DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS
170

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170

KOIT REIMAND

Autoimmunity in reproductive failure: A study on associated autoantibodies and autoantigens

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Department of General and Molecular Pathology, University of Tartu, Estonia

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Supervisor: Raivo Uibo, MD, PhD, Professor of Immunology, University of Tartu, Estonia

Reviewers: Helle Karro, MD, PhD, Professor of Obstetrics and Gynecology, University of Tartu, Estonia

Martti Laan, MD, PhD, Senior Research Fellow, Medical Faculty, University of Tartu, Estonia

Opponent: Olli Vainio, MD, PhD, Professor, Department of Medical Microbiology, University of Oulu, Finland

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Paper I: participation in: the study design, conducting the laboratory experiments, data analysis and writing the paper

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ABBREVIATIONS

3βHSD	3-beta-hydroxysteroid dehydrogenase 2
AA	Amino acids
ACA	Adrenocortical antibodies
AMA	Antimitochondrial antibodies
ANA	Anti-nuclear antibodies
APA	Antiphospholipid antibodies
APS-1	Autoimmune polyendocrine syndrome type 1
APS-2	Autoimmune polyendocrine syndrome type 2
ARA	Anti-reticulin antibodies
β2GPI	β2-glycoprotein I
CA	Carbonic anhydrase
cDNA	complementary DNA
CPM	Counts (readings) per minute
CYP11A1	Cytochrome P450 cholesterol side-chain cleavage enzyme
CYP17	Cytochrome P450 steroid 17-alpha-hydroxylase
CYP1A2	Cytochrome P450 1A2
CYP21A2	Cytochrome P450 steroid 21-hydroxylase
EIU	Enzyme-immunological units
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
FMR1	Fragile X mental retardation 1
FSH	Follicle stimulating hormone
GST	Glutathione S-transferase
IA-2	Islet cell antigen 512; protein-tyrosine phosphatase
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IIF	Indirect immunofluorescence assay
LH	Luteinizing hormone
LKMA	Liver/kidney microsomal antibodies
Mtsga10	Mice TSGA10
OD	Optical density
PCA	Parietal cell antibodies
PCO	Polycystic ovaries
PCOS	Polycystic ovary syndrome
PID	Pelvic inflammatory disease
POF	Premature ovarian failure
RIP	Radioimmunoprecipitation
RU	Relative units
SCA	Steroid-producing cell antibodies
SMA	Smooth muscle antibodies
T1D	Type 1 Diabetes

TBS-MT buffer	Tris-buffered saline buffer with skimmed milk and Tween-20 addition
TMA	Thyroid microsomal antibodies
TSGA10	Testis-specific protein 10

I. INTRODUCTION

Infertility, defined as the biological inability or diminished ability to conceive and have children, depends upon both partners in a couple. Infertility is referred to as the couple's inability to conceive after a one year of regular sexual intercourse without contraception (Gnoth *et al.* 2005). Infertility may also refer to the inability to achieve a livebirth, but the term infecundity also refers to this condition (Healy *et al.* 1994). Infertility is a global phenomenon among populations with reproductive abilities, a lot of couples experience infertility at some time in their lives. The prevalence of infertility has a broad range of 3.5–26.5%, but is mostly between 10–15%, and an estimated median is 9% (Larsen 2003; Boivin *et al.* 2007; Ombelet *et al.* 2008). A wide spectrum of factors can cause infertility. Gender conditions are the major cause with approximately one-third of cases of infertility resulting from female specific factors, another around a third of cases is resulting from male specific factors, which are mostly sperm disorders (Healy *et al.* 1994). A combination of factors inherent in both partners causes *circa* 20% of infertility cases and 15–30% remain unexplained, and is known as idiopathic infertility (Healy *et al.* 1994; Evers 2002).

The causes of idiopathic cases have begun to be understood during recent decades and in most cases immune system derangements are shown to be involved. Most importantly autoimmune mechanisms, i.e. immune reactions to the cells in reproductive organs or relevant biomolecules, appear to play a central role. Despite cellular immune reactions being core determinants of autoimmune tissue destruction, their development nearly always is accompanied by relevant autoantibodies which can be more conveniently used as markers of autoimmunity. Research has identified some autoantibodies related to reproductive failure and infertility. But judging by the variety of clinical symptoms associated with unexplained reasons of infertility, among both women and men, there are still many autoantibodies remaining to be indentified.

The present study had three main aims: (i) to examine the common autoantibodies in women with reproductive failure as circumstantial evidence for an involvement of autoimmunity; (ii) clarify the situation of autoantigenic targets in female hypogonadism associated with premature ovarian failure and autoimmune polyendocrine syndrome type 1; and (iii) identify new testicular autoantigens besides the known ones.

2. LITERATURE REVIEW

2.1. Reproductive failure and autoimmunity relations

The relationship between autoimmunity and reproduction has long been recognized (Scott 1966; Shulman 1971). In some cases, as in infertility caused by antisperm autoantibodies (Bohring and Krause 2003) or by ovarian autoimmunity in premature ovarian failure (Forges *et al.* 2004), the targets of autoimmunity are directly related to reproduction or located in the reproductive organs. On the other hand, infertility or subfertility can be indirectly related to autoimmunity, as in the case of infertility initiated by thyroid autoimmunity (Poppe *et al.* 2008).

The woman's contribution to successful reproduction – from egg cell fertilization to childbirth – is more sophisticated than the man's contribution. Therefore, health status is more likely to significantly influence female fertility. Some common autoimmune diseases like thyroid diseases (Poppe *et al.* 2006a; Poppe *et al.* 2006b), celiac disease (Ludvigsson *et al.* 2005; Ludvigsson *et al.* 2006) or presence of phospholipids antibodies do not affect male fertility but do have negative influence on female fertility (Ludvigsson *et al.* 2005; Cervera and Balasch 2008). Autoimmune disorders have a tendency to develop in women during their reproductive years (Cervera and Balasch 2008).

2.2. Female reproductive failure and autoimmunity

2.2.1. The common causes of female infertility

The most prominent as well-known causes of female infertility are ovulatory dysfunction, endometriosis, tubal factor infertility (tubal occlusion) and either uterine or cervical factors (Healy *et al.* 1994).

Ovulatory disorders form a heterogenic group. The primary defect, leading to oligo- or anovulation, may lie somewhere in the hypothalamic-hypophyseal-ovarian axis, in the chain of coordinative interactions, which is needed to be functional, as well as attuned, for female fertility. The functional classification of ovulatory disorders is divided into five categories: hypergonadotrophic hypogonadism; hypogonadotrophic hypogonadism; hyperprolactinaemia; normogonadotrophic oligo-anovulation and 'miscellaneous' (The ESHRE Capri Workshop Group 2002). Premature ovarian failure (POF) is defined as hypergonadotrophic hypogonadism (more detailed definition in section 2.3.1). A normogonadotrophic ovulatory disorder, polycystic ovary syndrome (PCOS) is the most common cause of oligo-anovulation or anovulation related infertility and is also the most common endocrine disorder in women of reproductive age (Yildiz *et al.* 2008). PCOS is a complex, multifaceted, heterogeneous disorder,

where most patients have insulin resistance and hyperinsulinemia (Ovalle and Azziz 2002), hyperandrogenism, ovulatory dysfunction, polycystic ovaries, and is diagnosable after the exclusion of related ovulatory or other androgen excess disorders (Mathur *et al.* 2008). PCOS definition by the Rotterdam 2003 criteria (Betterle *et al.* 2004) requires at least two of three features for diagnosis: chronic anovulation, polycystic ovaries, and either or both clinical and biochemical signs of hyperandrogenism. The prevalence of PCOS in women of reproductive age is 5–10% (Agrawal *et al.* 2004). Women with PCOS are prone to anovulatory infertility, according to the PCOS Rotterdam 2003 definition anovulatory infertility may be as prevalent as 65–80% (Hart 2007).

Endometriosis, an estrogen-dependent disorder, is the presence of endometrial tissue outside of the uterine cavity that induces a chronic inflammatory reaction (Kennedy *et al.* 2005) The estimated prevalence of endometriosis in fertile women is 0.5–5% (but even as high as 20%) and 25–50% in infertile women (Matarese *et al.* 2003; Ozkan *et al.* 2008).

Tubal factor infertility is mainly due to dysfunction of the fallopian tubes. The most common cause of this, if endometriosis induced cases are excluded, is pelvic inflammatory disease (PID) (Mardh 2004). PID is caused mostly by sexually transmitted pathogens like *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and *Mycoplasma genitalium* (The ESHRE Capri Workshop Group 2002; Haggerty 2008). The incidence of infertility following acute PID may vary from 6–60% (The ESHRE Capri Workshop Group 2002). Tubal factor infertility accounts for 11–30% of all cases of infertility (Evers 2002).

However, a significant proportion of infertility cases remain enigmatic. Despite advances in diagnosis, infertility remains unexplained in 25–30% of fully investigated couples (Evers 2002). Beside the issue of how autoimmunity is related to well-defined infertility diagnostic entities, the role and significance of autoimmunity in cases of unexplained infertility remain unclear (Gleicher *et al.* 2002; Cervera and Balasch 2008).

2.2.2. Reproductive failure: autoimmunity and associated autoantibodies

Women are more prone to autoimmune diseases than men and this is also reflected in female infertility. Autoimmunity is shown to be associated with certain types of POF and recurrent pregnancy loss. As more or less specific markers of relevant autoimmunity, several autoantibodies, mostly IgG and less frequently IgA or IgM, are the focus of interest.

Antiphospholipid antibodies (APA) are long time known to be associated frequently with recurrent pregnancy loss. These autoantibodies bind to several membrane phospholipids, which are covered by beta 2-glycoprotein I (β2GPI) or other phospholipid-binding proteins. Most frequently anti-β2GPI and anti-cardiolipin are used as diagnostic antibodies. However, controversial data has accumulated about the role of APA in female infertility (Gleicher *et al.* 2002;

Cervera and Balasch 2008). Meta-analysis of relevant studies, including therapeutical intervention series, tends to deny the significance of APA to either clinical pregnancy or live-birth (Cervera and Balasch 2008). Meanwhile, there are incomparabilities between the studies, e.g. differences in the patient selection criteria and antibody assessment, which reduce the adequacy of comparisons (Cervera and Balasch 2008). Furthermore, the redox-unmasking phenomenon must be taken into account in the assessment of APA (McIntyre 2004).

Endometriosis revealed as a disease related to immune system dysfunction, of which the most prominent dysfunction is the impaired function of natural killer cells subgroups. These natural immunity disturbances are inter-related to the characteristics of autoimmunity (Matarese *et al.* 2003). Research suggests candidate autoantigens for endometriosis are carbonic anhydrases (Kiechle *et al.* 1994; D'Cruz *et al.* 1996), alpha-enolase (Walter *et al.* 1995), endometrial transferrin and alpha 2-HS glycoprotein (Mathur 2000).

Ovarian antibodies are often detected in the peripheral circulation of women with POF and unexplained infertility (Fenichel *et al.* 1997; Luborsky *et al.* 2000; Luborsky 2002). The assays for these antibodies have mostly used ovarian homogenates as the characterization of the corresponding molecular targets is still being developed. Gonadotropin antibodies, anti-FSH and anti-LH, are indicated as separate markers; IgA-class autoantibodies against to a specific FSH epitope were more prevalent in endometriosis and PCOS (Haller *et al.* 2005) and gonadotropin antibodies are shown to occur in unexplained infertility patients without previous gonadotropin treatments (Shatavi *et al.* 2006).

Thyroid autoimmunity, mostly through causing hypothyroidism, influences ovarian function before and during pregnancy (Poppe *et al.* 2006a; Poppe *et al.* 2008). Importantly, the prevalence of coeliac disease in infertile women is three times higher than in the general population, particularly in the subgroup with unexplained infertility, but malabsorption of some essential components of food is unverified in the studied cases (Collin *et al.* 1996; Meloni *et al.* 1999). However, Jackson *et al.*'s recent study of coeliac disease in unexplained fertility cases (Jackson *et al.* 2008) casts some doubt on the findings of previous related research.

