paari Eesti kohta. Paari viljatust põhjustavad võrdselt nii naisepoolsed kui ka mehepoolsed põhjused. Enamik neist põhjustest ei viiks paari viljatuseni, kui see esineks vaid ühel partneritest, kuid võib olla oluliseks infertiilsuse põhjuseks, kui ka teisel partneril esineb subfertiilsus. Täielik viljatus ehk steriilsus leitakse vähemalt ühel partneritest vaid 3–15% viljatutest paaridest. Ligikaudu 20%-l viljatutest paaridest jääb aga viljatuse põhjus selgusetuks.

Mehepoolse viljatusega kaasnevad peaaegu alati muutused sperma parameetrites, mida hinnatakse põhiliselt spermatosoidide arvu, morfoloogia ja liikuvuse alusel. Pooltel juhtudel esineb nö. idiopaatiline sperma patoloogia, kus selle kliiniline põhjus jääb selgusetuks. Rasket sperma patoloogiat võivad põhjustada kromosomaalsed häired, aga ka ühe geeni mutatsioonid ning spermatosoidide DNA kahjustused. Sperma patoloogiat esineb veel varikotseele, infektsioosse või autoimmuunse orhiidi, endokriinse hüpogonadismi, krüptorhismi jm. haiguste korral.

Naisepoolset viljatust võivad põhjustada mitmed suguorganite ja/või suguteede haigused, häired neuroendokriinsüsteemis ning immuunsüsteemi regulatsioonis, kuid ka sisuliselt igasugune tervet organismi haarav üldseisundi oluline halvenemine. Tubaarne viljatus on Ida-Euroopa ja Kesk-Aasia maades, kuid ka Eestis kõige sagedamini esinev viljatuse põhjus. Tubaarse viljatuse all mõeldakse munajuhade osalisest või täielikust sulgusest, munajuha limaskesta kahjustusest või munasarja ümbritsevatest liitelistest protsessidest tingitud viljatust. Munajuhad võivad saada kahjustada endometrioosi haigel, kuid kõige levinumaks põhjuseks on naise väikevaagna põletik sugulisel teel levivate haiguste põdemise tagajärjel. Urogenitaalne klamüdioos on sugulisel teel levivate haiguste põhjuseks 20–40% juhtudest ning esineb koos gonorröaga 25–50% neist. Erinevatel andmetel viib väikevaagna põletik viljatuse kujunemiseni 6–60% juhtudel. Lääne-Euroopas ja Põhja-Ameerikas on kuni 10–20% naisepoolsest viljatusest tingitud endokrinoloogilistest põhjustest. Anovulatsioon võib olla tingitud hüperprolaktineemiast aga ka hüpogonadotroopsest hüpogonadismist, mille üheks põhjuseks on kehakaalu langus. Hüpergonadotroopse hüpogonadismi enamlevinud põhjus on varajane menopaus või enneaegne munasarjade puudulikkus (POF), esinedes vastavalt 5%-l ja 1–2%-l kõikidest

naistest. POF on heterogeenne haigus, mida üle pooltel juhtudel seostatakse munasarja koe autoimmuunse kahjustusega. Hüperandrogeneemilise anovulatsiooni kõige sagedamaks põhjuseks on polütsüstiliste munasarjade sündroom (PCOS), esinedes 4–10% reproduktiiveas naistest. Endometrioosi võib esineda 10–20% reproduktiiveas olevates naistest, kuid ~50% viljatutest naistest. Umbes pooled endometrioosi põdevatest naistest kannatavad ka viljatuse all. Ehkki raskema endometrioosiga kaasneb väikevaagna elundite anatoomia muutus, on viljatus I–II astme endometrioosi puhul tingitud eelkõige häiretest immuunsüsteemi regulatsioonis. Umbes 20–30%-l paaridest esineb nn. seletamatu infertiilsus, lähtudes infertiilsuse eelpool nimetatud definitsioonist. Enamus nendest paaridest rasestuks eeldatavasti ilma ravita pikema perioodi kui aasta jooksul ja vaid ~30% jääks viljatuks ka kuni 10 aasta möödudes. Seetõttu arvatakse, et osa seletamatust viljatusest moodustavad terved paarid, kes vajavad rasestumiseks lihtsalt pikemat aega, osa aga kannatab seni diagnoosimatu viljatuspõhjuse all. Immuunsüsteemi regulatsiooni häireid on peetud üheks võimalikuks viljatuse põhjuseks seletamatu viljatuse juures.

