MEDICAL RESEARCH CENTER
IMMUNCULUS

CLINICAL TRIAL PROTOCOL

STUDY NAME:

Evaluation of pregnancy course and outcome by means of the diagnostic «ELI-P-Complex» test for preconception care in women with history of adverse pregnancy outcome.

Multi-central, randomized, parallel-group trial

VERSION DATE: 4 April 2008

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CLINICAL TRIAL SUMMARY

TITLE	Evaluation of pregnancy course and outcome by means of the diagnostic «ELI-P-Complex» test for preconception care in women with history of adverse pregnancy outcome.
STUDY OBJECTIVES	**Primary objective**: To demonstrate the efficacy of "Eli-P-Complex" testing in preconception care for increasing the frequency of healthy children birth in women with history of adverse pregnancy outcome. **Secondary objective**: To demonstrate the efficacy of "Eli-P-Complex" testing in preconception care for improving the pregnancy outcome in women with history of adverse pregnancy outcome. **Other objectives:** To demonstrate the efficacy of "Eli-P-Complex" testing in preconception care for reducing the complications and improving the pregnancy course in women with history of adverse pregnancy outcome.
STUDY DESIGN	Multi-central, randomized, parallel-group trial
STUDY POPULATION	Main selection criteria: 1. Written informed consent obtained 2. Women aged 17 to 45, **AND** 3. Presence of the history of adverse pregnancy outcome: a) Miscarriage (2 or more) **OR** b) Missed miscarriage **OR** c) Anembryonic pregnancy **(**blighted ovum) **OR** d) Fetal death (stillbirth) **OR** e) Intrauterine growth restriction **OR** f) Premature birth **OR** g) Fetal and Congenital malformations Main exclusion criteria: 1. Women of childbearing age with no history of adverse pregnancy outcome. 2. Women diagnosed with infertility. 3. Presence of other condition (medical, psychological, social, or geographical) that would either interfere with participation in the trial or lead to inability to complete the trial. Expected number of patients: Approximately 4,000 (2,000 per study group or more)

CLINICAL TRIAL SUMMARY

INVESTIGATIONAL PRODUCT Lab technique	Diagnostic kit ELI-P-Complex used for semi-quantitative assess of the individual 'profiles' of the blood serum content of 12 autoantibodies-markers ELISA method
STUDY PLAN	Investigation phases: • Group A women were tested with ELI-P Complex during the preconception preparation before pregnancy. • In Group A women, the abnormalities, originally detected by ELI-P-Complex test, are confirmed by means of routine diagnostic methods. Individual treatment is conducted in accordance with the identified abnormalities. To assess the effectiveness of the treatment the ELI-P-Complex testing is performed one more time. • Both groups (Group A and Group B) were tested with ELI-P-Complex during the first, second and third trimester.
EVALUATION CRITERIA	Efficacy endpoints: Primary: Occurrence of one of the following Clinical Events: • Newborns with Apgar score of 6 points or less • Premature infant (Low birth weight) • Intrauterine growth restriction • Perinatal asphyxia • Congenital malformations Secondary: Occurrence of one of the following Clinical Events: • Miscarriage • Fetal death (stillbirth) • Missed miscarriage • Anembryonic pregnancy **(**blighted ovum) • Premature birth Other: Occurrence of one of the following Clinical Events: • Threatened miscarriage • Placental abruption • Pre-eclampsia, eclampsia • Intrauterine hypoxia • Intrauterine growth restriction • Fetal malformation • Polyhydramnios • Oligohydramnios

CLINICAL TRIAL SUMMARY

STATISTICAL CONSIDERATIONS	Analysis of the frequency of healthy children birth, pregnancy outcome and the amount of pregnancy complications comparing 2 groups with significance level 0.05 will perform using the SPSS 21.0.0 for Windows (IBM, NY, USA) and STATA/SE 13.0 software (STATA Corp., Texas, USA). Indicators (positive predictive value (PPV), sensitivity, specificity) of ELI-P-Complex test will calculate for pregnancy complications. The relative risk of adverse pregnancy outcome and of an unhealthy child birth will be calculated by groups A and B comparison.
DURATION OF STUDY PERIOD (per patient)	The estimated study duration that served as the assumption for sample size calculations is 75 months. All patients will be followed from randomization until the study end date, which will occur when the last patient has been followed for 15 months, based on 60-month recruitment period.