2.3. Premature Ovarian Failure (POF) and ovarian autoimmunity

2.3.1. Definition and clinical features of POF

POF is an unphysiological cessation of menses before the age of 40 years (de Moraes-Ruehsen and Jones 1967; Goswami and Conway 2005). The hallmarks are anovulation (primary or secondary amenorrhoea), hypogonadism (reduced estrogen steroid hormone levels) and elevated gonadotrophins (serum levels of FSH are disproportionately higher than LH; FSH greater than 40 IU liter^{-1} is

pathognostic) (Hoek *et al.* 1997). The prevalence of women suffering from clinically manifested POF is estimated as 0.9–1.1% (Coulam *et al.* 1986; Luborsky *et al.* 2003). In addition to well-defined and apparent POF, there is proposed the condition of premature ovarian aging (POA), which has an estimated prevalence of 10% (Nikolaou and Templeton 2004). While Gleicher *et al*. propose that POF is the most severe form of POA (Gleicher *et al.* 2009b), Welt suggests using primary ovarian insufficiency (POI) for being semantically more exact than POF, as POI encompasses the entirety of ovarian dysfunctions ranging from infertility to classic POF (Welt 2008). For practical reasons, however, POF is the contemporary term of reference.

The etiology of POF is heterogeneous as a broad spectrum of causes may lead to its development: chromosomal, genetic, autoimmune, metabolic/enzymatic (*e.g.* galactosaemia), toxic (*e.g.* smoking), infectious (*e.g.* mumps virus) and iatrogenic (Goswami and Conway 2005). However, in most POF cases, entitled idiopathic POF, mechanisms causing ovarian insufficiency remain unrecognized (Wittenberger *et al.* 2007). In the context of the chromosomal category, X-chromosomal aberrations constitute the majority, e.g. X monosomy in Turner syndrome, X-chromosomal deletions and translocations (Christin-Maitre *et al.* 1998). In the context of POF, several studies suggest the X-linked gene *FMR1* (fragile X mental retardation 1) is the most prevalent genetic cause of POF (Wittenberger *et al.* 2007; Gleicher *et al.* 2009a). At the same time Bachelot *et al.* find in a POF cohort study that *FMR1* related pathogenesis could be connected to only 4% of POF cases (Bachelot *et al.* 2009). Likewisely X-chromosomal factors several autosomal genes, involved in ovarian and follicular development, are causatives in relatively few POF patients, such as mutations of the FSH receptor gene, *BMP15*, *NR5A1* (van Dooren *et al.* 2009) and the autoimmune regulator (*AIRE*) gene (Laml *et al.* 2002).

2.3.2. Autoimmune background and autoantibodies in POF

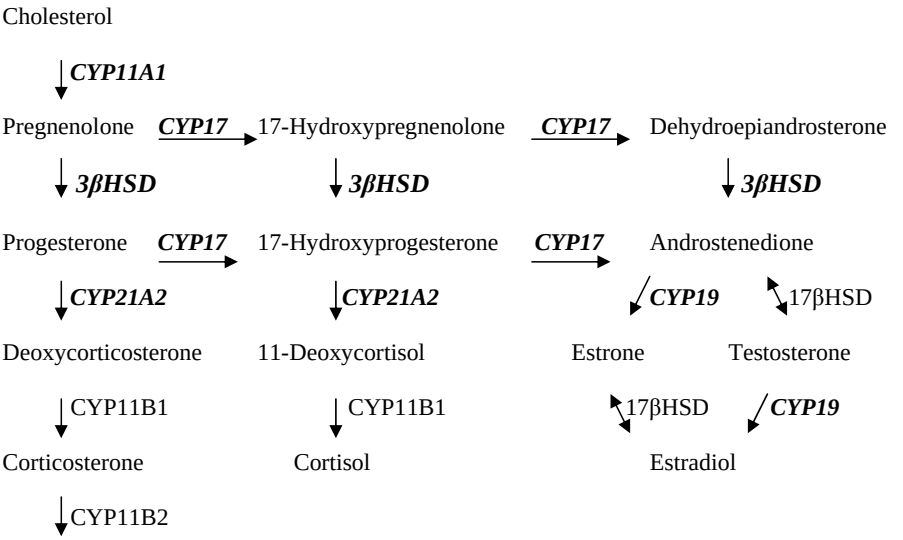
The majority of POF cases are diagnosed as idiopathic but the proportion of these arising from ovarian autoimmunity has long been hypothesized. The available data suggest that autoimmune mechanisms may be involved in pathogenesis in up to 30% of POF cases (Conway *et al.* 1996). HLA association consensus is not defined in isolated or idiopathic POF. Circumstantial evidence of ovarian autoimmunity in POF includes lymphocytic infiltration in ovarian follicle, frequent POF association with several other autoimmune diseases and familial tendency of POF (Fenichel *et al.* 1997; Hoek *et al.* 1997; Luborsky 2002). The POF association with Addison's disease is long known (Turkington and Lebovitz 1967; Irvine *et al.* 1968; Forges *et al.* 2004) and the most proven is the association of POF with adrenal autoimmunity and adrenal autoantibodies (Hoek *et al.* 1997; Falorni *et al.* 2002; Husebye and Løvås 2009). A number of other organ-specific and non-specific autoimmune diseases are occasionally associated with POF, with rates of occurrence varying according to the research,

i.e. 10–55% (Forges *et al.* 2004). However, frequent associations only occur with cases of thyroid diseases, Addison's disease and autoimmune polyendocrine syndrome type 1 as well as type 2 (APS-1 and APS-2, respectively) (Forges *et al.* 2004). Amongst autoimmune disease associations with POF, thyroid disorders are the most frequent, at 12–33% of POF patients (Betterle *et al.* 1993; Forges *et al.* 2004), while adrenal autoimmunity association is a great deal less frequent, 2–4% (Bakalov *et al.* 2005; Goswami and Conway 2005) but the association is robust (Hoek *et al.* 1997). Therefore autoimmunity in POF can be divided into two categories. First, POF cases associated with adrenal autoimmunity, which forms approximately 4% of all POF cases (Bakalov *et al.* 2002). As adrenal insufficiency, a potentially fatal disorder, manifests several years after the occurrence of POF (Turkington and Lebovitz 1967; Bakalov *et al.* 2005), the screening for adrenal autoimmunity is advocated for all spontaneous POF patients. True histologically confirmed autoimmune oophoritis in POF patients is so far firmly demonstrated only when POF is associated with adrenal autoimmunity or insufficiency (Bakalov *et al.* 2005). POF occurs in approximately 10% of female patients who have Addison's disease or APS-2 (Betterle *et al.* 1993; Betterle *et al.* 2004). In APS-patients, POF frequency is even more prevalent, 17–60% (Ahonen *et al.* 1990; Perheentupa 2006), but APS-1 is very rare.

The second group comprises POF cases associated with any other autoimmune diseases in absence of Addison's disease. A recently published large cohort study of 357 POF patients, which excluded karyotype abnormalities, like Turner's syndrome and iatrogenic etiology, shows a prevalence of 10.1% for associated autoimmune diseases, 2.3% for ovarian autoantibodies and 1.2% for adrenal autoantibodies (Bachelot et al. 2009).

The major autoantibodies in adrenal autoimmunity associated POF are proved as steroid-producing cell antibodies (SCA). SCA are directed against cytoplasmic antigens of steroid-producing cells in adrenal, gonads and placenta. SCA are detected as the distinctive fluorescence patterns within these tissue sections by indirect immunofluorescence assay (Hoek *et al.* 1997). The major molecular targets of SCA were identified as autoantigens in early nineties (Chen *et al.* 1996; Falorni *et al.* 2002; Dal Pra *et al.* 2003): the triad of steroidogenic enzymes from cytochrome P450 family: 21-hydroxylase (CYP21A2) (Winqvist *et al.* 1992), 17-hydroxylase (CYP17) (Krohn *et al.* 1992) and cholesterol side-chain cleavage (CYP11A1) (Winqvist *et al.* 1993).

All three enzymes are present together only in adrenal cortex tissues, CYP17 and CYP11A1 are present in gonadal steroid-producing cells (in ovaries and testes), but in the placenta only CYP11A1 is expressed (Uibo *et al.* 1994a). Among patients who have POF with adrenal autoimmunity, autoantibodies against these three target antigens are found in more than 90% of these cases, in contrast to approximately 10% of patients with non-adrenal autoimmunity and POF (Dal Pra *et al.* 2003). As evolvement of adrenal failure years later after POF manifestation is distinctive for APS-2, the future development of adrenal autoimmunity is impossible to exclude from the latter group.



Short name	Alternative names
CYP11A1	Cytochrome P450 cholesterol side-chain cleavage enzyme, P450scc
3βHSD	3-beta-hydroxysteroid dehydrogenase
CYP17	Cytochrome P450 steroid 17-alpha-hydroxylase, P450c17
CYP21A2	Cytochrome P450 steroid 21-hydroxylase, P450c21
CYP11B1	Cytochrome P450 steroid 11-beta-hydroxylase, P450c11
CYP11B2	Cytochrome P450 aldosterone synthase
CYP19	Cytochrome P450 aromatase
17βHSD	17-beta-hydroxysteroid dehydrogenase

Figure 1. Involvement of steroidogenic enzymes in the major pathways of steroid hormone biosynthesis. Enzymes are indicated by their short names, ***autoantigenic enzymes are in bold italics.***

Histological studies, in which ovarian biopsy showed the presence of oophoritis in almost all autoantibody-positive patients and the absence of signs of lymphocytic infiltration in autoantibody-negative patients have demonstrated the extreme accuracy of these steroidogenic enzyme autoantibody tests for autoimmune POF (Hoek *et al.* 1997; Bakalov *et al.* 2005).

In patients with idiopathic POF but with the absence of adrenal autoimmunity, the signs of endocrine autoimmunity are frequent: the most prevalent findings are thyroid (14%) and parietal cell (4%) antibodies (Hoek *et al.* 1997). These autoantibodies are also common in the normal population and, therefore, their significance regarding POF is moderate. The estimated co-occurrences of type 1 diabetes (T1D) and myasthenia gravis (or their diagnostic markers) with POF are both 2% (Hoek *et al.* 1997).

Circulating ovarian autoantibodies, investigated by different assays, have been reported in serum of both POF and infertility patients, and being reactive with granulosa cells, ovarian 'microsomal' antigens, thecal cells, zona pellucida and oocytes but few molecular autoantigens were characterized (Luborsky and Tung 2006). FSH and LH receptor antibodies, blocking or activating gonadotropin receptors in ovarial cells, are studied, but there is no clear evidence for them to be pathognostic in POF (Luborsky 2002).

Furthermore, an additional steroid cell enzyme, 3-beta-hydroxysteroid dehydrogenase (3βHSD) is reported as a target of SCA autoantibodies in POF (Arif *et al.* 1996). The enzyme is essential in the pathways of most steroids but does not belong to the P450 family (Figure 1). The study by Arif *et al*. indicates a fairly high prevalence of 3βHSD autoantibody (21%), assayed by immunoblot, in idiopathic POF (Arif *et al.* 1996). None of these POF cases had adrenalitis and only minority had other autoimmune disease, however, the 3βHSD autoantibody prevalence was relatively high (5%) also in the healthy control group. A later study using radioimmunoprecipitation (RIP) assay with enhanced analytical sensitivity provided a better diagnostic specificity and revealed 12% prevalence of anti-3βHSD in idiopathic POF patients who did not have Addison's disease (Arif *et al.* 2001). This study did not specify whether or not these patients had other autoimmune disease or autoantibodies accompanied with POF (Arif *et al.* 2001). Falorni *et al*. found a prevalence of 3βHSD autoantibody in 8% of Addison-related POF patients, none in the non-adrenal autoimmune disease related POF, 3% in isolated and idiopathic POF groups and 1% in healthy women (Falorni *et al.* 2002). Accordingly, the autoimmune background of idiopathic POF, especially in relation to the central steroidogenic enzyme 3βHSD, has still remained unclear.

2.4. Autoimmune polyendocrine syndrome type I (APS-I) and reproductive failure

2.4.1. Basic and clinical characteristics of APS-I

APS-1 is a rare monogenic autosomal recessive disorder caused by mutations of *AIRE* gene (Aaltonen *et al.* 1997; Nagamine *et al.* 1997). Clinical diagnosis of APS-1 requires the presence of at least two out the three main hallmarks of the syndrome: mucocutaneous candidosis, adrenocortical insufficiency and hypoparathyroidism (Perheentupa 2006). A broad spectrum of other clinical features of APS-1, mostly proved as autoimmune, may appear in childhood continuing into adolescence. The list of the APS-1 characterized components includes several endocrine and other disorders, among which the failure of gonadal function, especially in women, is one of the commonest (Ahonen *et al.* 1990; Perheentupa 2006; Husebye *et al.* 2009). Overall, the syndrome phenotype is extremely divergent. There is large variation in the disease components present and in the sequence of appearance over time (Perheentupa 2006; Lintas *et al.*

2008), which makes diagnosis of this rare syndrome more difficult. Therefore autoantibodies against interferon-alpha and interferon-omega, shown to be sensitive and sufficient specific markers for APS-1, have been suggested for use in the diagnostic process (Meager *et al.* 2006; Husebye *et al.* 2009); however these tests are not accessible for routine clinical studies. The HLA-association is shown with the individual disease components but not with the syndrome itself (Perheentupa 2006).

