

Putative Predictors of Antibodies Against Follicle-Stimulating Hormone in Female Infertility: A Study Based on *In Vitro* Fertilization Patients

Kadri Haller^{1,2}, Andres Salumets^{2,3,4,5}, Marina Grigorova⁵, Ija Talja¹, Liina Salur¹, Marie Christine Béné⁶, Maris Laan⁵, Raivo Uibo¹

¹Department of Immunology, Institute of General and Molecular Pathology, Centre of Molecular and Clinical Medicine, University of Tartu, Biomedicum, Tartu, Estonia;

²Department of Obstetrics and Gynecology, University of Tartu, Tartu, Estonia;

³Nova Vita Clinic, Centre for Infertility Treatment and Medical Genetics, Haabneeme, Viimsi, Harjumaa, Estonia;

⁴Estonian Biocentre, Tartu, Estonia;

⁵Institute of Molecular and Cell Biology, University of Tartu, Tartu, Estonia;

⁶Laboratoire d'Immunologie, Faculté de Médecine & CHU de Nancy, Université Henri Poincaré, Vandoeuvre lès Nancy, France

Keywords

Anti-FSH and common autoantibodies, follicle-stimulating hormone, FSH β -subunit gene, HLA-DQB1

Correspondence

Raivo Uibo, Department of Immunology, Institute of General and Molecular Pathology, Centre of Molecular and Clinical Medicine, University of Tartu, Ravila Str. 19, Biomedicum, Tartu 50411, Estonia.
E-mail: raivo.uibo@ut.ee

Submitted November 1, 2006;
revised November 26, 2006;
accepted November 27, 2006.

Citation

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

doi:10.1111/j.1600-0897.2006.00462.x

Problem

We have previously demonstrated the presence of naturally occurring antibodies against follicle-stimulating hormone (FSH) in patients with endometriosis and polycystic ovary syndrome (PCOS). Here, we investigated the parameters associated with anti-FSH antibodies in *in vitro* fertilization (IVF) patients.

Methods of study

The following parameters were studied in 135 patients: peripheral FSH levels, FSH β -subunit gene (*FSHB*) haplotypes, history of previous IVF, and susceptibility to autoimmune reactions in general [seven common autoantibodies (against nuclear antigens on human and rodent substrates, smooth muscle, gastric parietal cells, β 2-glycoprotein I, cardioli-pin, and thyroid peroxidase) and *HLA-DQB1* alleles].

Results

Although the anti-FSH levels were higher in patients when compared with controls, those higher levels were not associated with *FSHB* haplotypes. The anti-FSH IgM associated with (i) the levels of FSH in women with male and tubal factor infertility; (ii) the history of IVF in patients with PCOS, endometriosis, and unexplained infertility; and (iii) the production of common autoantibodies among all IVF patients. The anti-FSH IgA associated with *HLA-DQB1**03. The anti-FSH IgG correlated with the values of anti-FSH IgA and IgM.

Conclusion

Anti-FSH may be naturally occurring antibodies associated with peripheral FSH concentrations, but increased in infertile women with dysregulation of immune reactions and repeatedly performed IVF.

Introduction

Autoimmune mechanisms are involved in different forms of female infertility.¹ Additionally, there is evidence of activation of the immune system after seminal contact² and production of pregnancy-favoring antibodies.^{3–5}

Autoimmunity is associated with a dysbalance of various components of the immune response and with the development of autoantibodies directed against normal host antigens. The susceptibility to autoimmune reactions is regulated at several levels.⁶ The proliferation of mature T-lymphocytes in response to either self- or foreign antigenic stimuli is affected by the nature and strength of antigenic ligand-MHC stimulation.^{6,7} HLA class II molecules influence the stability of the antigenic-peptide-HLA complex in an allele-specific manner affecting the induction of central tolerance.⁶ As revealed by the studies on anti-insulin autoimmunity in the murine models of diabetes, the stimulation provided by antigenic ligand-MHC stimulation could also be modulated by genetic variations of the insulin gene, potentially influencing the gene expression in the thymus.^{8,9} Tissue-specific autoimmunity appears to be additionally dependent on local factors, including infection-related tissue damage,⁶ iatrogenic manipulations,¹⁰ and the level of autoantigen in periphery.^{11,12} Thus, the expansion of cells responding to low-affinity ligands (self-antigen) or anomalies in the deletion of high-affinity autoreactive T cells can lead to autoimmune reactions.⁷ Female infertility with various etiologies such as endometriosis, polycystic ovary syndrome (PCOS) or unexplained infertility, together with repeatedly unsuccessful *in vitro* fertilization (IVF) attempts has been characterized by immunological alterations as well as with an increased production of multiple autoantibodies.^{1,11,13,14} We have previously shown that there are naturally occurring antibodies against follicle-stimulating hormone (FSH), which are, however, predominantly present in patients with endometriosis and PCOS.¹⁵ The intriguing question of what associates the production of anti-FSH antibodies and female infertility stems directly from this context.