Immuunsüsteemi aktivatsiooni ning reaktsiooni oma kudede suhtes hoitakse ära tsentraalse ning perifeerse tolerantsuse mehhanismide poolt. Häired tolerantsuse kujunemises ning immuunsüsteemi vastuses võivad viia autoimmuunsuse tekkeni. Küpsete T-rakkude proliferatsioon vastusena oma või võõrantigeenile sõltub antigeense peptiidi ja peamise koesobivuskompleksi (HLA) sobivusest ja stabiilsusest. HLA II klassi molekulide sobivus aga antigeense peptiidiga on alleelspetsiifiline. Samuti võivad antigeeni geeni variatsioonid mõjutada tsentraalse tolerantsi efektiivsust, mõjutades antigeeni ekspressiooni tüümuses. Koespetsiifilist autoimmuunset reaktsiooni soodustavad omakorda lokaalsed faktorid, nagu näiteks koe kahjustus põletike tagajärjel või antigeeni liigne produktsioon. Autoagressiivsete T-rakkude kloonide aktivatsioon ja proliferatsioon viib autoimmuunsuse kujunemisele.

Autoimmuunsed mehhanismid ja suurenenud autoantikehade produktsioon kaasnevad paljude naisepoolse viljatuse põhjustega, nagu POF, endometrioos, PCOS, seletamatu viljatus, ning esinevad patsientidel, kellel on kehavälise viljastamise protseduurid (IVF) korduvalt ebaõnnestunud. Erineva põhjusega viljatutel patsientidel on kirjeldatud endomeetriumi, munasarja (AOA), tuuma (ANA), silelihaskoe vastaste (SMA), antifosfolipiid-antikehade (APA), kilpnäärme (TMA või anti-TPO) ja teiste organ-spetsiifiliste ja organ-mitte-spetsiifiliste autoantikehade esinemist. Välja arvatud AOA ovulatsiooni häirete ning endomeetriumi vastased antikehad endometrioosi korral, ei ole aga viljatutel naistel leitud autoantikehad tavaliselt spetsiifilised viljatusele, ega viljatust põhjustavale günekoloogilisele haigusele. Sageli ennustab autoantikehade arv, mitte aga antikeha tüüp, viljatusravi tulemuslikkuse. Seetõttu on hakatud rääkima üldisest autoimmuunsest aktivatsioonist naisepoolse viljatuse juures, ja mitte niivõrd ei seostata infertiilsust ühe konkreetse autoantikeha esinemisega. Seega kujutab autoimmuunsusega kaasnev viljatus sarnaselt klassikalistele

autoimmuunhaigustele endast T-lümfotsüütide polüklonaalset aktivatsiooni ja autoantikehade produktsiooni.

Munasarja koe vastased autoimmuunreaktsioonid on tõenäoliselt suunatud mitmete erinevate antigeenide vastu. Nii nagu autoantikehad teistegi autoimmuunhaiguste puhul, on ka osad AOA antikehad seotud otsese munasarjakoe kahjustusega ning mõned antikehad on vaid kaudseks rakulise immuunreaktsiooni markeriks. Võimalike immunoglobuliin G (IgG) tüüpi AOA antigeensete struktuuridena on kirjeldatud munarakku või *zona pellucida*'t, steroidogeenseid ensüüme granuloosa ja teeka rakkudes ning gonadotropiine ja nende retseptoreid.

