

**ANTIBODIES AND AUTOIMMUNE DISEASES  
IN RELATION TO REPRODUCTIVE FAILURES**

Summary of PhD Thesis

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## INTRODUCTION

Autoantibodies, autoimmune conditions are frequent causes underlying unsuccessful attempts for having a child. In a significant part of cases these are not recognised as the affected women have usually no other signs or symptoms suggesting an autoimmune disease.

A couple is considered sterile if there is a complete inability to conceive or produce an offspring. The infertility defined by the absence of conception after 24 months of regular, unprotected intercourse. Of couples in reproductive age, 8 to 10 % are sterile and 15 to 20 % are infertile. Various genetic, hormonal, gynaecological, andrological, and immunological factors may underlie both conditions, but in about 10 to 20 % of cases there is no detectable cause. In sterile/infertile (st/if) female patients auto-antibodies may be present which often escape detection, as affected patients have no signs or symptoms suggesting an autoimmune disease. However, sterility/infertility in women of childbearing age can be considered, particularly in the presence of autoantibodies, as a sign of a potential autoimmune disease. By detecting and appropriately treating autoimmune diseases/antibodies as latent pathogenetic factors underlying gestational morbidity, a part of sterility/infertility has become extinguishable.

## AIMS OF THE INVESTIGATION

**The aim of our study was to examine the antibodies and autoimmune diseases in relation to reproductive failures.**

1. The first aim was to establish the incidence of antiphospholipid antibodies (aPLs) which are included into the antiphospholipid syndrome (APS) diagnostic criteria (Sydney, 2004.) such as anticardiolipin antibody (aCL) and anti-beta-2 glycoprotein I antibody (a $\beta$ 2GPI) among the femal patients with reproductive failures.
2. The second aim was to detect some less frequent aPLs neither included into the diagnostic criteria such as anti-Annexin V antibody (aANX), anti-phosphatidyl serine antibody (aPS), and anti-prothrombin antibody (aPT) by our femal patients.
3. The third aim of our study was to diagnose APS among patients with aPLs when clinical criteria were also taken into consideration and to apply anti-coagulant treatment among them.
4. The fourth aim was to examine femal patients for the systemic autoimmune diseases apart from APS.

5. The fifth aim of our study was to establish the diagnoses of organ-specific autoimmune diseases such as autoimmune thyroiditis and celiac disease and to use their adequate treatment among patients with reproductive failures.

6. The sixth aim was to detect a-sperm antibody in femal patients and to investigate its role in reproductive failures.

## PATIENTS AND METHODS

### Patients

On the Department of Internal Medicine of Zala County Hospital during 88 months (between September 2004 and December 2011) 500 female patients (mean age:  $31.4 \pm 4$  years) were enrolled to our study, including 167 and 333 patients (33 % and 67 %) with sterility and infertility respectively. Any other causes underlying the sterility/infertility could be excluded or have already been eliminated previously in all of them. In addition to history taking and physical examination, our patients underwent the following tests: assay of antinuclear antibody (ANA) by immunofluorescent (IF) method, as well as enzyme-linked immunosorbent assays (ELISA) of anti-double-stranded DNA antibody (anti-dsDNA) (cut off: 25 - 120 U/mL), anticardiolipin antibody (aCL) (cut off: 10 - 100 MPL/mL and 10 - 104 GPL/mL), antibodies against extractable nuclear antigens (ENA-Profile) ( $> 1.2$  U/mL) such as anti-histidyl-tRNA synthetase antibody (anti-Jo-1), anti-ribonucleoprotein antibody (anti-RNP), antibody to Sm-proteins (anti-Sm), antibody to 3-component non-histone proteins (anti-SS-A), antibody to phosphoprotein of RNA-polimerase III cofactor (anti-SS-B), anti-thyroid peroxidase antibody (anti-TPO) (cut off: 0 - 30 U/mL), anti-phosphatidyl serine antibody (aPS) ( $> 15$  U/mL), anti-prothrombin antibody (aPT) ( $> 10$  U/mL), anti-beta-2 glycoprotein 1 antibody (a $\beta$ 2GPI) ( $> 10$  U/mL), anti-Annexin V antibody (aANX) ( $> 5$  U/mL), anti-tissue-transglutaminase antibody (anti-tTG) (cut-off: 0-7 U/mL), and anti-sperm antibody (a-sperm). We also examined 500 male patients (they are partners of our female patients) (mean age:  $38.5 \pm 3$  years) for CD, who underwent the tests of anti-tTG (cut-off: 0-7 U/ml) by ELISA. Furthermore, our anti-tTG positive female and male patients underwent deep duodenal biopsy for histological examination in order to confirm the diagnosis of CD and we carried laboratory tests out by them too, such as hemoglobin (Hgb) (123-153 g/L), hematocrit (Htc) (0.36-0.46 L/L), serum iron (Fe) (8.7-27.0  $\mu$ mol/L), serum ferritin (15-150 ng/mL), serum vitamin B12 (197-866 pg/mL), serum folic acid (3.1-17.5 ng/mL), serum total protein (65-80 g/l), serum albumin (33-50 g/L), serum immunoglobulin G (IgG) (7-16 g/L), se