Here, our objective was to study putative predictive parameters associated with higher production of anti-FSH antibodies in women with different causes of infertility, mainly focusing on (i) peripheral FSH levels; (ii) polymorphisms of the FSH β -subunit gene (*FSHB*), which codes for the receptor-binding and hormone specificity determining subunit; (iii) history of ovarian

punctures in previous IVF attempts; and (iv) the general propensity to develop autoimmune reactions.

Methods

Patients

The Ethics Committee of the University of Tartu approved the study and informed consent was obtained from all participants after the nature of the study was explained to them. The study group consisted of 135 women (mean age 34.0, S.D. = 4.8 years old) who were planning IVF at Nova Vita Clinic (Harjumaa, Estonia) between July 2004 and October 2005. The causes of infertility were as follows: tubal factor infertility ($n = 56$), male factor infertility ($n = 30$), PCOS ($n = 21$), endometriosis ($n = 12$), unexplained infertility ($n = 11$) and infertility due to the other reasons ($n = 5$). Among the IVF patients, 65.9% ($n = 89$) women were starting their first IVF procedure, while 19.3% ($n = 26$), 8.1% ($n = 11$) and 6.7% ($n = 9$) patients had already undergone 1, 2 and 3 or more IVF attempts, respectively. Serum and EDTA-collected peripheral blood samples were obtained from all participants between day 3 and 5 of the spontaneous menstrual cycle before starting the ovarian stimulation with exogenous FSH. In addition, none of the patients participating in this study had received non-IVF FSH treatment before entering the study. The pregnancy rate (positive chorionic gonadotropin test) and clinical pregnancy rate per embryo transfer were 40.3% (50/124) and 29.8% (37/124), respectively.

Endometriosis and PCOS were diagnosed as reported elsewhere.¹⁵ Tubal factor infertility due to occlusion of the fallopian tubes was diagnosed either by hysterosalpingography or diagnostic laparoscopy.¹⁶ The main cause for tubal occlusion was an episode of pelvic inflammatory disease. A couple was diagnosed to have male factor infertility when the woman was lacking any reason for infertility, while her partner experienced decreased semen quality.¹⁷ Other causes for infertility were endometrial hyperplasia, myoma uteri, and ovulatory dysfunction. Unexplained infertility was assumed if a woman was lacking any of the abovementioned reasons for infertility and her partner was shown to have normal semen quality, but the couple had experienced infertility of >1 year.

Serum samples from the healthy female blood donors ($n = 85$, mean age 44.9, S.D. = 10.7 years old) were collected as described elsewhere.¹⁵

Detection of Anti-FSH and Common Autoantibodies

Indirect ELISA with purified FSH (Fostimon® 75, IBSA, Pambio-Noranco, Switzerland) as antigen was used to detect separately anti-FSH antibodies of immunoglobulin (Ig) G, IgA and IgM isotypes, with an adapted protocol derived from our previous study.¹⁵ Polystyrene 96-well plates (MaxiSorp™, Nunc, Roskilde, Denmark) were coated with 18 ng/mL FSH (Fostimon®; IBSA) solution in carbonate-bicarbonate buffer (40 mM Na₂CO₃, 60 mM NaHCO₃, pH 9.6), at 100 µL per well. Plates were incubated for 48 hr at +4°C, washed with borate buffered saline (BBS, 200 mM H₃BO₃, 75 mM NaCl, pH 8.4) and blocked with 5% bovine serum albumin (Sigma, Saint Louis, MO, USA) in 0.5% Tween-20 in BBS (blocking buffer) for 1 hr at +37°C. The plates were divided into three sections for each antibody subclass and serum samples dilutions of 1:100 in blocking buffer were added, followed by incubation for 0.5 hr at +37°C. Horseradish peroxidase-conjugated rabbit anti-human IgG, IgA and IgM (Dako, Glostrup, Denmark) were diluted 1:1000 (IgA and IgM) and 1:5000 (IgG) in blocking buffer, distributed as 100 µL per well, followed by incubation for 1 hr at +37°C. The plates were washed three times after each incubation step. Color was developed by adding 100 µL/well of the substrate 3,3',5,5'-tetramethylbenzidine (Sigma, St Louis, MO, USA) 0.1 g/L in 0.1 M acetate-citrate buffer (80 mM C₂H₃O₂Na, 30 mM C₆H₈O₇ H₂O, 1 mM EDTA-Na₂, pH 4.5), and blocked after 10 min with 50 µL/well 2 N H₂SO₄. The optical density (OD) at 690 nm was subtracted from OD at 450 nm, and the difference was recorded as signal. Antibody levels were expressed as optical density (OD) ratios to the internal calibrator, calculated as follows – [(IgG, IgA or IgM sample mean OD – IgG, IgA or IgM blank OD)/(IgG, IgA and IgM calibrator median OD – IgG, IgA or IgM blank OD)]. The control wells contained all the assay components but the serum sample. The internal calibrator was a pool of sera from 200 healthy fertile women used routinely in clinically available tests.

