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Immune activation in female infertility: Significance of autoantibodies and inflammatory mediators

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Immune activation in female infertility: Significance of autoantibodies and inflammatory mediators

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To my family

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Paper IV. Participation in: study design, experimental work and data analysis.

ABBREVIATIONS

1-DE	One-dimensional gel electrophoresis
2-DE	Two- dimensional gel electrophoresis
ACA	Anti-cardiolipin antibodies
AEA	Anti-endometrial antibodies
AMA	Anti-mitochondrial antibodies
ANA	Anti-nuclear antibodies
AOA	Anti-ovarian antibodies
APA	Anti-phospholipid antibodies
APO-1	Apoptosis antigen 1
ART	Assisted reproductive technology
ASRM	American Society for Reproductive Medicine
ASA	Antisperm antibodies
ATA	Anti-thyroid antibodies
β2-GPI	β 2-glycoprotein I autoantibodies
Bcl-2	B cell leukemia 2
CD44(v6)	Cluster of differentiation 44 variant 6
CI	Confidence interval
COS	Controlled ovarian stimulation
DTT	1,2-dithio-DL-threitol
ESHRE	European Society for Human Reproduction & Embryology
Fas	Apoptosis stimulating fragment
FSH	Follicle stimulating hormone
G-CSF	Granulocyte-colony stimulating factor
GnRH	Gonadotropin-releasing hormone
hCG	Human chorionic gonadotropin
HEp-2	Human epithelial cell line type 2
HRP	Horseradish peroxidase
ICSI	Intracytoplasmic sperm injection
IEF	Isoelectric focusing
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IIF	Indirect immunofluorescence
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
IVF	*In vitro* fertilization
LH	Luteinizing hormone
MCP-1	Monocyte chemotactic protein 1
MIP-1	Macrophage inflammatory protein
MW	Molecular weight
OD	Optical density
OPU	Oocyte pick-up
OR	Odds ratio

PCA	Parietal cell autoantibodies
PCOS	Polycystic ovary syndrome
PID	Pelvic inflammatory disease
POF	Premature ovarian failure
POI	Primary ovarian insufficiency
PVDF	Polyvinylidene difluoride
RAFS	Reproductive autoimmune failure syndrome
RANTES	Regulated on activation, normal T cell expressed and secreted
RIFS	Reproductive immune failure syndrome
sAPO-1	Soluble apoptosis antigen 1
SD	Standard deviation
SMA	Smooth muscle autoantibodies
SPC	Spontaneous menstrual cycle
STD	Sexually transmitted diseases
sTREM	Soluble triggering receptor expressed on myeloid cells
TFI	Tubal factor infertility
TGF	Transforming growth factor
Th	T helper lymphocyte
TMA	Thyroid microsomal autoantibodies
TNF	Tumor necrosis factor
TREM	Triggering receptor expressed on myeloid cells
Tris	2-amino-2-(hydroxymethyl)propane-1,3-diol
WHO	World Health Organization

1. INTRODUCTION

Fertility is an essential component of reproductive health. Unfortunately, infertility has become a global medical and social problem affecting approximately 9% of reproductive-aged couples worldwide (Boivin et al., 2007; Mascarenhas et al., 2012). Population decline, together with a decrease in birth rates, is under special attention in all Europe, including Estonia (The ESHRE Capri Workshop Group, 2010). Nevertheless, due to discrepancies in infertility definitions used and deficient knowledge about characteristics and pathways related to impaired reproduction, infertility research has remained a discounted area of global health (The ESHRE Capri Workshop Group, 2004; Mascarenhas et al., 2012).

Human assisted reproduction technology (ART) has become a widely used treatment option for infertile couples with various causes, with *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) as the most successful procedures. Despite improvements in fertilization and pregnancy rates, the overall success rates for IVF and ICSI have remained low (Ferraretti *et al.*, 2012). The outcome of IVF or ICSI procedure is highly dependent on the effectiveness of controlled ovarian stimulation (COS), wherefore multiple biomarkers and scoring systems have been developed to evaluate the successfulness of COS (Haller *et al.*, 2008; Altmäe *et al.*, 2011; Alviggi *et al.*, 2012). As such, follicular fluid constituents have been measured as they correspond to the local environment during oocyte maturation. It is relevant, that follicular fluid sample material is easily obtained during oocyte retrieval. However, the research on finding more effective and optimal follicular fluid biomarkers is ongoing (Revelli *et al.*, 2009; Nel-Themaat and Nagy, 2011).

Underlying mechanism for IVF failure can be immune activation, including autoimmunity (Gleicher, 2002). Several autoantibodies are more frequently detected in patients with IVF treatment failure compared to women with successful IVF treatment, among them autoantibodies directed to fertility-specific tissues (Pires, 2010; Zhang *et al.*, 2012; Ying *et al.*, 2012). Although the presence of corresponding antibodies has been shown to associate with IVF pregnancy failure, there is still a lot of inconsistency about their impact on infertility pathogenesis (Carp *et al.*, 2012).

The current study was carried out in the Department of Immunology, Institute of Biomedicine and Translational Medicine, University of Tartu, Estonia and took place in good collaboration with colleagues from the Nova Vita Clinic, Centre for Infertility Treatment and Medical Genetics, Estonia; Department of Obstetrics and Gynecology, University of Tartu, and Laboratoire d'Immunologie, Faculté de Médicine, Université Henri Poincaré, Nancy, France. Research on infertility related immune impairments has been conducted in our laboratory since 1990's, with emphasis on autoantibody repertoire in women with various causes of reproductive failure. To bring new beneficial insight into the role of immune system impairments in female infertility, the current thesis focused on: (1) assessing the prevalence of common and infertility related autoantibodies in the blood serum in women with reproductive failure and evaluating the

influence of these autoantibodies on IVF treatment results; (2) measuring cytokines and other biomarkers from the follicular fluid of infertile women and evaluating their associations on oocyte and embryo quality as well as pregnancy results following IVF.

2. REVIEW OF LITERATURE

2.1. Infertility

Reproduction is a foundation for all living nature. According to World Health Organization's (WHO) definition of health, a healthy person should be free to decide if or when to have children (Preamble to the Constitution of the World Health Organization, 1948). Unfortunately, approximately 9% of reproductive-aged couples are affected by infertility worldwide. The prevalence of infertility has a broad range of 5–15% for more and less developed countries (Boivin *et al.*, 2007). By definition, infertility is a couple's inability to conceive in 12 months, with regular sexual intercourse and without using any contraceptives (Practice Committee of American Society for Reproductive Medicine, 2008). Causes for infertility usually divide equally in-between men and women in the population, often affecting both partners at the same time. In up to 15% of the cases, though, no clinically detectable cause is found (Forti and Krausz, 1998). A great deal of personal choices in life, including the will for childlessness and delayed parturition contribute to reduced fertility rates in Europe. However, genetic factors and medical conditions affecting endocrinological, gynaecological and immunological health reduce fecundity (The ESHRE Capri Workshop Group, 2010). Moreover, since diagnostic methods used today still leave many cases undetected, mechanisms of reproductive failure remain poorly understood (The ESHRE Capri Workshop Group, 2004).

2.1.1. Female infertility

The etiology of female infertility can be defined under multiple diseases (Table I) (Forti and Krausz, 1998). However, the most influential physiological component is the age of a woman. Similarly, genetic propensity, environmental factors, including smoking the cigarettes and substance abuse, malnutrition and over- or underweight play also a great role (The ESHRE Capri Workshop Group, 2002).

Table I. Non-genetic causes of female infertility (based on Forti and Krausz, 1998)

Ovulatory dysfunction
Premature ovarian failure (POF)
Polycystic ovary syndrome (PCOS)
Tubal obstructions
Tubal factor infertility (TFI)
Endometriosis
Vaginal and cervical factors
Uterus abnormalities
Unexplained infertility

Ovulatory dysfunction

Impairments in the endocrine system can lead to anovulatory infertility either with hypo-, hyper-, or normal levels of gonadotropic hormones. During hypogonadotropic hypogonadism, the reduced secretion of pituitary follicle stimulating hormone (FSH) and luteinizing hormone (LH) hinder ovulation. This condition is often seen in underweight women but also in excessively exercising female athletes. Elevated levels of FSH indicate ovarian failure, mainly, because of depletion of the oocytes, or, in rare cases, because of resistant ovary syndrome (The ESHRE Capri Workshop, 1996; The ESHRE Capri Workshop Group, 2002). When elevated serum FSH level is associated with amenorrhea and hypoestrogenism before the age of 40, the diagnosis of premature ovarian failure (POF) is assigned. Lately a pathophysiologically more precise term, primary ovarian insufficiency (POI), is preferred (Welt, 2008). The new term describes more accurately the state of ovarian condition, since varying degree of ovarian function is still preserved in some of the patients and, moreover, a small proportion of women with POF diagnosis may still spontaneously conceive and deliver (Tsigkou *et al.*, 2008; Welt, 2008). The estimated prevalence of POF in women by the age 40 is approximately 1%. Two major mechanisms of POF are follicle dysfunction and follicle depletion. The etiology of POF, however, remains mostly unknown and a variety of possible causes, including genetic, environmental and iatrogenic, have been proposed (Kokcu, 2010). Besides, an autoimmune basis has been detected in almost half of the POF cases as these patients have: (1) associated autoimmune diseases, such as Addison's disease or thyroid autoimmunity; (2) autoantibodies to steroid-producing cells and/or other ovarian antigens and (3) lymphocytic oophoritis (Hill *et al.*, 1990; Lebovic and Naz, 2004; Carp *et al.*, 2012; Kokcu *et al.*, 2012).

Women with normogonadotropic anovulation reveal serum levels of FSH and LH within normal range. Normogonadotropic anovulation can often be caused by adrenal hyperandrogenism or polycystic ovary syndrome (PCOS) (The ESHRE Capri Workshop, 1996; The ESHRE Capri Workshop Group, 2002). Although PCOS is the leading endocrine disorder in women, with prevalence up to 15%, there is still a lot unknown in the etiology of this disease. The clinical expression of this syndrome can be variable, generally including oligo- or anovulation, hyperandrogenism and polycystic ovaries. These women may suffer from subfertility due to the impact of obesity, hyperinsulinemia or –androgenism and endocrine irregularities on folliculogenesis and endometrial receptivity. Furthermore, because of the factors listed, these patients are more prone to develop pregnancy associated complications, like gestational diabetes or hypertension. Insulin resistance, commonly seen in obese PCOS patients, increases the risk of developing type II diabetes in these women (Fauser *et al.*, 2012). Additionally, autoimmune thyroiditis is significantly more prevalent in PCOS patients than in controls without PCOS (Janssen *et al.*, 2004). Therefore, an autoimmune implication has been suspected in the etiopathogenesis of PCOS cases that are associated with other autoimmune diseases, as these patients are

also positive for several autoantibodies (Luborsky, 2002; Petríková and Lazúrová, 2012).

Tubal obstructions

Tubal factors account for 25–35% of infertility cases. Causes for damaged fallopian tubes include pelvic inflammatory disease (PID), endometriosis and pelvic surgery. As a result, the transport of either spermatozoids or fertilized oocyte is impaired or even impossible (The Practice Committee of the American Society for Reproductive Medicine, 2006b). PID is a widely spread significant sequelae of sexually transmitted diseases (STD) among non-pregnant women of reproductive age. Long-term complications due to PID can be tubal factor infertility (TFI), ectopic pregnancy and chronic pelvic pain. Long term complications are mostly associated with the coinfection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* (Sweet, 2011). The induction of proinflammatory cytokines [tumor necrosis factor (TNF)-α and interleukin (IL)-1] after chlamydia or gonococci infection can damage the epithelium of the fallopian tubes causing thereby loss of function. In addition, hydrosalpinx fluid contains prostaglandins, leukotrienes and lymphocytes that can eventually have deleterious inflammatory effects also on the uterine environment, influencing thereby negatively implantation of an embryo (Meyer *et al.*, 1997; Camus *et al.*, 1999; Maisey *et al.*, 2003; Bontis and Theodoridis, 2006). Further evidence of deviations in the regulation of local immune response in TFI patients is provided by the significantly higher levels of serum autoantibodies, which may also harm the function of fallopian tubes and endometrium (Van Voorhis and Stovall, 1997; Stern *et al.*, 1998; Choudhury and Knapp, 2001; Reimand *et al.*, 2001).

Endometriosis, a chronic inflammatory disease characterized by the presence of endometrial glands and stroma outside the uterus, affects up to 10% of reproductive age women. The main manifestations are pelvic pain and infertility (Burney and Giudice, 2012). Diverse causes for reduced fertility have been described in these patients, as they have disturbances in folliculogenesis, functional disorders in fallopian tubes and impaired implantation of the embryo into the endometrium (Halis and Arici, 2004; de Ziegler *et al.*, 2010). Endometriosis is also characterized by inflammatory status of the immune system, for example impaired natural killer cell activity and macrophage function. Overexpression of proinflammatory and embryotoxic cytokines in the peritoneal fluid creates an inflammatory milieu not only in the peritoneum but also in the eutopic endometrium (de Ziegler *et al.*, 2010; Burney and Giudice, 2012). Besides, a high concordance of autoimmune diseases is observed in infertile women with endometriosis, as well as significantly higher prevalence of autoantibodies against endometrium, ovary and phospholipids, to name a few. Therefore, endometriosis is sometimes considered as an autoimmune disease (Matarese *et al.*, 2003; Burney and Giudice, 2012).

Unexplained infertility

Unexplained infertility is a diagnosis made by exclusion, when standard investigations used in everyday clinical approach (tests of ovulation, tubal patency and semen analysis) are normal in an infertile couple (The Practice Committee of the American Society for Reproductive Medicine, 2006a). The prevalence of unexplained infertility in infertile women varies from 21–26%, increasing with patients age (Maheshwari *et al.*, 2008). The causes for unexplained infertility are heterogeneous. Female age is thought to be the major influencing factor (The ESHRE Capri Workshop, 1996; Maheshwari *et al.*, 2008), but immune system impairments (Putowski *et al.*, 2004; De Carolis *et al.*, 2010), genetic factors (Altmäe *et al.*, 2009) and subclinical endocrinological diseases (Dmowski, 1995) have been suspected as much. In patients with immune system impairments, discrepancy between the T helper lymphocyte (Th)1-Th2 responses towards the pregnancy not favoring Th1 profile has been detected. Consequent imbalance in cytokine profile may in one's turn lead to changes in uterine natural killer cell number and activation status, which may additionally contribute to implantation failure in unexplained infertility patients (Perricone *et al.*, 2008; De Carolis *et al.*, 2010).

2.1.2. Male infertility

To diagnose male factor infertility, the male partner is studied by his medical history and physical examination, including semen analysis according to standards set by the WHO (Cooper *et al.*, 2010; World Health Organization, 2010). The most common cause for male infertility is varicocele, affecting around 20% of men in general population and up to 40% of infertile men (Nagler and Grotas, 2009). It is a condition that involves dilatation of scrotal veins. Links between varicocele and testicular dysfunction have remained obscure, with venous reflux and testicular temperature elevation as one of the possible culprits (The Practice Committee of American Society for Reproductive Medicine, 2008). However, increase in seminal levels of proinflammatory cytokines and oxidative stress as well as reduced total antioxidant capacity have been suspected as much (Nallella *et al.*, 2004; Moretti *et al.*, 2009). Besides, in the pathophysiology of varicocele impaired spermatogenesis because of autoimmunity has been proposed (Gilbert *et al.*, 1989; Naughton *et al.*, 2001). Infertility in the male due to immunological causes is mostly associated with antisperm antibodies (ASA), which can develop as a result of testicular damage, infection or inflammation. As a consequence, sperm antigens are able to pass through blood-testis barrier and may activate corresponding antigen-specific T- and B lymphocytes. ASA can affect sperm quality and fertilization capacity by causing sperm agglutination, inhibiting sperm mobility and impairing sperm capacitation and acrosome reaction. In the female, ASA can additionally disrupt sperm-oocyte fusion, act embryotoxic or hamper embryo implantation by

binding to the hatching embryo (Koide *et al.*, 2000; Choudhury and Knapp, 2001; Chamley and Clarke, 2007).

Male infertility can additionally be caused by congenital genetic factors, for example anomalies at the chromosomal or DNA level. The first group includes abnormalities in the number of sex chromosomes, with XXY as the commonest, microdeletions of the Y chromosome and structural anomalies. Monogenic defects leading to male infertility are mostly autosomal recessive, including congenital bilateral absence of the *vas deferens* and hormonal deficiencies due to defects in hormone synthesis or the respective receptor. Similarly, hypogonadism, STD and testicular cancer can lead to male infertility (The ESHRE Capri Workshop, 1996; The ESHRE Capri Workshop Group, 2002).

2.2. *In vitro* fertilization (IVF)

Assisted reproductive technology has become a hopeful treatment for infertile patients. Since the birth of the first IVF baby in 1978 over 5 million babies have been born worldwide using ART (Steptoe and Edwards, 1978; ESHRE, 2013). Introduction of ICSI in 1992 as a treatment for severe male infertility was a major breakthrough in the field of ART and quickly became a widely used procedure in many other indications for assisted reproduction (The ESHRE Capri Workshop Group, 2007). Approximately 947 IVF procedures per million inhabitants were performed in Europe in year 2008 and about 0.5–4.6% of all infants born were ART-babies. The ratio of ICSI to conventional IVF has remained 2 to 1 (Ferraretti *et al.*, 2012).

The process of these two treatments can be divided into 3 stages. The first phase is COS. During this step, ovaries are stimulated with administrating exogenous FSH following either gonadotropin-releasing hormone (GnRH) antagonists' or agonists' protocol, to mature multiple oocytes (Macklon *et al.*, 2006). At the second stage, cumulus-oocyte complexes are collected by ultrasound-guided transvaginal follicle aspiration, followed by insemination or injection with spermatozoa either in case of conventional IVF or ICSI, respectively. The oocytes are routinely monitored to confirm fertilization 16–18 h later and assessed for embryo cleavage a day after. As the final phase, selected embryo(s) are transferred to the uterus 2 or 3 days after fertilization. In order to avoid the development of multiple pregnancy, only 1 or 2 embryos are transferred at the time (Salumets *et al.*, 2003).

The fertilization rate is quite high, reaching up to 80% depending on the ART method used (Rienzi *et al.*, 2012). Still, implantation rate remains as low as 10–15% (Macklon *et al.*, 2006) wherefore the mean pregnancy rate per embryo transfer procedure remains around 30% for both, IVF and ICSI (Ferraretti *et al.*, 2012). The putative outcome of IVF or ICSI is largely dependent on the success of the COS, which is why besides embryo morphology evaluation multiple parameters or scoring systems are used to evaluate the effectiveness of COS (Salumets *et al.*, 2001; Salumets *et al.*, 2003; Haller *et al.*,

2008; Altmäe *et al.*, 2011; Rienzi *et al.*, 2011; Alviggi *et al.*, 2012). Nevertheless, there are no direct diagnostic tests to measure the quality of folliculogenesis, not to mention the physiologic function of the fallopian tubes or mechanisms of embryo implantation (The ESHRE Capri Workshop Group, 2004). Therefore, the need remains to discover more effective biomarkers along with more effective protocols to predict the success of IVF treatment (Macklon *et al.*, 2006; Nel-Themaat and Nagy, 2011).

As indicated above, the relatively low implantation rates in IVF and ICSI may additionally be contributed by immune system interference. Supraphysiological concentrations of sex steroids that are achieved during COS are associated with reduced implantation rates after IVF embryo transfer (Simon *et al.*, 1995; Macklon *et al.*, 2006). According to one hypothesis, they may exert an immunomodulatory effect through the hypothalamic–pituitary–gonadal axis (Beagley and Gockel, 2003; Cutolo *et al.*, 2004). Secondly, the enhancing role of estrogens on autoimmunity may lead to autoantibody formation often seen in IVF patients, which can be detrimental for implantation (Ansar Ahmed *et al.*, 1985; Birdsall *et al.*, 1996; Geva *et al.*, 1997; Stern *et al.*, 1998; Beagley and Gockel, 2003; Cutolo *et al.*, 2004). Further on, microtrauma during oocyte retrieval could induce the production of anti-ovarian autoantibodies (AOA) that can lead to poor IVF outcome (Geva *et al.*, 1997; Monnier-Barbarino *et al.*, 2003; Forges *et al.*, 2006; Haller *et al.*, 2007). However, debate over immune system interference in IVF and ICSI is ongoing and subject to further investigation.

2.3. The anatomy and physiology of the human ovary

Human ovary possesses two main functions: production of germ cells and biosynthesis of steroid hormones (Palma *et al.*, 2012). Ovary is surrounded by a connective tissue capsule called *tunica albuginea*. The interior part of the ovary, called ovarian stroma, consists of two layers: ovarian *cortex* and *medulla*. Ovarian *medulla* contains blood vessels, nerves and endocrine cells. The *cortex* may contain ovarian follicles in the different developmental stages, such as resting, maturing and atretic follicles as well as *corpus luteum* and its remnants (Figure 1) (Fritsch, 2008).