immunoglobulin A (IgA) (0.7-4.0 g/L), se immunoglobulin M (IgM) (0.4-2.3 g/L). Finally, our anti-TPO positive females have been studied for serum thyroid-stimulating hormone (TSH) (0.4-3.2 mU/L), serum free tri-jodide-thyronine (FT3) (2.23-6.50 pmol/L) and free thyroxin (FT4) (9.14-23.81 pmol/L).

## **Methods**

### Enzyme-linked immunosorbent assays (ELISA):

Anti-dsDNA was measured by ELISA technique. The surface of a polystyrene microtitration plate was sensitized for DNA overnight. On the next day, following three washings with PBS + 0.05 % Tween, samples were allotted to the wells of the plate doubled in 40x dilution. After 1-hour-long incubation and triple washings, anti-humane IgG/HRP (DAKO AS, Denmark) was pipetted into the wells in 6.000x dilution. Following a further 1-hour-long incubation and washing, o-phenylenediamine-H<sub>2</sub>O<sub>2</sub> substrate was applied, and then the color reaction was set by allotting 4N sulfuric acid. Results were measured at 492 nm with Labsystems MS Multiskan ELISA photometer and given in µg/mL based on a calibration curve for IgG.

Auto-antibodies against ENA and ENA subtypes (SS-A, SS-B, Sm, RNP, Jo-1) as well as antibodies against TPO, tTG, sperm, and aPLs (aCL, aβ2GPI, aPS, aPT, aANX) were detected by a commercially available ELISA test (AUTOSTAT II ENA-Screen, anti-SS-A, anti-SS-B, anti-Sm, anti-RNP, anti-Jo-1, anti-TPO, anti-tTG, a-sperm, aCL, aβ2GPI, aPS, aPT, aANX; Cogent-Hycor, Peniciuk, UK), in accordance with the instructions of the manufacturer.

### Immunofluorescent method (IF):

ANA was detected on HEp-2 cell substrate (Human Epithelioma Type 2 Cells - CCL - 23 - American TypeCulture Collection) with a standard indirect immunofluorescent technique. By using a laboratory-cultured cell line, cell preparations were made and fixed in acetone. Patient sera were applied in 40x dilution, and the bound auto-antibodies were detected with anti-humane IgG/FITC (The Binding Site, UK). Preparations were evaluated with a Leica Diaplan fluorescent microscope, at 500x magnification. Patterns of IF were created due to binding of ANA to various components of the cell nucleus.

Routine blood tests, TSH, FT3, FT4 and serum immunoglobulins were measured as a part of routine diagnostics, by using of Sysmex XP-1800I, Vacuette SRS 100/II, Modular MODU P8000 and Modular E170 systemes.

## **I. ANTIPHOSPHOLIPID ANTIBODIES AND ANTIPHOSPHOLIPID SYNDROME IN RELATION TO REPRODUCTIVE FAILURES**

### **Aims**

The aim was to establish the prevalence of aPLs which are included into the APS diagnostic criteria (Sydney, 2004.) such as aCL, a $\beta$ 2GPI, and detect some less frequent aPLs neither included into the diagnostic criteria such as aANX, aPS, and aPT, as well as to diagnose APS.

### **Results**

Twofold positive aCL was demonstrated in 27/500 patients (5.0 %) (st/inf: 4/23). When clinical criteria were also taken into consideration, 3/27 primary antiphospholipid syndrome (PAPS) could be diagnosed. In 4/27 cases the APS was associated with the presence of another antibodies, such as ANA, anti-dsDNA, anti-SS-A, anti-TPO, a-sperm, however these women didn't suffered from another autoimmune disease, accordingly secondary antiphospholipid syndrome (SAPS) couldn't be confirmed, but they were observed towards SAPS. In further 20/27 cases the clinical picture did not fulfil the criteria of APS as 15 infertile patients were examined already after the first or second abortion prior to Week (W) 10 of gestation, and further 5 patients were sterile (what is not even included in the system of criteria for APS).

In addition to the twofold positive aCL, less frequent antiphospholipid antibodies (aPLs) including a $\beta$ 2GPI, aPS or both were present in 2/27, 3/27 and 2/27 patients, as well as aANX in 2/27, aANX with together aPS in 1/27 and aPS along with aPT in 1/27 patients respectively.