TABLE OF CONTENTS

1. BACKGROUND 9
2. INVESTIGATIONAL PRODUCT 11
    2.1. Description 11
    2.2 Evaluation of ELI-P-Complex test results 13
3. STUDY OBJECTIVES 14
    3.1 Primary Objective 14
    3.2 Secondary Objective 14
    3.3 Other Objectives 14
4. STUDY DESIGN 14
5. STUDY STEERING COMMITTEE 14
6. SELECTION OF PATIENTS 15
    6.1 Inclusion criteria 15
    6.2 Exclusion criteria 15
7. STUDY PLAN 15
8. ASSESSMENT OF INVESTIGATIONAL PRODUCT 16
    8.1 Efficacy criteria 16
        8.1.1 Primary criterion 16
        8.1.2 Secondary criterion 16
        8.1.3 Other criteria 17
    8.2 Definitions of components of the primary efficacy endpoint 17
        8.2.1 Newborns with Apgar score of 6 points or less 17
        8.2.2 Premature infant (Low birth weight) 18
        8.2.3 Intrauterine growth restriction 18
        8.2.4 Perinatal asphyxia 18
        8.2.5 Congenital malformations 19
    8.3 Definitions of secondary efficacy endpoint 19
        8.3.1 Miscarriage 19
        8.3.2 Fetal death (stillbirth) 20
        8.3.3 Missed miscarriage 20
        8.3.4 Anembryonic pregnancy (blighted ovum) 21
        8.3.5 Premature birth 21
    8.4 Definition of other efficacy endpoint 21
        8.4.1 Threatened miscarriage 21
        8.4.2 Placental abruption 22
        8.4.3 Pre-eclampsia, eclampsia 22
        8.4.4 Intrauterine hypoxia 23
        8.4.5 Intrauterine growth restriction 24
        8.4.6 Fetal malformation 25
        8.4.7 Polyhydramnios 25
        8.4.8 Oligohydramnios 25
9. STATISTICAL ANALYSES 25
10. ETHICAL ISSUES 26
11. STUDY MONITORING 26
12. PROPERTY RIGHTS 27
13. DATA PROTECTION 27
14. SPONSOR AUDITS AND INSPECTIONS BY REGULATORY AGENCIES 27

15. CLINICAL TRIAL RESULTS 27
16. PUBLICATIONS 28
17. FINANCING 28
18. REFERENCES 29
19. ANNEXES 32
ANNEX 1. ELI-P-COMPLEX KIT. APPLICATION MANUAL 32
ANNEX 2. REGULATIONS FOR BLOOD SAMPLING AND SPECIMEN PREPARATION FOR EXAMINATION 39
ANNEX 3. ELI-P-COMPLEX TEST CHART 40
ANNEX 4. INTERPRETATION ALGORITHM OF THE ELI-P-COMPLEX TEST RESULTS HISTOGRAM (AUTOIMMUNOGRAM) 41
ANNEX 5. INTERPRETATION OF ELI-P-COMPLEX DATA (GENERAL ALGORITHMS) 43
ANNEX 6. THE STEERING COMMITTEE 44
ANNEX 7. THE UNIFIED CRITERIA FOR PATIENT EXAMINATION RECOMMENDED AFTER ELI-P-COMPLEX TEST 45
ANNEX 8. FETAL WEIGHT PERCENTILES THROUGHOUT GESTATION 49
ANNEX 9. DOPPLER ULTRASOUND EVALUATION 50
ANNEX 10. ULTRASOUND BIOMETRY OF THE FETUS 59
ANNEX 11. ULTRASOUND DIAGNOSIS OF FETAL ANOMALIES 64
ANNEX 12. CLINICAL TRIAL CONCENT FORM 66