During fetal development primordial germ cells, that have colonized the gonadal tissue, undergo extensive mitotic division. By the 20th week of gestation their number reaches up to 6 million cells. From there on, the ovarian reserve starts to continuously decrease throughout a woman's reproductive life span, first by oogonial atresia, but from puberty on, also by folliculogenesis. Therefore, at birth, about 1 million primordial follicles are present in the ovarian cortex and only 300,000–400,000 remain at puberty (Oktem and Oktay, 2008). Changes in the hypothalamic-pituitary axis negative feedback lead to initiation of folliculogenesis and onset of puberty (Messinis, 2006). Folliculogenesis starts with development of primordial follicles, surrounded by a single layer of

flat granulosa cells, into primary follicles, surrounded by a single layer of cuboidal granulosa cells. Granulosa cells further stratify: (1) into cumulus granulosa cells surrounding oocyte and forming cumulus-oocyte complex, and (2) into mural granulosa cells, which form the inner lining of the follicular basal lamina. Thus a secondary follicle is formed. Also, the thecal layer, which contains androgen-producing cells, starts to form around the granulosa. Mature Graafian follicle is distinguished from secondary follicle by the antrum filled with fluid. During ovulation, the cumulus-oocyte complex is released and follicle starts to develop into *corpus luteum*. Progesterone, synthesized by *corpus luteum*, is essential for maintaining the menstrual cyclicity and early pregnancy (Fritsch, 2008; Matsuda *et al.*, 2012).

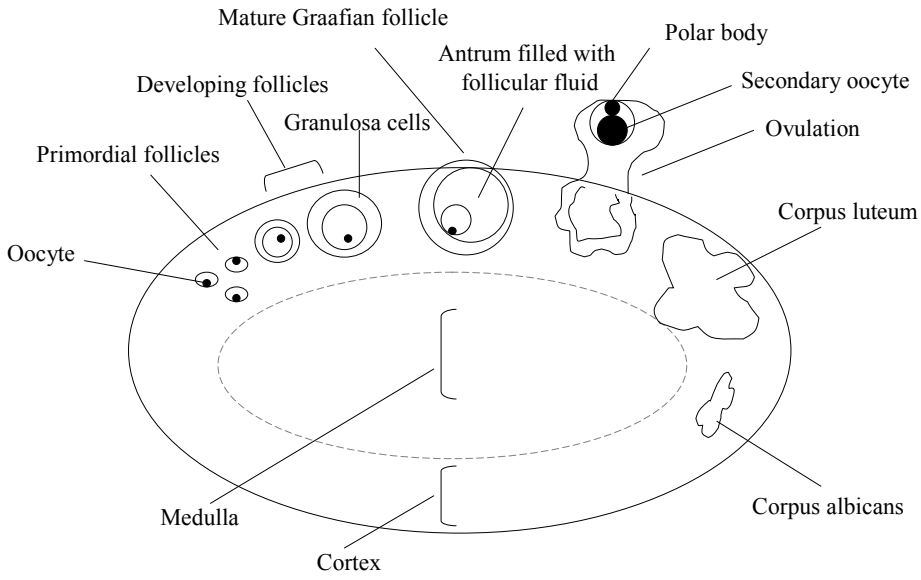

Figure 1. Ovarian structure and follicular development.

2.3.1. Regulation of the follicular cycle with emphasis on granulosa cells

Follicular development is regulated by various endocrine, paracrine and autocrine factors (Figure 2). The survival of preovulatory follicles is mediated through gonadotropins, estrogens, growth factors and cytokines, to name a few. Whereas the apoptosis stimulating fragment (Fas)/Fas ligand, TNF-α and B cell leukemia (Bcl)-2 family proteins stimulate follicular apoptosis (Revelli *et al.*, 2009; Matsuda *et al.*, 2012).

Hormones

Two critical hormones of the female reproductive cycle are pituitary gonadotropins FSH and LH. Their secretion is stimulated by GnRH from the hypothalamus. In women, FSH is responsible for antral stage follicular growth and selection of the dominant follicle. FSH also promotes estradiol synthesis by granulosa cells. LH is responsible for stimulating androgen production by theca cells and ovulation of the dominant follicle (Gougeon, 2010; Baerwald *et al.*, 2012). Gonadotropins act synergistically with estradiol, which is produced by granulosa cells. Estradiol influences ovarian folliculogenesis through the negative feedback mechanism of the hypothalamic–pituitary system, where estradiol decreases FSH release. Additionally, estradiol is important in the positive feedback mechanism, where it sensitizes pituitary to GnRH (Messinis, 2006). All in all, estradiol promotes folliculogenesis, increases the expression of gonadotropin receptors and inhibits granulosa cell apoptosis and subsequent follicular atresia (Matsuda *et al.*, 2012).

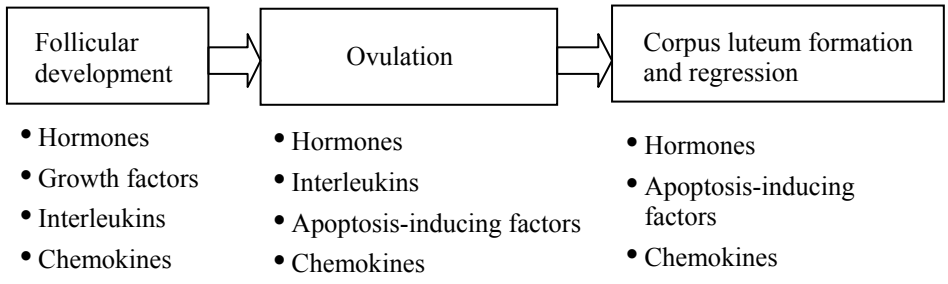

Figure 2. Regulators of the ovarian cycle in follicular fluid.

Growth factors

The follicular fluid contains multiple growth factors secreted from the granulosa cells with insulin-like growth factor (IGF)-I as the most essential and well described. IGF-I supports granulosa cell proliferation and inhibits apoptosis. It also takes part of dominant follicle selection by increasing the responsiveness to gonadotropins of the more developed follicle (Revelli *et al.*, 2009; Matsuda *et al.*, 2012). The mammalian transforming growth factor (TGF)-β superfamily has over 40 proteins, including activins, inhibins, bone morphogenetic proteins and growth differentiation factors, that are involved in cell proliferation, growth and differentiation (Knight and Glister, 2006; Trombly *et al.*, 2009). TGF-β additionally influences cell migration and production of other growth factors. Follicular fluid TGF-β is important in follicular growth, meet oocyte quality and embryo implantation, since higher levels of TGF-β in the follicular fluid of IVF patients show positive associations with the number of fertilized oocytes and pregnancy achievement (Fried and Wramsby, 1998). Another growth factor

produced by granulosa cells is granulocyte-colony stimulating factor (G-CSF), which plays important roles in proliferation and terminal differentiation of neutrophils, reduction of the production of proinflammatory cytokines in activated macrophages, and endothelial cell proliferation and migration (Barreda *et al.*, 2004). In follicular fluid, a role for G-CSF in the oocyte maturation has been proposed by several studies (Salmassi *et al.*, 2004; Ostanin *et al.*, 2007). Moreover, Ledee and colleagues suggested the level of follicular fluid G-CSF to be used as a biomarker for oocyte selection for ART (Lédée *et al.*, 2011).

Interleukins

Cytokines as the modulators of the immune system, also participate in the regulation of the ovarian cycle by supporting follicular growth as well as guiding the infiltration and activation of leucocytes necessary for ovulation and tissue remodeling during follicular rupture, luteinization and luteolysis (Büscher *et al.*, 1999; Revelli *et al.*, 2009). For example IL-6 may contribute to oocyte maturation, since lower levels of IL-6 in the preovulatory follicular fluid were associated with IVF pregnancy failure (Kawasaki *et al.*, 2003; Bedaiwy *et al.*, 2007). On the other hand, higher levels of IL-6 have been detected in women with ovarian hyperstimulation syndrome and endometriosis (Rizk *et al.*, 1997; Garrido *et al.*, 2000). Therefore, the exact physiological role of IL-6 in ovarian physiology is worth to investigate (Kawasaki *et al.*, 2003). IL-1β, another pro-survival factor that rescues granulosa cells from apoptosis, is also one of the main cytokine mediators in follicular rupture (Kaipia and Hsueh, 1997; Vassiliadis *et al.*, 2005; Matsuda *et al.*, 2012). Besides, IL-1β is a potent regulator of local inflammation and is involved in the activation and migration of lymphocytes and endothelial cells (Terranova and Rice, 1997; Gérard *et al.*, 2004; Kanaji *et al.*, 2011).

Several other cytokines have been measured in follicular fluid, but their role in the follicular cycle still remains controversial. IL-18, formerly known as interferon (IFN)-γ inducing factor, is a potent mediator of innate and adaptive immune responses (Nakanishi *et al.*, 2001; Vujisic *et al.*, 2006). In the mouse ovary, IL-18 and its receptor are involved in maturation of the cumulus-oocyte complex and ovulation (Tsuji *et al.*, 2001). In humans, preovulatory follicular fluid levels of IL-18 correlate with the number of retrieved oocytes (Gutman *et al.*, 2004). However, the function of IL-18 in human ovarian physiology has remained greatly understudied. IL-18 shares a synergistic action with IL-12, a powerful regulator of cell-mediated immune responses (Langrish *et al.*, 2004). IL-12 has been thought of as a Th1 cytokine, with the ability to down-regulate important processes for a successful pregnancy, such as Th2 responses and angiogenesis (Gazvani *et al.*, 2000). Therefore, a negative influence of IL-12 on reproductive physiology has been suggested by some investigators (Gazvani *et al.*, 2000; Bedaiwy *et al.*, 2007; Lédée *et al.*, 2008), while others argue against it (Gallinelli *et al.*, 2003). Together with IL-12, IL-23 represents the bridge between innate and adaptive immune responses. These two cytokines share many similarities in structure and source of production because of the p40 common

subunit. IL-23 is a key player in controlling acute infections, chronic inflammatory autoimmune diseases and holds a substantial role in memory responses of T-cells (Langrish *et al.*, 2004). To date, there are only few studies that have detected IL-23 or IL-12/IL-23 common subunit p40 from follicular fluid (Vujisic *et al.*, 2006). However, studies in mice suggest a connection between IL-23 and the length of estrous cycle (Enright *et al.*, 2011).

Interferons

The importance of IFNs secreted into the follicular fluid in ovarian function has remained unknown. The type I interferon, IFN-α is mostly known for its antiviral activity. It can also exert the induction of apoptosis and suppression of cellular growth (Rizza *et al.*, 2010). Also, IFN-α is suggested to favor folliculogenesis (Zidovec Lepej *et al.*, 2003; Lee *et al.*, 2009). The type II interferon, IFN-γ is a proinflammatory cytokine related to inflammatory processes in autoimmune diseases (Damsker *et al.*, 2010). In follicular fluid, levels of IFN-γ show contradictory associations. Higher follicular IFN-γ levels have been associated with increased oocyte quality, successful IVF cycles as well as early cleaved embryos (Ostanin *et al.*, 2007; Lédée *et al.*, 2008). There are also studies, where no associations between follicular IFN-γ and embryo or IVF parameters were detected (Cerkiene *et al.*, 2008).

Apoptosis-inducing factors

Follicular atresia is a degenerative process which occurs through an apoptotic mechanism. It can take place at any maturation time point of folliculogenesis, however, majority of the follicles undergo apoptosis during the antral stage (Matsuda *et al.*, 2012). Apoptotic factors are important in maintaining tissue homeostasis and remodeling in the cycling ovary. Follicular atresia is initiated within mural granulosa cells by Fas/Fas ligand system. Interestingly, the Fas system also plays a part in oocyte maturation (Sarandakou *et al.*, 2003; Matsuda *et al.*, 2012). Both Fas and Fas ligand are expressed in granulosa cells of preantral and antral follicles with an increasing level along the follicular maturation (Matsuda *et al.*, 2012). Human apoptosis antigen (APO)-1 is a transmembrane receptor but exists also in a soluble form (sAPO-1). Interestingly, when binding with Fas ligand the transmembrane receptor induces apoptosis, whereas the soluble form inhibits it by preventing death signal transduction (Sarandakou *et al.*, 2003). Similarly, TNF-α can induce apoptosis in follicular granulosa cells or stimulate cell proliferation instead, by binding either to TNF receptor 1 or 2, respectively (Matsuda *et al.*, 2012). One of the pathways for death ligand/death receptor signaling is the mitochondria-mediated apoptosis by Bcl-2 family members. These proteins regulate germ cell as well as somatic cell apoptosis in the follicles (Sasson *et al.*, 2002; Matsuda *et al.*, 2012).

Chemokines

Ovulation is considered as an inflammation-like process in a sense that it involves increased vascular permeability, immune cell infiltration, expression of proinflammatory cytokines and swelling of the follicular tissue (Machelon *et al.*, 2000). Before ovulation, infiltration of granulocytes, monocytes and T lymphocytes into the area surrounding the preovulatory follicle is needed for subsequent luteinization and luteolysis. For this, multiple chemokines are secreted into the follicular fluid by granulosa cells (García-Velasco and Arici, 1999; Machelon *et al.*, 2000). A potent chemotactic cytokine, IL-8, that activates neutrophilic granulocytes, is additionally involved in promoting cell proliferation and angiogenesis, both of which are prerequisites for normal ovarian function (Runesson *et al.*, 1996; Połeć *et al.*, 2011). Proinflammatory cytokines IL-1β and TNF-α both induce the production and secretion of IL-8 (Runesson *et al.*, 1996). High intrafollicular levels of IL-8, detected in women undergoing IVF, imply to an inflammatory reaction in progress (Runesson *et al.*, 1996; Büscher *et al.*, 1999). Chemokines of the α-chemokine family: the two macrophage inflammatory proteins (MIP)-1α and MIP-1β, regulated on activation, normal T cell expressed and secreted (RANTES) and monocyte chemotactic protein (MCP)-1, are all potent chemoattractants for monocytes and T lymphocytes (García-Velasco and Arici, 1999). Both MIP-1s are proinflammatory chemokines, involved in acute and chronic inflammatory host responses, immune response modulation and regulation of tissue homeostasis (Maurer and von Stebut, 2004). Although MIP-1α is produced by ovarian theca and granulosa cells, the follicular fluid levels in normal folliculogenesis are low (Dahm-Kähler *et al.*, 2006). In contrast, lower levels of MIP-1β are indicative to poor-quality embryos after IVF, referring to a role of this factor in oocyte maturation (Ostanin *et al.*, 2007). In addition to monocytes and T lymphocytes, infiltration of eosinophils and mast cells is essential for ovulation and formation of the *corpus luteum*. Chemoattractant RANTES is involved in the chemotaxis or activation of all of these leukocytes making it the fundamental chemokine of ovulatory processes (Schall *et al.*, 1990). In follicular granulosa cells, the production of RANTES is induced by TNF-α secretion (Aust *et al.*, 2000; Machelon *et al.*, 2000). Different from RANTES, the production of MCP-1 is promoted by IL-1β (Dahm-Kähler *et al.*, 2006), but also by LH and human chorionic gonadotropin (hCG) (Arici *et al.*, 1997). The latter may point to the implication of MCP-1 in timely follicular rupture (García-Velasco and Arici, 1999). The involvement of MCP-1 in ovulation and oocyte maturation is indicated by Kawano and colleagues, who found significantly higher levels of MCP-1 in follicles containing mature oocytes (Kawano *et al.*, 2001).

Other factors

Novel follicular fluid markers that might be related to follicular development are cluster of differentiation (CD)44 variant (v)6 and triggering receptor expressed on myeloid cells (TREM)-1. CD44, a transmembrane receptor shed into the follicular fluid, is expressed on cumulus granulosa cells at the time of ovulation

(Ohta *et al.*, 1999; Ohta *et al.*, 2001). Different forms of CD44 are involved in cell adhesion, migration, infiltration, proliferation and cytokine signaling (Ponta *et al.*, 2003). The isoform CD44v6 has been linked with cell motility and apoptosis resistance in cancer, but to our knowledge CD44v6 has not been studied in the follicular fluid before (Yu *et al.*, 2010; Jung *et al.*, 2011). TREM-1 has mostly been studied as a marker for active and chronic inflammation in bacterial infections. It is a transmembrane glycoprotein that belongs to the immunoglobulin (Ig) superfamily. As a result of TREM-1 activation, various proinflammatory cytokines and chemokines are produced, TNF-α, IL-1β, IL-6 and IL-8 among them (Bouchon *et al.*, 2000). In a previous study we discovered TREM-1 expression in follicular granulosa cells (Kõks *et al.*, 2010). Additionally, TREM-1 levels have been detected in the myometrium, umbilical cord blood and amniotic fluid (Matoba *et al.*, 2009; Youssef *et al.*, 2009; Kusanovic *et al.*, 2010), but to our knowledge TREM-1 has never been studied in follicular fluid. Interestingly, levels of TREM-1 from the bronchoalveolar lavage are in correlation with the severity of non-infectious tissue damage (Bingold *et al.*, 2011). Since follicular rupture is also a non-infectious process that involves tissue damage, a role for TREM-1 in ovulation facilitation might be expected.

Taken together, ovarian folliculogenesis is a long and complex process in which both endocrinological and immunological factors play significant roles. Ovulation induction in ART can lead to follicular asynchrony, which is one of the reasons why more precise markers for assessing follicular maturity are needed (Pellicer *et al.*, 1987). Besides, strict IVF legislatives in some countries increase the pressure to reduce overexpression of embryos (Soini *et al.*, 2006). Since follicular fluid can easily be obtained during oocyte aspiration, several cytokines, growth factors and related proteins in the follicular fluid and in the ovary have been studied to use them as markers for oocyte selection and predicting IVF success rate (Revelli *et al.*, 2009). However, it has become clear that commitment to one specific marker can lead to a dead end situation, wherefore the holistic approach of systems biology seems more appealing. With the development of new high-throughput methods, that allow simultaneous measuring of many analytes at once from little sample volume, the identification of whole metabolomes or immunomes has never been easier (Revelli *et al.*, 2009; Wallace *et al.*, 2012).

2.4. Function and structure of the human endometrium

Endometrium is the inner mucosal membrane of the uterus with the main function to provide an optimal environment for embryo implantation. It is also important in tissue clearance and regeneration during menstruation and provides a first line of defense against invading pathogens. On the other hand, endometrium must preserve tolerance against allogeneic sperm and the semi-allogeneic fetus. Therefore, a delicate balance between initiating immune response and tolerance must be maintained in the endometrium (Young and Loy, 2005).

Based on their involvement in the menstrual cycle two layers can be distinguished in the endometrium: the functionalis and the basalis (Figure 3). The functional layer undergoes cyclical changes and is shed if no embryo implantation occurred. It is composed of a single-layered epithelium that contains ciliated and secretory cells, and stroma, which is a specialized connective tissue that has the capability to decidualize. The basal layer is not shed during menstruation and is therefore the source of cyclical regeneration of the functionalis. An average menstrual cycle takes around 28 days and can be divided into 3 stages. It begins with the menstrual phase, during what the functional layer is sloughed off. The following proliferative stage marks the restoration of the functionalis under the influence of estrogen. The levels of estrogen peak at day 14, on the same day when ovulation should occur in a 28 day cycle. After ovulation the newly formed *corpus luteum* starts to produce progesterone which transforms the endometrium to its third stage, the secretory phase, and prepares it for embryo implantation. The short period on days 20–24 of the cycle, when the uterus is receptive for implantation, is called „the implantation window". If embryo implantation does not occur, levels of estrogen and progesterone start to decrease and the functional layer starts to degenerate, followed by menses (Young and Loy, 2005; Fritsch, 2008).

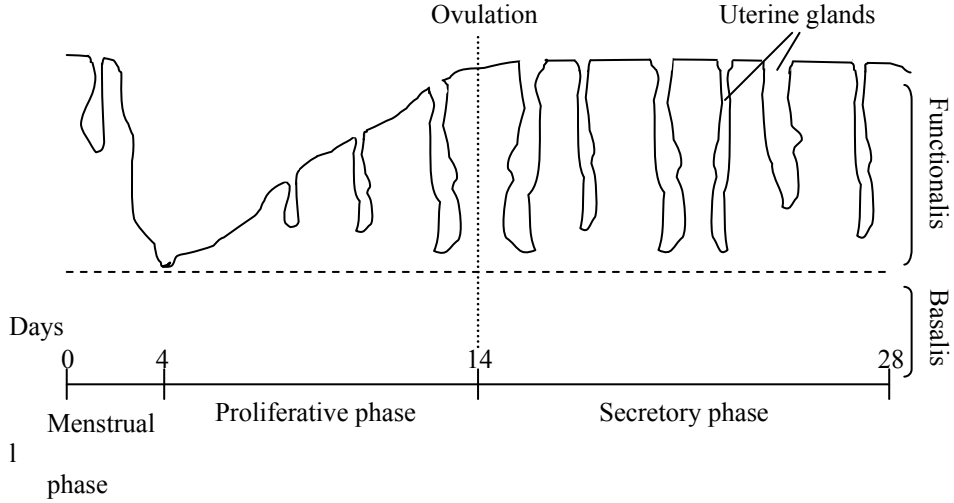

Figure 3. Structure of the human endometrium and phases of the menstrual cycle.

2.5. Reproductive immune failure syndrome

The significantly increased prevalence of autoimmune abnormalities seen in infertile women compared to fertile controls led Gleicher and el-Roeiy in year 1988 to propose the definition of reproductive autoimmune failure syndrome (RAFS) to describe polyclonal autoimmune activation in women suffering from

infertility, recurrent pregnancy loss and pregnancy complications (Gleicher and el-Roeiy, 1988). Recently Gleicher updated the acronym to RIFS (reproductive immune failure syndrome) indicating a general immune activation in these women, that includes alterations in the lymphocyte population and cytokine levels as well as autoimmune reactions (Gleicher, 2002).

