

# Application of ELI-P-Complex Method in Clinical Obstetrics

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## Problem

Evaluation of embryotropic natural auto-antibodies (a-Abs) of IgG class in blood serum of women planning pregnancy or in that of already pregnant women is necessary in order to predict the course of gestation and pregnancy outcomes.

## Method of study

The ELISA-based probability of pathology in pregnancy (ELI-P)-complex method is based on the enzyme-linked immunosorbent assay technology. We used it to evaluate serum samples of 380 women with complicated obstetric anamnesis (COA) by measuring contents/affinity (immunoreactivity; IR) of a-Abs interacting with eight specific antigens related to mechanisms of gestation process. The control group was presented by 30 healthy women without COA. For each woman we carried out a pre-gestational investigation and, if necessary, performed a standard antibacterial/antiviral treatment and correction of endocrine state.

## Results

We found that 92.6% of investigated women with COA were characterized by deviations in serum IR of the examined a-Abs. In contrast, in the control group there were no immune deviations related to the examined a-Abs. A direct relation was revealed between normal or deviated serum IR of investigated a-Abs and physiological or pathological pregnancy development and pregnancy outcome.

## Conclusion

We recommend ELI-P-complex as a screening and monitoring tool for predicting pregnancy outcomes and providing proper treatment therapies.

## Introduction

Nearly 30% of pregnancies end in death of a zygote or an early embryo,<sup>1</sup> 10–15% of pregnancies are interrupted at later stages (miscarriages, intrauterine deaths) or result in unhealthy newborns.<sup>2</sup> What is the main cause of these failures? Do genetic or epi-

genetic reasons prevail? To attempt to answer these questions, we need to determine preventive measures for poor pregnancy outcomes.

One of the most common reasons of embryo malformation is the disturbance in immune regulation.<sup>1</sup> It is clear that intrauterine development of semiallogeneic fetus (not rejected by maternal

organism during 40 weeks), directly depends on immune system of a pregnant woman. Not only different interleukins provide regulatory influence of the gestation process but also embryotropic natural auto-antibodies (a-Abs) of the IgG class. Surplus or insufficient production of such a-Abs negatively influences the gestation process.<sup>3–6</sup> Usually, serum contents of embryotropic a-Abs in healthy women are maintained within specific concentration ranges.<sup>2</sup> In contrast, in 75–95% of women suffering from habitual miscarriages or other forms of pregnancy complications, long-lasting abnormalities of a-Abs may be found.<sup>2</sup> Immune deviations do not necessarily lead to poor pregnancy outcomes, but sharply increase the probability of miscarriages, fetal deaths, or developmental malformations.<sup>2</sup> Marai et al.<sup>7</sup> found a significant association between recurrent miscarriages and a panel of a-Abs to aTPO, aTG and anti-ENA. Shoenfeld et al.<sup>8</sup> determined a number of a-Abs associated with reproductive failure and Cohen et al.<sup>9</sup> reported on steady deviations of a-Abs' levels in infertile women. The latter are often referred to extracorporeal fertilization (ECF) and usually with no success. Interestingly, the deviations in contents of a-Abs have been revealed in 80–90% of women who used ECF.<sup>2</sup>

Another important issue is the influence of maternal immune deviations upon the health of her child. Changes in a-Abs contents do not necessarily lead to miscarriages or intrauterine deaths, but practically always negatively influence the health state of a newborn.<sup>3</sup>

Our investigation aims to re-evaluate the ELI-P-complex method and to expand its practical application. ELI-P-complex measures serum immunoreactivity (IR) which is an integral parameter of the content/affinity of corresponding a-Abs<sup>2,5</sup> against choriogonadotropin, dsDNA,  $\beta$ 2-glycoprotein I,  $\beta$ 2-glycoprotein I-binding Abs, Fc-fragment of IgG, collagen IV, S100, and MP-65. This test is recommended by the Russian Ministry of Health for diagnostic and prognostic investigation of planning pregnancy or already pregnant women. Preliminary results of our investigations, which do not include data about a-Abs against choriogonadotropin, dsDNA,  $\beta$ 2-glycoprotein I,  $\beta$ 2-glycoprotein I-binding Abs, were described earlier.<sup>2</sup>

This work was approved by the ethical committee of The Kazan State Medical Academy (Kazan, Russia).