Indirect immunofluorescence was used to assess common autoantibodies to nuclear antigens (ANA-H and ANA-R on human and rodent substrates, respectively), smooth muscle (SMA) and gastric parietal cells (PCA). Results were considered positive for samples showing reactions starting from low titers (higher than 1:10).¹³ In-house ELISA was used to detect antibodies against β2-glycoprotein

I (B2-GPI)¹³ and cardiolipin (ACA)¹⁸ with the results expressed in enzyme-immunological units. Antibodies against thyroid peroxidase (anti-TPO) were detected using the ImmunoCAP technology according to the manufacturer's instructions (UniCAP, Phadia OY, Finland). Results were expressed as negative or positive. The presence of common autoantibodies was defined when a patient presented reactivity to at least one of the seven autoantigens investigated.

Genotyping of *FSHB* and *HLA-DQB1*

Haplotypes of the *FSHB* gene were detected by restriction fragment length polymorphism analysis of the haplotype tagging single nucleotide polymorphisms (SNP) of *FSHB*: +1234 C/A (rs594982) and +1736 C/T (rs6169).¹⁹ Haplotype no. 1 (HAP1) was determined by the presence of +1234 C and +1736 T, and haplotype no. 13 (HAP13) consisted of +1234 A and +1736 C alleles.¹⁹ *HLA-DQB1* typing for *02, *03 (0301 or 0302), *06 (0602 or 0603) or X as a non-defined allele was performed using hybridization of lanthanide-labeled allele-specific oligonucleotide probes with the PCR-amplified gene product (Delfia®; Wallac, Perkin-Elmer Life Sciences, Boston, MA, USA).²⁰

Statistical Analyses

The R2.3.1 A Language and Environment (Free Software Foundation, Boston, MA, USA) was used for *t*-test, proportion test, Pearson's linear correlation, linear regression and logistic regression. The blood donors participated as a reference group in the comparisons of anti-FSH antibody values between the IVF patients and the healthy women. The associations between anti-FSH antibodies and predicting parameters were further studied within the IVF patients. In addition, IVF patients with PCOS, endometriosis, unexplained and other causes of infertility (PEU) were studied separately from the women with male and tubal factor infertility (MTF). *P*-values <0.05 were considered statistically significant.

Results

Anti-FSH Antibodies in IVF Patients Compared with Healthy Women

The mean levels (±S.D.) of anti-FSH antibodies in the IVF patients and the healthy controls are shown in Table I. Linear regression analysis adjusted by the

Table 1 The levels of anti-follicle-stimulating hormone (FSH) antibodies in *in vitro* fertilization (IVF) patients and controls