Uuringud gonadotropiinide ja nende retseptorite vastaste antikehade tähtsusest munasarjavastases immuunsuses on olnud vastukäivad. Arvatakse, et retseptorit blokeerivad autoantikehad võivad reageerida ka hormooni endaga. Luteiniseeriva hormooni (LH) retseptori vastaseid antikehi on kirjeldatud kuni 30%-l IVF patsiendil ja kuni 50%-l viljatutel endometrioosi haigetel. Hiljem identifitseeriti aga AOA positiivsete POF haigete vereseerumis antikehi, mis reageerisid folliikuleid stimuleeriva hormooni (FSH) β-alaühikuga, kusjuures LH vastane reaktsioon puudus neil patsientidel täiesti. Need antikehad reageerisid erinevate epitoopidega üle terve β-alaühiku, kuid kõikidel patsientidel esines reaktsioon 78. ja 93. aminohappe vahelise immunodominantse regiooni (V14D) vastu. Vastav piirkond FSH β-alaühikul määrab aga ära hormooni kinnitumise spetsiifilisuse tema retseptorile. FSH vastaste antikehade võimet inhibeerida FSH hormooni toimet on näidatud varasemalt meestel. Seni pole aga teada FSH vastaste IgG, ega ka IgA või IgM tüüpi antikehade esinemist erinevate viljatuspõhjustega naistel ega ka mitte tervetel naistel. Ei ole ka teada faktoreid, mis võiksid soodustada FSH vastaste antikehade teket või kas nimetatud antikehad võiksid mõjutada FSH poolt stimuleeritud follikulogeneesi ja seega ka viljatuse ravi tulemuslikkust IVF-l. Samuti tekib küsimus, kas raseduse kui loomuliku follikulogeneesi supressiooni ajal muutub FSH vastaste antikehade produktsioon. Rasedusaegne anovulatsioon on tingitud alanenud FSH ja LH vastusest gonadotropiine vabastava hormooni (GnRH) toimele.

FSH kuulub hüpofüüsis toodetud glükoproteiinsete hormoonide (GPH) hulka, mis koos LH-ga reguleerib gonaadide funktsiooni. Üle parakriinsete faktorite mõjutab FSH juba preantraalsete folliikulite arengut, kuid antraalsete folliikulite areng ja kasv sõltub kriitiliselt FSH toimest. GPH-d on heterodimeerid, mis moodustuvad kõikidele selle perekonna hormoonidele ühisest α-alaühikust ning unikaalsest ja hormooni spetsiifilisust määravast β-alaühikust. FSH β-alaühikut kodeeriv geen (*FSHB*) asub 11. kromosoomis ja koosneb kolmest eksonist. Haplotüübi analüüs on näidanud, et Eesti populatsioonis eristub selgelt 2 tuumhaplotüüpi, mille sagedus Eesti populatsioonis on umbes 90%. Need kaks tuumhaplotüüpi arvatakse mõjutavat naise rasestumise kiirust, kuid ei ole teada, kas need haplotüübid on seotud ka FSH vastase autoimmuunsusega sõltuvalt geenikspressiooni erinevusest tüümuses.

FSH-d kasutatakse follikulogeneesi stimuleerimiseks IVF ravis. IVF on kujunenud kõige efektiivsemaks viljatuse ravi meetodiks erinevate viljatuspõhjuste korral. IVF ravi tulemuslikkus sõltub otseselt follikulogeneesi stimulatsiooni tulemuslikkusest. IVF programmi raames stimuleeritakse munasarju eksogeense FSH-ga, mida nimetatakse kontrollitud ovariaalseks hüperstimulatsiooniks (COH). COHi tulemusena küpseb korraga ühe folliikuli asemel kuni 15–20 folliikulit. Klassikalise IVF protseduuri raames inkubeeritakse munarakke progressiivselt liikuvate spermatosoididega. Teatud juhtudel aga kasutatakse spermatosoidi süstimist otse munaraku tsütoplasmasse (ICSI). Ühe patsiendi kumulatiivne rasestumise tõenäosus korduvate IVF-idega on ~70%, kuid ühe IVF tsükli kohta on rasestumistõenäosus vaid kuni 30%. Üheks võimalikuks IVF ebaõnnestumise põhjuseks peetakse autoantikehade esinemist naisel. Teisest küljest aga võib korduv IVF protseduuri läbimine olla üheks riskifaktoriks suurenenud autoantikehade tekkele. Seega on oluline uurida faktoreid, mis võivad mõjutada ovariaalset vastust standardsele FSH stimulatsioonile.