2.4.2. Immunological features of APS-1

APS-1 related research has already provided for biomedical sciences many significant implications and knowledge for understanding and resolving the enigma of the immune system and autoimmunity phenomena. The causative molecular pathology of APS-1 lies in the mutations of *AIRE* gene (Aaltonen *et al.* 1997; Nagamine *et al.* 1997), thereby paralyzing the central tolerance mechanisms in thymus. AIRE protein acts in thymus as a specialized transcription factor, allowing medullary thymic epithelial cells and dendritic cells to commit promiscuous gene expression (Peterson *et al.* 2008). This process consists in of expressing a large number of very distinct genes from the whole genome, including tissue-restricted proteins that are normally present only in restricted cell or tissue types in specialized peripheral organs, e.g. insulin in pancreatic beta-cells. Promiscuous gene expression in medullary thymic epithelial cells all together provides the aggregate resource of antigenic peptide fragments from virtually all the 'self' proteins of the organism (Holländer and Peterson 2009). These 'self' peptides can now be presented by MHC-molecules to T-lymphocyte precursors, developing in thymus. If a thymocyte's T-cell receptor and the presented 'self' peptide's interaction is sufficiently affine, the potentially autoimmune thymocyte is directed to apoptosis. This negative selection process of central tolerance is defective due to *AIRE* mutations in APS-1 patients.

T-lymphocytes are shown to contribute more than autoantibodies to induce autoimmune damage of endocrine targets in autoimmune endocrine diseases. Target tissue infiltrations by pathogenic autoreactive T-lymphocytes are similarly shown in APS-1 cases. However, the APS-1 hallmark is also the presence of extremely high levels of the corresponding autoantibodies, targeted mostly against intracellular key enzymes in the endocrine system (Söderbergh *et al.* 2004; Bensing *et al.* 2007). The large variation in the syndrome components is also reflected in a wide variety of autoantibodies, which can be found in the patients' blood. Many of the organ-specific autoantigens of APS-1 are identified by the approach of cDNA expression library immunoscreening with APS-1 patients' sera, known to contain autoantibodies against the cells of interest in endocrine glands or other tissues (Table 1). Some of these autoantigens, for example CYP17 (Dal Pra *et al.* 2003), are known targets of autoimmunity outside the APS-1 phenomenon. This underlines the usefulness of APS-1

patients' sera to detect novel autoantigens that represent molecular targets for autoimmune tissue destruction in APS-1, but also in autoimmune diseases related to single tissue or particular cell type destruction.

Table 1. Autoantigens identified by cDNA library immunoscreening with APS-1 patients' sera

Study	Screened cDNA library origin	Identified Autoantigens	Autoantibody-associated disease components or affected tissues in APS-1
Krohn *et al.* 1992	Human adrenal	Cytochrome P450 17-alpha-hydroxylase (CYP17)	Adrenal, gonads
Rorsman *et al.* 1995	Rat insulinoma	Aromatic-L-amino-acid decarboxylase	Liver, beta-cells in pancreas
Ekwall *et al.* 1998	Human duodenum	Tryptophan hydroxylase	Liver, intestinal dysfunction
Hedstrand *et al.* 2000	Human scalp	Tyrosine hydroxylase	Alopecia
Hedstrand *et al.* 2001	Human scalp	Transcription factors SOX9 and SOX10	Vitiligo
Bensing *et al.* 2007	Human pituitary	TDRD6 (tudor domain containing protein 6), mostly expressed in testis	No known associations with clinical manifestations
Alimohammadi *et al.* 2008	Human parathyroid	NALP5 (NACHT leucine-rich-repeat protein 5)	Hypoparathyroidism, ovarian insufficiency
Alimohammadi *et al.* 2009	Bovine cDNA library	KCNRG (putative potassium channel regulator)	Respiratory symptoms

2.4.3. APS-1: hypogonadism and autoantibodies

The prevalence of hypogonadism and ovarian failure in APS-1 female patients is age related: Perheentupa found a prevalence of 35% in women up to 15 years old, 53% in 16 to 20 years old and 69% in 21 to 40 years old (Perheentupa 2006). Ovarian failure is associated with adrenal failure (Perheentupa 2006), the pattern similar to POF with APS-2 syndrome. SCA and autoantibodies against the steroidogenic enzymes CYP11A1, CYP17, CYP21A2 are associated and good predictors of ovarian failure in APS-1 patients (Peterson *et al.* 1997; Betterle *et al.* 1998; Söderbergh *et al.* 2004).

Testicular failure is less prevalent in APS-1 patients, occurring in 8% of men aged up to 20 years old and 28% of 21–40 years old, and is heterogeneous: hypogonadotrophic testicular failure, azoospermia with otherwise normal sexual function, primary testicular atrophy were diagnosed (Perheentupa 2006). In contrast to female patients, there is no such clear-cut association with gonadal dysfunction with the presence of SCA in male patients. Only half of APS-1 male patients with hypogonadism have the autoantibodies to CYP11A1 or CYP17 (Uibo *et al.* 1994a; Söderbergh *et al.* 2004). Whilst male infertility in APS-1 can be successfully treated with immunosuppressant (Tsatsoulis and Shalet 1991), studies on male infertility-related autoimmunity and the search for relevant testicular autoantigens has not provided as much in-depth knowledge as for female hypogonadism in APS-1 (Söderbergh *et al.* 2004). Still, studies on the characterization of testicular autoantigens, specific for APS-1, are well justified.

3. AIMS OF THE STUDY

The aims of the present study were:

1. To investigate the character and prevalence of common autoantibodies as general markers of autoimmune disorders in women with reproductive failure of various clinical backgrounds;

2. To investigate the autoantigenic targets in female hypogonadism associated with POF and APS-1;

3. To search autoantigenic targets in male autoimmune hypogonadism, using sera from male patients with APS-1.

4. MATERIALS AND METHODS

4.1. Groups of patients and controls

The sources of clinical material analyzed in this study came from University hospitals in three countries, Estonia, the UK and Finland and formed the bases for three separate but linked studies, Papers I, II and III, (see Table 2). The three studies analyzed sera from a total of 344 patients and 579 healthy controls. The first study (Paper I) using a range of autoantibodies for characterization the autoimmune profiles of women with diverse causes of infertility. The second study (Paper II) focused on 3βHSD autoantibodies in women with POF either idiopathic or associated with APS-1. The third study focused on autoantigenic targets associated with male autoimmune hypogonadism in APS-1. Physicians at clinics of the University Hospitals of Tartu, Tampere and Helsinki and the Sheffield Teaching Hospitals conducted the clinical investigations and made the diagnoses. All sera used for antibody investigations were stored at least at minus 20°C. Table 2 presents the distribution by key medical characteristics of patients and controls involved in the three studies.

4.1.1. Patients and control groups for investigation of autoantibodies in females with reproductive failure (Paper I)

This study group consisted of total 108 consecutive female patients with infertility (Group AI, Table 2): 16 patients with polycystic ovaries (PCO), 20 patients with polycystic ovary syndrome (PCOS), 38 with endometriosis, and 34 patients either with chronic anovulation, luteal phase insufficiency or unexplained infertility. All the patients were seen at Tartu University Hospital. The duration of their infertility was at least one year and the mean age of the women was 27.5 years (age range 17–43 years).

Diagnoses of PCO and PCOS were performed by clinical, hormonal and endoscopical investigations. Transabdominal ultrasonographic examinations, employing Adams *et al.*'s ultrasonographic definition (Adams *et al.* 1986), using either the full-bladder technique or transvaginal ultrasound imaging were performed between days 1 and 10 of the patients' menstrual cycle or after irregular bleeding. PCO was diagnosed as a condition with ovaries of normal or enlarged size filled with 10 or more cysts (diameter 2–8 mm), but without the stromal tissue increase, clinical symptoms and hormonal changes, which are characteristic signs of PCOS.

Table 2. Study and Control Groups, Sources of Research Material, Institutions, Identified Autoantibodies and Methods

Study group	Group details	Institutions of collections origin	Major autoantibodies identified and *Method of identification*
PAPER I			
AI) Women with heterogeneous reproductive failure	Estonian Ages 17–43 years Mean age 27.5 y. (n=108)	University of Tartu, Women's Clinic	Common autoantibodies[1] ***by IIF*[2]**; anti-CA[1] and anti-β2GPI[1] ***by ELISA*[2]**; SCA ***by IIF*[2]**
BI) Healthy Control group – women	Randomly selected from volunteers, Estonian, Ages 17–43 years, Mean age 31.0 y. (n=392)	University of Tartu, Department of Immunology (Biobank)	
PAPER II			
AII) Women with heterogeneous POF[3] reproductive failure	British; (n=48)	University of Sheffield clinics	SCA ***by IIF*[2]** Anti-3βHSD[1] ***by RIP*[2]**
BII) Disease control group – Women with autoimmune diseases APS-1[3], APS-2[3], T1D[3], Addison's disease,	British and Finnish; (n=90)	University of Sheffield and University of Tampere clinics	
CII) Healthy control group of women	British and Finnish volunteers (n=71)	University of Sheffield and University of Tampere clinics	
PAPER III			
AIII) Women and Men with APS-1	Finnish (Men, n=40 Women, n=26)	University of Helsinki and University of Tampere clinics	Anti-TSGA10[1] ***by RIP*[2] *and immunoblot***
BIII) Disease Control Group - Women and Men with Addison's disease	Estonian, (Men, n=13 Women, n=19)	University of Tartu, Department of Immunology (Biobank)	
CIII) Healthy control group of women and men	Finnish and Estonian volunteers and blood donors (Men, n=70 Women, n=46)	University of Helsinki and University of Tartu clinics	

Notes:
1 – ANA, SMA, PCA, TMA, ARA, AMA, LKMA; CA= Carbonic anhydrase; β2GPI = β2-glycoprotein I; 3βHSD = 3-beta-hydroxysteroid dehydrogenase; SCA = Steroid-producing cell antibodies; TSGA10 = Testis-specific protein 10
2 – ***IIF = Indirect immunofluorescence assay, ELISA = Enzyme-linked immunosorbent assay; RIP = Radioimmunoprecipitation***
3 – POF = Premature ovarian failure; APS-1 = Autoimmune polyendocrine syndrome type 1; APS-2 = Autoimmune polyendocrine syndrome type 2; T1D = Type 1 Diabetes.

Endometriosis was diagnosed according to The American Fertility Society classification (American Fertility Society 1985). Laparoscopic investigation of endometriosis included thorough pelvic examination, with multiple biopsies of any abnormal tissue. The diagnosis for luteal phase defect was established on basal body temperature and progesterone values of plasma.

The diagnoses in the unexplained infertility group were done *per exclusionem*. The methods of microbiological analyses were used for exclusion of sexually transmitted diseases as cause of infertility; laparoscopic examination for exclusion of tubal factor and andrologist consultation with semen analysis for exclusion of any possible male factor of infertility..

Sera from 392 women from a general population sample, based on Uibo *et al.*'s exploration (Uibo *et al.* 1995), were randomly selected as a control group (Group BI, Table 2). The mean age of this group was 31.0 years (age range 17–43 years). Commonly analyzed autoantibodies (referred to by clinical immunologists as common autoantibodies) like anti-nuclear antibodies (ANA), smooth muscle antibodies (SMA), parietal cell antibodies (PCA), thyroid microsomal antibodies (TMA), anti-reticulin antibodies (ARA), antimitochondrial antibodies (AMA) and liver-kidney microsomal antibodies (LKMA) were detected from amongst these sera. A randomly selected control group of 31 of these 392 sera was selected for the tests of SCA, β2GPI and carbonic anhydrase (CA) autoantibodies.