The aCL could be detected on a single occasion in 34/500 women (6.8 %) (st/inf: 17/17), including 2/34 patients where PAPS and 1/34 patients where SAPS was suggested on the base of the clinical picture.

Among one-time aCL-positive patients a $\beta$ 2GPI, aPS, aPT and aANX could be detected in 1/34, 9/34, 2/34 and 1/34 patients, as well as a $\beta$ 2GPI with aANX and a $\beta$ 2GPI with aPS and aPS with aANX could be established in 2/34, 1/34 and 1/34 patients.

Considering the laboratory criteria of APS we studied the occurrence the a $\beta$ 2GPI antibody. Fifteen of the 500 (3.0 %) (st/inf: 4/11) patients was a $\beta$ 2GPI positive. We detected this antibody in 9/15 females with aCL-positive and 6/15 patients was aCL-negative.

The a $\beta$ 2GPI was established one-time in 10/15 cases and twice in 5/15 cases in addition to we could find 2/5 aCL-negative females in twofold a $\beta$ 2GPI-positive group. According to the

clinical criteria of APS we could confirm 1 PAPS-patient (she was twofold aCL-positive too) and 2 SAPS-patients, among them 1 was also one-time aCL-positive and 1 patient was only twice a $\beta$ 2GPI-positive.

Among 439 aCL-negative women, we found in 76/439 (17.3 %) patients not only a $\beta$ 2GPI antibody, but also such less frequent antibodies of APS (for instance aPS, aPT, aANX) too which not included in its laboratory criteria. The a $\beta$ 2GPI antibody was one-time positive in 2/76 cases and twofold positive in 1/76 case.

The aPS in itself was detected in 45/76 women, aPS with a $\beta$ 2GPI and aPS with aPT antibodies were verified equally in 1-1/76 patient. In six of 76 women aPS was detectable with together aANX antibody. We found the aPT in 1/76 case respectively and aPT with a $\beta$ 2GPI also in 1/76 woman. The aANX antibody alone was established in 16/439 patients and aANX along with a $\beta$ 2GPI was in 1/76 case. Finally, aPS with together aPT and aANX antibodies was detectable in 1/76 patient.

Summing up the results of our investigations - taking the clinical and laboratory criteria of APS into consideration - we could demonstrate the diagnosis of APS in 11/500 (2.2 %) patients, there are 5/11 PAPS-patients and 6/11 SAPS-patients among them.

#### Treatment in the follow-up period:

By the aPLs-positive patients we used the anticoagulant treatment, namely:

1. Administration of acetylsalicylic acid (ASA) (100 mg/day orally up to week 28 of gestation) alone, when the patient was aPLs-positive but the clinical picture and/or laboratory criteria did not fulfil the diagnostic criteria of APS or the patient rejected the anticoagulant injection.
2. Administration of maintenance dose low molecular weight heparin (LMWH) (100 U/kg/day subcutaneous up to the delivery) alone, when the patient was allergic to acetylsalicylic acid or the pregnant suffered from hematoma around fetus or hemorrhage from uterus.
3. Administration of ASA (100 mg/day orally up to week 28 of gestation) plus maintenance dose LMWH (100 U/kg/day subcutaneous up to the delivery).

In cases of SAPS or the patients with aPLs and antibodies of systemic autoimmune diseases and/or a-sperm antibody we completed the anticoagulant treatment with prednisolone (20 mg/day orally in the first trimester and then tapered to a maintenance dose of 5 mg/day) too. When patients suffered from autoimmune thyroiditis (and/or latent hypothyroidism) the anticoagulant cure was completed levothyroxine too.

Among the twofold aCL-positive patients 22 conceptions were developed, itemized: 2/22 abortions (ABs) and 19/22 deliveries such as 15/19 spontaneous (sp) developed pregnancies and 4/19 pregnancies apropos of in vitro fertilization (IVF) / insemination (insem). In addition 1/22 pregnancy is currently in progress. Furthermore 3 females were pregnant twice and there were 2 twin-pregnancies.

In the group of females with once-time aCL positivity we could detect 15 pregnancies, namely 3/15 ABs, 12/15 child-births (8/12 sp developed pregnancies and 4/12 IVF/insem pregnancies) and 1 twin-pregnancy.

When the aCL antibody was negative, but a $\beta$ 2GPI or other less frequent antibody of APS (for instance aPS, aPT, aANX) was positive, in the follow-up period there were 52 conceptions: 13/52 ABs, 32/52 deliveries (27/32 sp developed pregnancies and 5/32 IVF/insem pregnancies), 7/52 pregnancies are currently in progress (6/7 sp developed pregnancies, 1/7 IVF/insem pregnancy). In addition 3 women were pregnant twice and 5 pregnancies resulted twins.