Uurimistöö eesmärgid

1. Võrrelda IgG, IgA ja IgM tüüpi FSH vastaste antikehade taset naistel, kes on erineva põhjuse tõttu viljatud ning kellel esineb häireid munasarjade funktsioonis vastavate antikehade tasemega tervetel mitte-rasedatel naistel ning raseduse ajal.
2. Välja selgitada võimalikud faktorid, mis soodustavad FSH vastaste antikehade produktsiooni.
3. Teha kindlaks kuidas mõjutab COH FSH vastaste antikehade taset veres võrrelduna antikehade tasemega follikulaarvedelikus.
4. Hinnata FSH vastaste antikehade potentsiaalset võimet takistada FSH seostumist retseptorile.
5. Analüüsida FSH vastaste antikehade mõju follikulogeneesile, hinnates COH tulemuslikkust IVF protseduuris.

Uuritavad ja meetodid

Kokku uuriti 233 IVF patsienti erineva viljatuse põhjusega Nova Vita Kliinikust, Viimsist, 178 mitte-IVF patsienti endometrioosi ja PCOS-ga ning 75 rasedat naist SA TÜK Naistekliinikust ja 85 veredoonorit Kuressaare Haigla verekeskusest. Uuritavateks materjalideks oli kõigil vereseerum ning genoomne DNA ja follikulaarvedelik IVF patsientidel.

FSH, V14D, β 2-glükoproteiini ja kardiolipiini vastased antikehad määrati ensüümikaudse immunosorptsioonimeetodil (ELISA). ANA (inimese HEp2 rakuliini ja närilise maksa-neeru-mao koe preparaatidel), TMA, SMA ja mao

parietaalraku vastased antikehad määrati kaudse immunofluorestsentsmeetodi abil. Anti-TPO määrati fluoroensüümikaudsel immunosorptsioonimeetodil ning spermatosoidide vastased antikehad immunofluorestsentsmeetodi abil voolutsütomeetrial. Genoomne DNA puhastati sooladega sadestamise meetodil. *FSHB* geeni haplotüübid määrati polümeraasahelreaktsioonil (PCR) üles paljundatud produkti restriktsiooni fragmentide pikkuse analüüsi meetodil. *HLA-DQB1* geeni *03 ja *06 klassi alleelid määrati PCRi produkti hübridiseerimise teel lantanoididega märgistatud alleelspetsiifiliste sondidega. Statistiline analüüs teostati R-statistika keelt ja keskkonda kasutades. Kõikidelt uuritavatelt oli saadud informeeritud nõusolek.

Uurimistöö peamised tulemused ja järeldused

FSH vastased antikehad olid määratavad tervete mitte-rasedate naiste vereseerumis. IgG ja IgM tüüpi FSH vastaste antikehade tase langes raseduse korral ja seda oli märgata juba raseduse esimese kolmandiku lõpuks. Oluliselt kõrgemates väärtustes esinesid FSH vastased antikehad viljatute naiste vereseerumis. IgA tüüpi antikehade taseme tõus esines mitte-IVF patsientidel, kellel diagnoositi endometrioos või PCOS, kuid kõigi kolme alaklassi antikehade tase oli kõrgem erineva viljatuspõhjusega IVF patsientide grupis võrreldуna mitte-rasedate kontrollisikute antikehade tasemetega.

Suurenenud FSH vastaste antikehade produktsioon IVF patsientidel ei olnud seotud *FSHB* geeni tuumhaplotüüpidega. Kõrgemad FSH vastaste IgM antikehade väärtused olid seotud (i) FSH hormooni tasemega perifeerses veres patsientidel, kelle viljatus oli tingitud tubaarsest faktorist või mehepoolsest põhjusest, (ii) varasemalt läbi tehtud IVF protseduuridega patsientidel, kelle viljatuse põhjus seisnes endometrioosis, PCOS-is või jäi põhjus selgusetuks, (iii) samaaegselt teiste autoantikehade esinemisega vereseerumis kõikidel IVF patsientidel. IgA tüüpi FSH vastased antikehad esinesid kõrgemates väärtustes patsientidel, kellel esines *HLA-DQB1*03* alleel ning nende antikehade väärtused korreleerusid spermatosoidide pinnaantigeenide vastu suunatud IgA tüüpi antikehade väärtustega PCOS, endometrioosi ja seletamatu viljatusega IVF patsientide vereseerumis. IgG tüüpi FSH vastaste antikehade väärtused korreleerusid aga nii IgA kui IgM tüüpi FSH vastaste antikehade väärtustega.