| Group (n) | Age (years) (P-value) | Anti-FSH IgG | | Anti-FSH IgA | | Anti-FSH IgM | |
|--------------------|-----------------------|--------------------|--------------------------|--------------------|--------------------------|--------------------|--------------------------|
| | | Value ^a | r (P-value) ^b | Value ^a | r (P-value) ^b | Value ^a | r (P-value) ^b |
| MTF (86) | 34.0 ± 4.6 (<0.0001) | 0.79 ± 0.73 | 0.29 (0.006) | 0.71 ± 0.39 | 0.23 (<0.001) | 2.68 ± 1.14 | 0.66 (<0.001) |
| Tubal factor (56) | 34.0 ± 4.2 (<0.0001) | 0.69 ± 0.62 | 0.19 (0.071) | 0.67 ± 0.33 | 0.19 (0.001) | 2.61 ± 1.02 | 0.61 (0.004) |
| Male factor (30) | 34.2 ± 5.2 (<0.0001) | 0.96 ± 0.88 | 0.46 (<0.001) | 0.79 ± 0.47 | 0.31 (<0.0001) | 2.82 ± 1.34 | 0.82 (0.001) |
| PEU (49) | 33.9 ± 5.2 (<0.0001) | 0.71 ± 0.48 | 0.19 (0.021) | 0.62 ± 0.23 | 0.13 (0.006) | 2.55 ± 1.01 | 0.49 (0.019) |
| PCOS (21) | 32.1 ± 4.5 (<0.0001) | 0.58 ± 0.22 | 0.08 (0.578) | 0.62 ± 0.23 | 0.15 (0.072) | 2.55 ± 0.70 | 0.54 (0.052) |
| Endometriosis (12) | 34.0 ± 4.7 (<0.0001) | 0.81 ± 0.38 | 0.30 (0.082) | 0.72 ± 0.17 | 0.23 (0.016) | 3.07 ± 1.51 | 1.07 (0.002) |
| Unexplained (11) | 35.6 ± 5.4 (<0.0001) | 0.92 ± 0.85 | 0.42 (0.021) | 0.56 ± 0.27 | 0.08 (0.444) | 2.38 ± 0.72 | 0.39 (0.268) |
| Other causes (5) | 36.7 ± 6.2 (<0.0001) | 0.56 ± 0.20 | 0.07 (0.792) | 0.51 ± 0.20 | 0.02 (0.900) | 1.68 ± 0.50 | -0.31 (0.530) |
| Total (135) | 34.0 ± 4.8 (<0.0001) | 0.76 ± 0.65 | 0.26 (0.006) | 0.68 ± 0.34 | 0.20 (<0.001) | 2.63 ± 1.09 | 0.63 (<0.001) |
| Controls (85) | 44.9 ± 10.8 | 0.49 ± 0.31 | Reference | 0.52 ± 0.23 | Reference | 1.96 ± 0.99 | Reference |

Data are presented in means ± S.D. IVF patients with PCOS, endometriosis, unexplained and other causes of infertility (PEU) were studied separately from the women with male and tubal factor infertility (MTF).

^aAntibody values are expressed as a sample optical density (OD) ratio to the pool OD.

^bStudy groups were compared with the controls by using age adjusted linear regression models (*r*, regression coefficient) and *P* < 0.05 with Bonferroni correction was considered as statistically significant difference.

age of women revealed significantly higher values of anti-FSH antibodies in all IVF patients or separately in PEU or in MTF groups compared with the reference group. The age of woman was not significantly associated with the level of any type of anti-FSH. The values of anti-FSH antibodies did not differ between the IVF patients with PEU and MTF, as revealed from age adjusted logistic regression model [adjusted odds ratios (ORs) for IgG 0.89, *P* = 0.703, IgA 0.44, *P* = 0.168 and IgM 0.94, *P* = 0.724].

The levels of anti-FSH IgG were in good correlation with the levels of anti-FSH IgA and IgM (Pearson's correlation coefficients 0.31, *P* = 0.004 and 0.22, *P* = 0.046, respectively) among the reference group, but not among the IVF patients with PEU or MTF (data not shown). Anti-FSH IgA was not correlated with anti-FSH IgM among any of the study groups (data not shown).

Relationship Between Anti-FSH Antibodies and *FSHB* Haplotypes

The distribution of *FSHB* core haplotypes among IVF patients was as follows: 23.4% were homozygous for HAP1, 45.3% were HAP1/HAP13 and 31.3% were homozygous for HAP13. The logistic regression model adjusted by the age was unable to detect any significant association between *FSHB* haplotypes and different anti-FSH autoantibodies within the group

of all IVF patients (adjusted ORs for IgG 0.88, *P* = 0.669, IgA 1.55, *P* = 0.497 and IgM 0.92 *P* = 0.647) or if PEU and MTF patients were studied separately (data not shown).

Association Between anti-FSH Antibodies and Peripheral FSH Levels

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

Effect of Previous IVF Attempts on Anti-FSH Antibodies

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

Production of Anti-FSH in Association with the General Propensity to Autoimmune Reactions

Potential susceptibility of patients to autoimmunity was assessed by the presence of common autoantibodies in low titers in relation to the *HLA-DQB1* alleles. Among the IVF patients, 43.3% carried at least one allele of the *HLA-DQ B1*03* class (0301 and/or 0302) and 37.0% had at least one allele of the *HLA-DQB1*06* class (0602 and/or 0603). The prevalence of common autoantibodies among IVF patients with different causes of infertility is shown in Table II, representing separate autoantibodies as follows: ANA-H 13.2%, ANA-R 12.4%, anti-TPO 11.0%, SMA 8.8%, B2-GPI 8.1%, ACA 7.4% and PCA 3.6%.