## Materials and methods

### Antigens

Human choriogonadotropin, salmon dsDNA, Fc-fragment of rabbit's IgG, and bovine collagen IV were obtained from Sigma-Aldrich (St. Louis, MO, USA). Other antigens were purified chromatographically in our laboratory as described.<sup>4</sup> Human  $\beta$ 2-glycoprotein I (phospholipide-binding serum protein) was purified from fresh human sera. IgG Abs against  $\beta$ 2-glycoprotein I, used for detection of corresponding antiidiotypic Abs, were purified from sera of rabbits immunized by human  $\beta$ 2-glycoprotein I. The S100 protein, which participates in the regulation of apoptosis of different cell types and in regulation of the nervous tube development, was purified from fresh bovine brain. The MP-65 protein, which belongs to the family of adhesines and participates in regulation of intercellular contacts and general morphogenesis, was obtained in the similar manner. Purified antigens were adsorbed into the wells of 96-well immunoplates (Nunc maxisorp, Nunc A/S, Roskilde, Denmark) as described.<sup>2</sup>

### Evaluation of Serum IR

The study of patients' serum IR was performed using the ELI-P-complex method as described.<sup>5</sup> In summary, the investigated serum samples were diluted 1:200 in 0.05% Tween-20 phosphate-buffered saline and placed into the wells preliminary coated with the selected antigens. The standard enzyme-linked immunosorbent assay (ELISA) protocol followed. Serum IR of each sample was calculated in conditional units (CU) (in accordance with the recommendations by Kalsi and Isenberg<sup>6</sup>) to an internal standard optical density using the following equation:

$$IR = \frac{OD_{P(AG_n)} \times 100}{OD_{st}} - 100, \quad (1)$$

where  $OD_{P(AG_n)}$  is the optical density of patient sera with antigen ( $AG_n$ ) and  $OD_{st}$  is the optical density of an internal standard with the same antigen ( $AG_n$ ).

In other words, the averaged data of each analyzed serum sample were expressed as a percentage normalized to internal standard optical density (set at 100%).<sup>6</sup> Direct and specific dependency between serum IR and content of corresponding a-Abs was experimentally confirmed earlier by concurrent

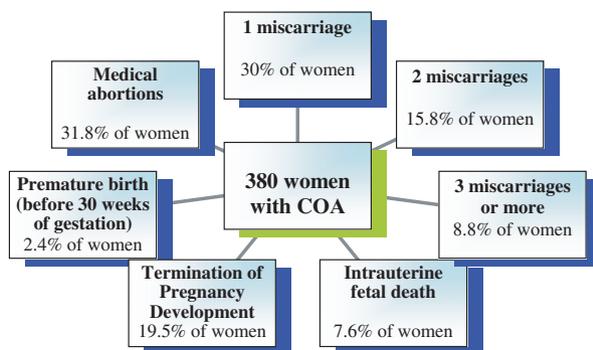
'quenching' of IR by a specific antigen.<sup>5</sup> Allowable physiological limits of serum IR with each of the ELI-P-complex antigens were calculated earlier as  $-30...+20$  CU (70%...120%) to the internal standard IR. This range was confirmed by an observation that the serum IRs of at least 95% of thoroughly investigated clinically healthy women of fertile age ( $n = 220$ ) fell within the indicated range.<sup>10</sup> All analysed samples and the standard sample were tested in triplicate. Coefficients of intro-assay (ELISA) variation were evaluated by running three samples seven times in one assay. Coefficients of inter-assay variation were determined by measuring the same samples in six consecutive assays. Determined coefficients of ELISA intro-assay variation were  $<7\%$  and coefficients of inter-assay variation were  $<11\%$  in all tests performed.

### Evaluation of Blood Coagulability

Blood samples were obtained from cubital vein in the morning before food intake. Concentration of fibrinogen, activation time of recalcification and partial thromboplastine, prothrombin index, anti-thrombin III activity, fibrinolytic activity of the blood plasma, and platelets aggregation were analysed by the commonly used methods.<sup>11</sup>

### Patients

We investigated serum IR of corresponding a-Abs in 410 non-pregnant women. Each woman underwent the informed consent process and reserved the right to withdraw from the study at any stage and/or refuse any procedure during any stage of the investigation or treatment.