Eksogeense rekombinantse FSH manustamise järgselt vähenes oluliselt IgG tüüpi FSH vastaste antikehade tase vereseerumis. Samuti langes IgA tüüpi FSH vastaste antikehade tase oluliselt, samas kui IgM tüüpi antikehade tase jäi COH vältel muutumatuks. Sarnaselt FSH vastastele antikehadele vähenes vereseerumis detekteeritavate teiste uuritud IgG tüüpi autoantikehade arv.

Follikulaarvedeliku FSH vastaste IgG antikehade tase oli seotud FSH vastaste IgG antikehade tasemega vereseerumis ja tõusis folliikuli diameetri kasvades. IgA tüüpi FSH vastaste antikehade tase oli samuti seotud vastavate

antikehade väärtustega vereseerumis, kuid ei olnud seotud folliikuli mõõtmetega. IgM tüüpi FSH vastaste antikehade väärtused follikulaarvedelikus olid võrreldes vereseerumis olevate antikehade väärtustega äärmiselt madalad, ja nende tase follikulaarvedelikus ei sõltunud vastavate antikehade väärtusest vereseerumis ega ka folliikuli mõõtmetest. Follikulaarvedelikus määratud FSH vastased IgG ja IgA antikehade väärtused olid positiivses korrelatsioonis munasarjade stimulatsiooniks kasutatud FSH kogusega ning FSH väärtusega follikulaarvedelikus.

Kõik seerumi FSH vastaste antikehade alaklassid korreleerusid antikehade väärtustega, mis reageerisid 78.–93. aminohappe vahelise piirkonnaga FSH β-alaühikul (V14D). Vastav piirkond määrab teadaolevalt ära FSH seostumise spetsiifilisuse tema retseptorile. FSH ja V14D vastaste antikehade korrelatsioon oli kõige nõrgem IgG tüüpi antikehade puhul. Uuritavatest gruppidest korreleerusid FSH ja V14D vastaste antikehade väärtused kõige enam endometrioosi ja PCOS patsientide hulgas võrrelduna korrelatsiooniga kontrollisikute või rasedate naiste hulgas.

Munarakkude kogumise päeval mõõdetud seerumi FSH vastaste IgG ja IgA antikehade, kuid mitte IgM antikehade väärtused olid lineaarses seoses halvema COH tulemuslikkusega. Tulemuste põhjal leiti parim FSH vastaste IgG ja IgA antikehade piirväärtus (>1,0 ELISA testi tulemusena) mis seostus madala COH tulemuslikkusega (≤3 saadud munaraku).

Seega, antud töö tulemuste põhjal võib järeldada, et FSH vastased antikehad on eelkõige loomulikud antikehad, mille produktsioon on muuhulgas allutatud rasedusaegse immuunsüsteemi regulatsiooni mehhanismidele. Vereseerumi IgA tüüpi FSH vastased antikehad võivad kajastada limaskestade vastust tolerantsuse induktsioonile sperma antigeenide suhtes. FSH vastaste IgM antikehade tase assotsieerub FSH hormooni tasemega veres ning panustab koos limaskestade poolt indutseeritud IgA antikehadega tsirkuleerivate IgG tüüpi FSH vastaste antikehade tootmisse.

Lisaks võib järeldada, et naisepoolse viljatusega kaasneb FSH vastaste antikehade suurenenud produktsioon. IgG ja IgM tüüpi FSH vastaste antikehade produktsioon IVF patsientidel võib olla seotud autoimmuunsete reaktsioonide aktivatsiooniga või korduvalt teostatud IVF protseduuridega. Kõrgemates väärtustes esinevad IgA tüüpi FSH vastased antikehad võivad olla seletatud aga näiteks genitaaltrakti limaskesta poolt vahendatud immunoloogilise tolerantsuse mehhanismide häirega. Vereseerumi IgG ja IgA tüüpi FSH vastased antikehad võiksid olla täiendavaks COH tulemuslikkuse prognostiliseks markeriks lisaks senistele kliinilistele parameetritele. FSH vastased antikehad, mis on suunatud aminohapete 78 ja 93 vahelise regiooni vastu hormooni β-alaühikul, võivad mõjutada FSH kinnitumist oma retseptorile ning seeläbi omada rolli viljatuse kujunemisel. Tulemustest selgus, et lisaks vereseerumile on FSH vastased IgG, IgA ja vähesel määral IgM tüüpi antikehad määratavad ka follikulaarvedelikus ning et veres tsirkuleerivad IgG tüüpi FSH vastased antikehad kontsentreeruvad preovulatoorsesse folliikulisse.