To study the associations between the level of anti-FSH antibodies and the presence of common autoantibodies, we used the linear regression analysis adjusted by the age and *HLA-DQB1*03* and **06* allele. Anti-FSH IgA levels were positively associated with the presence of the *HLA-DQB1*03* allele among all IVF patients ($r = 0.44$, $P = 0.034$) and separately in PEU patients ($r = 0.17$, $P = 0.022$), but not in MTF patients ($r = 0.13$, $P = 0.152$). The levels of anti-FSH IgM were in strong positive correlation with the production of other autoantibodies among all IVF patients (data not shown) and separately in MTF ($r = 0.70$, $P = 0.011$) but not in patients with

Table II The presence of common autoantibodies among *in vitro* fertilization (IVF) patients

| IVF patient groups (n) | Prevalence of autoantibody-positive ^a , n (%; 95% confidential interval) |
|------------------------------|---|
| MTF (86) | 30 (34.9, 25.1–46.0) |
| Tubal factor (56) | 18 (32.1, 20.6–46.1) |
| Male factor (30) | 12 (40.0, 23.2–59.2) |
| PEU (49) | 24 (49.0, 34.6–63.5) ^A |
| PCOS (21) | 9 (42.9, 22.6–65.6) |
| Endometriosis (12) | 7 (58.3, 28.6–83.5) ^{A, B} |
| Unexplained infertility (11) | 4 (36.4, 12.4–68.4) |
| Other causes (5) | 4 (80.0, 30.0–98.9) ^{A, B, C} |
| Total (135) | 54 (40.0, 31.8–48.9) |

^aAntibody-positivity was determined when a patient presented at least one of seven (ANA-R, ANA-H, SMA, PCA, ACA, B2-GPI and anti-TPO) autoantibodies measured. IVF patients with PCOS, endometriosis, unexplained and other causes of infertility (PEU) were studied separately from the women with male and tubal factor infertility (MTF). Statistically significant difference compared with the MTF (A), the tubal (B) or to the male factor (C) infertility (proportion test, $P < 0.05$).

PEU ($r = 0.14$, $P = 0.678$). The model could not reveal significant associations between anti-FSH IgG antibodies and the presence of common autoantibodies or *HLA-DQB1*03*06* alleles among all IVF patients and neither separately in PEU nor in MTF (data not shown).

Discussion

The current complex study on the associations of multiple parameters potentially involved in infertility helped to identify the putative factors favoring the emergence of anti-FSH antibodies in IVF patients.

Our results revealed higher values of anti-FSH IgG, IgA and IgM antibodies in the infertile patients compared with the reference group in an age-independent fashion. However, the differences in antibody values between the patients and the control group were statistically relevant but still rather small, meaning that these antibodies were also common among healthy women and could therefore merely represent examples of naturally occurring antibodies. These data support our previously published results¹⁵ despite of (1) the use of a different antigen source in the detection of anti-FSH antibodies; and (2) different selection of patients. Our previous study included patients with endometriosis or PCOS instead of infertile patients with different etiologies appointed

to IVF treatment. Although the current study group consisted of infertile women who were indicated for IVF, the serum samples were obtained before the administration of exogenous FSH. Thirty-four per cent of patients had had at least one previous IVF procedure, but at least 3 months had past since the last FSH ovarian stimulation. The further analysis demonstrated no significant differences in anti-FSH antibody levels between the combined groups of patients with tubal and male factor infertility compared with the women with PCOS, endometriosis, unexplained infertility and female infertility due to the other causes. These data together indicate that infertility itself, rather than the cause of infertility, could be a predictive factor for the emergence of anti-FSH antibodies.

Female infertility has been shown to be associated with a higher occurrence of autoantibodies.^{1,10,11,13,14} Except disease-specific autoantibodies described in case of endometriosis,^{21,22} autoantibodies detected in infertile patients^{1,10,11,13,14} are usually not specific to infertility or to the gynecological diseases leading to infertility. Thus, a general immune dysbalance and activation of autoimmune processes are expected to be characteristic for female infertility.²³ Here we assessed a potential susceptibility of patient to autoimmunity by the presence of at least one of seven common autoantibodies in relation to the *HLA-DQB1* alleles. We demonstrated that anti-FSH IgM were associated with the production of common autoantibodies in IVF patients. Our results along the ones from the literature discussed above indicate, that the production of anti-FSH IgM could be related to a general propensity to autoimmunity in infertile women.