4.1.2. Patients and control groups for establishing the prevalence of 3-beta-hydroxysteroid dehydrogenase (3βHSD) autoantibodies with POF (Paper II)

This study analyzed serum samples taken from 48 consecutive Caucasian women with POF, 1992–1996, at Sheffield Teaching Hospitals, in the UK (Group AII, Table 2). The age range was 17–67 years, the mean age was 36 years (+/- 11 years) and 8 of the 48 women were older than 40 years. This study defined POF as hypergonadotropic amenorrhoea diagnosed on at least two occasions and amenorrhea occurring for at least six months. There was no apparent cause of POF or any associated disease in 30 of the group; 7 had an iatrogenic or genetic (Turner's syndrome) etiology, and 11 had an associated disease (Addison's disease in 6, autoimmune thyroid disease in 5). A Disease Control Group (Group BII, Table 2) was formed consisting of sera from women with: isolated Addison's disease (33 British Caucasians and 5 Finns), APS-1 (15 Finns), APS-2 (9 Finns) newly diagnosed T1D (28 Finns). In addition a Healthy Control Group (Group CII, Table 2) of volunteers was formed comprising 48 British Caucasians and 23 Finns.

4.1.3. Patients and control groups for investigation of the autoantigenic targets of male autoimmune hypogonadism (Paper III)

This study used sera from 66 Finns with APS-1 (40 men, 26 women) as the study group (Group AIII, Table 2). Testicular hypofunction (azoospermia or testicular atrophy) was diagnosed in 5 of the men and ovarian dysfunction (primary or secondary amenorrhoea) was diagnosed in 12 of the women. Autoantibodies against either or both CYP17 and CYP11A1, tested by RIP assay with recombinant autoantigens (Peterson *et al.* 1997), were found in 1 of the 5 men diagnosed with testicular hypofunction and in all 12 women diagnosed with hypogonadism.

This study required two control groups. The first, the Disease Control Group (Group BIII, Table 2) analyzed sera from Estonians with autoimmune Addison's disease (13 men and 19 women). The second, the Healthy Control Group (Group CIII, Table 2) analyzed sera from Finnish volunteers (20 men) and 96 Estonian blood donors (50 men and 46 women).

Six APS-1 male patient sera were selected from these serum samples of Group AIII for cDNA library immunoscreening. Five were selected on the basis of clinical signs of hypogonadism and one for giving a weak positive immunofluorescence reaction with testis tissue substrate. Two of the selected sera had been previously tested by RIP (Peterson *et al.* 1997) to be positive for autoantibodies to CYP11A1, and one of them was also positive for CYP17.

4.2. Methods used for identifying common autoantibodies

4.2.1. Indirect immunofluorescence assay for detection of autoantibodies

Indirect immunofluorescence (IIF) method was used to assess common autoantibodies: ANA, SMA, PCA, TMA, ARA, AMA and LKMA. Rat liver and kidney, mice stomach and human thyroid tissue in 5 μm cryostat sections were used as antigenic substrates. SCA were detected on monkey ovary and adrenal tissue sections (Bio-Systems, Spain) as well as on cryostat sections of human (0-group) placenta. Sera were diluted 1:5 (SCA) or 1:10 and 1:100 (common autoantibodies). As a secondary antibody, FITC-conjugated rabbit *anti*-human IgG (DAKO, Denmark) in a 1:40 dilution was explored. Test results were viewed using a Jenalumar UV microscope (eyepiece 6.3×, objective 25×). All tests included known autoantibody positive and negative sera. An internal quality assessment scheme at Department of Immunology, University of Tartu, audited the performance of all antibody tests and an external quality assessment of UK NEQAS (Sheffield, UK) audited the tests for ANA, SMA, PCA, TMA, ARA and AMA.

4.2.2. ELISA for detection of autoantibodies

The study used the University of Tartu, Department of Immunology's in-house ELISA to detect antibodies against β2GPI and CA. A polystyrene 96 well microtiter plate (Combiplate 8, Biohit, Finland) was coated with a 5 μg/ml β2GPI solution (Crystal Chem. Inc., Chicago, IL) in borate buffered saline (200 mM H_3BO_3, 75 mM NaCl, pH 8.4) at 50 μl per well. After overnight incubation at +4°C, wash and blocking with 0.5% bovine serum albumin and 0.4% Tween-20 for 1.5 hours at room temperature took place. The sera diluted at 1:100 were inserted to react for 2 hours at room temperature. After incubation with alkaline phosphatase-conjugated anti-human IgG (DAKO, Denmark) for 1 hour at room temperature the color was developed by adding substrate, *p*-nitrophenyl phosphate 1 g l^{-1} in 1 M diethanoleamine buffer (pH 9.8), and the absorbances were read at 405 nm with 492 nm subtraction. Antibody levels were expressed in enzyme-immunological units (EIU), calculated as follows – [(sample OD−negative control OD)/(positive control OD−negative control OD)×100], EIU values of from 10–29 were counted as positive, and those above 30 as strongly positive.

ELISA for anti-CA was performed as described by D'Cruz *et al*. (D'Cruz *et al.* 1996). Microtitre plates were coated with 5 μg/ml of human CA type I and II (Sigma Comp., USA) overnight at +4°C. After blocking, diluted 1:100 patients' sera were incubated overnight. Subsequent steps – incubation with secondary antibody, color development and measuring, antibody levels expression in EIU – followed the same pattern as for the anti-β2GPI assay described above. The study used only type I CA due to the high rate of non-specific reactions with type II CA in the healthy control group (Group BI, Table 2).

4.3. Methods used in determination of autoantibodies against 3βHSD

The human 3βHSD partial cDNA encoding C-terminal 287 amino acids (AA) from a total of 373 was a gift from Dr. B. Murry (University of Texas, Dallas). In order to get the full-length cDNA, the missing N-terminal fragment of the human 3βHSD cDNA was amplified from human placental cDNA by PCR with 3βHSD-specific primers. The restriction enzymes *Eco*RI and *Kpn*I were used for fragment fusion and subcloning to vector pGEM3, yielding two constructs: pGEM3βHSD (containing full-length cDNA) and pGEM3βHSD-C (containing 287 AA from the C-terminus encoding cDNA). Both plasmid constructs were sequenced with T7 primer to confirm the correct orientation and reading frame of the cDNAs. The pGEM3βHSD and pGEM3βHSD-C were transcribed with T7 ribonucleic acid polymerase and translated into [35S]-cysteine (Amersham International, Aylesbury, UK) labelled protein with an *in vitro* transcription-translation kit (TNT-kit, Promega Corp., Madison, WI) according to the manufacturer's instructions. The translation products were further purified by

Sephadex G-50 column chromatography and were analyzed by SDS-PAGE and autoradiography.

In each assay the translation mixture of the labeled protein (20,000–50,000 cpm) was suspended in 50ml RIP buffer [20 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 0.1% Triton X-100, and 10 mg/ml aprotinin] and incubated with diluted serum (1:10 in RIP buffer) for an hour at room temperature. 50 µl of Sepharose Fast Flow protein G (Pharmacia Biotech, Uppsala, Sweden) 20% slurry in RIP buffer was added and incubated for an hour at room temperature with shaking. The immune complexes were washed four times by centrifugation and analyzed by SDS-PAGE with autoradiography. Rabbit IgG-enriched antiserum against human 3βHSD was obtained from Dr. Ian Mason (University of Edinburgh, Edinburgh, UK) and has been proved to be specific for 3βHSD.

IIF assay for SCA was performed as described by Uibo *et al.* (Uibo *et al.* 1994a). Human adrenal, placenta, ovary at follicular stage, and monkey (*Macaca fascicularis*) testis tissues were used as antigenic substrates. Cryostat sections (6 µm) were prepared and incubated with test sera at dilutions of 1:5 to 1:100 and were visualized by fluorescent secondary antibody (FITC-conjugated rabbit antihuman IgG, DAKO Corp., Copenhagen, Denmark).

4.4. Methods used for investigation of the autoantigenic targets, associated with male autoimmune hypogonadism in APS-I

4.4.1. Immunoscreening of testis cDNA expression library

Human testis cDNA library in lambda-TriplEx vector (Clontech, Palo Alto, CA, USA) was screened by plating out 2×10^6 plaque-forming units. For library immunoscreening, six sera from male APS-1 patients were selected according to clinical data and absence of other known autoantibodies reactive with human testis (CYP17, CYP11A1). The selected six sera were pooled to get the final dilution of 1:50 for every serum in 20mM Tris-HCl (pH 7.5), 150 mM NaCl buffer containing 0.25% NaN_3, 5% skimmed milk and 0.05% Tween-20 (TBS-MT). In order to reduce the non-specific background due to presence of E.coli antibodies, sera were preabsorbed with sonicated lysate of the bacteriophage plating bacteria (E.coli XL-1 Blue). This TBS-MT buffer was used for the nitrocellulose filter (NitroBind 0.45µm, Osmonics, Minnetonka, MN, USA) blocking, for secondary antibody dilutions as for washing steps. After overnight incubation with the primary antibody and subsequent extensive washings, the filters were incubated with biotinylated anti-human IgG, supplemented with HRP-Avidin D (both from Vector Laboratories, Burlingame, CA, USA). Prior to color development with 4-chloro-1-naphthol, the filters were extensively washed with TBS-MT and once with TBS. The positive clones were picked up from the plates and repeatedly re-screened until pure clone isolates were

obtained. The false-positive bacteriophage isolates were excluded by testing all clones with the secondary antibody alone and comparing the results with replicas obtained by immunoscreening. The positive bacteriophage lambda-TriplEx clones were converted to plasmid pTriplEx clones as specified in the library manual. The DNA inserts of bacteriophage clones were sequenced by ABI PRISM 310 with a BigDye sequencing kit (PE Applied Biosystems, Foster City, CA, USA) and the sequences were identified by similarity search with BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

4.4.2. Molecular cloning of TSGA10 cDNA

The testis cDNA library immunoscreening yielded several clones, containing TSGA10 cDNA fragments but none of full length. Therefore, normal male testis RNA pool was reverse transcribed by a FirstStrand cDNA synthesis kit (Fermentas, Vilnius, Lithuania). The full-length TSGA10 cDNA was amplified from the testis cDNA with the TSGA10-specific primers and Pfu DNA polymerase (Fermentas, Vilnius, Lithuania), and further cloned into two expression vectors, pGEX1ZT-SH3 (a gift from Dr. Kalle Saksela, Univ. of Tampere) and pcDNA3.1/myc-His(-)B (Invitrogen, Carlsbad, CA, USA). The constructs were verified by restriction enzyme analysis and DNA sequencing from both directions of the inserted fragments. The [S-35]-radiolabeled *in vitro* transcribed-translated TSGA10 protein was produced with pcDNA3.1-TSGA10 plasmid and TNT T7-coupled reticulocyte lysate system (Promega, Madison, WI, USA), followed by purification with Sephadex G-25 column (NAP-5 columns, Amersham Biosciences, Uppsala, Sweden). For bacterial expression as a GST-fusion protein, the E.coli strain DH5 transformed with pGEX-TSGA10 plasmid was induced by 1 mmol/L isopropyl-beta-D-thiogalactopyranoside. The bacterial pellet was lysed by ultrasonication and the recombinant GST-fusion protein was purified by Glutathione Sepharose (Amersham Biosciences, Uppsala, Sweden).

4.4.3. Immunoassays for the detection of TSGA10 autoantibodies: immunoblotting and radioimmunoprecipitation test

Immunoblotting with purified TSGA10+GST fusion protein was performed using Mini-Protean II and Mini Trans-Blot apparatuses (BioRad, Hercules, CA, USA), 0.45μm pore nitrocellulose membrane (Hybond-ECL, Amersham Biosciences, UK) and membrane blocking with 4% skimmed milk in TBS. The membrane strips were incubated with 1:100 sera dilutions overnight at +4°C. Rabbit polyclonal anti-GST antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at dilution 1:1000 was used as a positive control for the recombinant protein. After the washing steps, the strips were treated with alkaline phosphatase-conjugated secondary antibodies against human IgG (rabbit) or

against rabbit immunoglobulins (swine; both from DAKO, Glostrup, Denmark) and the immune complexes were visualized by BCIP/NBT color development.