Polyclonal B-cell activation in infertile women is reflective of a broad-based autoimmune activation, which generally leads to the presence of several common autoantibodies that are routinely used in diagnosis of different systemic autoimmune diseases, such as antiphospholipid syndrome and systemic lupus erythematosus (Geva *et al.*, 1997; Gleicher, 2002; Gleicher *et al.*, 2002; Carp *et al.*, 2012). The prevalence of organ-non-specific antibodies, especially antiphospholipid antibodies (APA), is one of the most studied immunological risk factor for recurrent implantation failure in IVF treatment (Coulam and Acacio, 2012). Targets of APA are negatively charged phospholipids and their cofactors. They include antibodies against cardiolipin (ACA) and its cofactor β2-glycoprotein I (β2-GPI), which are used for diagnosing antiphospholipid syndrome. Although the prevalence of APA is increased in women with recurrent reproductive failure compared to control population, the importance of APA in infertility remains controversial (Hammadeh *et al.*, 2002; Buckingham and Chamley, 2009). Furthermore, other common organ-non-specific autoantibodies, like anti-nuclear (ANA) and smooth muscle autoantibodies (SMA), have been detected in women with various causes of infertility, such as PCOS, TFI, endometriosis and unexplained infertility (Geva *et al.*, 1997; Van Voorhis and Stovall, 1997; Reimand *et al.*, 2001). Although some studies have shown associations between the presence of ANA and subsequent IVF treatment failure (Stern *et al.*, 1998; Ying *et al.*, 2012), the presence of these antibodies rather reflects a more general immunological activation (Geva *et al.*, 1997; Gleicher, 2002). Thyroid autoimmunity has been linked with increased abortion and IVF failure (Bussen *et al.*, 2000; Poppe *et al.*, 2008; Zhong *et al.*, 2012). Cross-reactivity of anti-thyroid antibodies (ATA) with *zona pellucida* and placental antigens may hamper fertilization and embryo implantation processes (Twig *et al.*, 2012). Moreover, ATA have been detected in the follicular fluid, where they might reduce oocyte quality by antibody mediated cytotoxicity (Monteleone *et al.*, 2011). Also, ATA may serve as markers for an immunologic defect at the T lymphocyte level which can contribute to reduced fecundity (Geva *et al.*, 1997; Poppe *et al.*, 2008).

The presence of organ-specific autoantibodies peculiar for infertility or to a gynecological disease leading to infertility may, however, directly contribute to reduced fecundity in these patients (Gobert *et al.*, 1992; Pires, 2010; Kokcu *et al.*, 2012). Autoimmune attack on the ovaries through AOA can lead to ovarian dysfunction in patients with POF, PCOS, endometriosis and unexplained infertility (Luborsky, 2002; Forges *et al.*, 2004; Petrikova and Lazurova, 2012). AOA are a heterogeneous group of antibodies that recognize several antigenic targets in the ovary, such as granulosa and theca cells, *zona pellucida*, oocyte cytoplasm, *corpus luteum*, but also gonadotropins and their receptors (Haller *et al.*,

2005; Monnier-Barbarino *et al.*, 2005; Haller *et al.*, 2007; Pires, 2010). Endometrial autoimmunity with the presence of anti-endometrial antibodies (AEA) is characteristic to most patients of endometriosis (Mathur *et al.*, 1982; Fernández-Shaw *et al.*, 1993). These autoantibodies have also been found in women suffering from TFI, unexplained infertility and PCOS (Palacio *et al.*, 1997; Palacio *et al.*, 2006). Although the prevalence of AEA does not correlate with the severity of endometriosis, it related to infertility in these patients (Fernández-Shaw *et al.*, 1993). The possible detrimental contribution of AEA to female reproduction might lay in interference with embryo implantation processes (Fernández-Shaw *et al.*, 1993; Gajbhiye *et al.*, 2008). To date, the nature of majority of AEA antibodies is still mostly unknown, since only a few AEA epitopes have been identified so far: α-enolase, transferrin and fetuin-A (Walter *et al.*, 1995; Pillai *et al.*, 1996; Lang and Yeaman, 2001).

3. AIMS OF THE STUDY

The general objective of this study was to assess the presence and significance of humoral immune system activation in serum and follicular fluid of IVF patients.

Accordingly, the specific aims were as follows:

1. To detect and compare the presence of common autoantibodies in the blood serum of infertile women of different etiologies.
2. To assess the anti-endometrial autoreactivity in sera of patients with TFI and endometriosis. To evaluate the association of detected autoantibodies with IVF treatment outcome. To identify endometrial antigen(s) related to the cause of infertility and IVF treatment outcome.
3. To examine the ovarian follicular content of infertile women by measuring proinflammatory cytokines and other related factors in the follicular fluid. To interpret these findings according to the cause of infertility and infertility treatment outcome.
4. To measure the level of follicular fluid and serum soluble (s)TREM-1 in infertile patients with different causes. To assess associations between the level of sTREM-1 and infertility etiology as well as IVF treatment parameters.

4. MATERIALS AND METHODS

4.1. Subjects and IVF

The studies recruited women from the Nova Vita Clinic, Estonia, who underwent IVF treatment during the time period from 2004 to 2010. All patients had been infertile for at least a year before entering the study. Clinical investigations as well as diagnoses of infertility were performed by physicians of the Nova Vita Clinic. The four study groups consisted of women with diverse causes for reproductive failure. In cases where the woman lacked known reasons for infertility while in her partner decreased semen quality was detected (World Health Organization, 1999) the diagnosis of male factor infertility was assigned. TFI due to occluded fallopian tubes was diagnosed either by hysterosalpingography or by diagnostic laparoscopy (Forti and Krausz, 1998). The main cause for tubal occlusion was an episode of infection (PID). Endometriosis was confirmed by diagnostic laparoscopy according to the classification of American Society for Reproductive Medicine (ASRM) (American Society for Reproductive Medicine, 1997). The diagnosis for PCOS was determined according to The Rotterdam European Society for Human Reproduction & Embryology (ESHRE)/ASRM-sponsored PCOS consensus group diagnostic criteria (The Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group, 2004). Unexplained infertility was assumed when the woman lacked any of the abovementioned reasons for infertility and her partner had normal semen quality, still the couple had suffered from infertility for more than a year. Study groups are further characterized in Table II. Supplemental clinical and IVF treatment parameters for study III and IV are shown in Paper III Table 2 and Paper IV Table I.

For ovarian hormonal stimulation GnRH agonist (Diphereline; Ipsen Pharma. Biotech, Paris, France) or antagonist (Cetrotide; Merck Serono, Geneva, Switzerland) was administered with recombinant FSH (Gonal-F, Merck Serono or Puregon, Schering-Plough, Kenilworth, NJ, US). IVF or ICSI was conducted at 4–6 h after oocyte retrieval, the resulting embryos were cultured up to 48 h. The indications for ICSI were either male factor infertility or previous oocyte fertilization failure. Good quality embryos were identified by the presence of at least four blastomeres and ≤20% fragmentation, the rate of good quality embryos was calculated as the proportion (%) of good quality embryos out of all fertilized oocytes. In most of the cycles, two embryos were chosen for embryo transfer. Clinical confirmation of intrauterine pregnancy was made using an ultrasound scan at the 6th or 7th week after embryo transfer.

Table II. Details of study groups and methods used

Study group	Mean age ± SD (years)	Period of collection	Study material	Hormonal stimulation protocol	Fertilization method	Method of identification
Study I (*n* = 129)						
Male factor infertility (*n* = 29) Tubal factor infertility (*n* = 56) Endometriosis (*n* = 12) PCOS (*n* = 21) Unexplained infertility (*n* = 11)	33.0 ± 5.5	2004–2005	SPC OPU	GnRH agonist (*n* = 7) and antagonist (*n* = 122)	IVF or ICSI	IIF for common autoantibodies; ELISA for β2-GPI and ACA
Study II (*n* = 190)						
Tubal factor infertility (*n* = 159) Endometriosis (*n* = 31)	33.8 ± 4.3	2004–2006	OPU	GnRH antagonist	IVF or ICSI	1- and 2-DE with immunoblot for AEA
Study III (*n* = 153)						
Male factor infertility (*n* = 67) Tubal factor infertility (*n* = 44) Endometriosis (*n* = 23) PCOS (*n* = 8) Unexplained infertility (*n* = 7) Other reasons (*n* = 4)	33.3 ± 4.5	2007–2010	Follicular fluid	GnRH antagonist	ICSI	Flow cytometry for biomarker detection

Study group	Mean age ± SD (years)	Period of collection	Study material	Hormonal stimulation protocol	Fertilization method	Method of identification
Study IV (*n* = 110)						
Male factor infertility (*n* = 48) Tubal factor infertility (*n* = 30) Endometriosis (*n* = 20) PCOS (*n* = 7) Unexplained infertility (*n* = 5)	32.8 ± 4.6	2007–2010	Follicular fluid OPU	GnRH antagonist	ICSI	ELISA for sTREM-1

PCOS – polycystic ovary syndrome; Other reasons – patients with other causes of infertility, such as endometrial hyperplasia, myoma uteri, ovulatory dysfunction, autoimmune diseases or chronic infections; SD – standard deviation; SPC – sera obtained from day 3–5 of patients' spontaneous menstrual cycle; OPU – sera obtained at the day of oocyte pick-up; IIF – indirect immunofluorescence assay; ELISA – enzyme-linked immunosorbent assay; 1- and 2-DE– one and two dimensional gel electrophoresis; β2-GPI – antibodies against β2-glycoprotein I; ACA – anti-cardiolipin antibodies; AEA – anti-endometrial antibodies; sTREM-1 – soluble triggering receptor expressed on myeloid cells 1

4.2. Methods used for autoantibody detection from serum samples

4.2.1. Detection of common autoantibodies

All autoantibody tests are clinically available in Autoimmunity laboratory, Hospital of Tartu University and have periodically been subjected to external quality assessment by UK NEQAS (Sheffield, UK). Indirect immunofluorescence (IIF) was used to detect the following autoantibodies: ANA-H and ANA-R on human HEp-2 cell line (Human Epithelial Cell Line type 2; Immuno Concepts®, Sacramento, CA, US) and rodent antigen, respectively, SMA, parietal cell (PCA), thyroid microsomal (TMA) and mitochondrial (AMA) (Reimand *et al.*, 2001). As antigenic substrates 5 μm cryostat sections of rat liver and kidney, commercial HEp-2 cells, mouse stomach and human thyroid tissue were used in case of certain antibody test. Sera were diluted 1:10 and 1:40 (ANA-H) or 1:100 (other antibodies). As a secondary antibody, a 1:40 dilution of fluorescein isothiocyanate conjugated rabbit anti-human IgG (DAKO, Glostrup, Denmark) was applied. Test results were explored using a UV microscope (Olympus BX50F4, Tokyo, Japan) by the laboratory specialist. The antibody levels were expressed as negative or as positive at lower (1:10) or higher (1:40 for ANA-H and 1:100 for other antibodies) titers. Every test included known autoantibody positive and negative sera as corresponding controls.

In-house enzyme-linked immunosorbent assay (ELISA) was used to detect antibodies against β2-GPI and ACA. Polystyrene 96 well microtiter plates (Nunc MaxiSorp, Roskilde, Denmark) were coated overnight at +4°C with 5 μg/ml β2-glycoprotein I (Crystal Chem, Chicago, IL, US) solution in borate buffered saline (200 mM H_3PO_3, 75 mM NaCl, pH 8.4) or 50 μg/ml cardiolipin (Sigma, Glostrup, Denmark) solution in 95% ethanol, followed by washing and blocking of the plates with 0.5% (β2-GPI) or 10% (ACA) bovine serum albumin and 0.4% Tween-20 in borate buffered saline. After incubation with sera at 1:100 (β2-GPI) or 1:50 (ACA) dilution, alkaline phosphatase-conjugated anti-human IgG (DAKO, Glostrup, Denmark) was applied in dilution 1:1000 as a secondary antibody. Color was developed by adding 100 μl (β2-GPI) or 50 μl (ACA) substrate, *p*-nitrophenyl phosphate 1 g/l in 1 M diethanolamine buffer (pH 9.8). Absorbencies were read at 405 nm with 492 nm subtraction. Antibody levels were expressed in enzyme-immunological units (EIU) calculated according to optical density (OD) values as follows: $[(OD_{sample}-OD_{negative\ control})/ OD_{positive\ control}-OD_{negative\ control})x100]$. Cut-off values for positive and strongly positive results were 10 and 30 EIU (β2-GPI) or 30 and 60 (ACA). Every test included known autoantibody positive and negative sera as corresponding controls.

4.2.2. Detection of anti-endometrial antibodies (AEA) and identification of their targets

A pool of human endometriotic tissue from 5 infertile patients, aged 31–35, was used as antigenic substrate. The histology of all biopsy specimens corresponded to the mid-secretory phase of the receptive endometrium. Tissue samples were first homogenized mechanically with Dounce homogenizer followed by sonication for 4x15 s at 60 W (Bandelin Sonoplus HD 2200; Berlin, Germany). In 1-dimensional gel electrophoresis 1-(DE) samples were solubilized in 2-amino-2-(hydroxymethyl)propane-1,3-diol (Tris)-HCl buffer containing: 3% sodium dodecyl sulfate; 10% glycerol; 0.1 M 1,2-dithio-DL-threitol (DTT); 0.02% bromophenol blue and 6.25 mM Tris-HCl, pH 6.8. For 2-DE a different solubilization buffer was used [5 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 100 mM DTT and 40 mM Tris-HCl, pH 9.5]. 1-DE was performed on a 5–20% gradient gel using SE-600 vertical electrophoresis system (Hoefer, San Francisco, CA, US) (Utt *et al.*, 2002), and the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane using a semi-dry electro-blotter (Hoefer). Membranes were blocked twice, first with a 0.2 M ethanolamine/glycine buffer containing 0.25 mM polyvinyl-pyrrolidone and 25% methanol, and then with the same buffer containing 0.14% Tween 20, 25% methanol and 0.5% gelatin hydrolysate. Sera diluted to 1:100 were applied to membrane strips and incubated overnight at +4^{o}C under constant shaking. As a secondary antibody, horseradish peroxidase (HRP)-conjugated, rabbit polyclonal antibodies against human IgA or IgG (Dako, Glostrup, Denmark) were used at a 1:500 dilution. To detect background antibody reactions, one strip was incubated only with secondary HRP-conjugated antibodies. Color was developed in a 50 mM sodium acetate buffer (pH 5.0) containing 0.04% 3-amino-9-ethylcarbazole and 0.015% hydrogen peroxide. Reactions only detected by control staining with secondary HRP-conjugated antibodies were excluded from further analysis.

The isoelectric focusing (IEF) was carried out to further substantiate results detected with 1-DE. For this, 7-cm Immobiline DryStrips (GE Healthcare, Piscataway, NJ, US) with a linear pH gradient of 3–10 in an IPGphor IEF system (Amersham Pharmacia Biotech, Uppsala, Sweden) were used. After 12 h of rehydration, the following focusing parameters were applied: a current of 50 μA per strip and a voltage increase over 8 h from 100 V to 8000 V. The IEF was finished at total of 25–30 kVh. For the second dimension separation, the strips were placed on top of 10% polyacrylamide gels in a Mini-PROTEAN 3 Cell Vertical Electrophoresis System (Bio-Rad Laboratories, Hercules, CA, US). Following electrophoresis, gels were either stained with PageBlue Protein Staining Solution (Fermentas, Vilnius, Lithuania) or blotted onto PVDF membranes, as described above. The gels and blotted membranes were scanned with a GS-710 Imaging Densitometer (Bio-Rad). The 2-DE images were analyzed with Melanie software version 3 (Bio-Rad).

For antigen identification protein spots stained with PageBlue that corresponded to serum antibody reactions on the two-dimensional immunoblots were analyzed with mass spectrometry by trypsin degradation (Wilm *et al.*, 1996). Mascot 2.2.04 Unix software (Matrix Science, London, UK) was used to identify the obtained peptide fragmentation spectra in the IPI_human human_20081009 database, which considered the following modifications: carbamidomethylation, deamidation, N-terminal acetylation and methionine oxidation. The search was carried out together with decoy search that included the following parameters: precursor accuracy of 5 p.p.m. and fragment accuracy of 0.8 Da. The theoretical isoelectric point and molecular weight for each identified protein was calculated with the ExPASy Compute web tool (http://www.expasy.org/tools/pi_tool.html). For anti-α-enolase antibody validation rabbit polyclonal antibodies (0.03 μg⁄ml) against human a-enolase isoform (ENO1; Abcam, Cambridge, UK) in combination with HRP-labeled goat anti-rabbit IgG antibodies (Dako, Glostrup, Denmark) were used.

4.3. Methods used for biomarker detection from follicular fluid

4.3.1. Flow cytometry analysis for multianalyte testing

In total, 16 biomarkers (divided into two 8-plexes) were evaluated from each individual follicular fluid sample with a commercially-available FlowCytomix Human Basic Kit Assay (Bender Medsystems, Vienna, Austria). The first 8-plex consisted of: IL-23, sAPO-1/Fas, MIP-1β, MIP-1α, CD44(v6), IL-8, G-CSF, and RANTES. The second 8-plex consisted of IL-12p70, IFN-γ, MCP-1, IL-6, IFN-α, IL-18, IL-1β, and TNF-α. Before sample processing, each follicular fluid was thawed and centrifuged at 450 *g* for 10 min and the supernatants were used for analysis. Microspheres coated with biomarker specific antibodies were incubated together with sample material and biotin-conjugated secondary antibody mixture. After washing, streptavidin-phycoerythrin solution was added for the second incubation. Quantization measurements were performed by flow cytometer instrument FC 500 and accompanying CXP Software (Beckman Coulter, CA, US). For calculations the FlowCytomix Pro 2.3 Software was used (Bender Medsystems). Standard curves for each biomarker were generated with manufacturer-supplied references. The concentration of a biomarker was calculated as mean fluorescent intensity divided by single median standard curve and expressed in pg/ml.

4.3.2. Enzyme-linked immunosorbent assay for soluble triggering receptor expressed on myeloid cells (sTREM-1) detection

To evaluate the levels of sTREM-1 from serum and follicular fluid samples Quantikine® ELISA assays (R&D Systems, Inc., Minneapolis, MN, US) were

used. Microplates pre-coated with monoclonal antibody specific for sTREM-1 were first incubated with sample fluid and secondly with sTREM-1 conjugate. The final incubation with Substrate Solution was stopped by adding Stop Solution (both solutions provided by manufacturer). The color reaction was measured at 450 nm with correction at 540 nm. For each set of samples standard curves were generated with manufacturer-supplied references. The concentration of sTREM-1 was expressed in pg/ml.

4.4. Statistical methods

The R2.3.1 A Language and Environment software (Free Software Foundation, Boston, MA, US) was used for statistical analysis. Altogether in the four studies, the following statistical methods were used: differences between study groups were evaluated using *t*- and proportion test, paired *t*-test, Mann-Whitney U-test, and chi-square tests with the Yates' correction. Correlations between the variables were evaluated by Pearson's correlation test, crude and adjusted simple regression and logistic regression analysis. In Papers I and II women with TFI were used as a reference group as the cause for their infertility was believed to be more related to anatomical abnormalities than immune system impairments. Based on results from the first two studies, male factor infertility patients were chosen to be more suitable for a reference group in Papers III and IV. The selection of a specific test and adjusted parameters are further explained at the results. A p-value of < 0.05 was considered statistically significant.

4.5. Ethical considerations

The studies were approved by the Ethics Committee of Medical Research of the University of Tartu. All voluntary participants signed the written informed consent. Personally identifying data was kept apart from data relating to sample material.

5. RESULTS

5.1. The presence of common autoantibodies in the serum of infertile women before and after ovarian stimulation (Paper I)

Antibodies to mitochondrial antigens were not detected in any of the patients. In women with endometriosis and PCOS antibody positivity was more frequent for at least one antibody at a lower titer (endometriosis only) and higher titer, compared to TFI patients (Table III). We also analyzed associations between the prevalence of certain autoantibodies at spontaneous menstrual cycle and the cause of infertility using logistic regression analysis adjusted for age and the number of previous IVF procedures. We found higher prevalence of 1:10 SMA to be associated with male factor infertility [adjusted odds ratio (OR) = 20.45, p = 0.018], endometriosis (adjusted OR = 37.29, p = 0.008) and the number of previous IVF procedures (adjusted OR = 2.87, p = 0.013). In addition, higher frequency of 1:10 ANA-R was detected in unexplained infertility patients (adjusted OR = 8.79, p = 0.038) than in the reference group. When autoantibody levels were measured after COS, the levels of SMA, PCA, TMA, ACA and β2-GPI antibodies had remained unchanged. However, the levels of ANA-H were significantly decreased (proportion test between the patients with decreased and increased antibody levels, $p < 0.05$).

Table III. Common autoantibodies in serum* of *in vitro* fertilization patients

Cause of infertility (*n*)	**Antibodies at low titer [a], % (*n*, 95% CI)**	**Proportion test (p-value)**	**Antibodies at high titer [b], % (*n*, 95% CI)**	**Proportion test (p-value)**
Tubal factor infertility (56)	30.4 (17, 19.2-44.3)	Reference	10.7 (6, 4.4-22.6)	Reference
Male factor infertility (29)	37.9 (11, 21.3-57.6)	N.S.	3.4 (1, 0.2-19.6)	N.S.
Polycystic Ovary Syndrome (21)	42.9 (9, 22.6-65.6)	N.S.	23.8 (5, 9.11-47.5)	< 0.05
Endometriosis (12)	58.3 (7, 28.6-83.5)	< 0.05	33.3 (4, 11.3-64.6)	< 0.05
Unexplained infertility (11)	36.4 (4, 12.4-68.4)	N.S.	18.2 (2, 3.2-52.2)	N.S.