Lastly, among patients with together more aPLs 8 conceptions occurred such as 1/8 AB, 6/8 labours (4/6 sp developed pregnancies and 2/6 IVF/insem pregnancies), 1/8 pregnancy is currently in progress and 1 twin-pregnancy was presented.

## **II. ANTIBODIES OF THE SYSTEMIC AUTOIMMUNE DISEASES IN RELATION TO REPRODUCTIVE FAILURES**

### **Aims**

The aim was to examine female patients for the systemic autoimmune diseases apart from APS and determine prevalence of autoantibodies such as ANA, anti-dsDNA, anti-Jo-1, anti-Sm, anti-RNP, anti-SS-A and anti-SS-B.

### **Results**

The auto-antibodies for the systemic autoimmune diseases could be established in 151/500 (30.2 %) patients.

In 66/500 (13.2 %) females these antibodies were detected without other auto-antibodies for instance aPLs, anti-tTG and anti-TPO. Among them we found ANA in itself in 52/66 women, furthermore ANA with anti-Sm, ANA with anti-SS-A and ANA with anti-Jo-1 antibodies were detectable equally in 1-1-1/66 patient. We verified ANA with together anti-SS-A and anti-Sm antibodies and ANA with together anti-SS-A and anti-SS-B antibodies in 1-1/66 case.

The anti-SS-A antibody could be establish in 2/66 patients, in addition to anti-SS-B, anti-Jo-1 and anti-RNP antibodies were found in 1-1-1/66 female respectively. In 3/66 patients the anti-dsDNA antibody in itself was presented. Finally, anti-RNP along with anti-SS-A and anti-SS-B antibodies were detectable in 1/66 patient.

In 85/500 (17.0 %) women the auto-antibodies of the systemic autoimmun diseases could be verify with together aPLs or auto-antibodies for organ-specific autoimmune diseases or a-sperm antibody. We could find mostly ANA (64/85), anti-dsDNA (9/85), and anti-SS-A (7/85) auto-antibodies with each other and/or aPLs and/or antibodies of the organ-specific autoimmune diseases and/or a-sperm antibody. Finally, in three of the 85 cases anti-Sm and in 2/85 females anti-SS-B antibody could be establish along with further antibodies.

#### Treatment in the follow-up period:

In cases of patients with antibodies of systemic autoimmune diseases we applied not only prednisolone (20 mg/day orally in the first trimester and then tapered to a maintenance dose of 5 mg/day) but also anticoagulant treatment (the same as in the aPLs-positive patients) too. Thanks to application of these medications we could detected 29 conception among these patients, itemized: 5/29 ABs, 22/29 child-births (18/22 sp developed pregnancies and 4/22 IVF/insem pregnancies), 2/29 pregnancies are currently in progress (1/2 sp developed pregnancy, 1/2 IVF/insem pregnancy). Moreover 3 women were pregnant twice and 1 pregnancy resulted twins.

### **III. AUTOIMMUNE THYROIDITIS AND THYROID DISFUNCTION IN RELATION TO REPRODUCTIVE FAILURES**

#### **Aims**

The aim was to verify the presence of autoimmune thyroiditis and thyroid dysfunction among female patients with reproductive failures.

#### **Results**

The anti-TPO as the organ-specific auto-antibody of the autoimmune thyroiditis was detectable in 71/500 (14.2 %) women, among them 52/71 patients were euthyroid and the further 19/71 patients suffered from latent hypothyroidism. Among the 19/71 patients suffered from latent hypothyroidism the anti-TPO in itself was detected in 9/19 women and we verified in 10/19 cases another sort of antibodies along with anti-TPO.

In 18/52 euthyroid cases the anti-TPO antibody could be established in itself, moreover in this group we studied other auto-antibodies of the systemic autoimmune and organ-specific autoimmune diseases, aPLs and a-sperm antibody too. In our study, we could detect anti-TPO with together ANA in 5/52 patients, in 11/52 cases not only anti-TPO and ANA antibodies were detectable but also other antibodies. Investigating of antibodies of the systemic autoimmune diseases except the ANA in 3/52 women we could confirm anti-TPO antibody along with anti-dsDNA, anti-Sm and anti-SS-A.

As far as aPLs are concerned, including aCL and aANX antibodies were present equally in 2-2/52 patients, as well as a $\beta$ 2GPI and aPS antibodies were verified in 1-1/52 case with together anti-TPO and in 3/52 cases we observed coexistence of two aPLs too.

In respect to the organ-specific auto-antibodies, the anti-tTG with anti-TPO was verified in 1/52 case. Last but not least, anti-TPO with a-sperm antibody could be detect in 2/52 patients furthermore anti-TPO with together a-sperm and aCL antibodies was detected in 1/52 case.