The radiolabelled TSGA10 protein (40 000 cpm per sample) in 50 µl RIP buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.4, 5 mM EDTA, 0.05% Tween-20, 1% gelatine hydrolysate) and human serum samples (50 µl 1:10 diluted) were mixed in 1.5 ml microcentrifuge tubes and then incubated at room temperature for 15 minutes. Then 50 µl protein G Sepharose (Amersham Biosciences, Uppsala, Sweden) 15% slurry in RIP buffer (vol/vol) was added to each sample and incubated in overhead mixing for 45 minutes. Samples were washed six times with 1 ml RIP buffer by centrifugation and aspiration. Finally, the test tube bottoms containing protein G Sepharose were sliced into scintillation vials, scintillation fluid was added and scintillation counted by Wallac Guardian 1414 (Wallac OY, Turku, Finland). For the positive control we used the serum from an APS-1 patient that was included in the pool of sera used for library immunoscreening and showed the highest reactivity, whereas the negative control was a TSGA10 autoantibody negative APS-1 serum. Samples were analyzed in duplicates and the results were expressed as a TSGA10 radioimmunoprecipitation relative units (RU, $((\text{cpm sample} - \text{cpm}_{\text{negative control}})/(\text{cpm}_{\text{positive control}} - \text{cpm}_{\text{negative control}})) \times 100$). An index of 6 units was chosen as the upper limit of normal results based on the results of the healthy controls.

4.4.4. Immunofluorescence with testicular tissue

The TSGA10 positive and TSGA10 negative sera reactivity toward testicular tissue in IIF was evaluated at sera dilutions in 1:10 in phosphate buffered saline. Rat testicular tissue 5 µm cryosections was used as antigenic substrate. Secondary antibody, rabbit anti-human IgG conjugate with Alexa 488 fluorophore (A21218, Molecular Probes Inc., Eugene, OR) was used in 1:400 dilution.

4.5. Statistical methods

Fisher's exact test and Wilcoxon Mann-Whitney (U-) test were used for statistical analysis with the R language and environment for statistical computing (R Development Core Team 2006). A χ^2-test was used for statistical analysis of estimating the prevalence of antibodies in female reproductive failure and differences were read as significant if the value of '*P*' was less than 0.05.

4.6. Ethical aspects

The Ethics Committee of Medical Research of the University of Tartu and the relevant ethics committees of the other hospitals from where the study material originated approved the studies. Informed consent was obtained from all the participants in the research programme.

5. RESULTS

5.1. The prevalence and character of autoantibodies in women with reproductive failure (Paper I)

The most significant outcome of the study was that women in the reproductive failure group (Group AI) have significantly more non-organ-specific common autoantibodies (40.7%) than in the age matched control group (Group BI) women (14.8%) measured by indirect immunofluorescence assay using a serum dilution of 1:10. The most frequently detected autoantibodies in reproductive failure Group AI were ANA (13.9%) and SMA (27.8%) and in control Group BI ANA (3.6%) and SMA (5.1%). These paired values were significantly different ($P<0.005$). The autoantibody TMA occurred in two of Group AI's 108 patients (1.9%), which did not differ significantly from TMA frequency among the control Group BI (3.8%, $P>0.05$). No cases with AMA and LKMA were revealed, whereas PCA were detected in one patient ($P>0.05$ in comparison to the control Group BI).

SCA antibodies were detected in only one female patient with PCOS and primary infertility. This serum showed autoantibody reactivity with placental, but not with adrenal or ovarian tissue sections, at a serum dilution of 1:5.

Anti-β2GPI were found in eight cases (7.4%), including two INF patients without other autoantibodies. Anti-CA were revealed in nine (8.3%) cases including PCOS, E and INF patients. There were no significant differences either in anti-β2GP-I or anti-CA frequencies between patients with PCO, PCOS, E and INF ($P>0.05$). Clinical data comparisons with these antibody assay results did not show any significant associations.

5.2. 3βHSD autoantibodies in POF and APS-1 patients (Paper II)

When evaluating the prevalence of SCA and 3βHSD autoantibodies in POF (Group AII) and APS-1 (Group BII), 2 of the 48 (4%) POF patients had SCA in their sera; in addition, 2 patients had adrenocortical antibodies (ACA). Both SCA-positive patients and one ACA-positive patient had Addison's disease. A second ACA-positive POF patient had autoimmune hypothyroidism. SCA were found in 11 of 15 (73%) patients with APS-1 (Group BII) whereas SCA was not found in any of the Healthy Control Group CII.

The *in vitro* translated and [35S]-labeled full-length and C-terminal 3βHSD products appeared as the expected 42-kDa and 31-kDa bands, respectively, in SDS-PAGE and autoradiography. As a positive control, rabbit antiserum against human 3βHSD clearly recognized both 35S-labeled 3βHSD proteins in an immunoprecipitation assay. During immunoprecipitation with human sera, one SCA-positive POF serum (Group AII) precipitated the full-length 3βHSD

product (1 of 41 idiopathic POF; prevalence 2.4%). A second SCA-positive serum as well as the remainder of the 46 POF in Group AII sera remained negative. In addition, 3 of 15 (20% prevalence) APS-1 sera that were all positive for SCA (Group BII) reacted with the full-length 3βHSD product. None of the 71 sera of the Healthy Control Group BII was able to precipitate the 3βHSD product. When the 287 AA long C-terminal part of the 3βHSD protein was used in immunoprecipitation, autoradiography did not reveal any positive bands, indicating that the epitopes (or residues) needed for autoantibody binding (or conformational epitope folding) locate within the 86 AA from the N-terminal part of the protein. As 10 APS-1 sera (Group BII) were positive for anti-CYP17 and 12 APS-1 sera (Group BII) were positive for CYP11A1 antibodies, POF patient sera (Group AII) were analyzed for the presence of both CYP17 and CYP11A1 antibodies. However, no antibody reactivity with these sera was observed with CYP17 and CYP11A1 autoantigens.

5.3. Testis expressed protein TSGA10 as target of autoantibodies in APS-1 patients (Paper III)

The immunoscreening of human testis cDNA library with APS-1 male sera pool yielded altogether ten recombinant λ-phage clones. Inserts of the clones were sequenced and identified by database searches. Eight of the ten positive clones contained cDNA fragments identical to human testis specific gene TSGA10 mRNA (accession NM 025244) as revealed by DNA sequence similarity analysis with BLAST program. Entire inserts of these eight clones corresponded to the mRNA coding regions and all fragments ended at the XbaI restriction enzyme site that was located within the cDNA. As during the cDNA library construction, the XbaI digestion was used for the cloning of the 3' ends of inserts, all purified clones lacked the sequence encoding the last 132 C-terminal AA. At their 5' ends, the clones were different by insert size with the shortest fragment encoding for 175 AA and the longest for 494 AA from the total 698 AA of full-length TSGA10 protein. In addition, an alternative splicing was detected in two of the cDNA clones as one fragment lacked the exon 15 and another lacked the exon 11.

TSGA10 full-length cDNA was amplified with the gene-specific primers from the human testis mRNA sample and cloned further to pGEX1ZT expression vector as to pcDNA3.1 expression vector. The latter was transcribed with T7 RNA polymerase and the *in vitro* translated and [S-35]-radiolabeled product appeared as expected as 82 kDa band after SDS-PAGE and subsequent autoradiography.

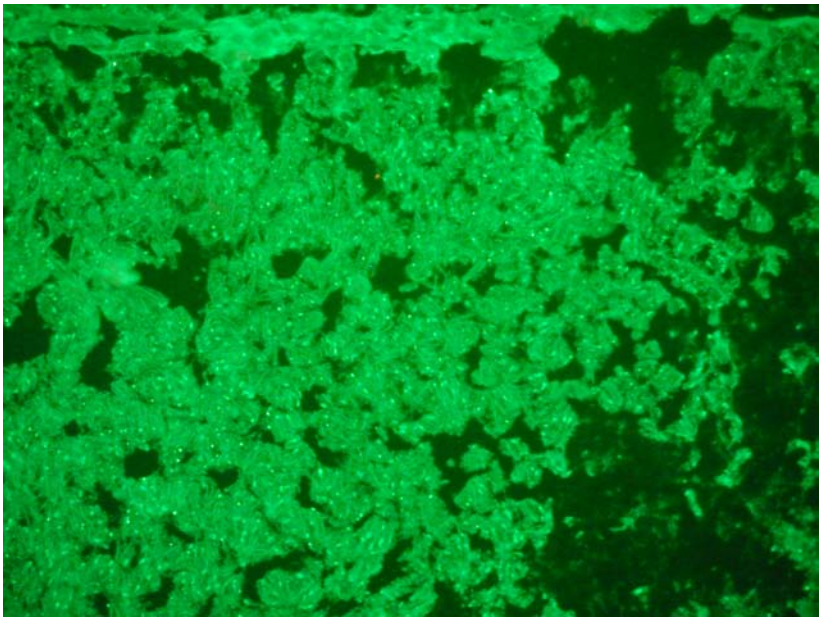
Bacterially expressed and glutathione-affinity purified GST-TSGA10 fusion protein was used as an antigen in immunoblotting with 5 APS-1 male sera (Group AIII) and 34 sera from healthy controls (Group CIII). In addition to one recombinant 108 kDa (TSGA10 82 kDa + GST 26 kDa) protein band several smaller bands appeared in Coomassie stained SDS-PAG. According to

immunoblot analysis there were no differences between the APS-1 (Group AIII) and the healthy control (Group CIII) sera immunoreactivity, indeed the majority of the control sera demonstrated the same pattern of immunoreactivity as APS-1 sera.

Radioimmunoprecipitation analysis of serum samples from three (7.5%) male and two (7.7%) female APS-1 patients (Group AIII) showed antibody reactivity against full-length TSGA10 protein above the cut-off value of 6 units of index. Higher index values were revealed in TSGA10 antibody positive males (92, 60 and 20 RU) than females (11 and 10 RU). All the sera of the healthy control group (CIII), of either Finnish or Estonian origin, gave negative values and the frequency of antibodies was significantly different from that in APS-1 Group AIII ($p=0.0055$). Also there was a statistically significant difference in the RIP index values between APS-1 and the healthy control group ($p= 0.039$).

None of the three TSGA10 antibody-positive APS-1 male patients in Group AIII had any clinical signs suggesting testicular failure, but on the other hand, none of the five APS-1 males with clinical signs of hypogonadism had antibody reactivity against TSGA10. However, one of the TSGA10 antibody positive males had these autoantibodies in all four sera available from a 26 year follow-up medical record. Indirect immunofluorescence of his sera and of a serum from another patient with antibodies against TSGA10 revealed a speckled staining pattern in the cytoplasm of spermatocytes/spermatids on testicular tissue (Figure 2). Although this was distinguishable from patterns seen with a number of TSGA10 antibody negative sera, other sera with TSGA10 antibodies did not reveal such definite immunofluorescence characteristics. The second TSGA10 antibody positive patient had sera from 18 year period and all three samples contained these antibodies; the third TSGA10 antibody positive male patient had, according to a 25 year follow-up medical record, developed antibodies at the age of 33 years. One of the two TSGA10 antibody positive APS-1 female patients (Group AIII) had secondary amenorrhea at the age of 18.5 years and gonadal insufficiency. None of the other 11 female APS-1 patients with hypogonadism had TSGA10 antibodies. Also, there was no statistically significant correlation between TSGA10 antibody positivity and gonadal failure or antibodies to CYP11A1 and CYP17 in the studied sera. Hence the presence of TSGA10 antibodies does not have any correlation with testicular or ovarian failure or autoimmune hypogonadism markers in APS-1 patients.