The female infertility has often been studied in the context of IVF. The follicular puncture performed in IVF, in particular, can induce the production of anti-ovarian antibodies (AOA).¹⁰ Patients with AOA have often antibodies against FSH, as one of the major autoantigen for the pool of autoantibodies forming AOA.²⁴ In concordance with these data, we showed that the level of anti-FSH IgM was higher in the patients who had undergone previous IVF procedures. The association was revealed among IVF patients who were suffering from PCOS, endometriosis, unexplained infertility and infertility due to the other causes, but not among the women with tubal or male factor infertility. These results encourage us to speculate, that repeatedly performed ovarian punctures do not enhance anti-ovarian autoimmunity

unless a patient's infertility is caused by the diseases associated with disturbances in immune regulation.^{1,10,11,13,14} However, simply based on the association study performed here, we cannot substantiate whether the antibodies themselves may cause the need for multiple IVF procedures, or alternatively, the use of IVF procedure *per se* may enhance the production of anti-FSH.

In our study, the production of anti-FSH IgM antibodies was also associated with peripheral FSH hormone levels (i.e. with the amount of hormone produced), but only among IVF patients with tubal and male factor infertility. However, several conditions have been reported where the production of autoantibodies is associated with the elevated level of autoantigen, such as elevated FSH levels and AOA in premature menopause.¹¹ Similarly, autoantibodies and insulin levels in pancreatic β cells are correlated in murine autoimmune diabetes models.¹² In the current study, the level of FSH remained between the reference values for the majority of patients, with only five patients (3.7%) having FSH level above the reference value (>21.7 IU/L). Moreover, the anti-FSH IgM was correlated with the level of peripheral FSH in the patients with rather lower level of hormone and infertility caused by other than immune system dysregulation – the patients with tubal and male factor infertility. These results support our idea of anti-FSH antibodies being the primarily naturally occurring antibodies. This hypothesis is further supported by the discussion provided by Thomas (2001) who concluded that physiological hormone levels remain below a critical threshold for the stimulation of relevant autoimmune reactions.²⁵

The receptor-binding and hormone specificity determining β -subunit of FSH hormone is coded by *FSHB* gene at the 11p13.²⁶ Haplotype analysis has revealed two most prevalent variants of *FSHB* gene – HAP1 and HAP13, covering together about 90% of Estonians.¹⁹ Similarly to insulin gene polymorphisms affecting central tolerance through the level of gene expression in thymus,⁸ we were looking for an association between the two *FSHB* core haplotypes¹⁹ and autoimmunity against FSH. As we could not detect such relationship, we suggest that either these SNPs do not affect gene expression in the thymus during central tolerance induction, or that *FSHB*-associated autoimmunity to FSH depends on *HLA-DQB1* allelic variants other than those evaluated in the current study.

The production of anti-FSH IgA is probably related to different factors than those involved in the production of anti-FSH IgM. Anti-FSH IgA were associated with the presence of the *HLA-DQB1*03* allele but not with the cause of infertility, the history of previous IVF attempts or the presence of other autoantibodies. The cervical mucosa along with that of the endometrium most probably plays a major part in the induction of the mucosal tolerance to paternal antigens.^{27,28} In this context, it is interesting to refer to the published associations between the *HLA-DQB1*03* allele, and the presence of the sperm-immobilizing antibodies in cervical secretions.²⁹ Therefore, it would be tempting to speculate that anti-FSH IgA could not be autoantibodies but alloantibodies triggered by seminal FSH,^{30,31} and originating from mucosal response, as discussed previously.¹⁵ The reasons for an increased production of this particular IgA isotype of antibodies in IVF patients, however, remain unclear.

Correlation analysis of anti-FSH antibody values showed that the levels of anti-FSH IgM and IgA correlated both with the values of anti-FSH IgG. There are some indirect evidence that anti-FSH IgG antibodies may, however, further worsen female fecundity by reducing the FSH functionality.^{32,33} An important question of whether anti-FSH antibodies influence the outcome of IVF treatment, comes up from the current study and is also the priority of our ongoing studies.