In our results we noticed 18/500 (3.6 %) women suffering from latent hypothyroidism without anti-TPO antibody and what is more among them there are 9/18 patients without any antibodies. However in further 9/18 females with latent hypothyreosis we could find other antibodies such as ANA, anti-dsDNA, aCL, aPS, anti-tTG, a-sperm.

#### Treatment in the follow-up period:

Among patients suffered from latent hypothyroidism with or without anti-TPO antibody we could cure with levothyroxine (starting with a dose of 25  $\mu$ g/day orally, and adjusted according to the TSH-hormone findings when required).

Among 9/18 patients with latent hypothyroidism without anti-TPO and other antibodies 1 patient with levothyroxine plus ASA had got 2 child-births (sp developed pregnancies) and in this group levothyroxine alone resulted further 3 deliveries (sp developed pregnancies).

In cases of patients with autoimmune thyroiditis and latent hypothyroidism along with antibodies of systemic autoimmune diseases and a-sperm we could 14 conceptions were presented, from them there were 4/14 ABs, 8/14 labour (5/8 sp developed pregnancies and 3/8 IVF/insem pregnancies), 2/14 pregnancies are currently in progress. Moreover 1 female was pregnant twice and 1 pregnancy resulted twins.

When anti-TPO antibody was positive and the patients with euthyroidism, in the follow-up period 19 conceptions were verified: 3/19 ABs, 16/19 child-briths (13/16 sp developed pregnancies and 3/16 IVF/insem pregnancies). In addition 2 women were pregnant twice and 2 pregnancies resulted twins.

## IV. CELIAC DISEASE IN RELATION TO REPRODUCTIVE FAILURES

### Aims

The anti-tTG antibody for celiac disease (CD) was examined in female and male patients (they are partners of our female patients), then anti-tTG positive patients underwent deep duodenal biopsy for histological examination in order to confirm the diagnosis of CD and we carried laboratory tests out by them too.

### Results

Among male patients 4/500 (0.8 %) anti-tTG positivity has been demonstrated; histology was pathognomic for CD in all of them and the examination of sperm showed normal parameters in all cases.

Among the women we could detect anti-tTG in 11/500 (2.2 %) cases of whom the diagnosis of celiac disease was histologically confirmed in 8/500 (1.6 %) patients.

### Treatment in the follow-up period:

In the follow-up period success of gluten-free diet we could verify 3/8 miscarriages, 3/8 child-births (2/3 sp developed pregnancies and 1/3 IVF/insem pregnancies) and 2/8 pregnancies are currently in progress.

### Case reports

#### Case 1

In May 2009 we examined the 30-year-old female patient after 5 unsuccessful inseminations. Her medical history included treatment for bronchial asthma in childhood and permanent constipation. Her laboratory findings (Hgb: 134 g/l; Htc: 0.41 l/l; serum Fe: 7.4 µmol/l; serum ferritin: 28.7 ng/ml; serum vitamin B12: 344 pg/ml; serum folic acid: 20 ng/ml; serum total protein: 69 g/l; serum albumin: 45 g/l) showed slight iron deficiency and selective IgA deficiency (serum IgA: 0.67 g/l; serum IgG: 10.89 g/l; serum IgM: 0.84 g/l). The immunoserological tests demonstrated the positivity of anti-tTG (23 U/ml) and aCL (it could be detected only on one occasion, it turned to negative already after 6 weeks). Based on the subtotal villous atrophy confirmed by histological examination, we diagnosed CD at which we recommended further observation for selective IgA deficiency and APS. The patient kept a strict gluten-free diet and received also ASA (100 mg/day orally) therapy.

## **Case 2**

The 34-year-old male patient, examined in May 2009, had no complaints. However, his laboratory findings (Hgb: 143 g/l; Htc: 0.44 l/l; serum Fe: 4.9  $\mu$ mol/l; serum ferritin: 3.4 ng/ml; serum vitamin B12: 389 pg/ml; serum folic acid: 2.86 ng/ml; serum total protein: 63 g/l; serum albumin: 41 g/l) indicated iron and folic acid deficiency and slight hypoproteinemia. Of his immuno-serological results, anti-tTG (76.1 U/ml) has proven to be positive and the aPS showed positivity on one occasion. Histological examination demonstrated total villous atrophy. The findings related to the sperm of this male patient with CD showed normal values concerning all examined parameters including the number, shape and motion of his sperm cells. The patient started gluten-free diet.

Curiously, the partner of the above male patient with CD was a female patient with CD, APS, and selective IgA deficiency, also diagnosed by us. Their genetic testing detected HLA-DQ2 homozygous state in both of them. The couple with gluten sensitivity commonly continued keeping a strict gluten-free diet. In November 2009 - as no spontaneous conception occurred up to that time - she underwent IVF which ended with no success.