**Fig. 1** Complications of obstetric anamnesis in women from the main group.

**Table 1** Immunoreactivity (IR) Subgroups. Main Group before Pregnancy

Subgroup	N	Description
1	28	Normal ranges of serum IR against investigated antigens
2	64	Abnormally low serum IR
3	213	Abnormally elevated serum IR
4	75	Combined changes in serum IR <sup>a</sup>

<sup>a</sup>Meanings of serum IR against some antigens were below and against others – above the normal limits; most typical cases were characterized with elevated IR against dsDNA and  $\beta$ 2-Glycoprotein I coupled with abnormally low IR against choriogonadotropin and Fc-fragments.

In accordance with personal anamnesis data, all women were attributed either to the control group ( $n = 30$ ; average age  $27 \pm 5.5$ ), which is the group of women without complications in obstetric anamnesis (COA), or to the main group ( $n = 380$ ; average age  $28.5 \pm 5.7$ ), i.e. women with COA (miscarriages, intrauterine fetal death, etc.; details of cases are presented in Fig. 1). We did not find an evident anamnestic indication for a direct cause of complication (infection, endocrine, genetic, etc.) in any of the observed patients.

For every woman in the control group before, as well as during the followed pregnancy, serum IR against any of the used antigens was within physiological norm that is, in the range between  $-30$  CU and  $+20$  CU. Women in the main group were assigned into four subgroups based on their serum IRs data (Table 1): 1, normal ranges of serum IR against investigated antigens; 2, abnormally low serum IR; 3, abnormally elevated serum IR; 4, combined abnormally high and low serum IR.

Pregnancy was planned by 99 women out of 380 in the main group and by all 30 women in the control group. For each woman we carried out a pre-gestational investigation, including routine blood and urine tests, and blood coagulability. Local signs of inflammatory processes in distal portion of reproductive tract (colpites) were revealed in 30% of investigated women of the main group and in 10% of the control group. Chronic adnexitis and chronic endometritis in stages of remission were observed respectively in 5% and 3% of patients. Herpes simplex virus and cytomegalovirus were detected in 60–70% of women in both groups. 15–20% of women were identified as asymptomatic carriers of

**Table II** Immunoreactivity (IR) Subgroups. Pregnant Women from the Main Group

Subgroup	N	Description
P-1	16	Normal ranges of serum IR against investigated antigens
P-2	16	Abnormally low serum IR
P-3	17	Abnormally elevated serum IR
P-4	27	Combined changes in serum IR

mycoplasma and ureaplasma infections. Asymptomatic bacteriuria was revealed in 40–50% of women in both groups. All patients characterized by an increased blood coagulability were treated by antiaggregants (fraxiparine, aspirin and/or curantyl). Menstrual dysfunctions were not identified in the control group but were typical for 8% of women in the main group. Pregravid treatment was performed when necessary using standard antibacterial and antiviral therapy (prescriptions of antibiotics, valtrex, immunostimulating drugs, i.e. amixin, licopid). To correct endocrine functions we prescribed oral contraceptives, which were also used in a few cases to correct thyroid functions and elevated production of prolactin.

Pregnancy onset was registered 6–9 months after the first investigation of the serum IRs in all 30 women in the control group and in 76 out of 99 women in the main group. At 6–9 weeks of pregnancy serum IRs in all women was again investigated by the ELI-P-complex method. In accordance with the serum IRs data, all pregnant women were attributed to one of the following subgroups: P-1, P-2, P-3, P-4 (Table II).

Statistical analysis of obtained data was performed using STATISTICA 6.0 (StatSoft, Inc., Tulsa, OK, USA). Formulated statistical hypotheses were designed based on the probability levels of  $P < 0.05$  or  $P < 0.01$ .

## Results

All women without complications in individual obstetric history were characterized by normal serum IR related to each of the investigated a-Abs. At the same time, the immune deviations were observed in 92.6% of women in the main group (with COA). Only in 7.2% of women with COA all investigated parameters of serum IRs were within normal ranges.