In conclusion, our results suggest that anti-FSH antibodies may be naturally occurring antibodies associated with peripheral FSH concentrations and produced in higher levels in infertile women. The production of anti-FSH IgM and IgG antibodies was not associated with *FSHB* gene haplotypes, but could be related to a general propensity to autoimmunity or to previous IVF treatments. The elevated values of anti-FSH IgA in IVF patients could be explained by somewhat different mechanisms including a genetically determined failure in mucosal tolerance in the genital tract. Regardless of the origin of the stimulation of anti-FSH antibody production in infertile women, the impact of this phenomenon on the outcome of infertility treatment deserves further evaluation.

Acknowledgments

This study was supported by the Estonian Science Foundation (Grants no. 6498, 6514 and 5796), the

Estonian Ministry of Education and Science (Core grants no. 0182582Cs03, 0182586s03 and 0182721s06), the Wellcome Trust International Senior Research Fellowship (Grant no. 070191/Z/03/Z), the Howard Hughes Institute International grant (Grant no. 55005617), Estonia-France Parrot grant and a grant from UHP-BQRI in Nancy. We thank Dr T. Forges from the Centre d'Assistance Médicale a la Procréation, Maternité Régionalé et Universitaire, Nancy Cedex, France for providing the antigen for anti-FSH assay. K. Teesalu MSc, A. Kaldmaa, K. Koppel and E. Prans from the Department of Immunology of the University of Tartu, Estonia and C. Mathieu from the Laboratoire d'Immunologie du CHU, Nancy, France are acknowledged for their methodological and laboratory assistance. H. Pisarev from the Department of Epidemiology and Biostatistics of the University of Tartu, Estonia is greatly acknowledged for her advice with statistical analysis. We also thank Dr E. Talving, Dr P. Karits and K. Rohlta from the Nova Vita Clinic, Centre for Infertility Treatment and Medical Genetics, Estonia for collecting patient data. This study was partly presented at the 16th European Congress of Immunology – ECI, September 6–9, 2006, Paris, France.

References

- 1 Geva E, Amit A, Lerner-Geva L, Lessing JB: Autoimmunity and reproduction. *Fertil Steril* 1997; 67:599–611.
- 2 Szekeres-Bartho J, Barakonyi A, Miko E, Polgar B, Palcovics T: The role of γ/δ T cells in the fetomaternal relationship. *Semin Immunol* 2001; 13:229–233.
- 3 Tremellen KP, Valbuena D, Landeras J, Ballesteros A, Martinez J, Mendoza S, Norman RJ, Robertson SA, Simon C: The effect of intercourse on pregnancy rates during assisted human reproduction. *Hum Reprod* 2000; 15:2653–2658.
- 4 Hegde UC, Ranpura S, D'Souza S, Raghavan VP: Immunoregulatory pathways in pregnancy. *Indian J Biochem Biophys* 2001; 38:207–219.
- 5 Robertson SA, Bromfield JJ, Tremellen KP: Seminal 'priming' for protection from pre-eclampsia – a unifying hypothesis. *J Reprod Immunol* 2003; 59: 253–265.
- 6 Nepom GT, Kwok WW: Molecular basis for HLA-DQ associations with IDDM. *Diabetes* 1998; 47:1177–1184.
- 7 Muraro PA, Douek DC: Renewing the T cell repertoire to arrest autoimmune aggression. *Trends Immunol* 2006; 27:61–67.