12. ACKNOWLEDGEMENTS

This study was carried out in the Immunology Group, Institute of General and Molecular Pathology, University of Tartu, Estonia from 2002 to 2007. The work was supported by the Estonian Science Foundation (grants no. ETF4631 and ETF6514) and the Estonian Ministry of Education and Reseach (core grant no. SF0182586s03).

A part of the study was performed at the Laboratoire d'Immunologie, Faculté de Médecine & CHU de Nancy, Université Henri Poincaré, Nancy, France (February – March, 2004 and December 2004 – January 2005; supported by the Estonia-France Parrot grant, scholarships of Centre of Molecular and Clinical Medicine, University of Tartu, scholarship of Andreas and dr. Elmerice Traks, scholarship of Kristjan Jaak by Archimedes Foundation, and a grant from UHP-BQRI in Nancy), at the Institute of Molecular and Cell Biology, University of Tartu, Estonia (February – July 2005; supported by the SF0182721s06, ETF5796), and since March 2006, my studies were supported by the Department of Obstetrics and Gynecology, University of Tartu, Estonia (SF0182582Cs03 and ETF6498). Majority of study samples and clinical data were collected from and supported by the Nova Vita Clinic, Centre for Infertility Treatment and Medical Genetics, Viimsi, Estonia.

I would like to thank all of those who believed in me and encouraged me to start and to continue this work and made my research possible.

I would like to express my deepest respect and gratitude to:

My supervisor

Professor Raivo Uibo, for introducing me this fascinating field of immunology, for specialized expertise and for his friendly professional guidance to exert and develop a productive criticism and discussions; for continuous encouragement and help during these years, and for a never failing belief in me. I am very grateful for the incredible opportunity he gave me, to learn so much.

Referees

Docent Reet Mändar and Professor Aavo-Valdur Mikelsaar, I am grateful for the time they took with my work.

My co-authors and colleagues

Dr. Andres Salumets, for inspiring me to study this interesting field of assisted reproduction, for giving me the opportunity to incorporate in the scientific projects in Nova Vita Clinic and for being a faithful friend. Without his encouraging support and comprehensive help I would have never been able to finish my study.

I also thank him and his colleagues, especially Dr. Maire Peters and Ms. Anu Sikut, at the Department of Obstetrics and Gynecology, University of Tartu

for the collaboration, friendship and genuine interest in the progress of my work.

Dear colleagues from Immunology Group, University of Tartu, especially Dr. Kalle Kisand, Dr. Liina Salur, Ms. Ija Talja, Ms. Aili Sarapik and Ms. Külli-like Koppel for their co-operation, support and interesting discussions. I received a lot of preciuous moments, help and inspiration from everyone in the Immunology Group, and I thank them all for this wonderful time throughout these years.

Professor Marie Christine Béné and Mrs. Christine Mathieu at the Laboratoire d'Immunologie, Université Henri Poincaré, Nancy, France for the valuable experience, methodological advice and the wonderful time at the laboratory in Nancy.

Professor Helle Karro, Docent Kadri Matt and especially my dear friend Dr. Kristiina Rull from Women's Clinic, Tartu University Hospital, for collecting the samples of pregnant women, the support and encouragement.

Mrs. Heti Pissarev from the Department of Epidemiology and Biostatistics, University of Tartu for her valuable advice with statistical analysis.

Dr. Elle Talving, Dr. Peeter Karits and Ms. Kaie Rohtla from the Nova Vita Clinic, Centre for Infertility Treatment and Medical Genetics for mindful collecting of patient data.

Professor Maris Laan and Ms. Marina Grigorova at the Institute of Molecular and Cell Biology, University of Tartu for the productive collaboration.

Dr. Virge Nemvalts from Kuressaare Hospital, Estonia for her co-operation in collecting the samples of control women.

Others

Pilleriin, Kaidi and Kristofer, my dear friends, for their constant support and patience during all these years.

Finally, my most profound thanks are directed to my family — my parents Anne and Kristjan, my sister Tiina and brother Toomas with their families. I am grateful for their love and patience, and I acknowledge the inspiring academic atmosphere and encouragement during these long years. Without the support of my family this research would have never been completed.