A1



A2

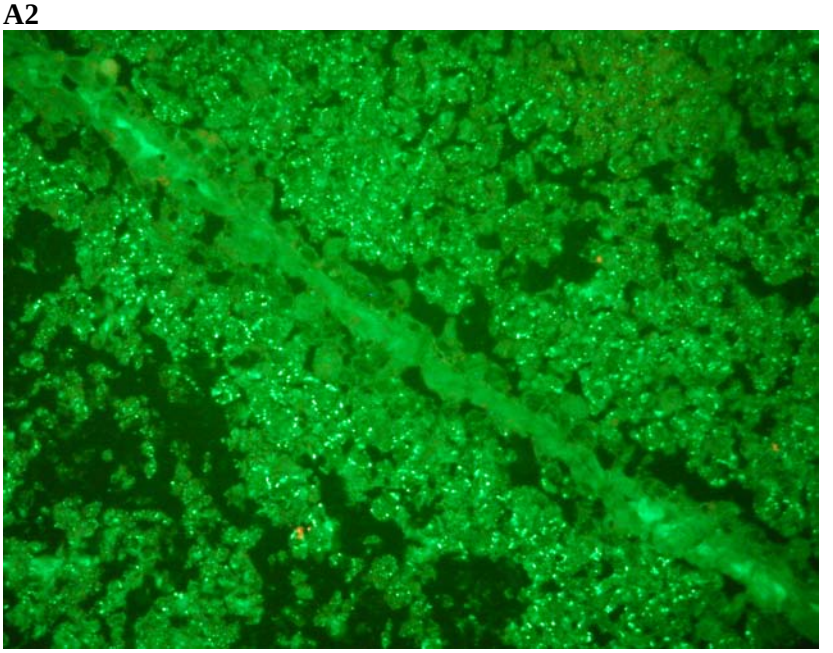
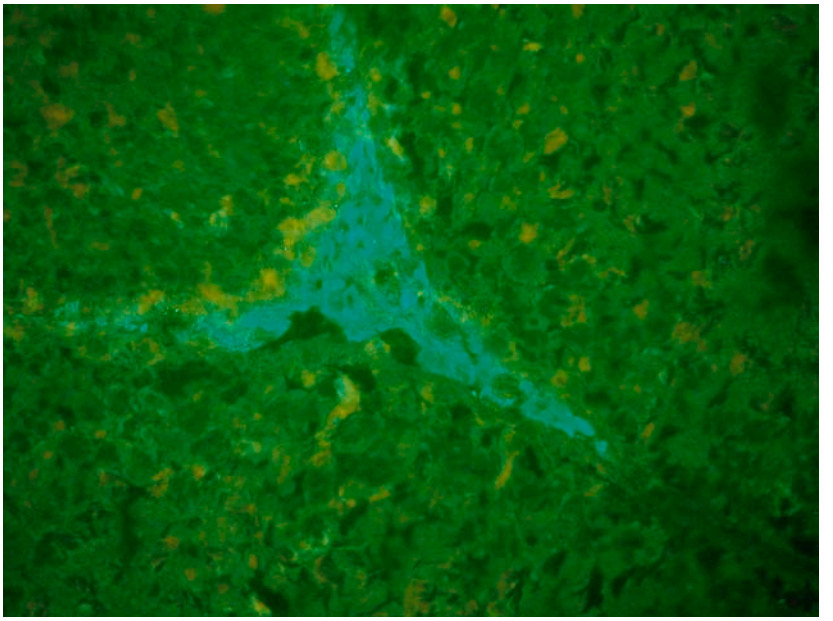


Figure 2a. Indirect immunofluorescence tests results with autoimmune polyendocrine syndrome type 1 (APS-1) sera. The antigenic substrate is rat testicular tissue, 40x magnification. (**A1, A2**) Two testis-reactive and TSGA10 antibody positive sera gave speckled staining pattern in spermatocytes/spermatids cytoplasm.

B



C

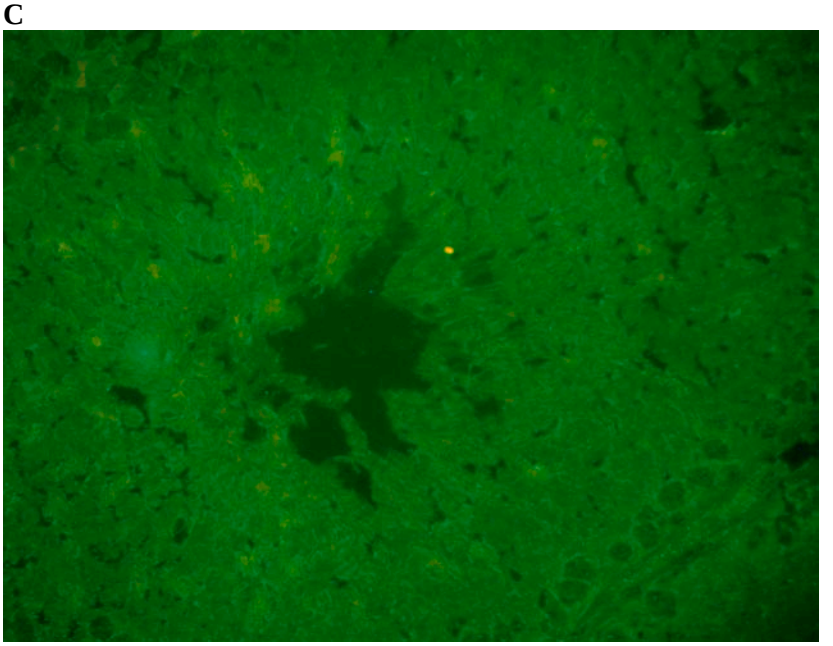


Figure 2b. Indirect immunofluorescence tests results with autoimmune polyendocrine syndrome type 1 (APS-1) sera. The antigenic substrate is rat testicular tissue, 40x magnification. (**B**) Testis-reactive but TSGA10 antibody negative serum with Leydig cell staining pattern; (**C**) Control with testis non-reactive and SCA-negative serum.

6. DISCUSSION

6.1. The prevalence of non-organ specific autoantibodies in female patients with reproductive failure

In this first study, we observed the differences in the frequency of occurrences of organ-non-specific autoantibodies – ANA and SMA – in sera of infertile women *versus* a healthy control group of women. As expected, the incidence of these autoantibodies was significantly higher in the sera of female patients with primary or secondary infertility than in the sera of women of the randomly selected population control group women.

The association of reproductive failure with autoimmunity has been recognized for a long time (Shulman 1971) and some types of autoantibodies have been shown to have a role in pathogenesis or characterized as diagnostic markers for several causes of reproductive failure (Shoenfeld *et al.* 2007; Cervera and Balasch 2008). But in the context of ANA and SMA associations with female infertility, there are fairly few specific ANA and SMA studies concerning distinct diagnostic entities, which may be either a primary or secondary cause or contribute to infertility. ANA is mostly studied in patients with systemic lupus erythematosus, for which ANA is diagnostic, but also occur in infertility associated with this disease.

In this study we used semi-quantified (with sera dilutions 1:10 and 1:100) indirect immunofluorescence assay to detect common autoantibodies and deemed a positive result in the instance of a specific fluorescence pattern appearing with 1:10 diluted sera. In routine diagnostics higher sera dilutions, 1:40 or 1:100, are regarded as diagnostically positive. If a serum gives a characteristic fluorescence pattern, only, in 1:10 titer but the pattern disappears in higher dilutions, the autoantibody positivity solely in 1:10 titer is presumed as non-diagnostic for SLE or other autoimmune diseases, thus dispensable in adults. A similar approach, accounting also with sub-diagnostic autoantibody findings, is shown to be rational in POF studies (Gleicher *et al.* 2009b). The presence of ANA and SMA at the sub-diagnostic level for rheumatic disease is, however, fairly common in the sera obtained from the general population. The prevalence of these 1:10 titer antibodies in the control group (BI) was 3.6% (ANA) and 5.1% (SMA), which compares favorably with Metsküla, *et al.*'s findings of 1:10 titer antibodies, 4.2% (ANA) and 5.7% (SMA), in a whole population study (Metsküla *et al.* 2002).

In the present study, we cannot say whether these non-organ-specific antibodies are developed as an epiphenomenon, accompanying immune system activation against specific autoantigens in reproduction-related tissues and thus being surrogate indicators of a possible autoimmune escalation, or by immune activation initiated by viral and bacterial inflammation. In the latter case, these autoantibodies are a transient reflection of a recent viral or bacterial infection, indicating natural autoimmunity that is still controllable by peripheral immuno-

tolerance mechanisms. In favor of the latter case is our finding that no autoantibodies directed against ovarian tissue were detected in these patients. Nevertheless, the indirect immunofluorescence test that was used for anti-ovarian antibody detection has a fairly low degree of sensitivity and may give a negative result in cases with low-level antibodies. Therefore, in further studies these sera should be investigated using more sensitive techniques like immunochemistry (Pires *et al.* 2007). The rationale of such studies is well justified by the results of Haller *et al.* (Haller *et al.* 2006) who also found elevated autoimmunity background in patients with unexplained infertility.

We also investigated whether anti-β2GPI and anti-CA can be used for the separation of patient groups with certain clinical characteristics. Anti-β2GPI has been shown to be associated with reproductive failure due to generalized anti-phospholipid syndrome (Blank and Shoenfeld 2009). Sometimes these antibodies are the only markers for autoimmunity in a subgroup of patients with long-lasting infertility (Carp and Shoenfeld 2007). We discovered anti-β2GPI at a similar frequency in all patient groups studied. These autoantibodies, however, have been demonstrated as the only markers of autoimmunity in two patients, showing that in these cases anti-β2GPI might be the cause of reproductive failure and demonstrating the importance of anti-β2GPI assessment in infertility clinics.

Anti-CA was revealed in 7.4% of all patients studied. As there were no significant differences of anti-CA frequencies between patients groups of INF, endometriosis, PCO or PCOS, we were unable to confirm the studies, in which these CA autoantibodies were thought to be characteristic for endometriosis (Kiechle *et al.* 1994; D'Cruz *et al.* 1996). Our results are coherent with studies where anti-CA has been demonstrated in various autoimmune diseases, such as primary biliary cirrhosis, Sjögren's syndrome and others (Itoh and Reichlin 1992; Kino-Ohsaki *et al.* 1996; Comay *et al.* 2000). The frequent association of endometriosis with other autoimmune and inflammatory diseases might explain the high percentage of anti-CA in patients with endometriosis. However, there might be CA isoenzymes, which are more specific targets for autoimmunity in endometriosis. Most interestingly, one of POCS patients with primary infertility had SCA antibodies, which were non-reactive with other known placental steroid-cell autoantigens and negative for common autoantibodies, β2GPI and CA autoantibodies. This finding supports the idea that there exist autoantibodies of unknown molecular specificity, which react with steroidogenic tissues.

This study indicates the higher prevalence of non-organ specific autoantibodies in women with reproductive failure is due to a variety of causes. The revealed autoantibodies might reflect the overall propensity to develop autoimmune reactions in such groups, associated with the development of autoimmune reactions to specific reproductive tissue target antigens, or the propensity to develop some other autoimmune diseases, which can indirectly influence and reduce a woman’s fecundity.

6.2. 3βHSD autoantibodies are rare in POF and more common in APS-1 patients' sera

The causes of female infertility form a broad spectrum. POF is a diagnostic entity, in which the role of autoimmunity in pathogenesis has been suspected for a long time and shown to be valid in certain cases. POF can be caused by a multitude of factors, such as genetic mutations, chromosomal abnormalities, iatrogenic, like irradiation or chemotherapy, and idiopathic causes. Consequently distinguishing primary autoimmune etiology from secondary autoimmune reactions, due to transient infections or iatrogenic and genetic reasons, has been difficult (Wheatcroft *et al.* 1994). More evidence about the links between of POF and autoimmunity are revealed in the instance of autoimmune Addison's disease accompanies POF (Bakalov *et al.* 2005). In this type of case, autoantibodies against steroid-producing cells are present, mostly in co-occurrence with autoantibodies to CYP21A2, the cytochrome P450 family steroidogenic enzyme expressed exclusively in adrenal cortex (Betterle *et al.* 2002; Falorni *et al.* 2002; Dal Pra *et al.* 2003).

The finding of autoantibodies to steroidogenic enzyme 3βHSD, not belonging to the P450 cytochrome family, in a relatively large proportion (21%) of POF cases (Arif *et al.* 1996) was intriguing and suggested a pathogenesis for idiopathic autoimmune ovarian failure that is distinct from that seen in APS-1 and Addison's disease. This study results indicate that the presence of autoantibodies to 3βHSD is rare (2%) among POF patients. However, there was a significant proportion (20%) of APS-1 patients, who had these autoantibodies in their sera as detected by RIP.

All four sera positive for anti-3βHSD antibodies precipitated the full-length protein, but not the truncated C-terminal polypeptide (encoding 287 AA from 372). This finding may infer that an immunodominant epitope locates most likely in the N-terminal region of 3βHSD that contains a long stretch (AA 25–75) of the predicted α-helix. The shared epitope region in one POF and three APS-1 cases may, in turn, reflect the partially common autoimmune mechanism in some idiopathic cases of POF and APS-1. However, research by Arif *et al.* (Arif *et al.* 2001) has shown that major epitopes of 3βHSD occur in both, C- and N-terminal fragments, in the regions bounded by AA 1–150 and 266–372. This discrepancy may explain their use of luciferase-fused proteins as antigen in RIP. But theoretically, any truncation or fusing with other polypeptide may influence the protein folding and therefore modify also immunoassay results.