In March 2010 another IVF was planned, however the menstruation of the female patient delayed and her pregnancy test indicated spontaneously conceived gravidity. The pregnant woman with CD underwent regular medical and gynecologic follow-ups and no overt state of deficiency (iron, vitamin B12, folic acid) could be detected during her pregnancy. In October 2010, in the 26<sup>th</sup> week of her pregnancy her anti-tTG has already proven to be negative (0.52 U/ml). In January 2011 she delivered a healthy newborn (birth weight: 2980 g) in the 38<sup>th</sup> week of her gestation.

## **V. ANTI-SPEM ANTIBODY IN RELATION TO REPRODUCTIVE FAILURES**

### **Aims**

The aim was to establish the prevalence of a-sperm antibody in female patient with reproductive failures.

### **Results**

The a-sperm antibody could be presentable in 49/500 (9.8 %) cases and it could be detected alone in 21/49 women. In further 28/49 patients a-sperm was concomitant with other

antibodies such as ANA, anti-dsDNA, aCL, aPS, aANX, anti-SS-A, anti-SS-B, anti-TPO, anti-tTG:

Treatment in the follow-up period:

When the patient was a-sperm positive alone or with ANA or anti-SS-A antibody, there were 19 conceptions such as: 2/19 ABs, 16/19 deliveries (13/16 sp developed pregnancies, 3/16 IVF/insem pregnancies) and 1/19 pregnancy is currently in progress furthermore we verified 1 twice-pregnant woman and 3 pregnancies resulted twins.

## **DISCUSSION OF THE DISSERTATION**

Of couples in reproductive age, 8 to 10 % are sterile and 15 to 20 % are infertile. A couple is sterile when no pregnancy occurs within 2 years. In case of infertility, conception takes place; however due to any case, no live neonate is born from the pregnancy/pregnancies. 1 to 2 % of women in childbearing age have habitual abortions, or at least 3 consecutive spontaneous miscarriages. Underlying both sterility and infertility, there may be various genetic, hormonal, gynecologic, andrologic or immunologic factors; however no detectable cause is found in a significant part of cases. (Auto)antibodies may be present in both members of the couples, causing failure of attempts to have children. Often these are not recognized, as the affected individuals have no signs or symptoms suggesting any autoimmune disease. Nevertheless, sterility/infertility in women of childbearing age who have autoantibodies is considered as a potential sign of an autoimmune disease.

Due to the importance of the issue, we believed that studying of the immunologic processes underlying sterility and infertility is worth to be the topic of a PhD thesis.

### **Antiphospholipid antibodies and antiphospholipid syndrome in relation to reproductive failures**

In our work with sterile/infertile women, we studied not only the presence of aCL and a $\beta$ 2GPI antibodies which are included in the diagnostic criteria of APS, but also the presence of further aPLs (aANX, aPS, and aPT) which are not among the criteria, but can often be found in the background of pathologic pregnancies.

Antibodies included in the laboratory criteria of APS (aCL, a $\beta$ 2GPI):

The aCL antibody could be detected on two occasions in 27/500 (5.0 %) (st./if.: 4/23) patients; in addition to aCL further aPLs were also present in 11/27 of them. The aCL

antibody could be observed on one occasion in 34/500 (6.8 %) (st./if.: 17/17) female patients; in this group other aPLs were found in addition to aCL in 17/34 patients. The presence of a $\beta$ 2GPI antibodies could be demonstrated in 15/500 (3.0 %) (st./if.: 4/11) patients (in 9/15 aCL-positive and in 6/15 aCL-negative women): a $\beta$ 2GPI proved to be positive once or twice in 10/15 and 5/15 patients respectively.

The following aPLs were observed among the aCL-negative patients:

Less frequent aPLs detected in 76/439 (17.3 %) of the 439/500 aCL-negative female patients: 3 a $\beta$ 2GPI; 45 aPS; 1 aPS + a $\beta$ 2GPI; 1 aPS + aPT; 6 aPS + aANX; 1 aPT; 1 aPT + a $\beta$ 2GPI; 16 aANX; 1 aANX + a $\beta$ 2GPI; 1 aPS + aPT + aANX.

We found aPL in a total of 137/500 women during the study; while taking both clinical and laboratory criteria of APS into consideration, the diagnosis of APS could be established in 11/500 (2.2 %) patients, including 5/11 and 6/11 patients with PAPS and SAPS respectively.