* Serum was obtained on the 3.–5. day of the patients' spontaneous menstrual cycle. [a] Antibody-positivity at the low titer was assessed by counting the number of positive tests at the following titers: 1:10 for ANA-R, ANA-H, SMA, TMA, PCA or ACA and β2-GPI present at least at the weak positive value. [b] Antibody-positivity at the high titer was assessed by counting the number of positive tests at the following titers: 1:100 for ANA-R, SMA, TMA; 1:10 for PCA; 1:40 for ANA-H or highly positive results for ACA and β2-GPI. CI – confidence interval; N.S. – statistically not significant ($p > 0.05$)

5.2. The prevalence and implication of serum AEA in patients with tubal factor infertility and endometriosis (Paper II)

We identified AEA reactivity on the endometrial homogenate in the molecular weight range of 10–100 kDa. To compare patient's personal antibody reactivity, individual AEA reactions were counted and combined into a total reaction number for each patient. An average ± SD of 13.7 ± 4.5 and 10.3 ± 3.5 IgA AEA reactions were detected in patients with TFI and endometriosis, respectively. According to adjusted logistic regression model, patients with TFI showed significantly more IgA AEA reactions compared to endometriosis patients (age and total number of IgG AEA adjusted OR = 1.2, $p < 0.001$). In comparison, the total reaction numbers of IgG AEA did not differ between the study groups (adjusted OR = 1.00, $p = 0.100$). An average of 20.8 ± 6.3 and 18.5 ± 4.8 IgG AEA was detected in TFI and endometriosis patients, respectively. However, certain individual IgA and IgG antibody reactions showed risk associations with the cause of infertility (Table IV).

Since no differences were detected between clinical pregnancy rates in women with TFI (38.7%) and endometriosis (33.3%) (age-adjusted OR = 1.66, $p = 0.297$), further analysis was conducted on the entire study group. The total reaction number for IgA AEA in pregnant and non-pregnant IVF patients was 13.2 ± 4.3 and 12.1 ± 4.7 reactions, respectively. The according numbers for IgG AEA were 20.4 ± 6.1 and 20.3 ± 6.5 reactions, respectively. Age-adjusted logistic regression model showed no correlation between IVF pregnancy outcome and the total number of IgA (adjusted OR = 0.95, $p = 0.257$) or IgG (adjusted OR = 1.0, $p = 0.921$) type AEA reactions. However, certain individual AEA reactions showed significant associations with IVF pregnancy failure (Table V).

According to data found in the literature, α-enolase (47 kDa) has previously been suggested as an antigenic target in endometriosis (Walter *et al.*, 1995). In our study, the AEA reactions against the 47-kDa endometrial antigen were preponderant in TFI patients (Table IV). Additionally, there was a difference between clinical pregnancy rates in patients with or without the 47-kDa IgA AEA reaction (Table V). Therefore, we aimed to validate α-enolase as an antigen for AEA reactions in infertile women in our study. For this, 1-DE and subsequent immunoblots with patients' sera and polyclonal α-enolase antibodies were compared. After finding an identical reaction in the 47-kDa region, endometrial antigens were separated using 2-DE (Figure 4a). The comparison of following

Table IV. Serum anti-endometrial antibodies that were associated with endometriosis and tubal factor infertility

Antigen MW kDa	Endometriosis (*n* = 31) % (*n*, 95% CI)	Tubal factor infertility (*n* = 159) % (*n*, 95% CI)	Association with disease: OR, p*
IgA AEA			
95	38.7 (12, 22.4–57.7)	11.3 (18, 7.0–17.5)	5.21, < 0.001[a]
73	48.4 (15, 30.6–66.6)	17.6 (28, 12.2–24.6)	4.59, 0.001[a]
70	16.1 (5, 6.1–34.5)	4.4 (7, 1.9–9.2)	4.84, 0.015[a]
55	3.2 (1, 0.2–18.5)	37.3 (60, 30.3–45.8)	1.06, 0.007[b]
52	48.4 (15, 30.6–66.6)	86.2 (137, 79.6–90.9)	1.15, < 0.001[b]
47	67.7 (21, 48.5–82.7)	84.9 (135, 78.2–89.9)	1.45, 0.015[b]
45	9.7 (3, 2.5–26.9)	35.8 (57, 28.5–43.9)	1.25, 0.012[b]
37	25.8 (8, 12.5–44.9)	50.9 (81, 42.9–58.9)	1.56, 0.023[b]
34	6.5 (2, 1.1–22.8)	43.4 (69, 35.6–51.5)	1.10, 0.002[b]
22	12.9 (4, 4.2–30.8)	35.8 (57, 28.5–43.9)	1.33, 0.015[b]
16	19.4 (6, 8.1–38.1)	70.4 (112, 62.6–77.3)	1.11, < 0.001[b]
14	6.5 (2, 1.1–22.8)	50.3 (80, 42.3–58.3)	1.08, < 0.001[b]
IgG AEA			
200	12.9 (4, 4.2–30.8)	40.3 (64, 32.6–48.3)	1.33, 0.013[b]
150	6.5 (2, 1.1–22.8)	41.5 (66, 33.8–49.6)	1.12, 0.004[b]
130	48.4 (15, 30.6–66.6)	59.7 (95, 51.7–67.4)	1.12, 0.004[b]
110	9.7 (3, 2.5–26.9)	59.1 (94, 51.0–66.8)	5.21, < 0.001[b]
45	19.4 (6, 8.1–38.1)	61.6 (98, 53.6–69.1)	1.14, < 0.001[b]
28	35.5 (11, 19.8–54.6)	86.8 (138, 80.3–91.5)	1.09, < 0.001[b]
24	90.3 (28, 73.1–97.5)	68.6 (109, 60.6–75.5)	4.74, 0.015[a]
20	90.3 (28, 73.1–97.5)	42.8 (68, 35.0–50.9)	13.32, < 0.001[a]

* Age adjusted logistic regression (OR – odds ratio, CI – confidence interval). [a] Association with endometriosis. [b] Association with tubal factor infertility. AEA – anti-endometrial antibodies; MW – molecular weight

Table V. Serum anti-endometrial antibodies that were associated with probability for *in vitro* fertilization pregnancy

IgA AEA				**IgG AEA**			
Antigen MW kDa	**Probability for pregnancy***			**Antigen MW kDa**	**Probability for pregnancy***		
	OR	95% CI	p		OR	95% CI	p
80	0.22	0.06–0.77	0.018	110	0.31	0.14–0.69	0.004
47	0.37	0.15–0.90	0.029	100	0.37	0.16–0.85	0.020
37	0.42	0.19–0.92	0.030	40	0.45	0.20–1.03	0.058
32	0.44	0.21–0.94	0.034	29	0.49	0.23–1.04	0.065
25	0.37	0.14–0.96	0.042	28	0.48	0.20–1.12	0.089
16	0.39	0.18–0.84	0.017	14	0.28	0.08–1.00	0.049

* Age adjusted logistic regression (OR – odds ratio, CI – confidence interval). AEA – anti-endometrial antibodies; MW – molecular weight

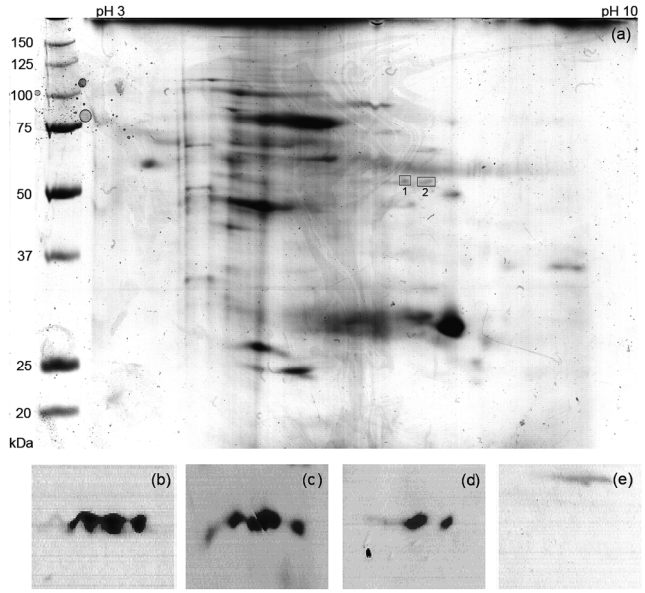

Figure 4. Endometrial homogenate resolved by isoelectric focusing in a pH range from 3–10 (a). Boxes 1 and 2 show spots identified as ENO1 isoforms of α-enolase. Immuno-blot comparison of the spots recognized: (b) the same region by ENO1 polyclonal antibodies; (c) a patient's serum IgA; (d) a patient's serum IgG; and (e) a patient's serum unreactive towards the 47 kDa antigen.

immunoblots with polyclonal α-enolase antibodies and sera either with or without IgA or IgG AEA reactions to the 47-kDa protein (Figure 4b–d) revealed up to three identical spots with different isoelectric point values (5.9–7.1). Two spots (Figure 4a, Boxes 1 and 2) were subjected to in-gel digestion and mass spectrometry analysis combined with sequence database searches with a significance threshold of $p < 0.001$ and an ion score cut-off of 20. In spot 1, twelve peptides were matched with sequence coverage of 28%. In spot 2, eleven peptides were matched with sequence coverage of 37%. In both of the spots ENO1 (IPI ID: IPI00465248) was identified.

5.3. Associations of follicular proinflammatory biomarker levels with infertility etiology and IVF treatment (Paper III)

The concentrations of the biomarkers detected by flow cytometry analysis are shown in Table VI. The associations between follicular fluid biomarker levels and infertility etiology were assessed using adjusted linear regression models and male factor infertility patients as a reference group. According to our results, women with TFI were characterized by significantly lower concentrations of follicular IL-1β (adjusted r = –12.6 pg/ml, p = 0.037) and IFN-α, when the status of smoking was included in the model (adjusted r = –13.9 pg/ml, p = 0.046). In endometriosis patients, higher levels of IL-23 were measured (adjusted by follicular number prior to stimulation r = 157.1 pg/ml, p = 0.025). Further, PCOS patients had significantly higher levels of CD44(v6) (age adjusted r = 2072.7 pg/ml, p = 0.010) and MIP-1α (adjusted for age, cause of infertility and follicular count in prestimulatory ovary r = 3111.7 pg/ml, p = 0.007). On the other hand, lower levels of CD44(v6) were characteristic to unexplained infertility (age adjusted r = –1888.4 pg/ml, p = 0.025). Additionally, we found active smoking to be related to elevated concentrations of CD44(v6) (adjusted for age and cause of infertility r = 1227.8 pg/ml, p = 0.019 vs. never-smokers) and sAPO-1/Fas (adjusted r = 464.9 pg/ml, p = 0.031vs. never-smokers). Likewise, the levels of follicular IL-23 were higher in women who had been or were smoking at present, when compared to never-smokers (adjusted for age and cause of infertility r = 107.6 pg/ml, p = 0.043). Interestingly, raised concentrations of IL-23 were also more characteristic to women experiencing secondary infertility rather than primary infertility (adjusted for the cause of infertility r = 94.6 pg/ml, p = 0.043).

Further we analyzed associations between follicular fluid biomarker concentrations and IVF treatment parameters. Our results indicated a positive correlation between the level of IL-12 and the following infertility treatment parameters: (1) the number of fertilized oocytes (adjusted r = 0.15 pg/ml per every additional two-pronuclear zygote, p = 0.007); (2) and the proportion of good quality embryos (adjusted r = 0.22 pg/ml per every additional embryo, p = 0.006), when the data was adjusted for age, cause of infertility and follicular size. We also

Table VI. Biomarker levels in the follicular fluid of women undergoing *in vitro* fertilization

	Male factor infertility (*n* = 67)[a]	**Tubal factor infertility (*n* = 44)**	**Polycystic Ovary Syndrome (*n* = 8)**	**Endometriosis (*n* = 23)**	**Unexplained infertility (*n* = 7)**	**Other reasons (*n* = 4)**	**Total (*n* = 153)**
Biomarkers (pg/ml)*							
IL-1β	0 (0–236.8)	0 (0–53.6)	0 (0–29.0)	0 (0–110.3)	0 (0–0)	0 (0–143.1)	0 (0–236.8)
IL-6	0 (0–18.7)	0 (0–10.7)	0 (0–16.2)	0 (0–37.2)	0 (0–0)	0 (0–8.4)	0 (0–37.2)
IL-12p70	0 (0–24.9)	0 (0–6.1)	0 (0–8.1)	0 (0–21.0)	0 (0–0)b	0 (0–8.1)	0 (0–24.9)
IL-18	311.0 (0–722.0)	290.2 (0–812.5)	463.4 (0–648.5)	283.3 (44.6–874.3)	199.1 (0–255.5)[b]	310.9 (110.8–767.0)	297.2 (0–874.3)
IL-23	282.3 (0–1069.0)	208.7 (0–1280.0)	237.4 (0–746.4)	388.8 (0–1160.0)	408.5 (0–557.8)	120.3 (0–260.3)	260.3 (0–1280.0)
IFN-α	0 (0–150.7)	0 (0–107.6)	0 (0–93.5)	0 (0–114.2)	0 (0–0)	0 (0–161.9)	0 (0–161.9)
IFN-γ	0 (0–111.2)	0 (0–74.5)	0 (0–60.4)	0 (0–111.2)	0 (0–0)	9.5 (0–147.5)	9.5 (0–147.5)
TNF-α	0 (0–30.7)	0 (0–10.8)	0 (0–5.3)	0 (0–21.0)	0 (0–0)	0 (0–58.8)	0 (0–58.8)
IL-8	307.3 (119.4–4857.0)	367.2 (117.4–1117.0)[b]	417.6 (236.9–1032.0)	473.6 (172.8–1879.0)[b]	424.3 (343.8–1472.0)	416.2 (172.8–2851.0)	371.2 (117.4–4857.0)
MIP-1α	143.6 (0–5766.0)	80.6 (0–15990.0)	555.8 (0–19840.0)	227.6 (0–18230.0)	52.3 (0–3383.0)	136.3 (0–1788.0)	130.8 (19840.0)
MIP-1β	52.3 (6.0–1254.0)	48.2 (11.5–433.2)	38.7 (17.59–96.4)	51.7 (17.1–120.9)	40.7 (36.8–64.5)	63.5 (25.7–967.0)	48.4 (6.0–1254.0)

	Male factor infertility (n = 67)[a]	Tubal factor infertility (n = 44)	Polycystic Ovary Syndrome (n = 8)	Endometriosis (n = 23)	Unexplained infertility (n = 7)	Other reasons (n = 4)	Total (n = 153)
Biomarkers (pg/ml)*							
MCP-1	1019.0 (594.2–2046.0)	1054.0 (416.1–2564.0)	1067.0 (56.4–15732.0)	1033.0 (373.8–2780.0)	992.9 (818.1–1265.0)	801.7 (198.4–1598.0)[b]	1016.0 (198.4–2780.0)
G-CSF	82.5 (0–2464.0)	48.1 (0–498.0)	104.1 (0–3156.0)	122.2 (0–4809.0)	118.5 (0–463.4)	23.7 (0–341.6)	89.7 (0–4986.0)
sAPO-1/Fas	129.0 (0–564.2)	169.4 (0–9589.0)	152.1 (0–4469.0)	94.9 (0–520.4)	96.8 (64.3–292.5)	98.8 (0–226.8)	129.0 (0–9589.0)
CD44(v6)	8426.0 (5063.0–14030.0)	8554.0 (5348.0–20610.0)	10750.0 (6394.0–12130.0)[b]	8219.0 (5535.0–11770.0)	66190.0 (5168.0–9348.0)[b]	6836.0 (6611.0–10200.0)	8219.0 (5063.0–20610.0)
RANTES	97.4 (0–705.1)	97.4 (0–908.3)	50.1 (0–189.3)	146.6 (0–438.8)	77.4 (12.2–182.7)	74.1 (2.6–1428.0)	97.4 (0–1428.0)

* Concentrations are provided as medians (minimum–maximum value). Differences between study groups: [a] Reference group; [b] Mann-Whitney U-test, $p < 0.05$. Associations between different parameters assessed by adjusted regression models are provided in the text

found higher levels of follicular MIP-1β to be more characteristic to women achieving intrauterine pregnancy when compared to hCG-negative patients (adjusted for age and cause of infertility r = 48.0 pg/ml, p = 0.047). Moreover, a positive association was revealed between the concentrations of follicular MIP-1β and IFN-α with the diameter of a follicle (adjusted r = 7.8 pg/ml, p = 0.037 and r = 2.4 pg/ml for every millimeter in diameter, p = 0.023, respectively), regardless of age or cause of infertility. The concentration of IL-8 in follicular fluid was positively associated with infertility treatment parameters, such as: (1) intrauterine pregnancy (adjusted for age, cause of infertility, rate of good quality embryos transferred and endometrial thickness r = 207.5 pg/ml, p = 0.051); (2) parity (adjusted for age and cause of infertility r = 150.6 pg/ml for every child born, p = 0.039); (3) the diameter of a follicle (adjusted for age and cause of infertility r = 40.2 pg/ml, for every additional millimeter in diameter p = 0.005); (4) but also with serum progesterone after ovarian stimulation (adjusted r = 4.7 pg/ml, p = 0.031). Similarly, the level of follicular IL-18 was positively associated with: (1) the number of fetuses detected by ultrasonography (adjusted for age, cause of infertility, number of embryos transferred, rate of good quality embryos among them and endometrial thickness r = 67.2 pg/ml for every additional fetus, p = 0.020); (2) increased parity (adjusted for age and cause of infertility r = 60.7 pg/ml for every child born, p = 0.038); (3) and the follicular diameter (adjusted for age and cause of infertility r = 13.1 pg/ml for every additional millimeter in diameter, p = 0.022).

5.4. Level of follicular fluid and serum sTREM-1 in infertile women (Paper IV)

Concentrations of sTREM-1 were measured with ELISA in the follicular fluid [52.4–232.5 pg/ml (mean ± SD, 140.4 ± 34.4)] and blood serum [45.7–242.5 pg/ml (115.6 ± 35.1)]. According to Pearson's correlation test, follicular fluid and serum levels of sTREM-1 were in good correlation with significantly higher levels in follicular fluid compared to serum (*t*-test = 5.24, p < 0.0001). In follicular fluid the levels of sTREM-1 in different patient groups were as follows: 150.3 ± 40.5 pg/ml in patients with male factor infertility; 139.8 ± 32.4 pg/ml in TFI; 124.1 ± 30.4 pg/ml in women with endometriosis; 148.0 ± 28.8 pg/ml in PCOS and 142.0 ± 15.2 pg/ml in unexplained infertility. When we compared these levels using age adjusted linear regression analysis, we found that women with endometriosis had significantly lower levels of follicular sTREM-1 compared to patients with male factor infertility (adjusted r = –25.7 pg/ml, p = 0.018). We found no further associations between the other patient groups and the reference group or with other clinical data and IVF parameters (data not shown).

In serum, following sTREM-1 concentrations were detected in different patient groups: 118.3 ± 39.8 pg/ml in male factor infertility patients; 120.3 ± 37.6 pg/ml in TFI; 96.9 ± 19.2 pg/ml in endometriosis; 128.7 ± 18.9 pg/ml in PCOS and 112.7 ± 26.2 pg/ml in patients with unexplained infertility. As in follicular

fluid, endometriosis patients had significantly lower levels of sTREM-1 in serum when compared to the reference group (age-adjusted regression analysis r = –22.1 pg/ml, p = 0.030). Additionally, adjusted simple regression analysis revealed an association between higher levels of serum sTREM-1 and lower rate of embryo quality among fertilized oocytes (adjusted for age and cause of infertility r = –0.3% of less good-quality embryos gained per one pg/ml increase of serum sTREM-1, p = 0.033). Subsequent logistic regression analysis marked out the cutoff value of serum sTREM-1 with specificity of 95% to be between 111.5 and 113.3 pg/ml (OR = 0.38, p = 0.048 and OR = 0.34, p = 0.028, respectively). This concentration interval distinguishes whether a patient will get less or more than 39% of embryos with good quality. No further significant associations were found between serum levels of sTREM-1 and other clinical data or IVF parameters.

6. DISCUSSION

6.1. Serum organ-specific and organ-non-specific autoantibodies in IVF patients

Higher incidence of autoantibodies in IVF patients compared to fertile controls is a common observation (Gleicher and el-Roeiy, 1988; Geva *et al.*, 1997; Van Voorhis and Stovall, 1997; Mettler *et al.*, 2004; Putowski *et al.*, 2004). Our results added further confirmation that autoimmune mechanisms are associated with a deteriorated uterine milieu in infertile patients. Also, in this study we indicated that the prevalence of AEA IgG and IgA is associated with IVF treatment results and we suggested α-enolase to be one of the antigens for AEA.

Some studies have shown the detrimental effect of certain autoantibodies, such as APA, on pre-implantation embryo, decidual or placental vascularization and embryo implantation (Geva *et al.*, 1997; Di Simone *et al.*, 2010). Similarly, a possible association between ANA and oocyte and embryo development as well as embryo implantation has been suggested, as women positive for ANA are more prone to implantation failure and early post-implantation loss (Geva *et al.*, 1997; Taniguchi, 2005; Ying *et al.*, 2012). Others have questioned these effects, mainly associations with embryonic development, since these autoantibodies are restricted to the maternal blood and do not come into contact with preimplantation embryos, especially in the IVF procedure. These authors have suggested that autoantibodies in infertile patients are markers for a broader immune dysfunction with reduced fecundity as one of the symptoms (Gleicher *et al.*, 2002; Dias *et al.*, 2006; Buckingham and Chamley, 2009).