Although having Addison's disease, the 3βHSD autoantibody-positive POF patient could not be classified as either APS-1 or APS-2 because she had no signs of candidiasis and hypoparathyroidism or T1D and autoimmune hypothyroidism. Furthermore, no mutations were found in her DNA in the two most common positions of the AIRE gene that together cover more than 80% of British APS-1 patients' mutations (Pearce *et al.* 1998).

In Arif *et al.*'s seminal study (Arif *et al.* 1996), of 48 POF cases none had associated Addison's disease and only 3 had autoimmune thyroid disease,

compared to 6 (associated Addison's disease) and 5 (autoimmune thyroid disease) in this study (Group AII). In Arif *et al.*'s subsequent study (Arif *et al.* 2001) a similar patter occurred: of 100 POF cases none had associated Addison's disease and 4 had autoimmune thyroid disease; from 12 3βHSD-reactive sera, 4 had thyroid autoantibodies and 1 of these presented with clinical autoimmune thyroid disease. This seems unlikely to explain the difference found in 3βHSD autoantibody frequencies, as 2 of the 3 hypothyroid POF patients in Arif *et al.*'s seminal study (Arif *et al.* 1996) had 3βHSD antibodies. The higher proportion of patients with associated autoimmune disorders in this study should, therefore, have increased the probabilities of finding 3βHSD antibodies. We included also 7 POF patients with iatrogenic or genetic etiology to study group (Group AII) since such patients may develop ovarian antibodies presumably arising secondary to ovarian damage (Wheatcroft *et al.* 1994), and we pondered whether these ovarian antibodies are directed against 3βHSD. We were unable to find 3βHSD autoantibodies in patients with T1D (Group BII), although a large number (23%) of such patients were reported in Arif *et al.*'s seminal study (Arif *et al.* 1996).

As the CYP17 and CYP11A1 enzymes are known to be targets of SCA, the reactivity of POF sera to these two enzymes were studied. It is known that the SCA seen in APS-1 is mainly due to antibody reactivity toward these two steroidogenic enzymes (Uibo *et al.* 1994b; Peterson *et al.* 1997). All three APS-1 patients sera with 3βHSD autoantibodies in this study (Group BII) also contained autoantibodies to CYP17 and CYP11A1, but none of the POF patients sera did. One SCA-positive but 3βHSD autoantibody-negative POF serum (Group AII) had no reactivity to CYP11A1 and CYP17, suggesting the existence of still uncharacterized SCA targets. As earlier pointed out (second paragraph of section 5.1), serum with SCA reactivity but without verifiable molecular target was revealed also in a patient with PCOS (Group AI).

In conclusion, our findings indicate that 3βHSD autoantibodies are rare in patients with POF. Accordingly, their significance as a diagnostic marker for autoimmune POF remains low. This is coherent with Falorni *et al.*'s study, in which 3βHSD autoantibody prevalence was 8% in Addison-related POF and 2% in POF patients without adrenal autoimmunity (Falorni *et al.* 2002). Falorni *et al.* also emphasized the low overall levels of these antibodies, because the highest 3βHSD autoantibody levels stay close to the qualitative set-point value. In consideration of the heterogenic essence of POF, the fairly low frequency of reported 3βHSD autoantibodies may just reflect a certain subset within POF, but the current data on the topic does not favour that idea. Also, complementary evidence like SCA reactivity elimination by absorption with 3βHSD or T-cellular autoreactivity studies are still lacking, indicating that the significance of 3βHSD autoantibodies needs further wide-scale research.

6.3. TSGA10 as a target of autoreactivity

APS-1 is a considerable public healthcare issue only in countries or regions with small populations, like Finland and Sardinia. APS-1 is, however, noteworthy as a model disease, being virtually a sole monogenic autoimmune syndrome. Research indicates a deeper understanding of APS-1 will aid in gaining a better understanding of the phenomenon of autoimmunity (Holländer and Peterson 2009; Kämpe 2009).

One standard of APS-1 studies is the identification of a number of organ-specific autoantigens. APS-1 is characterized by a multifaceted autoimmunity and presence of corresponding high-level autoantibodies in patients' sera. In this field a high level of success has been obtained since early 1990s (Krohn *et al.* 1992; Peterson *et al.* 2004; Fetissov *et al.* 2009). Some of these APS-1 autoantigens are later found also to be targets of autoreactivity in several isolated autoimmune diseases.

Among APS-1 female patients there is a strong correlation between the presence of hypogonadism and SCA, whereas CYP11A1 is the main antigenic target for such autoantibodies (Söderbergh *et al.* 2004). However, among male patients with testicular failure and APS-1, corresponding autoantibody association is much weaker (Uibo *et al.* 1994a). Accordingly, we hypothesized that in APS-1 in men, other autoantigens may be responsible for autoimmune testicular failure.

One of the aims of our study was to identify these targets of autoantibodies by testis cDNA expression library immunoscreening with APS-1 male hypogonadic patients' sera. The immunoscreening of cDNA library yielded isolation of clones containing *TSGA10* cDNA fragments. TSGA10 was shown to be specific for testicular tissue in the seminal study of Modarressi *et al.* in 2001, which describes a *TSGA10* gene (Modarressi *et al.* 2001).

The *in vitro* transcription/translation of the full-length cDNA of *TSGA10* and the protein immunoreactivity analysis by radioimmunoprecipitation assay enabled us to demonstrate the occurrence of autoantibodies to TSGA10 in 7.5% of patients with APS-1 (Group AIII), whereas healthy control sera (Group CIII) were negative. At the same time, no positive reactions were revealed with the patients' sera on immunoblotting using TSGA10 protein expressed and purified from *E.coli*. The discrepancy between the results of the two assays may be due to the recognition of the conformational TSGA10 epitopes in the immunoprecipitation assay with *in vitro* transcribed/translated antigen but not in the immunoblotting using antigen expressed in *E. coli*. Similar differences between RIP and immunoblot analysis with recombinant autoantigens have been reported in other studies (Tuomi *et al.* 1994; Herzog *et al.* 1999). As most of the epitopes, recognized by autoantibodies, are conformational (Mackay and Rowley 2004), RIP can be considered as a more suitable assay for autoantibody detection compared to the immunoblot. Surprisingly, both male and female sera reacted with TSGA10. Originally TSGA10 was identified as testis-specific protein with a key role in spermatogenesis (Modarressi *et al.* 2001; Modarressi

et al. 2004). Subsequent studies, however, indicated its over-expression in various types of malignant tumors and actively dividing foetal tissues (Tanaka *et al.* 2004; Theinert *et al.* 2005). Thereafter, Behnam *et al.* present evidence for a broader distribution of TSGA10 in embryogenesis but also in some normal tissues, especially in neural crest derivates and actively dividing post-mitotic cells (Behnam *et al.* 2006). Therefore, the development of autoantibodies against TSGA10 could not be limited to testicular autoimmunity, as seen by the absence of correlation between hypogonadism and TSGA10 autoantibody positivity in men. However, testicular failure in APS-1 is heterogeneous and rather difficult to diagnose (Perheentupa 2006). The presence of a high amount of TSGA10 autoantibodies in one APS-1 patient without testicular failure, with a 26-year follow-up medical record, supports the idea that losing tolerance to TSGA10 in APS-1 patients do not lead to progressive autoimmune destruction of testicular tissue. Malignancies, shown to induce TSGA10 antibodies (Tanaka *et al.* 2004), are discounted because the patients have undergone extensive clinical investigations throughout the study period.

Although new data about TSGA10 is steadily being accumulated, still insufficient is known about the functions of the protein. Experiments with mice homologue, Mtsga10 (NM 207228, 89% DNA and 94% AA level identity with human TSGA10), demonstrate predominant translation 65-kDa Mtsga10 protein in the postmeiotic phase in spermatogenesis (Modarressi *et al.* 2004). Full-length 65-kDa Mtsga10 protein appears to be processed to a 27-kDa structural protein of fibrous sheath in the principal piece of the sperm tail (Modarressi *et al.* 2004). At present, TSGA10, being predominantly expressed in testis and over-expressed in several malignant tumours, might be considered to belong to the cancer-testis antigen (Tanaka *et al.* 2004). Cancer-testis antigens can be considered functionally tumor-specific and attractive targets for tumor immunotherapy. The described role of TSGA10 in active cell division, differentiation and migration of cells, and interaction with hypoxia-inducible factor-1α (Hägele *et al.* 2006) provides several new study avenues for this protein.

The cause of TSGA10 antibodies development in APS-1 patients remains difficult to resolve. Antibodies were detected in a few patients without any other features to distinguish them from the other patients. Characteristically, sera of APS-1 patients contain a variety of specific autoantibodies, frequently in high titer. Some of these autoantibodies, like the against enzymes CYP21A2 and GAD65, are commonly detected in APS-1 patients such as those with isolated organ-specific autoimmune diseases. On the other hand, some autoantibodies such as the against aromatic L-amino acid decarboxylase, CYP1A2 and a putative potassium channel regulator KCNRG, are found almost exclusively in APS-1 (Clemente *et al.* 1997; Dalekos *et al.* 2003; Dal Pra *et al.* 2004; Alimohammadi *et al.* 2009). The present study does not clarify whether or not TSGA10 autoantibodies are exclusive for APS-1. The prevalence of TSGA10 antibodies is approximately 7% in APS-1, which is fairly low but in the same magnitude of the prevalence as the autoantibodies IA-2 and CYP1A2 in APS-1

(Söderbergh *et al.* 2004). Similar to TSGA10, these autoantibodies do not correlate with any other specific clinical or immunologic features.

Importantly, TSGA10 antibodies were not revealed among patients with Addison disease that in some cases could be associated with autoantibodies reactive with tissues of reproductive organs (Betterle *et al.* 1999; Falorni *et al.* 2002). The mechanism of development and exact role of these antibodies remains to be evaluated in further studies. A particularly important assessment should be of these autoantibodies in the male partners of couples who have a male infertility factor. As the TSGA10 protein is shown to have a role in cell division, other autoimmune disease entities including diseases from non-organ-specific part of autoimmunity spectrum must also be screened for TSGA10 reactivity in the patients' sera.

6.4. Summary

The observational experience that aberrant autoimmunity can lead to premature ovarian failure and infertility has been known for decades. Organ-specific endocrine diseases, encompassing relevant autoantibodies, TMA, ACA and ovarian antibodies, may cause female infertility in cases of POF (Hoek *et al.* 1997). Many other antibodies, e.g. ANA, SMA and APA, are suggested to be more prevalent in infertile groups than healthy control female series (Cervera and Balasch 2008). As the statistical co-occurrence but not the causal association is shown in most cases, even the phrase 'immunological noise' is advised for use in describing minimal evidence of autoimmunity, increasing the risk toward premature ovarian ageing (Gleicher *et al.* 2009b).

However, pathogenetically important autoantibodies could be hidden behind this 'immunological noise'. This could be drawn from our study showing that infertility is quite frequently accompanied by different autoantibodies, showing the involvement of immunological mechanisms at least in a proportion of the cases. Nevertheless, in spite of the successful identification of target auto-antigens for immune reactions in some infertility cases, the major autoantigenic target involved in either or both the development and the natural cause of female and male infertility still remains to be discovered. Last, but not least, the combination of experimental studies in laboratory animals and those in human clinical material is the most promising to achieve clinically most important results, as shown recently by Hou *et al.*, who discovered an autoantigen semenogelin involved in the development of spontaneous chronic prostatitis in humans (Hou *et al.* 2009). In the search for new discoveries, there is now an enormous methodological arsenal, among which cDNA expression library screening, used in the present study, as well as phage display libraries and immunoassay followed by mass spectrometry (Conrad *et al.* 2010) have great potential for success. For these purposes, well selected and clinically characterized material gives steady basis of further successive studies, particularly in cases of male infertility.

7. CONCLUSIONS

1. Non-organ-specific common autoantibodies (ANA and SMA) are more prevalent among female patients with infertility than women in the general population. Although this prevalence might be due to a variety of causes, the incidence strongly supports the argument for autoimmune reactions being involved in some cases of female infertility.
2. In patients with premature ovarian failure, 3βHSD autoantibodies are rare and therefore their significance as a single diagnostic marker for premature ovarian failure is low. A few patients with POF or PCOS with primary infertility have autoantibodies to steroidogenic tissues in which the actual antigen target is unknown.
3. Autoantibodies against TSGA10 are present in a small proportion of APS-1 patients without any clinical association with gonadal failure in these patients.
4. The persistence of TSGA10 autoantibodies in sera of male APS-1 patients during 25 years follow up denotes a significant association with APS-1.
5. Molecular cloning of autoantigens using cDNA expression libraries prepared from human tissues deserves a successful method to discover novel antigenic targets, involved in autoimmune diseases. Radioimmunoprecipitation with *in vitro* transcribed/translated antigen could be advocated as a suitable method for the relevant innovative antibody detection, as has been shown by the example of TSGA10.