- 8 Vafiadis P, Ounissi-Benkhalha H, Palumbo M, Grabs R, Rousseau M, Goodyer CG, Polychronacos C: Class III alleles of the variable number of tandem repeat insulin polymorphism associated with silencing of thymic insulin predispose to type 1 diabetes. *J Clin Endocrinol Metab* 2001; 86:3705–3710.
- 9 Jasinski JM, Eisenbarth GS: Insulin as a primary autoantigen for type 1A diabetes. *Clin Dev Immunol* 2005; 12:181–186.
- 10 Gobert B, Barbarino-Monnier P, Guillet-May F, Béné MC, Faure GC: Anti-ovary antibodies after attempts at human in vitro fertilization induced by follicular puncture rather than hormonal stimulation. *J Reprod Fertil* 1992; 96:213–218.
- 11 Fénichel P, Gobert B, Carré Y, Barbarino-Monnier P, Hiéronimus S: Polycystic ovary syndrome in autoimmune disease. *Lancet* 1999; 353:2210.
- 12 Byersdorfer CA, Schweitzer GG, Unanue ER: Diabetes is predicted by the β cell level of autoantigen. *J Immunol* 2005; 175:4347–4354.
- 13 Reimand K, Talja I, Metsküla K, Kadastik Ü, Matt K, Uibo R: Autoantibody studies of female patients with reproductive failure. *J Reprod Immunol* 2001; 51: 167–176.
- 14 Matarese G, De Placido G, Nikas Y, Alviggi C: Pathogenesis of endometriosis: natural immunity dysfunction or autoimmune disease? *Trends Mol Med* 2003; 9:2.
- 15 Haller K, Mathieu C, Rull K, Matt K, Béné MC, Uibo R: IgG, IgA and IgM antibodies against FSH: serological markers of pathogenic autoimmunity or of normal immunoregulation? *Am J Reprod Immunol* 2005; 54:262–269.
- 16 Forti G, Krausz C: Clinical review 100 valuation and treatment of the infertile couple. *J Clin Endocrinol Metab* 1998; 83:4177–4188.
- 17 World Health Organization: WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction. Cambridge University Press, Cambridge, UK, 1999.
- 18 Khamashta MA, Hughes GRV: Detection and importance of anticardiolipin antibodies. *J Clin Pathol* 1993; 46:104–107.
- 19 Grigorova M, Rull K, Laan M: Haplotype structure of FSHB, the beta-subunit gene for fertility-associated follicle-stimulating hormone: possible influence of balancing selection. *Ann Hum Genet* 2007; 71:18–28.
- 20 Sjöroos M, Iitiä A, Ilonen J, Reijonen H, Lövgren T: Triple-label hybridization assay for type I diabetes-related HLA alleles. *Biotechniques* 1995; 18:870–877.
- 21 Mathur SP, Lee JH, Jiang H, Arnaud P, Rust PF: Levels of transferrin and alpha 2-HS glycoprotein in women with and without endometriosis. *Autoimmunity* 1999; 29:121–127.
- 22 Lang GA, Yeaman GR: Autoantibodies in endometriosis sera recognize a Thomsen–Friedenreich-like carbohydrate antigen. *J Autoimmun* 2001; 6:151–161.
- 23 Gleicher N: Antiphospholipid antibodies (aPL) affect in vitro fertilization (IVF) outcome. *Am J Reprod Immunol* 2001; 46:330–331.
- 24 Gobert B, Jolivet-Reynaud C, Dalton P, Barbarino-Monnier P, Faure CG, Jolivet M, Béné MC: An immunoreactive peptide of the FSH involved in autoimmune infertility. *Biochem Biophys Res Commun* 2001; 289:819–824.
- 25 Thomas JW: Antigen-specific responses in autoimmunity and tolerance. *Immunol Res* 2001; 23:235–244.
- 26 Fox KM, Dias JA, Van Roey P: Three dimensional structure of human follicle-stimulating hormone. *Molec Endocr* 2001; 15:378–389.
- 27 Robertson SA: Control of the immunological environment of the uterus. *Rev Reprod* 2000; 5: 164–174.
- 28 Geva E, Yovel I, Lerner-Geva L, Lessing JB, Azem F, Amit A: Intrauterine insemination before transfer of frozen-thawed embryos may improve the pregnancy rate for couples with unexplained infertility: preliminary results of a randomized prospective study. *Fertil Steril* 2000; 73:755–760.
- 29 Tsuji Y, Mitsuo M, Yasunami R, Sakata K, Shibahara H, Koyama K: HLA-DR and HLA-DQ gene typing of infertile women possessing sperm-immobilizing antibody. *J Reprod Immunol* 2000; 46:31–38.
- 30 Vasquez JM, Ben-Nun I, Greenblatt RB, Mahesh VB, Keel BA: Correlation between follicle-stimulating hormone, luteinizing hormone, prolactin, and testosterone with sperm cell concentration and motility. *Obstet Gynecol* 1986; 67:86–90.
- 31 Luboshitzky R, Kaplan-Zverling M, Shen-Orr Z, Nave R, Herer P: Seminal plasma androgen/oestrogen balance in infertile men. *Int J Androl* 2002; 25: 345–351.
- 32 Meyer WR, Lavy G, DeCherney AH, Visintin I, Economy K, Luborsky JL: Evidence of gonadal and gonadotropin antibodies in women with a suboptimal ovarian response to exogenous gonadotropin. *Obstet Gynecol* 1990; 75:795–799.
- 33 Reznik Y, Benhaim A, Morello M, Herlicoviez M, Ballet J-J, Mahoudeau J: High frequency of IgG antagonizing follicle-stimulating hormone – stimulated steroidogenesis in infertile women with a good response to exogenous gonadotropins. *Fertil Steril* 1998; 69:46–52.