We observed notably higher numbers of common serum autoantibodies in infertile patients with endometriosis and PCOS than in patients with TFI. Significantly increased prevalence of SMA was characteristic to women with endometriosis. Although SMA is primarily a marker for chronic viral infections, they have also been more frequently detected in women with ovulatory dysfunction, endometriosis, TFI and unexplained infertility (Nip *et al.*, 1995; Geva *et al.*, 1997; Van Voorhis and Stovall, 1997; Reimand *et al.*, 2001). Supposedly, SMA could alter the function of the fallopian tubes in these women (Taylor *et al.*, 1989). Higher levels of SMA additionally correlated with male factor infertility and the number of previous IVF procedures. These are associations not easily explained at first notice. However, in infertile couples where reduced fertility in the male has been diagnosed, undetected or subclinical infertility in the female cannot easily be excluded. It is therefore recommendatory that both partners should be investigated, since the probability for coexistence of reduced female fecundity is higher in these couples (The ESHRE Capri Workshop, 1996). Whether or not the IVF procedure itself could induce autoantibody production has long been debated without a clear consensus, since these antibodies could be part of the infertile state and cause the need for multiple IVF procedures by their nature (Gobert *et al.*, 1992; Fisch *et al.*, 1995; Geva *et al.*, 1997; Delgado Alves *et al.*, 2005).

Our finding that the presence of ANA was characteristic to unexplained infertility is in accordance with previous studies (Taylor *et al.*, 1989; Putowski *et al.*, 2004). ANA positivity is considered to be a marker for an autoimmune activation as it is a common finding in patients with autoimmune diseases (Geva *et al.*, 1997; Dias *et al.*, 2006). These results along with ours, allow suggesting that impairments in the immune system may be involved in the etiology of unexplained infertility (Gleicher, 1998; Putowski *et al.*, 2004; De Carolis *et al.*, 2010). It is also possible that these patients could be suffering from subclinical endometriosis or recurrent preimplantation pregnancy loss, two conditions where ANA are frequently detected (Dmowski, 1995; Geva *et al.*, 1997; Van Voorhis and Stovall, 1997; Coulam and Acacio, 2012).

Multiple researches have shown that AEA reactivity to a rather wide molecular weight range of antigenic targets is relevant to endometrial autoimmunity in cases of endometriosis (Mathur *et al.*, 1995; Gajbhiye *et al.*, 2008). The origin of these antibodies is explained by antigenic activation to eutopic or ectopic endometrium (Fernández-Shaw *et al.*, 1993). Also, oxidative stress can chemically modify lipids and proteins causing thereby a rise in "new epitopes" (Shanti *et al.*, 1999; Palacio *et al.*, 2006). Besides endometriosis, AEA have been detected in PCOS, unexplained infertility and in patients with tubal obstructions (Palacio *et al.*, 1997; Palacio *et al.*, 2006). Palacio suggested with his colleagues a pathogenetic involvement for AEA in TFI, but the repertoire of AEA in TFI has not been studied much (Palacio *et al.*, 1997). In our study, patients with TFI showed significantly more IgA AEA reactions compared to endometriosis patients. Given that IgA is a marker for local inflammation in mucosal tissues, our result was rather expected. Increased oviductal IgA levels can be caused by local inflammation in the fallopian tubes. Salpingitis can develop due to STD, although, we were unaware of any current infections among our IVF patients. Still, subclinical infections or sequelae of previous STD can similarly cause an inflammatory milieu in the fallopian tubes (Arraztoa *et al.*, 2002). Oviductal inflammation can eventually lead to an inflammatory milieu in the uterus, thereby reducing endometrial receptivity (Meyer *et al.*, 1997; Edi-Osagie *et al.*, 2004; Copperman *et al.*, 2006). Further on, as the mucosa of the fallopian tubes expresses genes similar to endometrial genes, AEA tissue cross-reactivity could be believed in TFI (Quayle *et al.*, 1998; Djahanbakhch *et al.*, 1999).

Additionally, our results provide further evidence that certain AEA reactions are associated with IVF pregnancy failure. The presence of AEA in endometriosis patients has been linked with impaired implantation and greater risk for miscarriage through a hindering effect on fertilization and preimplantation embryogenesis (Fernández-Shaw *et al.*, 1993; Randall *et al.*, 2007; Gajbhiye *et al.*, 2008; Randall *et al.*, 2009). From the few AEA antigens identified, antibodies to fetuin-A (formerly named α2-Hermans Schmidt glycoprotein) 64 kDa and transferrin 72 kDa are specific and relevant to endometrial autoimmunity (Mathur *et al.*, 1995; Pillai *et al.*, 1996; Mathur, 2000). Autoimmune reactivity to both of these targets may contribute to infertility in endometriosis: transferrin

through its role in the local regulation of ovarian function and fetuin-A through its importance in *zona pellucida* formation (Mathur, 2000). Additionally, autoantibodies to these antigens may inhibit sperm motility (Pillai *et al.*, 1998; Mathur, 2000).

We succeeded to identify AEA reactions against the 47-kDa endometrial antigen, preponderant in TFI patients, as α-enolase antibodies. Interestingly, the α-enolase IgA AEA were additionally associated with IVF pregnancy failure. Enolase is a highly conserved glycolytic enzyme, important not only in the glycolysis cycle, but other multiple functions in a cell, making it a ubiquitous enzyme (Pancholi, 2001; Kim and Dang, 2005). In vertebrates, enolase exists in three isotypes which can form homodimers or heterodimers: α-enolase is expressed in majority of tissues; β-enolase is muscle specific and γ-enolase can be found in neuronal and neuroendocrine tissues (Wegner *et al.*, 2010). The roles of α-enolase depend on its localization in the cell. For example, if expressed on cell surface, it can participate in inflammatory cell recruitment (Wygrecka *et al.*, 2009). Previously, α-enolase has been proposed as an antigen for AEA in endometriosis (Walter *et al.*, 1995) and for AOA in POF and unexplained infertility, referring to possible defective immunoregulation in the etiology of these diseases (Sundblad *et al.*, 2006; Edassery *et al.*, 2010). However, antibodies to α-enolase are not a disease specific marker, since they are also common to systemic autoimmune diseases as well as many inflammatory and degenerative diseases, such as rheumatoid arthritis, systemic lupus erythematosus and autoimmune polyglandular syndrome type 1 (Peterson *et al.*, 1996; Pancholi, 2001; Shih *et al.*, 2010; Wegner *et al.*, 2010). Regardless, levels of α-enolase antibodies can be used as prognostic markers for disease severity and prognosis (Shih *et al.*, 2010).

Polyclonal B-cell activation along with autoantibody production is only one of the hallmarks for RIFS, additionally including alterations in the lymphocyte population and cytokine levels (Gleicher, 2002). Moreover, peripheral autoantibodies may not always correspond to the local situation in the female reproductive tract (Geva *et al.*, 1997). Therefore, measuring cytokines that are produced and act locally, would give us a more relevant picture of immune system status in reproductive tissues at certain time points (Revelli *et al.*, 2009).

6.2. Proinflammatory cytokines and other immunologic markers in the follicular fluid of IVF patients

Follicular fluid is a metabolically active environment and contains various hormonal constituents such as hormones, growth factors and cytokines but also granulosa cells and leukocytes that are all important in oocyte maturation, subsequent fertilization and embryo development (Agarwal *et al.*, 2003). In a previous study we showed the expression of several immune response related genes in granulosa cells during COS in IVF treatment (Kõks *et al.*, 2010). In the current study, out of the 16 immunologic markers measured from the follicular

fluid, ten were associated with infertility etiology or IVF treatment outcome (Figure 5 and 6). A limitation of this part of our approach is that the follicular fluid samples were not opposed to the course of each oocyte following the fertilization for statistical analysis and the following results should be considered in the light of this.

We detected significantly lower concentrations of follicular IL-1β in women with TFI. This result was rather surprising since IL-1β is a local inflammatory factor upregulated in microbial infection, for instance (Maisey *et al.*, 2003). Furthermore, IL-1β is one of the main cytokine mediating follicular ovulation (Vassiliadis *et al.*, 2005). Our finding could therefore imply that the function of IL-1β in the fallopian tubes and ovaries is different and impaired folliculogenesis might contribute to the infertility in these patients. Malfunctional folliculogenesis in case of TFI is further supported by lower levels of IFN-α. The positive role for IFN-α in folliculogenesis has been suggested previously by others (Zidovec Lepej *et al.*, 2003; Lee *et al.*, 2009). We succeeded to confirm this positive function, by demonstrating the association between higher follicular IFN-α levels and follicular diameter.

Systemic inflammation is common to women with PCOS (Fauser *et al.*, 2012). According to this, a rise in the levels of proinflammatory mediators in follicular fluid can be expected. We found elevated levels of MIP-1α in follicular fluid of PCOS patients, which is implicated in inflammatory host responses (Maurer and von Stebut, 2004). In addition, granulosa cells of PCOS patients contain less apoptotic effectors and more cell survival factors than granulosa cells of healthy women (Onalan *et al.*, 2005; Das *et al.*, 2008). Respective imbalance could contribute to folliculogenesis defects commonly seen in women with PCOS (Fauser *et al.*, 2012). Our findings of higher concentrations of CD44(v6) in PCOS patients indirectly support this hypothesis, since CD44(v6) has been linked with apoptosis resistance (Yu *et al.*, 2010; Jung *et al.*, 2011). Correspondingly, lower levels of CD44(v6) detected in unexplained infertility patients could point to a higher ovarian follicular apoptosis rate in these women. This is in accordance with a previous report where granulosa cell apoptosis was significantly higher in unexplained infertility patients compared to TFI (İdil *et al.*, 2004). However, since there are no previous reports on CD44(v6) in female reproduction, the exact role of CD44(v6) in folliculogenesis should be further evaluated. Interestingly, we found that the status of active smoking was positively associated with elevated concentrations of follicular CD44(v6) as well as sAPO-1/Fas, another apoptosis inhibitor (Sarandakou *et al.*, 2003). Both of these associations are rather challenging to investigate, since smoking has previously been associated with excessive oxidative stress and increased granulosa cell apoptosis (Paszkowski *et al.*, 2002; Bordel *et al.*, 2006). Elevated levels of follicular CD44(v6) and sAPO-1/Fas may therefore reflect the human body's effort to compensate for apoptosis induction caused by smoking.

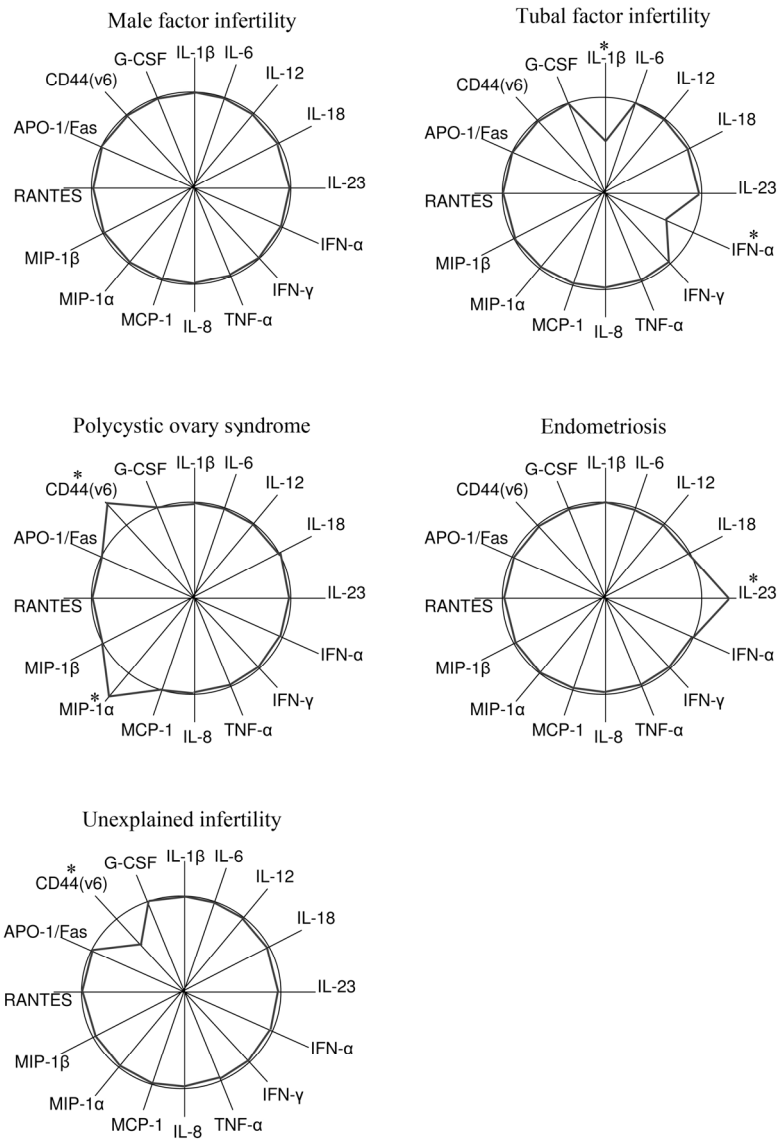

Figure 5. Follicular fluid proinflammatory biomarker profiles of women with different infertility etiologies. Data is presented as the proportion of the mean level of biomarkers in follicular fluid of male factor infertility, used as a reference. * Biomarkers that were significantly decreased or increased in the follicular fluid, when compared to the reference group ($p < 0.05$).

Higher follicular levels of IL-23 were characteristic to women with endometriosis and were additionally associated with smoking and secondary infertility. To date, only few studies have detected IL-23 or IL-12/IL-23 common

subunit p40 from the follicular fluid (Vujisic *et al.*, 2006). However, elevated levels of inflammatory cytokines in the follicular fluid of endometriosis patients are a common finding (Pellicer *et al.*, 1998; Carlberg *et al.*, 2000). Perhaps the inflammatory compliance in case of endometriosis, as concluded herein repeatedly, may attribute to poor oocyte quality and subsequent IVF failure (Vujisic *et al.*, 2006; Xu *et al.*, 2006). Besides, IL-23 is an important mediator in chronic inflammatory autoimmune diseases, indicating also to similar pathogenesis between this and endometriosis (Burney and Giudice, 2012).

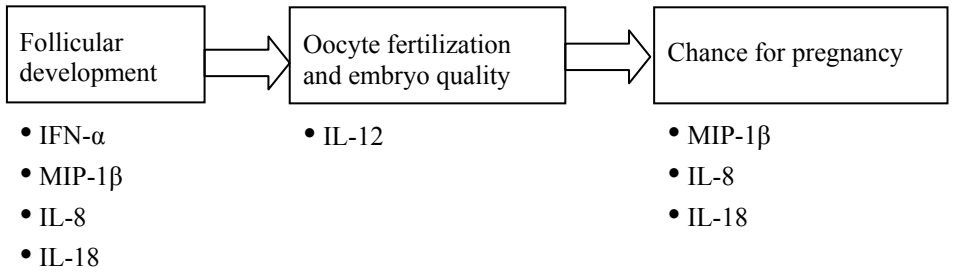

Figure 6. Schematic illustration of studied follicular fluid proinflammatory cytokines and chemokines that were positively associated with folliculogenesis and pregnancy achievement.

Previously, some researches demonstrated a negative association between follicular IL-12 and folliculogenesis, fertilization and pregnancy outcome (Gazvani *et al.*, 2000; Bedaiwy *et al.*, 2007; Lédée *et al.*, 2008), while others revealed no association at all (Gallinelli *et al.*, 2003). On the contrary, our results suggest an entirely positive correlation between follicular IL-12 level and the number of fertilized oocytes and embryo quality. Discrepancies between the results might be explained by differences in medical conditions recruited under the investigation or the way follicular fluid was handled, either pooled or studied separately. Also, different methods used for cytokine detection vary in their sensitivities and detection ranges. In fact, the mean ± SD levels of IL-12 detected by us (0.4 ± 2.8 pg/ml) were much lower than the ones reported previously: Bedaiwy *et al.* (24.45 ± 20.15 pg/ml in non-pregnant cycles versus 3.38 ± 10.6 pg/ml in pregnant cycles) and Ledee *et al*. (15.3 ± 6.2 pg/ml) (Bedaiwy *et al.*, 2007; Lédée *et al.*, 2008). These two studies suggested a deleterious function for IL-12 on female reproduction. Coming from that, we propose a dose-dependent role for IL-12 in folliculogenesis with a positive effect in lower concentrations and an adverse effect in higher levels. However, further studies are required to clarify this course.

Two of the chemokines analyzed, MIP-1β and IL-8, showed positive associations with follicular growth and chance for pregnancy. The role of MIP-1β

has not been profoundly studied in female reproduction before. Ostanin and colleagues detected lower MIP-1β levels in the follicular fluid of women with poor quality oocytes and failed IVF cycles. They therefore suggested a positive role for MIP-1β in folliculogenesis and pregnancy establishment (Ostanin *et al.*, 2007). We measured higher follicular MIP-1β levels in women who achieved intrauterine pregnancy and found a positive correlation with the size of the follicle, supporting further Ostanin's conclusions. In correspondence with previous results (Runesson *et al.*, 2000; Fujii *et al.*, 2003; Yoshino *et al.*, 2003; Malizia *et al.*, 2010), levels of IL-8 were positively associated with the follicular diameter. Yoshino and colleagues explained the link between the size of the follicle and IL-8 production by presenting a connection between the growing follicular diameter and increasing hypoxia in follicles, which in turn stimulates IL-8 secretion from granulosa cells (Yoshino *et al.*, 2003). Additionally, in agreement with previous studies (Büscher *et al.*, 1999; Gazvani *et al.*, 2000; Yoshino *et al.*, 2003), IL-8 was detected in all our patients. Since IL-8 can also be detected in the follicular fluid of unstimulated cycles (Runesson *et al.*, 2000), a role for IL-8 in normal follicular physiology has been proposed (Fujii *et al.*, 2003). Moreover, a potential use for follicular fluid IL-8 as an indicator of successful folliculogenesis and oocyte maturity has been suggested (Runesson *et al.*, 1996; Fujii *et al.*, 2003; Malizia *et al.*, 2010). However, our findings that the concentration of IL-8 in follicular fluid was positively associated with intrauterine pregnancy, parity and serum progesterone after COS, are different from previously published data, which have shown no correlations between these parameters (Runesson *et al.*, 1996; Büscher *et al.*, 1999; Gazvani *et al.*, 2000; Yoshino *et al.*, 2003). Similar to IL-12, the contradictory results of IL-8 in our study and in literature may come from different study design or higher levels of IL-8 operated in our study (Runesson *et al.*, 2000; Fujii *et al.*, 2003; Yoshino *et al.*, 2003; Malizia *et al.*, 2010) Interestingly, Morelli and colleagues found that lower serum levels of IL-8 were indicative of extrauterine pregnancy (Morelli *et al.*, 2008). The pregnancy favoring effect in serum and follicular fluid might be supported by growth-promoting and angiogenic role of IL-8 (Połeć *et al.*, 2011). Also, our results suggest an association between the levels of follicular IL-8 and serum progesterone with respect to other researchers, who demonstrated the role of IL-8 in formation of *corpus luteum* (Połeć *et al.*, 2011) and progesterone synthesis in the bovine (Shimizu *et al.*, 2012).

Regardless of the pleiotropic nature of IL-18, only a few studies have analyzed IL-18 in the follicular fluid (Nakanishi *et al.*, 2001). Based on a study on mice, Tsuji and colleagues noted a promoting role for IL-18 in normal ovarian functions, such as follicular development, ovulation and luteinization (Tsuji *et al.*, 2001). This was further supported by Gutman and colleagues, who detected a positive correlation between follicular fluid IL-18 concentrations and the number of retrieved oocytes after IVF procedure. They suggested a regulatory role of IL-18 in folliculogenesis (Gutman *et al.*, 2004). Our finding of a positive association between follicular IL-18 and the diameter of the follicle adds further support to this. In addition, higher levels of follicular IL-18

correlated positively with parity in our study. Although, Kilic and colleagues detected no connection between follicular levels of IL-18 and pregnancy establishment after IVF in women with unexplained infertility (Kilic *et al.*, 2009), Vujisic and colleagues suggested this association in unstimulated cycles (Vujisic *et al.*, 2006). To sum it up, the impact of follicular IL-18 in predicting IVF treatment outcome should be further studied.

The TREM-1 was discovered in year 2000 as a transmembrane Ig super-family receptor expressed on neutrophils and monocytes/macrophages (Bouchon *et al.*, 2000). The results of the current study showed that sTREM-1 was detectable in all follicular fluid samples, irrespective of the cause of infertility. This finding may indicate that TREM-1 is a physiologic constituent of the follicular fluid. Moreover, we detected a positive correlation between follicular fluid and serum sTREM-1 concentrations, with significantly higher levels in follicular fluid compared to serum. Also, our previous work showed TREM-1 expression in floating granulosa cells in infertile women undergoing IVF (Kõks *et al.*, 2010). Together, these results refer to local production of TREM-1 in the follicles (Salmassi *et al.*, 2004). Since ovulation can be regarded as an inflammatory process, upregulation of proinflammatory cytokines and chemokines might be needed for normal folliculogenesis, ovulation and luteolysis. Activation of TREM-1 via an immunoreceptor tyrosine-based activation motif (ITAM)-containing adaptor molecule increases the production on multiple proinflammatory cytokines and chemokines, such as TNF-α, IL-6, IL-1β, IL-8, MCP-1 and MIP-1α (Bouchon *et al.*, 2000; Derive *et al.*, 2010). The soluble form of TREM-1, on the other hand, is thought of as a decoy receptor capable of down-regulating the TREM-1 pathway and thereby decreasing the proinflammatory effect (Gibot *et al.*, 2004a; Sharif and Knapp, 2008). The reciprocal association detected in the current study between higher levels of serum sTREM-1 and lower rate of embryo quality among fertilized oocytes may therefore reflect the down-regulation of proinflammatory cytokines important in folliculogenesis.