LIST OF ABBREVIATIONS

a-Abs = Autoantibody
AC = Abdomial Circumference
AEDF = Absent End Diastolic Flow
AFI = Amniotic Fluid Index
BPD = Biparietal Diameter
CPR = Cerebroplacental Ratio
CRF = Case Report Form
CRL = Crown-Rump Length
DHEA-S = Dehydroepiandrosterone Sulfate
DIC = Disseminated Intravascular Coagulation
FL = Femur Length
FSH = Follicle-Stimulating Hormone
GCP = Good Clinical Practice
GI = Gastrointestinal tract
HC = Head Circumfrence
hCG = Human Chorionic Gonadotropin
HPV = Human Papilloma Virus
ICH = International Conference on Harmonization
IUGR = Intrauterine Growth Restriction
LH = Luteinizing Hormone
MCA = Middle Cerebral Artery
MSD = Mean gestational Sac Diameter
OFD = Occipital Frontal Diameter
PCR = Polymerase Chain Reaction
PI = Pulsatility Index
PSV = Peak Systolic Velocity
RCOG = Royal College of Obstetricians and Gynaecologists
REDF = Reversal of And Diastolic Flow
RI = Resistance Index
SD = Standard Deviations
UA = Umbilical Artery
US = Ultrasound scan
WHO = World Health Organization

1. BACKGROUND

Nearly 30% of pregnancies end in death of a zygote or an early embryo [1], 10–15% of pregnancies are interrupted at later stages (miscarriages, stillbirth) or result in unhealthy newborns [2]. Despite the significant progress made recently within different areas of obstetrics and gynaecology, the problem of adverse pregnancy outcome remains far from being resolved.

The issue is aggravated by the fact that couples experiencing an adverse pregnancy outcome may be at risk of having other adverse outcomes in future pregnancies. Adverse pregnancy outcome of any genesis causes severe psycho-emotional response in potential parents, and the doctor's role is to figure out and to explain the reason for the failure, as well as to conduct a reasonable preconception preparation to ensure the normal course of subsequent pregnancy to the highest possible level. The mission is quite difficult, and often it is just unfeasible.

Speaking of the most examined reasons that often provoke pregnancy loss, a significant impact of infectious processes in adolescence medical history should be noted, as well as a proved role of extragenital diseases in pregnant women [3]. Urogenital inflammatory diseases, especially sexually transmitted infections, are an important cause of adverse pregnancy outcome. Endocrine disorders, especially accompanied by luteal phase deficiency and hyperandrogenism, are of undeniable importance in the genesis of pregnancy loss [4]. It is accepted that 2-5% of pregnancy losses are due to chromosomal abnormalities [5], which necessitates cytogenetic examination of spouses. The relatively small frequency of adverse pregnancy outcomes is observed due to female genital organs anomalies [6].

A substantial cause that prevents early foetal development, is excessive production of prostaglandin E2 [7], lupus anticoagulant (LA), antibodies to human chorionic gonadotropin [8], macroglobulins involved in the regulation of fibrinolysis, coagulation, cellular and humoral immunity and other factors [9, 10].

Despite the availability of reasonably detailed information about etiological factors, still there is no consensus about the realization mechanisms of adverse pregnancy outcomes. It is not clear why the same damaging agents in some cases cause termination of pregnancy, while in others these do not affect its course. Lack of sufficient understanding of the pathogenesis of adverse pregnancy outcome determines empirical therapeutic interventions and want of definite tactics while conducting preconception preparation for women after pregnancy loss; that prevents from reducing the incidence of the pathology significantly. This necessitates the search for new approaches to the study of pregnancy loss issues.