However, we used anticoagulant therapy during the follow-up period not only in patients with confirmed APS, but also in those who did not fulfill the diagnostic criteria of the disease completely. Consequently, 22 pregnancies (2/22 ABs, 19/22 deliveries; 1/22 pregnancy is currently in progress) occurred in patients with aCL positivity on two occasions, and there were 15 pregnancies (3/15 ABs, 12/15 childbirths) in women who were aCL-positive on one occasion. In the group of aCL antibody-negative women who were at the same time carriers of one of the less frequent aPLs, a total of 52 conceptions occurred (13/52 ABs, 32/52 deliveries; 7/52 pregnancies are currently in progress). Finally, among aCL-negative women who were at the same time carriers of multiple less frequent sPLs, 8 pregnancies were observed (1/8 AB, 6/8 childbirths, 1/8 pregnancy is currently in progress). Having taken the successfully delivered pregnancies into consideration, we also demonstrated that ASA + LMWH therapy has proven to be the most effective treatment in patients with aPLs.

Summing up, it can be stated that the previously undetected APS may be an important etiologic factor underlying sterility/infertility; furthermore an appropriate therapy can terminate the adverse effects of aPLs that cause morbidities of pregnancy.

### **Antibodies of the systemic autoimmune diseases in relation to reproductive failures**

In addition to APS, we also looked in our female patients for other autoantibodies (ANA, anti-dsDNA, anti-Jo-1, anti-Sm, anti-RNP, anti-SS-A, anti-SS-B) suggesting the presence of other systemic autoimmune diseases.

The presence of autoantibodies was observed in a total of 151/500 (30.2 %) patients; these antibodies occurred as single in 66/151 patients, and in the remaining 85/151 cases they were

detected in association with aPLs and/or with antibodies of organ-specific autoimmune diseases (anti-tTG, anti-TPO).

In this group of patients we used not only immunosuppressive prednisolone therapy, but also anticoagulant therapy in several cases, due to which 29 conceptions occurred (5/29 ABs, 22/29 childbirths; 2/29 pregnancies are currently in progress).

The results of our study showing that ANA, anti-dsDNA and anti-SS-A antibodies, particularly together with aPLs, occur very frequently in patients with reproductive problems are in accordance with the international literature. Although we could not diagnose any systemic autoimmune disease among our patients, as the specific clinical signs and symptoms characteristic to those were not present, nevertheless anticoagulant therapy seems to be justified in the presence of autoantibodies, as this may make a successful childbirth possible.

### **Autoimmune thyroiditis and thyroid dysfunction in relation to reproductive failures**

We screened the women with reproductive failure also for thyroiditis and thyroid dysfunction.

#### Autoimmune thyroiditis:

Anti-TPO antibodies were found in a total of 71/500 (14.2 %) patients, including 52/71 euthyroid women and 19/71 patients with latent hypothyroidism. Among the 9/19 women with latent hypothyroidism, 9/19 had only anti-TPO, while in 10/19 other antibodies were also present in addition to the anti-TPO. In the group of euthyroid patients anti-TPO positivity alone occurred in 18/52, while aPLs, antibodies of systemic autoimmune diseases, anti-tTG and a-sperm antibodies could also be detected in 34/52 patients.

#### Thyroid dysfunction without autoimmune thyroiditis:

Among our patients, 18/500 (3.6 %) women had latent hypothyroidism without being a carrier of anti-TPO antibodies. Furthermore, no autoantibody at all could be found in 9/18 patients; in the other 9/18 patients ANA, anti-dsDNA, aPLs (aCL, aPS), anti-tTG and a-sperm antibodies could be observed in addition to the latent hypothyroidism.

Patients with latent hypothyroidism received levothyroxine substitution during the follow-up period; checks of TSH levels were performed in the euthyroid anti-TPO-positive women. Among the women with autoimmune thyroiditis, 14 pregnancies were observed (4/14 ABs, 8/14 childbirths; 2/14 pregnancies are currently in progress). In the anti-TPO-positive euthyroid women a total of 19 conceptions (3/19 ABs, 16/19 childbirths) occurred. A total of 5 childbirths occurred among patients who had no detectable anti-TPO or any other antibodies in association with their thyroid dysfunction.

Our results demonstrate that levothyroxine substitution should start already prior to conception in patients with latent or overt hypothyroidism and it should be continued, with close monitoring of TSH levels during pregnancy. As sterility/infertility may also be caused by autoimmune thyroiditis in a euthyroid patient, regular checking of TSH in TPO-positive women of reproductive age should be underlined; in these cases the upper limit of TSH is 2.5 U/L.

### **Celiac disease in relation to reproductive failures**

Determination of anti-tTG, required for the detection of CD was performed not only in our female patients but in their male partners as well, and then deep duodenal biopsy and laboratory tests seeking malabsorption were also done in the anti-tTG positive patients.

Among men, anti-tTG positivity was observed in 4/500 (0.8 %) patients, and the histological examination confirmed CD in all of them. Among women, anti-tTG positivity was detected in 11/500 (2.2 %), and the diagnosis of CD could be confirmed by histological examination in 8/500 (1.6 %) of them. As a result of a strict gluten-free diet, 8 pregnancies (3/8 ABs, 3/8 childbirths, 2/8 pregnancies are currently in progress) developed during the follow-up. No premature birth and no IUGR of the newborn babies occurred among the mothers with CD.