Although TREM-1 has been shown to be upregulated during bacterial infections and sepsis (Gibot *et al.*, 2004a; Gibot *et al.*, 2004b), recent data show that TREM-1 levels can also be upregulated during non-infectious tissue damage, such as acute pancreatitis, chronic inflammation in rheumatoid arthritis or traumatic lung contusion (Cavaillon, 2009; Ferat-Osorio *et al.*, 2009; Kuai *et al.*, 2009; Murakami *et al.*, 2009; Bingold *et al.*, 2011). These reports indicate that TREM-1 activation is not only initiated by pathogen-associated molecular patterns and Toll-like receptor activation, but can additionally be triggered by damage or danger associated molecular patterns via a Toll-like receptor independent way (Cavaillon, 2009; Bingold *et al.*, 2011). Non-infectious chronic inflammation is characteristic to endometriosis (Burney and Giudice, 2012). It was therefore surprising that endometriosis patients showed in our study significantly decreased sTREM-1 levels, both in follicular fluid and serum samples. This finding is not easily explained. However, when taken into consideration that the cell surface TREM-1 promotes inflammatory responses, but sTREM-1 contrarily possesses anti-inflammatory properties, our result may again point

towards a tendency for inflammatory milieu in the ovaries of endometriosis patients (Gibot *et al.*, 2004a).

In conclusion, infertile women with different etiologies who were included in the present study presented with elevated levels of autoantibody reactivity towards several organ-specific and organ-non-specific autoantigens. This adds further proof that impairment of the immune system is characteristic to infertile women undergoing IVF treatment. Although some of the antibodies detected were related to cause of infertility or IVF treatment outcome, their pathogenetic role still needs to be clarified, as only few of the related antigens have been identified so far. We managed to identify α-enolase as an antigen for AEA. However, since α-enolase antibodies are commonly found in other systemic autoimmune and inflammatory diseases, they more likely serve as indicators for a defective immunoregulation in case of infertility than markers for a specific disease underlying infertility. We additionally found either reduced or elevated levels of certain proinflammatory cytokines, chemokines and apoptosis regulators in the follicular fluid of IVF patients. Imbalance in the levels of immune system related mediators of folliculogenesis refer to ongoing impaired inflammatory reactions that can negatively affect folliculogenesis and subsequent IVF treatment outcome in these patients. On the other hand, some of the cytokines correlated positively with oocyte fertilization, embryo quality and pregnancy outcome after IVF treatment, implying thereby to their importance in normal ovarian function.

7. CONCLUSIONS

1. Autoimmune activation, defined by the presence of organ-specific or -non-specific autoantibodies in serum, was characteristic for women with infertility of different forms, with significantly higher number of common autoantibodies in endometriosis and polycystic ovary syndrome (PCOS) patients.

2. Anti-endometrial antibodies (AEA) were detected against a wide range of eutopic endometrial antigens in the sera of patients with tubal factor infertility (TFI) or endometriosis. The prevalence of certain IgA and IgG type AEA reactions varied between the study groups and had a predictive value for *in vitro* fertilization (IVF) treatment outcome. We identified α-enolase as one of the target antigens for AEA. The levels of α-enolase antibodies were more prevalent among TFI patients and were associated with pregnancy failure after IVF.

3. Different forms of infertility were associated with distinct intrafollicular pro-inflammatory cytokine profiles. Follicular fluid levels of some of the cytokines evaluated, such as IL-12, had positive correlations with oocyte fertilization and embryo quality, while the levels of others, including MIP-1β, IL-8 and IL-18, were related to successful pregnancy following IVF treatment.

4. Reportedly, this study was the first to describe soluble triggering receptor expressed on myeloid cells (sTREM-1) in follicular fluid. Soluble TREM-1 was detectable in all follicular fluid samples, irrespective of the infertility etiology. Endometriosis patients had significantly decreased sTREM-1 levels in their follicular fluid and serum samples. Additionally, serum sTREM-1 concentration was inversely associated with embryo quality.

This study provided further evidence on impairments of the immune system in infertile women undergoing IVF treatment and that immune system alterations may be involved in the infertility pathogenesis of these patients. Associations of certain AEA with lower chances for pregnancy could refer to antibody contribution to pregnancy failure in IVF patients. Changes in follicular fluid levels of main cytokines regulating folliculogenesis may imply to ongoing impaired inflammatory reactions that negatively affect folliculogenesis and subsequent IVF treatment outcome in these patients. Collectively, the knowledge about presence and spectra of autoantibodies and intrafollicular biomarkers in IVF patients has the potential for application in pre-IVF treatments to raise the chances of pregnancy and should be evaluated as such by future prospective studies.

8. FUTURE PROSPECTS

The present thesis, showing an immune activation in infertile women undergoing IVF, gives further information about impairments in immune system regulation. Resulting from the present study, it would be worthy to assess autoantibodies, as well as cytokines, from endometrial flushing. This would give a more relevant overview about endometrial condition before IVF embryo transfer, since autoantibody detection from serum may not always correspond to the local situation. It would also offer a new perspective on biomarker detection and non-invasive prognostic method for implantation failures. Also, our today´s results lead us to carry out *in situ* hybridization on human ovaries in the future. We will aim to confirm the production and secretion of cytokines measured in the present thesis from follicular granulosa cells.

SUMMARY IN ESTONIAN

Naise viljatusega kaasnev immuunsüsteemi aktivatsioon: autoantikehade ja põletikuliste mediaatorite tähendus

Infertiilsuse ehk viljatuse all mõistetakse olukorda, kus naisel ei ole õnnestunud rasestuda vähemalt 12 kuud kestnud regulaarse suguelu jooksul, rasedusest hoidumata. Lastetusega puutub kokku kuni 9% reproduktiivses eas paaridest. Viljatus võib olla põhjustatud nii naise- kui mehepoolsetest faktoritest, kuid ligi 15% juhtudest jääb põhjus selgusetuks. Naisepoolset infertiilsust võivad põhjustada hormonaalsetest häiretest tingitud anovulatoorsed menstruaaltsüklid, munajuhade ja emaka patoloogiad ning autoimmunoloogilised probleemid. Mehepoolne viljatus võib tuleneda hormonaalsetest või meessuguorganite arenguhäiretest ning autoimmunoloogilistest probleemidest, mis põhjustavad muutusi seemnerakkude arvus, liikuvuses ja morfoloogias.

Lähtuvalt viljatuse põhjusest kasutatakse erinevaid ravimeetodeid. Kõige efektiivsemaks neist on osutunud kehavälise viljastamise protseduur (IVF). Protseduuri esimeses etapis manustatakse naisele mõne päeva vältel folliikuleid stimuleerivat hormooni (FSH), et indutseerida munasarjades polüfollikulogenees, kus ühe folliikli asemel küpseb korraga enamasti 15–20 folliiklit. Küpsed munarakud kogutakse transvaginaalselt ja viljastatakse seemnerakkudega. Emakasse siiratakse 1–2 kõige kvaliteetsemat embrüot 2.–3. päeval pärast viljastamist. Ühe IVF ravitsükli kohta on keskmine rasestumistõenäosus 30%, kuigi munarakkude viljastumise protsent võib küündida 80-ni.

IVF ravi tulemuslikkus on otseselt sõltuv follikulogeneesi stimulatsiooni tulemuslikkusest, mille hindamiseks kasutatakse erinevaid parameetreid ja hindamiskriteeriume. Üheks võimaluseks on follikulaarvedeliku koostise määramine, kuna selles sisalduvad komponendid annavad relevantse ülevaate küpsevat munarakku ümbritsevast mikrokeskkonnast ning seeläbi ootsüüdi kvaliteedist. Lisaks on follikulaarvedelik kergesti kogutav munaraku aspiratsioonil. Ehkki siinkohal on publitseeritud mitmeid uurimistöid, jätkuvad endiselt uuringud efektiivsemate ja optimaalsemate biomarkerite leidmiseks, et nende põhjal paremini ennustada IVF ravi edukust. IVF ravi ebaõnnestumist on seostatud mitmesuguste organ-spetsiifiliste või -spetsiifikata autoantikehade esinemisega viljatute naiste vereseerumis. Need autoantikehad võivad mõjutada follikulogeneesi ja munarakkude küpsemist munasarjades, takistada viljastumist munajuhades või embrüo implantatsiooni emakas. Vastavate märklaudvalkude vastu suunatud immuunreaktsioonidest on praeguseks hetkeks kirjeldatud vaid mõned. Mitmed relevantsed autoantikehareaktsioonid on aga siiani täpsemalt identifitseerimata.

Uurimistöö eesmärgid

Käesoleva doktoritöö üldeesmärgiks oli määrata viljatute naiste vereseerumis ja follikulaarvedelikus humoraalse immuunsüsteemi aktivatsiooni markereid ning

hinnata nende tähenduslikkust viljatusravi tulemusele. Selleks seadsime järgmised alaeesmärgid:

1. Määrata organ-spetsiifiliste ja -spetsiifikata autoantikehade esinemist erineva viljatuse põhjusega naiste vereseerumis.
2. Määrata endometrioosi ja tubaarse viljatusega patsientide vereseerumist endomeetriumi vastaseid antikehi (AEA) ning hinnata nende autoantikehade esinemise seoseid IVF ravitulemustega. Lisaks identifitseerida olulisemad antigeenid.
3. Määrata proinflammatoorseid tsütokiine ja teisi biomarkereid viljatute naiste follikulaarvedelikus ning analüüsida saadud tulemusi vastavalt viljatuse põhjustele ja IVF ravitulemustele.
4. Mõõta viljatute naispatsientide vereseerumist ja follikulaarvedelikust müeloidsetel rakkudel ekspresseeruva aktivatsiooniretseptori lahustunud vormi (sTREM)-1 taset. Hinnata leitud tasemete seost viljatuse põhjuse ja IVF ravitulemustega.

Materjal ja meetodid

Uuringu esimeses osas moodustasid uuritavate grupi 129 IVF patsienti, kellel oli diagnoositud mehepoolne või tubaarne infertiilsus, endometrioos, polütsüstiliste ovaariumide sündroom (PCOS) või seletamatu viljatus. Patsientide vereseerumist määrati kaudsel immunofluorestsentsmeetodil (IIF) tuuma- (ANA), silelihaskoe- (SMA), parietaalrakkude- (PCA), kilpnäärme mikrosoomide- (TMA) ja mitokondrite (AMA) vastased autoantikehad ning ensüümikaudsel immunosorptsioonmeetodil (ELISA) kardiolipiini- (ACA) ja tema kofaktori β2-glükoproteiin I (β2-GPI) vastased autoantikehad.

Teises uuringuetapis olid uuritavateks 190 endometrioosi ja tubaarse infertiilsusega (TFI) naist, kelle vereseerumist detekteeriti immunoblotmeetodil IgG ja IgA tüüpi AEA. Antigeenide täpsemaks identifitseerimiseks kasutati kahe-dimensionaalset immunoblotanalüüsi koos tandem mass-spektromeetriaga.

Uuringu kolmandas osas oli uuritavaid kokku 153 IVF patsienti, kellel esines mehepoolne või tubaarne infertiilsus, endometrioos, PCOS, seletamatu viljatus või muud põhjused. Uuringumaterjaliks oli folliiklite punktsioonil saadud follikulaarvedelik. Patsiendi follikulaarvedelikust määrati voolutsütomeetri abil multiplex meetodil 16 erinevat biomarkerit: interleukiin (IL)-23, apoptoosi antigeen (APO-1)/Fas, makrofaagide põletikuvalk (MIP)-1α, MIP-1β, diferentseerumise klaster (CD)44 isovorm 6, IL-8, granulotsüütide kolooniaid stimuleeriv faktor (G-CSF), RANTES (*regulated on activation normal T-cell expressed and secreted*), IL-12 subühik 70, interferoon (IFN)-γ, monotsüütide kemotaktiline valk (MCP)-1, IL-6, IFN-α, IL-18, IL-1β ja tuumorinekroosifaktor (TNF)-α.

Neljandas uuringuetapis olid uuritavateks 110 IVF patsienti, kellel diagnoositi mehepoolne või tubaarne infertiilsus, endometrioos, PCOS või seleta-

matu viljatus. Uuringumaterjaliks olid patsiendi follikulaarvedelik ja vereseerum, millest määrati ELISA meetodil sTREM-1 taset.

Tulemused

Võrreldes TFI grupiga esines endometrioosi ja PCOS patsientide seas sagedamini vähemalt üks uuritud autoantikehadest. SMA esinemine oli iseloomulik endometrioosi ja mehepoolse infertiilsusega naistele ning korreleerus positiivselt eelnevate IVF protseduuride arvuga. Seletamatu viljatusega patsientidele oli iseloomulik ANA esinemine.

Endometrioosi ja tubaarse infertiilsusega patsientidel esines nii IgA kui IgG tüüpi AEA. IgA tüüpi AEA reaktsioone leiti sagedamini tubaarse viljatusega naiste vereseerumis ning teatud IgA ja IgG tüüpi antikeha reaktsioonide esinemine assotsieerus halvemate IVF ravitulemustega. Kasutades kahedimensionaalset immunoblotmeetodit koos mass-spektromeetriaga õnnestus identifitseerida ühe olulise antigeenina α-enolaasi isovorm (ENO1) molaarmassi piirkonnas 47 kDa. ENO1 vastaseid autoantikehi esines sagedamini tubaarse infertiilsusega patsientidel ja ebaõnnestunud IVF ravi korral.

Follikulaarvedelikust detekteeritud biomarkerite profiilid erinesid patsientide hulgas haiguspõhiselt. Tubaarse infertiilsusega patsientidel leiti oluliselt vähem tsütokiine IL-1β ja IFN-α. Endometrioosihaigetel oli follikulaarvedelikus tõusnud IL-23, PCOS patsientidel aga CD44(v6) ja MIP-1α tasemed. Seletamatu viljatusega naistel leiti oluliselt vähem CD44(v6). Peale selle leiti biomarkereid, mille esinemine oli seotud viljatusravi erinevate parameetritega. Näiteks oli IL-12 kontsentratsioon positiivses seoses viljastatud munarakkude arvu ja embrüo kvaliteediga. Folliikli diameetriga assotsieerusid IFN-α, MIP-1β, IL-8 ja IL-18 tasemed. Samuti leiti, et kõrgem MIP-1β, IL-8 ja IL-18 kontsentratsioon oli korrelatsioonis viljatusravi järgsete paremate rasedustulemustega.

Lisaks seerumile oli sTREM-1 detekteeritav follikulaarvedelikus. Seerumi ja follikulaarvedeliku sTREM-1 tasemed olid omavahelises positiivses korrelatsioonis ning võrreldes seerumiga oli follikulaarvedeliku sTREM-1 kontsentratsioon oluliselt kõrgem. Endometrioosihaigetel oli võrreldes mehepoolse viljatusega naistega sTREM-1 tase madalam nii follikulaarvedelikus kui seerumis. Lisaks leidsime pöördvõrdelise seose seerumi sTREM-1 kontsentratsiooni ja embrüo kvaliteedi vahel.

Järeldused

Käesoleva väitekirja tulemuste põhjal võib järeldada, et erineva põhjusega viljatutele naistele, kes läbivad IVF protseduuri, on iseloomulik üldine immuunsüsteemi aktivatsioon, millele viitab nii üldiste organspetsiifikata autoantikehade kui ka spetsiifiliselt reproduktiivorganite vastu suunatud autoantikehade esinemine vereseerumis. Seosed antikehade esinemise ja IVF ravi tulemuste vahel võivad osutuda tõhusateks markeriteks IVF ravi edukuse hindamisel. Follikulogeneesis osa-

levate põletikuliste tsütokiinide kontsentratsioonide erinevused võivad viidata follikulogeneesi häiretele, mille aluseks võivad omakorda olla muutused immuunsüsteemi põletikuvastastes reaktsioonides. Siinkohal uuritud follikulaarsed biomarkerid võivad leida kasutamist prognostiliste markeritena IVF ravi edukuse prognoosimisel. Nii seerumi autoantikehade kui follikulaarvedeliku biomarkerite patogeneetiline toime tuleks aga kinnitada vastavate prospektiivsete uuringutega.

REFERENCES

Agarwal A, Saleh RA and Bedaiwy MA. (2003) Role of reactive oxygen species in the pathophysiology of human reproduction. *Fertil Steril* 79: 829–843.

Altmäe S, Haller K, Peters M, Saare M, Hovatta O, Stavreus-Evers A, Velthut A, Karro H, Metspalu A and Salumets A. (2009) Aromatase gene (cyp19a1) variants, female infertility and ovarian stimulation outcome: A preliminary report. *Reprod Biomed Online* 18: 651–657.

Altmäe S, Hovatta O, Stavreus-Evers A and Salumets A. (2011) Genetic predictors of controlled ovarian hyperstimulation: Where do we stand today? *Hum Reprod Update* 17: 813–828.

Alviggi C, Humaidan P and Ezcurra D. (2012) Hormonal, functional and genetic biomarkers in controlled ovarian stimulation: Tools for matching patients and protocols. *Reprod Biol Endocrinol* 10: 9.

American Society for Reproductive Medicine. (1997) Revised American Society for Reproductive Medicine classification of endometriosis: 1996. *Fertil Steril* 67: 817–821.

Ansar Ahmed S, Penhale WJ and Talal N. (1985) Sex hormones, immune responses, and autoimmune diseases. Mechanisms of sex hormone action. *Am J Pathol* 121: 531–551.

Arici A, Oral E, Bukulmez O, Buradagunta S, Bahtiyar O and Jones EE. (1997) Monocyte chemotactic protein-1 expression in human preovulatory follicles and ovarian cells. *J Reprod Immunol* 32: 201–219.

Arraztoa JA, Rocha A, Varela-Nallar L, Velasquez L, Toro V, Cardenas H and Imarai M. (2002) IgA in the lumen of the human oviduct is not related to the menstrual cycle but increases during local inflammation. *Fertil Steril* 77: 633–634.

Aust G, Simchen C, Heider U, Hmeidan FA, Blumenauer V and Spanel-Borowski K. (2000) Eosinophils in the human corpus luteum: The role of RANTES and eotaxin in eosinophil attraction into periovulatory structures. *Mol Hum Reprod* 6: 1085–1091.

Baerwald AR, Adams GP and Pierson RA. (2012) Ovarian antral folliculogenesis during the human menstrual cycle: A review. *Hum Reprod Update* 18: 73–91.

Barreda DR, Hanington PC and Belosevic M. (2004) Regulation of myeloid development and function by colony stimulating factors. *Dev Comp Immunol* 28: 509–554.

Beagley KW and Gockel CM. (2003) Regulation of innate and adaptive immunity by the female sex hormones oestradiol and progesterone. *FEMS Immunol Med Microbiol* 38: 13–22.

Bedaiwy M, Shahin AY, AbulHassan AM, Goldberg JM, Sharma RK, Agarwal A and Falcone T. (2007) Differential expression of follicular fluid cytokines: Relationship to subsequent pregnancy in IVF cycles. *Reprod Biomed Online* 15: 321–325.

Bingold TM, Pullmann B, Sartorius S, Geiger EV, Marzi I, Zacharowski K, Wissing H and Scheller B. (2011) Soluble triggering receptor on myeloid cells-1 is expressed in the course of non-infectious inflammation after traumatic lung contusion: A prospective cohort study. *Crit Care* 15: R115.

Birdsall MA, Lockwood GM, Ledger WL, Johnson PM and Chamley LW. (1996) Antiphospholipid antibodies in women having in-vitro fertilization. *Hum Reprod* 11: 1185–1189.

Boivin J, Bunting L, Collins JA, Nygren KG. (2007) International estimates of infertility prevalence and treatment-seeking: potential need and demand for infertility medical care. *Hum Reprod* 22: 1506–1512.

Bontis JN and Theodoridis TD. (2006) Laparoscopic management of hydrosalpinx. *Ann N Y Acad Sci* 1092: 199–210.
Bordel R, Laschke MW, Menger MD and Vollmar B. (2006) Nicotine does not affect vascularization but inhibits growth of freely transplanted ovarian follicles by inducing granulosa cell apoptosis. *Hum Reprod* 21: 610–617.
Bouchon A, Dietrich J and Colonna M. (2000) Cutting edge: Inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. *J Immunol* 164: 4991–4995.
Buckingham KL and Chamley LW. (2009) A critical assessment of the role of antiphospholipid antibodies in infertility. *J Reprod Immunol* 80: 132–145.
Burney RO and Giudice LC. (2012) Pathogenesis and pathophysiology of endometriosis. *Fertil Steril* 98: 511–519.
Bussen S, Steck T and Dietl J. (2000) Increased prevalence of thyroid antibodies in euthyroid women with a history of recurrent in-vitro fertilization failure. *Hum Reprod* 15: 545–548.
Büscher U, Chen FC, Kentenich H and Schmiady H. (1999) Cytokines in the follicular fluid of stimulated and non-stimulated human ovaries; is ovulation a suppressed inflammatory reaction? *Hum Reprod* 14: 162–166.
Camus E, Poncelet C, Goffinet F, Wainer B, Merlet F, Nisand I and Philippe HJ. (1999) Pregnancy rates after in-vitro fertilization in cases of tubal infertility with and without hydrosalpinx: A meta-analysis of published comparative studies. *Hum Reprod* 14: 1243–1249.
Carlberg M, Nejaty J, Froysa B, Guan Y, Soder O and Bergqvist A. (2000) Elevated expression of tumour necrosis factor alpha in cultured granulosa cells from women with endometriosis. *Hum Reprod* 15: 1250–1255.
Carp HJ, Selmi C and Shoenfeld Y. (2012) The autoimmune bases of infertility and pregnancy loss. *J Autoimmun* 38: J266–274.
Cavaillon JM. (2009) Monocyte TREM-1 membrane expression in non-infectious inflammation. *Crit Care* 13: 152.
Cerkiene Z, Eidukaite A and Usoniene A. (2008) Follicular fluid levels of interleukin-10 and interferon-gamma do not predict outcome of assisted reproductive technologies. *Am J Reprod Immunol* 59: 118–126.
Chamley LW and Clarke GN. (2007) Antisperm antibodies and conception. *Semin Immunopathol* 29: 169–184.
Choudhury SR and Knapp LA. (2001) Human reproductive failure I: Immunological factors. *Hum Reprod Update* 7: 113–134.
Cooper TG, Noonan E, von Eckardstein S, Auger J, Baker HW, Behre HM, Haugen TB, Kruger T, Wang C, Mbizvo MT and Vogelsong KM. (2010) World Health Organization reference values for human semen characteristics. *Hum Reprod Update* 16: 231–245.
Copperman AB, Wells V, Luna M, Kalir T, Sandler B and Mukherjee T. (2006) Presence of hydrosalpinx correlated to endometrial inflammatory response in vivo. *Fertil Steril* 86: 972–976.
Coulam CB and Acacio B. (2012) Does immunotherapy for treatment of reproductive failure enhance live births? *Am J Reprod Immunol* 67: 296–304.
Cutolo M, Sulli A, Capellino S, Villaggio B, Montagna P, Seriolo B and Straub RH. (2004) Sex hormones influence on the immune system: Basic and clinical aspects in autoimmunity. *Lupus* 13: 635–638.