Most women who suffered habitual miscarriages ( $n = 207$ ) were characterized by abnormally high value of serum IR against S100 ( $m = 43$  CU;  $P < 0.05$ ) and relatively low IR against MP-65 ( $m = -23$  CU;  $P < 0.05$ ). A progressive increase in IR against dsDNA (from 3 CU in women with one miscarriage to 29 CU on average in women with 4–5 miscarriages;  $P < 0.05$ ) was also typical.

Most women with cases of terminated pregnancy development or intrauterine foetal death ( $n = 121$ ) were also characterized by abnormally high value of serum IR against S100 ( $m = 36$  CU;  $P < 0.05$ ). Repeated termination of pregnancy was accompanied by additional increase in serum IR against S100 (from 30 CU, two terminations, to 60 CU, four terminations;  $P < 0.05$ ), progressive increase of IR against dsDNA (from 20 to 73 CU,  $P < 0.05$ ), and IR against  $\beta$ 2-glycoprotein I (from 15 to 70 CU,  $P < 0.05$ ). Elevated levels of serum IR against MP65, collagen IV and Fc-fragment (40–65 CU on average) were also characteristic of these women.

For women who previously had medical abortions ( $n = 121$ ), elevated serum IR against S100 was typical ( $m = 56$  CU). The increase in the number of abortions led to an additional increase in IR against S100 (up to 72 CU in women with four or more abortions). Three or more abortions in anamnesis were also accompanied by an increase in IR against Fc-fragment, dsDNA,  $\beta$ 2-glycoprotein I and anti-idiotypic Abs against Abs to  $\beta$ 2-glycoprotein I ( $P < 0.05$ ). These data may indicate that abortions lead to long-term transformations, which include abnormal changes in the activity of many lymphocytes' clones.

Retrospective analysis of serum IR in women from the main group, who became pregnant, revealed that immune anomalies were observed before pregnancy in 100% of cases. Pregravid treatment was accomplished by the normalization of investigated immune parameters in 21% of observed women and decreased prominently the deviations in the majority of treated women. It should be noted that the management of correction in women with imbalance of different IRs (combinations of pathologically high and abnormally low IR) was the least effective. The investigated immune changes were better amendable in cases with more or less homogenous elevation or homogenous decrease in serum IRs against the used antigens. The development of pregnancy was directly related to the effectiveness of normalization of serum IRs. The rest of the results are summarized in Table III.

**Table III** Summary of Gestation and Pregnancy Outcomes by Subgroups

Group	Characteristic	Outcome
Control	No obstetric complications and normal serum IRs	We have not registered any notable complications during gestation period in all 30 women from the control group
P-1	Fully normalized serum IR	Only a small number of patients revealed minor complications during the course of gestation. A compensated risk of miscarriage was diagnosed in three cases (18.8%). Signs of chronic placental insufficiency were detected by ultrasound/dopplerometria in one case (6.3%)
P-2	Low serum IR after pregravid treatment	One pregnancy (6%) ended in miscarriage at 9 weeks of gestation. The risk of miscarriage was diagnosed in three cases (19%). Signs of chronic placental insufficiency were detected by ultrasound/dopplerometria in three cases (19%). Moderate gestosis was revealed in three patients (19%)
P-3	Elevated serum IR after pre-gravid treatment	Three pregnancies (18%) ended in miscarriages at 7–9 weeks of gestation. Risk of miscarriage was diagnosed in four cases (24%). Signs of chronic placental insufficiency were detected in six cases (35%). Moderate gestosis was revealed in three patients (18%)
P-4	Misbalance in serum IR after pregravid treatment	All patients had complications during the course of gestation. Five pregnancies (19%) ended in miscarriages at 7–10 weeks of gestation. Risk of miscarriage was diagnosed in 13 cases (48%); in three cases this problem was treatment-resistant and all three women were hospitalized repetitively (five times per pregnancy in average). Signs of chronic placental insufficiency were detected in 14 cases (52%): compensated form in 12 women (44%), and subcompensated form with fetal retardation in two women (7%). Moderate gestosis was revealed in eight patients (30%).