It cannot be ruled out that widely different by nature agents, eventually leading to adverse pregnancy outcome, actualize their effect through a unified

pathophysiological mechanisms. The immune system of the body can be directly related to such key mechanisms. Any adverse factors of external and internal environment (infectious diseases, numerous endocrine changes, radiation and toxic damage, pharmaceuticals, etc.), where their connection with developmental pregnancy disorders are reliably traced, first of all impact on the general status of immune system and on the functional status of its individual cellular and humoral elements [11]. And immune disorders are in turn the direct cause of the onset of various forms of adverse pregnancy outcomes.

Over the last few decades many researchers [12, 13] concluded that natural (physiological) antibodies of the IgG class involved in the regulation of the processes of embryogenesis (so-called embryotrophic autoantibodies) are continually present in a healthy woman. Influenced by various agents (inflammatory processes, endocrinopathies, toxic effects, etc.) the level of production of these autoantibodies may fluctuate. But changes in the level of embryotrophic autoantibodies beyond the physiologically acceptable limits, regardless of the specific reason that caused them, lead to pregnancy course disruption, foetal malformations, and even to foetal death [13].

Thus, alterations to normal production of regulatory embryotrophic autoantibodies in woman's body are perhaps the basic and earliest sign of woman’s reproductive function ill-being. In that case embryotrophic autoantibodies are not only the indicator of adverse pregnancy outcome, but, as these are directly involved in control of gestation and development of tissues and organs of embryo and foetus, these are, therefore, common causes of congenital malformations or pregnancy course standstill [13, 14].

It is the detection of serum content of embryotrophic autoantibodies involved in the control of the embryogenesis processes, that is the basis of diagnosis by means of the ELI-P-Complex test designed by LLC MRC "IMMUNCULUS".

It is quite difficult to identify the cause of adverse pregnancy outcome, and often it is just unfeasible. It takes a large number of laboratory tests and diagnostic procedures to have been performed; also a lot of time and resources have been spent. However, even in that case the cause of pregnancy loss can be not clarified.

ELI-P-Complex by means of just a single blood test gives a clear understanding of the cause of disorder and therefore turns the diagnostic search to the right direction that prevents spending money on others blindly conducted studies. The use of the test contributes to reducing the number of appointments with a doctor and hospitalizations, in-patient treatment time and amount of diagnostic investigations.

A large number of clinical studies indicate that the ELI-P-Complex test allows to predict the outcome of pregnancy and prevent such complications in the newborn as the birth of a premature baby, neurological disorders, diabetes, congenital malformations [15, 16, 17, 18, 19]. There is evidence that women with a history of

complications during previous pregnancies who were under the ELI-P-Complex Test control have a satisfactory next pregnancy more than five times than those who did not use the test [20]. At least 70% of children were born by women with the normal parameters of ELI-P-Complex method were evaluated as practically healthy persons at ages 4-6 years old. In opposite, no more than 15% children were evaluated as practically healthy if were born from women with steady deviated parameters of serum autoantibodies [21, 22].

The laboratory diagnostic test "Eli-P-Complex" development was completed in the late 90s. In the early 2000s, the test was modified, its sensitivity and specificity was increased. The first data regarding successful clinical application of the test appeared. Since that time, some countries outside the EU approved the clinical use of the test. In 2009, the Eli-P-Complex test was certified by the Institute for Certification and Testing "EUROCAT" (Germany) and got the CE certificate. Since then numerous clinical studies were carried out, however, there is still a lack of evidence-based clinical trials of the ELI-P Complex technology. This hinders the widespread clinical use of the test, despite the existing data of its application benefits.

This clinical study, performed with the compliance of Good Clinical Practice (GCP), on the basis of reliable data, enables understanding of effectiveness and importance of the ELI-P-Complex test for preconception care in women with history of adverse pregnancy outcome.