Dahm-Kähler P, Runesson E, Lind AK and Brännström M. (2006) Monocyte chemotactic protein-1 in the follicle of the menstrual and IVF cycle. *Mol Hum Reprod* 12: 1–6.

Damsker JM, Hansen AM and Caspi RR. (2010) Th1 and Th17 cells: Adversaries and collaborators. *Ann N Y Acad Sci* 1183: 211–221.

Das M, Djahanbakhch O, Hacihanefioglu B, Saridogan E, Ikram M, Ghali L, Raveendran M and Storey A. (2008) Granulosa cell survival and proliferation are altered in polycystic ovary syndrome. *J Clin Endocrinol Metab* 93: 881–887.

De Carolis C, Perricone C and Perricone R. (2010) NK cells, autoantibodies, and immunologic infertility: A complex interplay. *Clin Rev Allergy Immunol* 39: 166–175.

De Ziegler D, Borghese B and Chapron C. (2010) Endometriosis and infertility: Pathophysiology and management. *Lancet* 376: 730–738.

Delgado Alves J, Radway-Bright EL, Lee S, Grima B, Hothersall J, Ravirajan CT and Isenberg DA. (2005) Antiphospholipid antibodies are induced by in vitro fertilization and correlate with paraoxonase activity and total antioxidant capacity of plasma in infertile women. *Lupus* 14: 373–380.

Derive M, Massin F and Gibot S. (2010) Triggering receptor expressed on myeloid cells-1 as a new therapeutic target during inflammatory diseases. *Self Nonself* 1: 225–230.

Di Simone N, Di Nicuolo F, D'Ippolito S, Castellani R, Tersigni C, Caruso A, Meroni P and Marana R. (2010) Antiphospholipid antibodies affect human endometrial angiogenesis. *Biol Reprod* 83: 212–219.

Dias JA, Jr., de Oliveira RM and Abrao MS. (2006) Antinuclear antibodies and endometriosis. *Int J Gynaecol Obstet* 93: 262–263.

Djahanbakhch O, Saridogan E, Ertan Kervancioglu M, Mahmood T, Li L and Grudzinskas JG. (1999) Secretory function of the fallopian tube epithelial cells in vitro: A review. *Placenta* 20, Supplement 1: 87–104.

Dmowski WP. (1995) Immunological aspects of endometriosis. *Int J Gynaecol Obstet* 50 Suppl 1: S3–10.

Edassery SL, Shatavi SV, Kunkel JP, Hauer C, Brucker C, Penumatsa K, Yu Y, Dias JA and Luborsky JL. (2010) Autoantigens in ovarian autoimmunity associated with unexplained infertility and premature ovarian failure. *Fertil Steril* 94: 2636–2641.

Edi-Osagie EC, Seif MW, Aplin JD, Jones CJ, Wilson G and Lieberman BA. (2004) Characterizing the endometrium in unexplained and tubal factor infertility: A multiparametric investigation. *Fertil Steril* 82: 1379–1389.

Enright BP, Davila DR, Tornesi BM, Blaich G, Hoberman AM and Gallenberg LA. (2011) Developmental and reproductive toxicology studies in IL-12p40 knockout mice. *Birth Defects Res B Dev Reprod Toxicol* 92: 102–110.

ESHRE. (2013) *European Society for Human Reproduction & Embryology: ART fact sheet*. Available at: http://www.eshre.eu/ESHRE/English/Guidelines-Legal/ART-fact-sheet/page.aspx/1061.

Fauser BC, Tarlatzis BC, Rebar RW, Legro RS, Balen AH, Lobo R, Carmina E, Chang J, Yildiz BO, Laven JS, Boivin J, Petraglia F, Wijeyeratne CN, Norman RJ, Dunaif A, Franks S, Wild RA, Dumesic D and Barnhart K. (2012) Consensus on women's health aspects of polycystic ovary syndrome (PCOS): The Amsterdam ESHRE/ASRM-sponsored 3rd PCOS Consensus Workshop Group. *Fertil Steril* 97: 28–38 e25.

Ferat-Osorio E, Wong-Baeza I, Esquivel-Callejas N, Figueroa-Figueroa S, Duarte-Rojo A, Guzman-Valdivia-Gomez G, Rodea-Rosas H, Torres-Gonzalez R, Sanchez-

Fernandez P, Arriaga-Pizano L, Lopez-Macias C, Robles-Diaz G and Isibasi A. (2009) Triggering receptor expressed on myeloid cells-1 expression on monocytes is associated with inflammation but not with infection in acute pancreatitis. *Crit Care* 13: R69.

Fernández-Shaw S, Hicks BR, Yudkin PL, Kennedy S, Barlow DH and Starkey PM. (1993) Anti-endometrial and anti-endothelial auto-antibodies in women with endometriosis. *Hum Reprod* 8: 310–315.

Ferraretti AP, Goossens V, de Mouzon J, Bhattacharya S, Castilla JA, Korsak V, Kupka M, Nygren KG, Nyboe Andersen A, European IVF-monitoring, Consortium for European Society of Human Reproduction and Embryology. (2012) Assisted reproductive technology in Europe, 2008: Results generated from European registers by ESHRE. *Hum Reprod* 27: 2571–2584.

Fisch B, Fried S, Manor Y, Ovadia J, Witz IP and Yron I. (1995) Increased antiphospholipid antibody activity in in-vitro fertilization patients is not treatment-dependent but rather an inherent characteristic of the infertile state. *Am J Reprod Immunol* 34: 370–374.

Forges T, Monnier-Barbarino P, Faure GC and Béné MC. (2004) Autoimmunity and antigenic targets in ovarian pathology. *Hum Reprod Update* 10: 163–175.

Forges T, Monnier-Barbarino P, Guillet-May F, Faure GC and Bene MC. (2006) Corticosteroids in patients with antiovarian antibodies undergoing in vitro fertilization: A prospective pilot study. *Eur J Clin Pharmacol* 62: 699–705.

Forti G and Krausz C. (1998) Clinical review 100: Evaluation and treatment of the infertile couple. *J Clin Endocrinol Metab* 83: 4177–4188.

Fried G and Wramsby H. (1998) Increase in transforming growth factor beta1 in ovarian follicular fluid following ovarian stimulation and in-vitro fertilization correlates to pregnancy. *Hum Reprod* 13: 656–659.

Fritsch H. (2008) Female genital system. *Color atlas of human anatomy. Internal organs*. 5 th ed. Germany, US: Thieme Medical Publishers, 267–292.

Fujii A, Harada T, Yamauchi N, Iwabe T, Nishi Y, Yanase T, Nawata H and Terakawa N. (2003) Interleukin-8 gene and protein expression are up-regulated by interleukin-1beta in normal human ovarian cells and a granulosa tumor cell line. *Fertil Steril* 79: 151–157.

Gajbhiye R, Suryawanshi A, Khan S, Meherji P, Warty N, Raut V, Chehna N and Khole V. (2008) Multiple endometrial antigens are targeted in autoimmune endometriosis. *Reprod Biomed Online* 16: 817–824.

Gallinelli A, Ciaccio I, Giannella L, Salvatori M, Marsella T and Volpe A. (2003) Correlations between concentrations of interleukin-12 and interleukin-13 and lymphocyte subsets in the follicular fluid of women with and without polycystic ovary syndrome. *Fertil Steril* 79: 1365–1372.

García-Velasco JA and Arici A. (1999) Chemokines and human reproduction. *Fertil Steril* 71: 983–993.

Garrido N, Navarro J, Remohí J, Simón C and Pellicer A. (2000) Follicular hormonal environment and embryo quality in women with endometriosis. *Hum Reprod Update* 6: 67–74.

Gazvani MR, Bates M, Vince G, Christmas S, Lewis-Jones DI and Kingsland C. (2000) Follicular fluid concentrations of interleukin-12 and interleukin-8 in IVF cycles. *Fertil Steril* 74: 953–958.

Gérard N, Caillaud M, Martoriati A, Goudet G and Lalmanach AC. (2004) The interleukin-1 system and female reproduction. *J Endocrinol* 180: 203–212.

Geva E, Amit A, Lerner-Geva L and Lessing JB. (1997) Autoimmunity and reproduction. *Fertil Steril* 67: 599–611.

Gibot S, Kolopp-Sarda MN, Bene MC, Bollaert PE, Lozniewski A, Mory F, Levy B and Faure GC. (2004a) A soluble form of the triggering receptor expressed on myeloid cells-1 modulates the inflammatory response in murine sepsis. *J Exp Med* 200: 1419–1426.

Gibot S, Kolopp-Sarda MN, Bene MC, Cravoisy A, Levy B, Faure GC and Bollaert PE. (2004b) Plasma level of a triggering receptor expressed on myeloid cells-1: Its diagnostic accuracy in patients with suspected sepsis. *Ann Intern Med* 141: 9–15.

Gilbert BR, Witkin SS and Goldstein M. (1989) Correlation of sperm-bound immunoglobulins with impaired semen analysis in infertile men with varicoceles. *Fertil Steril* 52: 469–473.

Gleicher N. (1998) Autoantibodies in infertility: Current opinion. *Hum Reprod Update* 4: 169–176.

Gleicher N. (2002) Some thoughts on the reproductive autoimmune failure syndrome (RAFS) and Th-1 versus Th-2 immune responses. *Am J Reprod Immunol* 48: 252–254.

Gleicher N and el-Roeiy A. (1988) The reproductive autoimmune failure syndrome. *Am J Obstet Gynecol* 159: 223–227.

Gleicher N, Vidali A and Karande V. (2002) The immunological "wars of the roses": Disagreements amongst reproductive immunologists. *Hum Reprod* 17: 539–542.

Gobert B, Barbarino-Monnier P, Guillet-May F, Bene MC and Faure GC. (1992) Anti-ovary antibodies after attempts at human in vitro fertilization induced by follicular puncture rather than hormonal stimulation. *J Reprod Fertil* 96: 213–218.

Gougeon A. (2010) Human ovarian follicular development: From activation of resting follicles to preovulatory maturation. *Ann Endocrinol (Paris)* 71: 132–143.

Gutman G, Soussan-Gutman L, Malcov M, Lessing JB, Amit A and Azem F. (2004) Interleukin-18 is high in the serum of IVF pregnancies with ovarian hyperstimulation syndrome. *Am J Reprod Immunol* 51: 381–384.

Halis G and Arici A. (2004) Endometriosis and inflammation in infertility. *Ann N Y Acad Sci* 1034: 300–315.

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

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

Haller K, Salumets A and Uibo R. (2008) Anti-FSH antibodies associate with poor outcome of ovarian stimulation in IVF. *Reprod Biomed Online* 16: 350–355.

Hammadeh ME, Ertan AK, Zeppezauer M, Baltes S, Georg T, Rosenbaum P and Schmidt W. (2002) Immunoglobulins and cytokines level in follicular fluid in relation to etiology of infertility and their relevance to IVF outcome. *Am J Reprod Immunol* 47: 82–90.

Hill JA, Welch WR, Faris HM and Anderson DJ. (1990) Induction of class II major histocompatibility complex antigen expression in human granulosa cells by interferon gamma: A potential mechanism contributing to autoimmune ovarian failure. *Am J Obstet Gynecol* 162: 534–540.

İdil M, Çepni İ, Demirsoy G, Öcal P, Salihoğlu F, Şenol H, Elibol F and İrez T. (2004) Does granulosa cell apoptosis have a role in the etiology of unexplained infertility? *Eur J Obstet Gynecol Reprod Biol* 112: 182–184.

Janssen OE, Mehlmauer N, Hahn S, Offner AH and Gartner R. (2004) High prevalence of autoimmune thyroiditis in patients with polycystic ovary syndrome. *Eur J Endocrinol* 150: 363–369.

Jung T, Gross W and Zoller M. (2011) CD44v6 coordinates tumor matrix-triggered motility and apoptosis resistance. *J Biol Chem* 286: 15862–15874.

Kaipia A and Hsueh AJ. (1997) Regulation of ovarian follicle atresia. *Annu Rev Physiol* 59: 349–363.

Kanaji N, Sato T, Nelson A, Wang X, Li Y, Kim M, Nakanishi M, Basma H, Michalski J, Farid M, Chandler M, Pease W, Patil A, Rennard SI and Liu X. (2011) Inflammatory cytokines regulate endothelial cell survival and tissue repair functions via NF-kappaB signaling. *J Inflamm Res* 4: 127–138.

Kawano Y, Kawasaki F, Nakamura S, Matsui N, Narahara H and Miyakawa I. (2001) The production and clinical evaluation of macrophage colony-stimulating factor and macrophage chemoattractant protein-1 in human follicular fluids. *Am J Reprod Immunol* 45: 1–5.

Kawasaki F, Kawano Y, Kosay Hasan Z, Narahara H and Miyakawa I. (2003) The clinical role of interleukin-6 and interleukin-6 soluble receptor in human follicular fluids. *Clin Exp Med* 3: 27–31.

Kilic S, Moraloglu O, Yuksel B, Ozaksit G, Tasdemir N and Dogan M. (2009) IL-18 and IL-18 binding protein concentrations in serum and ovarian follicular fluid do not affect in vitro fertilization outcomes in obese, overweight, and non-obese patients. *J Reprod Immunol* 79: 211–214.

Kim JW and Dang CV. (2005) Multifaceted roles of glycolytic enzymes. *Trends Biochem Sci* 30: 142–150.

Knight PG and Glister C. (2006) TGF-beta superfamily members and ovarian follicle development. *Reproduction* 132: 191–206.

Koide SS, Wang L and Kamada M. (2000) Antisperm antibodies associated with infertility: Properties and encoding genes of target antigens. *Proc Soc Exp Biol Med* 224: 123–132.

Kokcu A. (2010) Premature ovarian failure from current perspective. *Gynecol Endocrinol* 26: 555–562.

Kokcu A, Yavuz E, Celik H and Bildircin D. (2012) A panoramic view to relationships between reproductive failure and immunological factors. *Arch Gynecol Obstet* 286: 1283–1289.

Kuai J, Gregory B, Hill A, Pittman DD, Feldman JL, Brown T, Carito B, O'Toole M, Ramsey R, Adolfsson O, Shields KM, Dower K, Hall JP, Kurdi Y, Beech JT, Nanchahal J, Feldmann M, Foxwell BM, Brennan FM, Winkler DG and Lin LL. (2009) TREM-1 expression is increased in the synovium of rheumatoid arthritis patients and induces the expression of pro-inflammatory cytokines. *Rheumatology (Oxford)* 48: 1352–1358.

Kusanovic JP, Romero R, Chaiworapongsa T, Mittal P, Mazaki-Tovi S, Vaisbuch E, Erez O, Gotsch F, Than NG, Edwin SS, Pacora P, Jodicke C, Yeo L and Hassan SS. (2010) Amniotic fluid sTREM-1 in normal pregnancy, spontaneous parturition at term and preterm, and intra-amniotic infection/inflammation. *J Matern Fetal Neonatal Med* 23: 34–47.

Kõks S, Velthut A, Sarapik A, Altmäe S, Reinmaa E, Schalkwyk LC, Fernandes C, Lad HV, Soomets U, Jaakma U and Salumets A. (2010) The differential transcriptome and ontology profiles of floating and cumulus granulosa cells in stimulated human antral follicles. *Mol Hum Reprod* 16: 229–240.

Lang GA and Yeaman GR. (2001) Autoantibodies in endometriosis sera recognize a Thomsen-Friedenreich-like carbohydrate antigen. *J Autoimmun* 16: 151–161.

Langrish CL, McKenzie BS, Wilson NJ, de Waal Malefyt R, Kastelein RA and Cua DJ. (2004) IL-12 and IL-23: Master regulators of innate and adaptive immunity. *Immunol Rev* 202: 96–105.

Lebovic D and Naz R. (2004) Premature ovarian failure: Think "autoimmune disorder" *Sexuality, Reproduction and Menopause* 2: 230–233.

Lédée N, Lombroso R, Lombardelli L, Selva J, Dubanchet S, Chaouat G, Frankenne F, Foidart JM, Maggi E, Romagnani S, Ville Y and Piccinni MP. (2008) Cytokines and chemokines in follicular fluids and potential of the corresponding embryo: The role of granulocyte colony-stimulating factor. *Hum Reprod* 23: 2001–2009.

Lédée N, Frydman R, Osipova A, Taieb J, Gallot V, Lombardelli L, Logiodice F, Petitbarat M, Fanchin R, Chaouat G, Achour-Frydman N and Piccinni MP. (2011) Levels of follicular G-CSF and interleukin-15 appear as noninvasive biomarkers of subsequent successful birth in modified natural in vitro fertilization/intracytoplasmic sperm injection cycles. *Fertil Steril* 95: 94–98.

Lee DL, Kim SH, Kim E, Chun SY and Kim TS. (2009) Interferon-alpha is involved in the luteinizing hormone-induced differentiation of rat preovulatory granulosa cells. *J Interferon Cytokine Res* 29: 801–808.

Luborsky J. (2002) Ovarian autoimmune disease and ovarian autoantibodies. *J Womens Health Gend Based Med* 11: 585–599.

Machelon V, Nomé F and Emilie D. (2000) Regulated on activation normal T expressed and secreted chemokine is induced by tumor necrosis factor-alpha in granulosa cells from human preovulatory follicle. *J Clin Endocrinol Metab* 85: 417–424.

Macklon NS, Stouffer RL, Giudice LC and Fauser BC. (2006) The science behind 25 years of ovarian stimulation for in vitro fertilization. *Endocr Rev* 27: 170–207.

Maheshwari A, Hamilton M and Bhattacharya S. (2008) Effect of female age on the diagnostic categories of infertility. *Hum Reprod* 23: 538–542.

Maisey K, Nardocci G, Imarai M, Cardenas H, Rios M, Croxatto HB, Heckels JE, Christodoulides M and Velasquez LA. (2003) Expression of proinflammatory cytokines and receptors by human fallopian tubes in organ culture following challenge with Neisseria gonorrhoeae. *Infect Immun* 71: 527–532.

Malizia BA, Wook YS, Penzias AS and Usheva A. (2010) The human ovarian follicular fluid level of interleukin-8 is associated with follicular size and patient age. *Fertil Steril* 93: 537–543.

Mascarenhas MN, Flaxman SR, Boerma T, Vanderpoel S and Stevens GA. (2012) National, regional, and global trends in infertility prevalence since 1990: A systematic analysis of 277 health surveys. *PLoS Med* 9: e1001356.

Matarese G, De Placido G, Nikas Y and Alviggi C. (2003) Pathogenesis of endometriosis: Natural immunity dysfunction or autoimmune disease? *Trends Mol Med* 9: 223–228.

Mathur SP. (2000) Autoimmunity in endometriosis: Relevance to infertility. *Am J Reprod Immunol* 44: 89–95.

Mathur S, Peress MR, Williamson HO, Youmans CD, Maney SA, Garvin AJ, Rust PF and Fudenberg HH. (1982) Autoimmunity to endometrium and ovary in endometriosis. *Clin Exp Immunol* 50: 259–266.

Mathur S, Butler WJ, Chihal HJ, Isaacson KB and Gleicher N. (1995) Target antigen(s) in endometrial autoimmunity of endometriosis. *Autoimmunity* 20: 211–222.

Matoba N, Yu Y, Mestan K, Pearson C, Ortiz K, Porta N, Thorsen P, Skogstrand K, Hougaard DM, Zuckerman B and Wang X. (2009) Differential patterns of 27 cord blood immune biomarkers across gestational age. *Pediatrics* 123: 1320–1328.

Matsuda F, Inoue N, Manabe N and Ohkura S. (2012) Follicular growth and atresia in mammalian ovaries: Regulation by survival and death of granulosa cells. *J Reprod Dev* 58: 44–50.

Maurer M and von Stebut E. (2004) Macrophage inflammatory protein-1. *Int J Biochem Cell Biol* 36: 1882–1886.

Messinis IE. (2006) From menarche to regular menstruation: Endocrinological background. *Ann N Y Acad Sci* 1092: 49–56.

Mettler L, Bachmann J and Schmutzler A. (2004) High prevalence of markers for immunological disorders in IVF patients. *Int J Gynaecol Obstet* 86: 59–60.

Meyer WR, Castelbaum AJ, Somkuti S, Sagoskin AW, Doyle M, Harris JE and Lessey BA. (1997) Hydrosalpinges adversely affect markers of endometrial receptivity. *Hum Reprod* 12: 1393–1398.

Monnier-Barbarino P, Jouan C, Dubois M, Gobert B, Faure G and Bene MC. (2003) Anti-ovarian antibodies and in vitro fertilization: Cause or consequence? *Gynecol Obstet Fertil* 31: 770–773.