A curiosity of our study: we could demonstrate previously unrecognized CD in both members of a couple, and then at a gluten-free diet they had, via spontaneous conception a healthy offspring in 2011. At a gluten-free diet, no positivity was shown in the child by periodic anti-tTG tests. With the examination of blood and tissue (umbilical cord and placenta) samples obtained from parents and child, we could make a contribution to an international genetic study of non-HLA genes underlying CD.

### **Anti-sperm antibody in relation to reproductive failures**

In our female patients, we also performed the examination of a-sperm antibodies which may be an emerging underlying etiologic factor of reproductive failure.

A-sperm could be detected in 49/500 (9.8 %) patients, of them no other antibody was present in 21/49, while in the further 28/49 women also autoantibodies could be observed in addition to a-sperm.

In a-sperm positive patients, with the use of prednisolone, and added anticoagulant therapy in some cases, 19 conceptions (2/19 ABs, 16/19 deliveries, 1/19 pregnancy is currently in progress) occurred.

Although a-sperm is a hetero-antibody in women, nevertheless the results of our study confirmed that it is frequently associated with autoantibodies (it has been observed mostly with ANA, anti-dsDNA, anti-TPO, and aPLs); therefore immunosuppressive therapy may be justified in patients with sterility/infertility .

In summary, it can be stated: the results of our series of examinations shed light on the fact that it is worthwhile to examine sterile/infertile couples for autoimmune diseases, autoantibodies, as the confirmation and appropriate treatment of these may lead to a successful childbirth. The results of our PhD work have been published in both Hungarian and international literature, and successful conception of 155 children occurred during our treatments. As far as we know, we published on this topic as first in Hungary.

### **NEW OBSERVATION**

1. As first in Hungary, we introduced an organized assessment of infertile couples for autoimmune diseases in an outpatient basis within the frame of specialist clinics of immunology.
2. Our outpatient clinics of this purpose attained not only a regional but also a trans-border role in a short time. The number of patients with reproductive failure examined and treated by us has reached, and currently exceeded 500. In addition to infertile couples from Zala County, a significant part of our patients arrived from Budapest, from all Trans-Danubian counties, on several occasions from even farther areas of the country (Csongrád, Bács-Kiskun, Hajdú-Bihar counties), moreover from abroad (Austria, Slovakia, Romania) as well.
3. During our activity we could establish a very good and successful professional relationship with the representatives of the partner specialties (Clinical Genetics, Obstetrics-Gynecology, Endocrinology, Gastroenterology, Laboratory Diagnostics), and we formed an interdisciplinary working group.
4. Our results obtained in relation to APS illuminate the necessity of an assessment of infertile women not only in pathological states which fulfill the diagnostic criteria of APS, but it is justified on the one hand already earlier (after one or two abortions in the first trimester, or also related to infertility), on the other hand in the presence of

other pathological states which are not included to the criteria (e.g. history of preeclampsia, premature delivery, low-weight newborn).

5. Our findings demonstrate that antibodies not included to the laboratory criteria of APS (aPS, aANX) have a significant relevance in the development of pregnancy-related morbidities, so that in the presence of these an appropriate anticoagulant therapy may result in childbirth.
6. Although the infertile women had no clinical symptoms suggesting autoimmune diseases (no systemic autoimmune disease, except APS, could be diagnosed); nevertheless their chances for childbirth were improved by low-dose corticosteroid therapy, administered – often in association with anticoagulation – due to their auto-antibody positivity.
7. Thyroid diseases occurred frequently among our patients; therefore we strongly recommend screening for autoimmune thyroiditis and thyroid dysfunction in women with reproductive failure.
8. By presenting the case of a couple with celiac disease, which is considered a rarity in Hungary, we wish to call attention to the fact that celiac disease in both women and men may be the underlying cause of pathological states of pregnancy, so that a screening of both members of the infertile couples is justified. Engaged in an international scientific research, with samples obtained from the parents and their offspring (sera and tissues taken from the placenta and the umbilical cord in relation to the delivery), we could made a contribution to a more exact elucidation of the genetic background of celiac disease.
9. Our study exemplifies well that anti-sperm antibodies appear often in association with other auto-antibodies, so that an administered anticoagulation and/or corticosteroid therapy may prove to be effective in this group of patients.
10. Our results support the fact that immunological assessment should be a part of the assessment of infertile couples, in addition the treatment and care of couples with demonstrated antibody positivity requires a multidisciplinary cooperation of the representatives of partner specialties in order to attain a successful childbirth.

The attachment bellow includes the tableau portraits of a part of the children successfully delivered as a result of our research and therapy.

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