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SUMMARY IN ESTONIAN

Autoimmuunsus reproduktiivhäirete korral: assotsieeruvate autoantikehade ja autoantigeenide uuring

Infertiilsuse ehk viljatuse all mõistakse üldjuhul olukorda kus heteropaari üheaastase regulaarse kaitsmata seksuaalelu jooksul naine ei rasestu/ei saa järglasi. Elu jooksul kogeb infertiilsust ligikaudu 10–15 protsenti paaridest. Viljatuse põhjuste spekter on lai, kõikidest juhtudest ligikaudu kolmandik on tingitud naise organismi poolsetest põhjustest ja teisel kolmandikul kaasustel on põhjuseks mehepoolsed faktorid. Ülejäänud juhtude hulgas on mõlemapoolsete faktorite koostoimetest tingitud viljatus ning ligikaudu viiendik juhtudest jääb nn idiopaatiliseks ehk selgusetu põhjusega. Mehepoolne viljatus on enamikel juhtudel väljendunud ka sperma parameetrite – seemnerakkude arv, ehitus ja liikuvus – kõrvalekallete kaudu. Naisepoolsete viljatuse põhjuste skaalal on olulisemad järgmised jaotused: erinevate põhjustega ovulatsioonihäired, endometrioos, munajuhade patoloogiaga seotud viljatus ja emaka/emakakaela faktoritest tingitud infertiilsus. Ovulatsioonihäirete alla kuuluvad muuhulgas enneaegne ovariaalne puudulikkus (*premature ovarian failure*, POF) ja polütsüstiliste ovaariumide sündroom (*polycystic ovary syndrome*, PCOS).

Autoimmuunsus on immuunsüsteemi – täpsemalt immuunsüsteemi adaptiivse, lümfotsüütide vahendatud osa – aktiveerumine organismi omaenese molekulaarsete struktuuride vastu. Sel moel immuunsüsteemi märklauaks sattunud kehaomaseid antigeene nimetatakse autoantigeenideks. Kuivõrd autoimmuunse konflikti eskaleerumise korral kaasneb autoreaktiivsete T-rakkude tekkele alati ka samade autoantigeenidega reageerivate autoantikehade teke, on mitmed vereseerumist hõlpsalt määratavad autoantikehad kasutusel vastavate autoimmuunhaiguste biomarkeritena.

Uurimistöö eesmärgid

Töö tehti eesmärkidega:

1. Uurida erinevate kliiniliste põhjuste tõttu tekkinud viljatusega naiste gruppides üldiste autoantikehade – kui autoimmuunsete kõrvalekallete indikaatorite – levimusmäärasid ja levimusmustreid.
2. Uurida munasarjade alatalitusega seotud autoimmuunsuse sihtmärke POF-i ja autoimmuunse polüglandulaarse I tüüpi sündroomi (APS-1) korral.
3. Leida autoimmuunse patogeneesiga testiste alatalituse tekkega seotud autoantigeene, kasutades APS-1 haigusega meeste seerumeid.

Materjal ja meetodid

Uurimustöös olid kasutusel 344 patsiendi ja 579 kontrollgrupi isiku vereseerumid. Patsientide gruppidesse kuulusid POF, PCOS, endometrioosist, munajuhadest tingitud või teadmata põhjusel viljatud naised; mõlemast soost APS-1 patsiendid; Addison'i tõve ja 1. tüüpi suhkruhaigusega patsiendid. Uuritavad seerumid koguti koos informeeritud nõusolekutega Tartu, Helsingi, Tampere ja Sheffield'i ülikoolide kliinikutes. Kontrollgruppide hulgas olid tervete vabatahtlike, veredoonorite ja rahvastiku-uuringu hulgast vanuse järgi standarditud tervete naiste grupp.

Autoantikehade määramiseks kasutati kaudset immunofluorestsentsmeetodit üldiste autoantikehade ja steroidraku antikehade (SCA) määramiseks; ensüüm-immunosorptsioon-analüüsi (ELISA) beeta-2-glükoproteiini ja süsinikanhüdraasi autoantikehade määramisel. Radio-immunopretsipitatsiooni meetod oli kasutusel 3βHSD (3-beeta-hüdroksüsteroidi dehüdrogenaas 2) ja valk TSGA10 vastaste antikehade määramisel, viimase puhul oli kasutusel ka immunoblot-analüüs. Radioimmunopretsipitatsioonil kasutatavate [S-35]-isotoop märgistusega antigeenid valmistati rakuvabas keskkonnas *in vitro* transkriptsiooni/translatsiooni meetodil. Lambda-bakteriofaagides asuv inimese testise cDNA ekspressiooniraamatukogu propageeriti *E.coli* kaudu ja immunoskriiniti APS-1 haigusega meeste seerumite abil. Leitud positiivsete bakteriofaagikloonide cDNA-inserdi olemus määrati DNA järjestamise tulemuste võrdlemisel DNA järjestuste andmebaasidega.

Uurimistöö peamised tulemused

Üldiste autoantikehade määramisel selgus et infertiilsete naiste seerumeis leidub märkimisväärselt sagedamini organspetsiifikata (ANA ja SMA tüüpi) autoantikehi võrreldes rahvastikuuuringu grupist vanuse järgi standarditud naiste seerumitega (40.7% *versus* 14.8%; P<0,005). Nende autoantikehade suurem levimus viljakust vähendavate haigustega naiste gruppides võib peegeldada suurenenud valmidust autoimmuunsete fenomenide tekkeks, sealhulgas viljatuse patogeneesis osalevate autoimmuunsete koekahjustusreaktsioonide tekkeks.

Autoantigeen 3βHSD-ga reageerivate antikehade levimuse määramisel selgus et POF haigetel esineb neid oluliselt harvemini, vaid ühel juhul 48-st, kui selle autoantigeeni esmaavastajate andmetel. Samas viieteistkümne APS-1 haige seerumis leidusid 3βHSD autoantikehad kolmel juhul. Mõlemas grupis olid kõik 3βHSD autoantikehade suhtes reaktiivsed seerumid positiivsed ka SCA antikehade suhtes. SCA ega 3βHSD autoantikehi ei leidunud 38 Addison'i tõve, 28 I tüüpi diabeedi ega 71 tervete inimeste rühma seerumites.

Inimese testise cDNA ekspressiooniraamatukogu immunoskriining kuue APS-1 meespatsiendi seerumi abil andis lõplikuks saagiseks kümme rekombinantset lambda-bakteriofaagi klooni. Neist kaheksa klooni cDNA-inserdi järjestused vastasid inimese geen *TSGA10* mRNA-le. Kuivõrd isoleeritud

kloonide cDNA-inserdid ei katnud TSGA10 täispikka mRNA-d, kloneeriti täispikka TSGA10 valku kodeerivad ekspressioonivektorid inimese testise RNA-st cDNA vahendusel polümeraasi ahelreaktsiooni abil. *E.coli* bakteris ekspresseeritud ja sealt afiinsuskromatograafiaga puhastatud TSGA10 valku immunoblot-meetodis antigeenina kasutades ei õnnestunud TSGA10-reaktiivseid antikehi APS-1 patsientide ega tervete inimeste seerumites leida. Rakuvabas keskkonnas *in vitro* transkribeeritud/transleeritud antigeeniga teostatud radioimmunopretsipitatsioon-analüüs sedastas TSGA10 autoantikehad APS-1 patsientide grupist kolme mehe (7,5% APS-1 meestest) ja kahe naise (7,7%) seerumeis. TSGA10 autoantikehade esinemise ja teiselt poolt gonaadide puudulikkuse kliiniliste, biokeemiliste ega immunoloogiliste biomarkerite vahel korrelatsiooni ei esinenud. Addison'i tõvega patsientide ja tervete isikute seerumites TSGA10 reaktiivseid antikehi ei leitud.

Järeldused

1. Üldiseid mitte-organspetsiilisi autoantikehi (ANA ja SMA) esineb oluliselt sagedamini infertiilsetel naistel kui üldrahvastiku naistel. See erinevus võib olla tingitud paljudest põhjustest, kuid on siiski oluliseks kaudseks tõendiks et autoimmuunsus on seotud naiste viljatuse mõnede juhtude tekkega.
2. Enneaegse ovariaalse puudulikkusega patsientide vereseerumeis on 3βHSD autoantikehad harvaesinevad ja on seetõttu iseseisva haigusmarkerina selle haiguse korral väheolulised. Mõnede enneaegse ovariaalse puudulikkusega patsientide või esmase viljatusega seotud PCOS patsientide seerumeis on sedastatavad steroide tootvate rakkudega reageerivad autoantikehad mille sihtmärgid molekulaarsel tasandil on veel selguseta.
3. Testistes rohkelt ekspresseeruva valgu TSGA10 vastased autoantikehad esinevad väikesel osal APS-1 patsientidel, seejuures puuduvad seosed nende antikehade leiu ja gonaadide talitlushäirete kliiniliste nähtude vahel.
4. TSGA10 autoantikehade püsimine APS-1 haigusega meeste seerumites kuni 25 aasta vältel märgib nende antikehade olulist seost selle haigusega.
5. Autoimmuunhaigustega seotud uute autoantigeenide leidmisel ja molekulaarsel kloneerimisel on inimkoe cDNA ekspressiooniraamatukogude immunoskriinimine edukas meetod. Radioimmunopretsipitatsioon *in vitro* transkribeeritud/transleeritud antigeeniga on soovitatav meetod vastavate autoantikehade määramiseks, nagu kinnitab käesolev TSGA10 uurimustöö.

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PUBLICATIONS

CURRICULUM VITAE

KOIT REIMAND

Born:	July 19, 1968 in Tartu, Estonia
Citizenship:	Estonian
Address:	University of Tartu, Institute of General and Molecular Pathology, Ravila 19, Tartu 50411, Estonia
Phone:	+372 737 4234
E-mail:	Koit.Reimand@ut.ee

Education

1983–1986	Anton Hansen Tammsaare Tartu Secondary School No. 1, Tartu, Estonia
1986–1993	University of Tartu, Faculty of Medicine, MD diploma
2009–2010	University of Tartu, Faculty of Medicine, PhD studies

Professional employment

1993–1995	Tartu University Maarjamõisa Hospital, Medical internship
1995–	University of Tartu, Institute of General and Molecular Pathology, Chair of Immunology, assistant

Scientific work

Main topics of research are related to autoantibodies as autoimmunity biomarkers, particularly in case of adrenocortical, steroid-producing cells and type I diabetes related autoimmunity; the mechanisms and autoantigens of gonadal autoimmunity.

Co-author of seven original publications.

Member of Estonian Society for Immunology and Allergology.

ELULOOKIRJELDUS

KOIT REIMAND

Sündinud: 19. juulil 1968 Tartus, Eestis
Aadress: Tartu Ülikool, üld- ja molekulaarpatoloogia instituut, Ravila 19, Tartu 50411 Eesti
Telefon: +372 737 4234
E-mail: Koit.Reimand@ut.ee

Haridus

1983–1986	Anton Hansen Tammsaare nimeline Tartu 1. Keskkool
1986–1993	Tartu Ülikool, arstiteaduskond, diplom arstiteaduses
2009–2010	Tartu Ülikool, arstiteaduskond, doktorant-ekstern arstiteaduses immunoloogia erialal

Erialane teenistuskäik

1993–1995	Tartu Ülikooli Maarjamõisa haigla, üldarstlik internatuur
1995– …	Tartu Ülikool, üld- ja molekulaarpatoloogia instituut, immunoloogia assistent

Teadustegevus

Teadustöö põhisuunad on olnud seotud organspetsiifiliste autoimmuunhaiguste korral esinevate autoantikehade kui haiguste biomarkeritega, eeskätt neerupealise koore ja steroide sünteesivate rakkude autoantikehad; 1. tüüpi suhkrutõve korral esinevad autoantikehad; gonaadide vastane autoimmuunsus ja selle molekulaarsed sihtmärgid.

Autor või kaasautor seitsmes teaduspublikatsioonis.

Eesti immunoloogide ja allergoloogide seltsi liige.

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