Monnier-Barbarino P, Forges T, Faure GC and Bene MC. (2005) Gonadal antibodies interfering with female reproduction. *Best Pract Res Clin Endocrinol Metab* 19: 135–148.

Monteleone P, Parrini D, Faviana P, Carletti E, Casarosa E, Uccelli A, Cela V, Genazzani AR and Artini PG. (2011) Female infertility related to thyroid autoimmunity: The ovarian follicle hypothesis. *Am J Reprod Immunol* 66: 108–114.

Morelli SS, Keegan DA, Krey LC, Katz J, Liu M and Noyes N. (2008) Early serum interleukin-8 evaluation may prove useful in localizing abnormally implanted human gestations after in vitro fertilization. *Fertil Steril* 90: 2068–2072.

Moretti E, Cosci I, Spreafico A, Serchi T, Cuppone AM and Collodel G. (2009) Semen characteristics and inflammatory mediators in infertile men with different clinical diagnoses. *Int J Androl* 32: 637–646.

Murakami Y, Akahoshi T, Aoki N, Toyomoto M, Miyasaka N and Kohsaka H. (2009) Intervention of an inflammation amplifier, triggering receptor expressed on myeloid cells 1, for treatment of autoimmune arthritis. *Arthritis Rheum* 60: 1615–1623.

Nagler HM and Grotas A. (2009) Varicocele. In: Lipshultz LI, Howards SS and Niederberger CS (eds) *Infertility in the male.* 4 th ed. Cambridge: Cambridge University Press, 331–361.

Nakanishi K, Yoshimoto T, Tsutsui H and Okamura H. (2001) Interleukin-18 is a unique cytokine that stimulates both Th1 and Th2 responses depending on its cytokine milieu. *Cytokine Growth Factor Rev* 12: 53–72.

Nallella KP, Allamaneni SS, Pasqualotto FF, Sharma RK, Thomas AJ, Jr. and Agarwal A. (2004) Relationship of interleukin-6 with semen characteristics and oxidative stress in patients with varicocele. *Urology* 64: 1010–1013.

Naughton CK, Nangia AK and Agarwal A. (2001) Pathophysiology of varicoceles in male infertility. *Hum Reprod Update* 7: 473–481.

Nel-Themaat L and Nagy ZP. (2011) A review of the promises and pitfalls of oocyte and embryo metabolomics. *Placenta* 32 Suppl 3: S257–263.
Nip MM, Taylor PV, Rutherford AJ and Hancock KW. (1995) Autoantibodies and anti-sperm antibodies in sera and follicular fluids of infertile patients; relation to reproductive outcome after in-vitro fertilization. *Hum Reprod* 10: 2564–2569.
Ohta N, Saito H, Kuzumaki T, Takahashi T, Ito MM, Saito T, Nakahara K and Hiroi M. (1999) Expression of CD44 in human cumulus and mural granulosa cells of individual patients in in-vitro fertilization programmes. *Mol Hum Reprod* 5: 22–28.
Ohta N, Saito H, Kaneko T, Yoshida M, Takahashi T, Saito T, Nakahara K and Hiroi M. (2001) Soluble CD44 in human ovarian follicular fluid. *J Assist Reprod Genet* 18: 21–25.
Oktem O and Oktay K. (2008) The ovary: Anatomy and function throughout human life. *Ann N Y Acad Sci* 1127: 1–9.
Onalan G, Selam B, Baran Y, Cincik M, Onalan R, Gunduz U, Ural AU and Pabuccu R. (2005) Serum and follicular fluid levels of soluble Fas, soluble Fas ligand and apoptosis of luteinized granulosa cells in PCOS patients undergoing IVF. *Hum Reprod* 20: 2391–2395.
Ostanin AA, Aizikovich BI, Aizikovich IV, Kozhin AY and Chernykh ER. (2007) Role of cytokines in the regulation of reproductive function. *Bull Exp Biol Med* 143: 75–79.
Palacio JR, Iborra A, Gris JM, Andolz P and Martínez P. (1997) Anti-endometrial autoantibodies in women with a diagnosis of infertility. *Am J Reprod Immunol* 38: 100–105.
Palacio JR, Iborra A, Ulcova-Gallova Z, Badia R and Martínez P. (2006) The presence of antibodies to oxidative modified proteins in serum from polycystic ovary syndrome patients. *Clin Exp Immunol* 144: 217–222.
Palma GA, Arganaraz ME, Barrera AD, Rodler D, Mutto AA and Sinowatz F. (2012) Biology and biotechnology of follicle development. *ScientificWorldJournal* 2012: 938138.
Pancholi V. (2001) Multifunctional alpha-enolase: Its role in diseases. *Cell Mol Life Sci* 58: 902–920.
Paszkowski T, Clarke RN and Hornstein MD. (2002) Smoking induces oxidative stress inside the graafian follicle. *Hum Reprod* 17: 921–925.
Pellicer A, Diamond MP, DeCherney AH and Naftolin F. (1987) Intraovarian markers of follicular and oocyte maturation. *J In Vitro Fert Embryo Transf* 4: 205–217.
Pellicer A, Albert C, Mercader A, Bonilla-Musoles F, Remohi J and Simon C. (1998) The follicular and endocrine environment in women with endometriosis: Local and systemic cytokine production. *Fertil Steril* 70: 425–431.
Perricone R, Perricone C, De Carolis C and Shoenfeld Y. (2008) NK cells in autoimmunity: A two-edg'd weapon of the immune system. *Autoimmun Rev* 7: 384–390.
Peterson P, Perheentupa J and Krohn KJ. (1996) Detection of candidal antigens in autoimmune polyglandular syndrome type I. *Clin Diagn Lab Immunol* 3: 290–294.
Petrikova J and Lazurova I. (2012) Ovarian failure and polycystic ovary syndrome. *Autoimmun Rev* 11: A471–478.
Pillai S, Zhou GX, Arnaud P, Jiang H, Butler WJ and Zhang H. (1996) Antibodies to endometrial transferrin and alpha 2-Heremans Schmidt (HS) glycoprotein in patients with endometriosis. *Am J Reprod Immunol* 35: 483–494.
Pillai S, Rust PF and Howard L. (1998) Effects of antibodies to transferrin and alpha 2-HS glycoprotein on in vitro sperm motion: Implications in infertility associated with endometriosis. *Am J Reprod Immunol* 39: 235–242.

Pires ES. (2010) Multiplicity of molecular and cellular targets in human ovarian autoimmunity: An update. *J Assist Reprod Genet* 27: 519–524.
Połeć A, Ráki M, Åbyholm T, Tanbo TG and Fedorcsák P. (2011) Interaction between granulosa-lutein cells and monocytes regulates secretion of angiogenic factors in vitro. *Hum Reprod* 26: 2819–2829.
Ponta H, Sherman L and Herrlich PA. (2003) CD44: From adhesion molecules to signalling regulators. *Nat Rev Mol Cell Biol* 4: 33–45.
Poppe K, Velkeniers B and Glinoer D. (2008) The role of thyroid autoimmunity in fertility and pregnancy. *Nat Clin Pract Endocrinol Metab* 4: 394–405.
Practice Committee of American Society for Reproductive Medicine. (2008) Definitions of infertility and recurrent pregnancy loss. *Fertil Steril* 89: 1603.
Preamble to the Constitution of the World Health Organization as adopted by the International Health Conference, New York, 19–22 June, 1946; signed on 22 July 1946 by the representatives of 61 States (Official Records of the World Health Organization, no. 2, p. 100) and entered into force on 7 April 1948.
Putowski L, Darmochwal-Kolarz D, Rolinski J, Oleszczuk J and Jakowicki J. (2004) The immunological profile of infertile women after repeated IVF failure (preliminary study). *Eur J Obstet Gynecol Reprod Biol* 112: 192–196.
Quayle AJ, Porter EM, Nussbaum AA, Wang YM, Brabec C, Yip KP and Mok SC. (1998) Gene expression, immunolocalization, and secretion of human defensin-5 in human female reproductive tract. *Am J Pathol* 152: 1247–1258.
Randall GW, Gantt PA, Poe-Zeigler RL, Bergmann CA, Noel ME, Strawbridge WR, Richardson-Cox B, Hereford JR and Reiff RH. (2007) Serum antiendometrial antibodies and diagnosis of endometriosis. *Am J Reprod Immunol* 58: 374–382.
Randall GW, Bush S, 2nd and Gantt PA. (2009) Serum and peritoneal fluid antiendometrial antibodies in assisted reproduction. *J Reprod Med* 54: 353–360.
Reimand K, Talja I, Metsküla K, Kadastik U, Matt K and Uibo R. (2001) Autoantibody studies of female patients with reproductive failure. *J Reprod Immunol* 51: 167–176.
Revelli A, Delle Piane L, Casano S, Molinari E, Massobrio M and Rinaudo P. (2009) Follicular fluid content and oocyte quality: From single biochemical markers to metabolomics. *Reprod Biol Endocrinol* 7: 40.
Rienzi L, Vajta G and Ubaldi F. (2011) Predictive value of oocyte morphology in human IVF: A systematic review of the literature. *Hum Reprod Update* 17: 34–45.
Rienzi L, Cobo A, Paffoni A, Scarduelli C, Capalbo A, Vajta G, Remohi J, Ragni G and Ubaldi FM. (2012) Consistent and predictable delivery rates after oocyte vitrification: An observational longitudinal cohort multicentric study. *Hum Reprod* 27: 1606–1612.
Rizk B, Aboulghar M, Smitz J and Ron-El R. (1997) The role of vascular endothelial growth factor and interleukins in the pathogenesis of severe ovarian hyperstimulation syndrome. *Hum Reprod Update* 3: 255–266.
Rizza P, Moretti F and Belardelli F. (2010) Recent advances on the immunomodulatory effects of IFN-alpha: Implications for cancer immunotherapy and autoimmunity. *Autoimmunity* 43: 204–209.
Runesson E, Boström EK, Janson PO and Brännström M. (1996) The human preovulatory follicle is a source of the chemotactic cytokine interleukin-8. *Mol Hum Reprod* 2: 245–250.
Runesson E, Ivarsson K, Janson PO and Brannstrom M. (2000) Gonadotropin- and cytokine-regulated expression of the chemokine interleukin 8 in the human preovulatory follicle of the menstrual cycle. *J Clin Endocrinol Metab* 85: 4387–4395.

Salmassi A, Schmutzler AG, Huang L, Hedderich J, Jonat W and Mettler L. (2004) Detection of granulocyte colony-stimulating factor and its receptor in human follicular luteinized granulosa cells. *Fertil Steril* 81 Suppl 1: 786–791.

Salumets A, Hydén-Granskog C, Suikkari AM, Tiitinen A and Tuuri T. (2001) The predictive value of pronuclear morphology of zygotes in the assessment of human embryo quality. *Hum Reprod* 16: 2177–2181.

Salumets A, Hydén-Granskog C, Mäkinen S, Suikkari AM, Tiitinen A and Tuuri T. (2003) Early cleavage predicts the viability of human embryos in elective single embryo transfer procedures. *Hum Reprod* 18: 821–825.

Sarandakou A, Malamitsi-Puchner A, Baka S, Rizos D, Hassiakos D and Creatsas G. (2003) Apoptosis and proliferation factors in serum and follicular fluid from women undergoing in vitro fertilization. *Fertil Steril* 79: 634–636.

Sasson R, Winder N, Kees S and Amsterdam A. (2002) Induction of apoptosis in granulosa cells by TNF alpha and its attenuation by glucocorticoids involve modulation of Bcl-2. *Biochem Biophys Res Commun* 294: 51–59.

Schall TJ, Bacon K, Toy KJ and Goeddel DV. (1990) Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. *Nature* 347: 669–671.

Shanti A, Santanam N, Morales AJ, Parthasarathy S and Murphy AA. (1999) Autoantibodies to markers of oxidative stress are elevated in women with endometriosis. *Fertil Steril* 71: 1115–1118.

Sharif O and Knapp S. (2008) From expression to signaling: Roles of TREM-1 and TREM-2 in innate immunity and bacterial infection. *Immunobiology* 213: 701–713.

Shih NY, Lai HL, Chang GC, Lin HC, Wu YC, Liu JM, Liu KJ and Tseng SW. (2010) Anti-alpha-enolase autoantibodies are down-regulated in advanced cancer patients. *Jpn J Clin Oncol* 40: 663–669.

Shimizu T, Kaji A, Murayama C, Magata F, Shirasuna K, Wakamiya K, Okuda K and Miyamoto A. (2012) Effects of interleukin-8 on estradiol and progesterone production by bovine granulosa cells from large follicles and progesterone production by luteinizing granulosa cells in culture. *Cytokine* 57: 175–181.

Simon C, Cano F, Valbuena D, Remohi J and Pellicer A. (1995) Clinical evidence for a detrimental effect on uterine receptivity of high serum oestradiol concentrations in high and normal responder patients. *Hum Reprod* 10: 2432–2437.

Soini S, Ibarreta D, Anastasiadou V, Ayme S, Braga S, Cornel M, Coviello DA, Evers-Kiebooms G, Geraedts J, Gianaroli L, Harper J, Kosztolanyi G, Lundin K, Rodrigues-Cerezo E, Sermon K, Sequeiros J, Tranebjaerg L, Kaariainen H, ESHG and ESHRE. (2006) The interface between assisted reproductive technologies and genetics: Technical, social, ethical and legal issues. *Eur J Hum Genet* 14: 588–645.

Steptoe PC and Edwards RG. (1978) Birth after the reimplantation of a human embryo. *Lancet* 2: 366.

Stern C, Chamley L, Hale L, Kloss M, Speirs A and Baker HW. (1998) Antibodies to beta2 glycoprotein I are associated with in vitro fertilization implantation failure as well as recurrent miscarriage: Results of a prevalence study. *Fertil Steril* 70: 938–944.

Sundblad V, Bussmann L, Chiauzzi VA, Pancholi V and Charreau EH. (2006) Alpha-enolase: A novel autoantigen in patients with premature ovarian failure. *Clin Endocrinol (Oxf)* 65: 745–751.

Sweet RL. (2011) Treatment of acute pelvic inflammatory disease. *Infect Dis Obstet Gynecol* 2011: 561909.

Zhong YP, Ying Y, Wu HT, Zhou CQ, Xu YW, Wang Q, Li J, Shen XT and Li J. (2012) Relationship between antithyroid antibody and pregnancy outcome following in vitro fertilization and embryo transfer. *Int J Med Sci* 9: 121–125.

Zidovec Lepej S, Vujisić S, Stipoljev F and Mazuran R. (2003) Interferon-alpha-like biological activity in human seminal plasma, follicular fluid, embryo culture medium, amniotic fluid and fetal blood. *Reprod Fertil Dev* 15: 423–428.

Taniguchi F. (2005) Results of prednisolone given to improve the outcome of in vitro fertilization-embryo transfer in women with antinuclear antibodies. *J Reprod Med* 50: 383–388.

Taylor PV, Campbell JM and Scott JS. (1989) Presence of autoantibodies in women with unexplained infertility. *Am J Obstet Gynecol* 161: 377–379.

Terranova PF and Rice VM. (1997) Review: Cytokine involvement in ovarian processes. *Am J Reprod Immunol* 37: 50–63.

The ESHRE Capri Workshop. (1996) Infertility revisited: The state of the art today and tomorrow. *Hum Reprod* 11: 1779–1807.

The ESHRE Capri Workshop Group. (2002) Physiopathological determinants of human infertility. *Hum Reprod Update.* 435–447.

The ESHRE Capri Workshop Group. (2004) Diagnosis and management of the infertile couple: Missing information. *Hum Reprod Update* 10: 295–307.

The ESHRE Capri Workshop Group. (2007) Intracytoplasmic sperm injection (ICSI) in 2006: Evidence and evolution. *Hum Reprod Update* 13: 515–526

The ESHRE Capri Workshop Group. (2010) Europe the continent with the lowest fertility. *Hum Reprod Update* 16: 590–602.

The Practice Committee of American Society for Reproductive Medicine. (2008) Report on varicocele and infertility. *Fertil Steril* 90: S247–249.

The Practice Committee of the American Society for Reproductive Medicine. (2006a) Effectiveness and treatment for unexplained infertility. *Fertil Steril* 86: S111–114.

The Practice Committee of the American Society for Reproductive Medicine. (2006b) The role of tubal reconstructive surgery in the era of assisted reproductive technologies. *Fertil Steril* 86: S31–34.

The Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group. (2004) Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). *Hum Reprod* 19: 41–47.

Trombly DJ, Woodruff TK and Mayo KE. (2009) Roles for transforming growth factor beta superfamily proteins in early folliculogenesis. *Semin Reprod Med* 27: 14–23.

Tsigkou A, Marzotti S, Borges L, Brozzetti A, Reis F, Candeloro P, Luisa Bacosi M, Bini V, Petraglia F and Falorni A. (2008) High serum inhibin concentration discriminates autoimmune oophoritis from other forms of primary ovarian insufficiency. *J Clin Endocrinol Metab* 93: 1263–1269.

Tsuji Y, Tamaoki TH, Hasegawa A, Kashiwamura S, Iemoto A, Ueda H, Muranaka J, Adachi S, Furuyama J, Okamura H and Koyama K. (2001) Expression of interleukin-18 and its receptor in mouse ovary. *Am J Reprod Immunol* 46: 349–357.

Twig G, Shina A, Amital H and Shoenfeld Y. (2012) Pathogenesis of infertility and recurrent pregnancy loss in thyroid autoimmunity. *J Autoimmun* 38: J275–281.

Utt M, Nilsson I, Ljungh A and Wadstrom T. (2002) Identification of novel immunogenic proteins of Helicobacter pylori by proteome technology. *J Immunol Methods* 259: 1–10.

Wallace M, Cottell E, Gibney MJ, McAuliffe FM, Wingfield M and Brennan L. (2012) An investigation into the relationship between the metabolic profile of follicular

fluid, oocyte developmental potential, and implantation outcome. *Fertil Steril* 97: 1078–1084.e1071–1078.
Walter M, Berg H, Leidenberger FA, Schweppe KW and Northemann W. (1995) Autoreactive epitopes within the human alpha-enolase and their recognition by sera from patients with endometriosis. *J Autoimmun* 8: 931–945.
Van Voorhis BJ and Stovall DW. (1997) Autoantibodies and infertility: A review of the literature. *J Reprod Immunol* 33: 239–256.
Vassiliadis S, Relakis K, Papageorgiou A and Athanassakis I. (2005) Endometriosis and infertility: A multi-cytokine imbalance versus ovulation, fertilization and early embryo development. *Clin Dev Immunol* 12: 125–129.
Wegner N, Lundberg K, Kinloch A, Fisher B, Malmstrom V, Feldmann M and Venables PJ. (2010) Autoimmunity to specific citrullinated proteins gives the first clues to the etiology of rheumatoid arthritis. *Immunol Rev* 233: 34–54.
Welt CK. (2008) Primary ovarian insufficiency: A more accurate term for premature ovarian failure. *Clin Endocrinol (Oxf)* 68: 499–509.
Wilm M, Shevchenko A, Houthaeve T, Breit S, Schweigerer L, Fotsis T and Mann M. (1996) Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. *Nature* 379: 466–469.
World Health Organization. (1999) WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction, UK: Cambridge University Press.
World Health Organization. (2010) WHO laboratory manual for the examination and processing of human semen, Switzerland: WHO Press.
Vujisic S, Lepej SZ, Emedi I, Bauman R, Remenar A and Tiljak MK. (2006) Ovarian follicular concentration of IL-12, IL-15, IL-18 and p40 subunit of IL-12 and IL-23. *Hum Reprod* 21: 2650–2655.
Wygrecka M, Marsh LM, Morty RE, Henneke I, Guenther A, Lohmeyer J, Markart P and Preissner KT. (2009) Enolase-1 promotes plasminogen-mediated recruitment of monocytes to the acutely inflamed lung. *Blood* 113: 5588–5598.
Xu H, Schultze-Mosgau A, Agic A, Diedrich K, Taylor RN and Hornung D. (2006) Regulated upon activation, normal T cell expressed and secreted (RANTES) and monocyte chemotactic protein 1 in follicular fluid accumulate differentially in patients with and without endometriosis undergoing in vitro fertilization. *Fertil Steril* 86: 1616–1620.
Ying Y, Zhong YP, Zhou CQ, Xu YW, Wang Q, Li J, Shen XT and Wu HT. (2012) Antinuclear antibodies predicts a poor IVF-ET outcome: Impaired egg and embryo development and reduced pregnancy rate. *Immunol Invest* 41: 458–468.
Yoshino O, Osuga Y, Koga K, Hirota Y, Yano T, Tsutsumi O, Fujimoto A, Kugu K, Momoeda M, Fujiwara T and Taketani Y. (2003) Upregulation of interleukin-8 by hypoxia in human ovaries. *Am J Reprod Immunol* 50: 286–290.
Young SL, Loy T. (2005) Normal cycling endometrium: molecular, cellular and histologic perspectives. In: Olive DL (ed) *Endometriosis in clinical practice.* Oxfordshire: Taylor and Francis, 1–12.
Youssef RE, Ledingham MA, Bollapragada SS, O'Gorman N, Jordan F, Young A and Norman JE. (2009) The role of Toll-like receptors (TLR-2 and -4) and triggering receptor expressed on myeloid cells 1 (TREM-1) in human term and preterm labor. *Reprod Sci* 16: 843–856.
Yu P, Zhou L, Ke W and Li K. (2010) Clinical significance of pAKT and CD44v6 overexpression with breast cancer. *J Cancer Res Clin Oncol* 136: 1283–1292.

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Eesti Immunoloogide ja Allergoloogide Seltsi liige.

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