13. PUBLICATIONS

CURRICULUM VITAE

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Education

1991–1994	Hugo Treffner High School
1994–2000	University of Tartu, Faculty of Medicine, Diploma of Medicine
2000–2001	Tartu University Hospital, Medical internship
2002–2007	University of Tartu, Faculty of Medicine, Immunology Group, PhD studies

Special courses

2003	15th European Immunology Congress EFIS2003, Rhodos, Greec.
2003	Summer School in Immunology "Translational medicine in the field of autoimmunity", Ljusterö, Sweden.
2004	1st Workshop in Reproductive Biomedicine, Tartu, Estonia.
2004	Ist International Conference on Basic and Clinical Immunogenomics. Budapest, Hungary.
2005	Pre-congress course "Early pregnancy" and 21st congress of ESHRE2005, Copenhagen, Denmark.
2005	1st Conference of European Society of Reproductive Immunology ESRADI2005, Colchester, England.
2006	Course in statistical practice in epidemiology using R, Tartu, Estonia.
2006	16th European Immunology Congress ECI2006, Paris, France.

Professional employment

2001–2007	University of Tartu, Institute of General and Molecular Pathology, Immunology Group, laboratory assistant
2006– …	University of Tartu, Department of Obstetrics and Gynecology, specialist in research
2007– …	University of Tartu, Institute of General and Molecular Pathology, Immunology Group, specialist in research

Scientific work

The main topics of the research have been the investigation of the immunological and genetic factors of female and male infertility, and previously also the genetic markers of autoimmune diabetes.

Ten original publications (excluding abstracts).

Member of Estonian Society of Immunology and Allergology.

Member of organizing committee of "The Second Workshop in Reproductive Biomedicine", May 17–19, 2007, Tartu, Estonia.

ELULOOKIRJELDUS

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Haridus

1991–1994	Hugo Treffneri nimeline gümnaasium
1994–2000	Tartu Ülikool, Arstiteaduskond, diplom arstiteaduses
2000–2001	Tartu Ülikooli Kliinikum, üldarsti internatuur
2002–2007	Tartu Ülikool, Arstiteaduse doktorant immunoloogia erialal

Erialane täiendus

2003	15. Euroopa immunoloogia konverents EFIS2003, Rhodos, Kreeka.
2003	Immunoloogia suvekool "Translational medicine in the field of autoimmunity", Ljusterö, Rootsi.
2004	Esimene rahvusvaheline reproduktiivbioloogia ja -meditsiini konverents, Tartu, Eesti.
2004	Esimene rahvusvaheline üld- ja kliinilise immuunogenoomika konverents, Budapest, Ungari.
2005	21. Euroopa inimese reproduktiiv- ja embrüoloogia ühingu poolt korraldatud kursus "Early pregnancy" ning konverents ESHRE2005, Kopenhaagen, Taani.
2005	Esimene Euroopa Reproduktiivimmunoloogia Ühingu konverents ESRADI2005, Colchester, Inglismaa.
2006	Kursus statistilised meetodid epidemioloogias tarkvara R abil, Tartu, Eesti.
2006	16. Euroopa immunoloogia konverents ECI2006, Pariis, Prantsusmaa.

Erialane teenistuskäik

2001–2007	Tartu Ülikool, Üld- ja molekulaarpatoloogia instituut, Immunoloogia teadusgrupp, laborant
2006– …	Tartu Ülikool, Sünnitusabi ja günekoloogia õppetool, spetsialist
2007– …	Tartu Ülikool, Üld- ja molekulaarpatoloogia instituut, Immunoloogia teadusgrupp, spetsialist

Teadustegevus

Teadustöö põhisuundadeks on olnud naisepoolse viljatuse immunoloogiliste ja nii naise- kui mehepoolse viljatuse geneetiliste tekkepõhjuste välja selgitamine ning varasemalt ka autoimmuunse diabeedi geneetiliste riskifaktorite hindamine.

Ilmunud on kümme teaduspublikatsiooni (v.a. teesid).

Eesti immunoloogide ja allergoloogide seltsi liige.

Teise rahvusvahelise reproduktiivbioloogia ja -meditsiini konverentsi kaaskorraldaja, Tartus, 17.–19. mai, 2007.

DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS

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