### Health of Newborns

All newborns from the control group, as well as from women in P-1 subgroup, were evaluated by pediatricians and received the Apgar score of 9–10 points. P-2 subgroup: three newborns received the Apgar score of 7–8 points and 12 newborns received the score of 9–10 points. P-3 subgroup: one newborn received the Apgar score of 5–6 points, three newborns received 7–8 points, and 10 newborns received 9–10 points. P-4 subgroup: five newborns received the Apgar score of 5–6 points, 13 newborns received the Apgar score of 7–8 points, and 11 newborns received the Apgar score of 9–10 points. Morphological and functional immaturity as well as perinatal encephalopathy (characteristic features of babies with low Apgar score rates) were typical for newborns from women in P-2, P-3, and especially P-4 subgroups but not from women in P-1 or the control group ( $P < 0.05$ ;  $P < 0.01$ ).

### Correlations between Changes in Serum IR and Haemodynamic Changes

The elevation in IR against dsDNA,  $\beta$ 2-glycoprotein I and corresponding anti-idiotypic Abs was revealed

in 44% of pregnant women from the main group. This situation was assessed as a typical sign of antiphospholipid syndrome.<sup>12</sup> Such cases were characterized by an increase of fibrinogen, accelerated spontaneous aggregation of platelets, prolongation of kaolin time and activated partial thromboplastin time. Similar changes were also typical of women with general increase in different IRs (polyclonal activation).

Abnormally high IR against choriogonadotropin (without notable increase in other IRs) was found in 16% of pregnant women. Such cases were usually characterized by normal haemostasiological parameters.

Abnormally low parameters of serum IR against all or most of the used antigens (polyclonal immunosuppression) were typical of 25% of pregnant women. These women were characterized by an increase of fibrinogen (35% of cases) or accelerated spontaneous aggregation of platelets, prolongation of kaolin time, activated partial thromboplastin time, and an increase of serum content of soluble complexes of monomeric fibrin (16% of cases).

In 16% of pregnant women we found an imbalance of serum IRs against different antigens. In most

typical cases (10 of 16) elevated IR against dsDNA and  $\beta$ 2-glycoprotein I were coupled with abnormally low IR against choriogonadotropin and Fc-fragments. Haemostasiological changes in every 10th woman were typical of a chronic disseminated intravascular coagulation syndrome.

In general, abnormal changes in serum IR, revealed by ELI-P-complex in 92.2% of cases, were coupled with haemostasiological changes, manifested as disseminated intravascular coagulation syndrome (49 cases), hyperfibrinigenaemia (5 cases), and prolongation of kaolin time (5 cases). In all 30 pregnant women from the control group, as well as in all women from the P-1 subgroup, haemostasiological parameters were normal.

## Discussion

In 380 investigated women with COA not a single case of preceded complications (miscarriage, intra-uterine death, etc.) was directly related to a specific cause. It indicates the complicated character of pathogenesis in most COA cases. On the other hand, nearly 93% of these women revealed different anomalies in serum IR against each or some of the used eight antigens. Hence, very different aetiological factors of COA (infections, endocrine problems, other metabolic dysfunctions, etc.), as well as combinations of factors may often be reflected by changes in the immune homeostasis.

Only 7.4% of women with COA (subgroup 1) were characterized by normal (physiological) serum IR against each of the used antigens. The obtained results well correspond to the conventional value of nearly 10% 'genetically based reproductive failures' and nearly 90% of genetically unrelated reproductive failures.<sup>2</sup> These values suggest that immunity unrelated genes/chromosomes aberrations could be the main cause of reproductive rebuff in women from subgroup 1. In other cases, reproductive failures were of seemingly epigenetic in nature, therefore, principally corrigible in contrast to genetic defects. It is indicative that at least three-fourths of early observed immune deviations were successfully normalized after a course of pregravid treatment, and the majority of the following pregnancies in these women were relatively propitious. These data can be considered as an additional indirect evidence of non-genetic causes of the reproductive failures in most cases. Hence we are faced with the following questions: is the modern practice of prognosis and

prophylaxis of pathology in pregnancy sufficiently effective? Can the partial redistribution of medical efforts and financial investments towards the detection and correction of immune-based deviations of fertility bring advantage by reducing the number of pathology in pregnancy and decreasing the number of unhealthy newborns? Our results support the latter: preliminary normalization of immune deviations (P-1 subgroup) resulted in physiologically normal course of pregnancy and the birth of practically healthy babies in most cases. In contrast, insufficient (incomplete) correction of immune deviations (subgroups P-2, P-3 and especially P-4) often led to complications in pregnancy and/or birth of unhealthy newborns.

Pregnant women with imbalance of IRs (P-4) were considered to be the most unfavourable subgroup. Such deviations were usually accompanied by different endocrine dysfunctions,<sup>2</sup> and perhaps are related to different influences of hormonal abnormalities upon different clones of lymphocytes.<sup>13</sup> Relatively low meanings of IRs against most antigens (P-2) corresponded to the presence of intracellular bacterial (mycoplasmas, chlamydia, etc.) or massive viral infection of herpetic group in a latent form.<sup>2</sup> Relatively high meanings of IRs (P-3) served as a sign of active inflammation due to acute infection (mostly bacterial) or exacerbation of a nidus of a chronic infection.<sup>2</sup>

Detailed mechanisms mediating the negative influence of immune deviations upon the gestation process should be elucidated. Nevertheless, the obtained data linking immune and hemostasiological changes may serve as a basis for some propositions. For example, any long-lasting active infection process (viral or bacterial) may be aetiologically related to antiphospholipid syndrome and accompanied, in particular, by abnormally elevated serum levels of a-Abs against  $\beta$ 2-glycoprotein I and DNA.<sup>12</sup> The mentioned a-Abs could be pathogenically related to haemostasiological changes<sup>12</sup> which, in turn, may often cause vascular problems in pregnant women and affect the gestation process. A surplus of anti-collagen IV a-Abs may indicate inborn or acquired defects of the connective tissue (A.B. Poletaev, unpublished observations in women and children with displastic interstitial/connective tissue defects). Changes in serum a-Abs against S100, typical of most women with COA, may indicate the presence of additional problems. It is known that proteins of the S100 family take part

in regulation of apoptosis.<sup>14</sup> On the other hand, morphologic transformations, which are the basis of the normal embryo development, depend on the well-ordered apoptotic events. Obviously, any deviations in the mechanism of apoptosis (for example related to abnormal production of a-Abs against S100) can be the cause of pregnancy failures or developmental malformations. In fact, HPV infection in women usually induces an abnormal increase in production of a-Abs against S100 (probably by molecular mimicry) and lead to 10–12 fold augmentation of inborn anomalies or embryo deaths/malformations.<sup>15</sup> The MP-65 protein belongs to the group of superficial membrane adhesions and partly to neural cell adhesion molecule (N-CAM). Steadily elevated serum IR against MP-65 in pregnant women may lead to different kinds of aplastic and displastic defects in a fetus.<sup>2,10</sup> Causes underlying changes in the production of a-Abs against MP-65 are unknown. A surplus of a-Abs against choriogonadotropin may be the reason for apparent deficiency in this hormone, which is important for placenta development and maturation. In turn, insufficient production of such a-Abs (specific a-Abs protect peptide hormones from premature proteolysis<sup>2</sup>), may lead to a true deprivation of pregnant women from choriogonadotropin. The increase in serum IR against Fc-fragments of IgG (rheumatic factor) may be considered as rather unspecific indicator of general immune activation.<sup>16</sup> Its influence upon pregnancy and fetal development should be elucidated; however, speculation about its possible adaptive function (a-Abs against Fc-fragments of immunoglobulins could be endogenous unspecific restrictors of inflammation) seems to be acceptable as a working hypothesis.

The above-mentioned propositions about the biological meaning of investigating a-Abs are no more than separate fragments of complex and poorly understood immune-related disturbances of the gestation process. Future studies in this vast area are very important for the development of new prophylactic and treatment measures.

In conclusion, ELI-P-complex improves on the basic ELISA limitation by allowing the detection of multiple antibodies simultaneously. Currently, the multiplexed assays using fluorescence microspheres technology are being used for the detection of anti-nuclear autoantibodies.<sup>17</sup> It would be of interest to compare the performance of the ELI-P-complex using ELISA and multiplexed assays.

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