2. INVESTIGATIONAL PRODUCT

2.1. Description

The ELI-P-Complex (from **E**nzyme-**L**inked-**I**mmuno-Test; **P** – from Pregnancy) is a cost-effective test based on user-friendly ELISA technique, intended both for research purposes and for clinical practice. ELI-P-Complex Kit is intended for measuring of specific serum immune reactivity (IR) which depends on serum content of twelve a-Abs of IgG class [23, 24].

The ELI-P-Complex test panel contains the following biomarkers: a-Abs against choriogonadotropin, ds-DNA, β2-Glycoprotein I, collagen II, Fc-fragment of IgG, insulin, thyroglobulin, S100, Spr-06, ANCA, TrM-03 and KiM-05:

1. **Human Chorionic Gonadotropin** (**hCG**) – is a peptide hormone which regulates the process of implantation of zygote, and is important for placenta formation and maturation. Excess of a-Abs against choriogonadotropin may be indication of endocrine disfunctions and may be the cause of the functional insufficiency of choriogonadotropin. The last may be reason for infertility and placenta malformation, and leads to feto-placental insufficiency [25, 26]. Besides excess of such a-Abs is typical for women with ovarian insufficiency, including cases of premature ovarian failure [27].

2. **dsDNA** – is the main component of cell nucleus. Rise of according a-Abs may indicate for activation of apoptosis (virus-induced mainly) [25].
3. Excess of a-Abs against **β2-Glycoprotein I** is marker of anti-phospholipid syndrome which may be the cause of thrombosis in placenta and other organs and leads to feto-placental insufficiency. Excess of such a-Abs may reveal embryotoxic effects [26, 28].
4. Excess of a-Abs against **Fc-fragment of IgG** (Rheumatoid factor) – is indicator of inflammatory processes of any localization. Excess of such a-Abs may reveal embryotoxic effects [25].
5. **Collagen II** – is the main protein of intercellular matrix. Excess of a-Abs to collagen may be sign of pathological changes in a connective tissue. Excess of such a-Abs may reveal embryotoxic effect [25].
6. Excess of a-Abs against **insulin** may indicate of forming insulin insufficiency because gestational diabetes or insulin-dependent diabetes type I. Excess of such a-Abs may be pathogenic factor for diabetic fetopathy [25].
7. Excess of a-Abs against **thyroglobulin** may be a sign of the existing or forming malfunction of thyroid gland [25].
8. **Proteins of S100 family** take part in regulation lot of cellular functions (including regulation of apoptosis). Excess of a-Abs against S100 may leads to deviations of general morphogenesis and cellular differentiation in embryo. S100 proteins participate in differentiation of neuroblasts of the neural tube and excess of a-Abs against S100 may be cause of deviation of the nervous system ontogenic formation. One of the most often reason for induction of excessive production of such a-Abs against is human papilloma virus infection (by molecular mimicry mechanisms). Therefore human papilloma virus infection in organism of pregnant women may lead to frequent death of embryo or to malformation of the nervous system of the foetus [25].
9. **Spr-06** – is one of important antigens of sperm membranes. Excess of a-Abs against Spr-06 may be cause of declining fertility in women and men and is biomarker of endometritis [25].
10. **TrM-03** – is one of important antigens of platelet membranes. Excess of a-Abs against TrM-03 is sign of trombocytopathy and may lead to the following deviations 1) excessive lysis of platelets which leads to thrombocytopenia and abnormal bleeding; 2) excessive aggregation of platelets (without their lysis) and frequent trombotic events [25].
11. Antigen **ANCA** expression increased in systemic or local vasculites (inflammation of internal wall of blood vessels). Excess of ANCA-specific a-Abs is typical sign of different forms of vasculites [25].
12. **KiM-05** – is one of important antigens of kidney cell membranes. Excess of a-Abs against KiM-05 should be considered as indication for inflammatory process in renal tissue [18, 25].

The ELI-P-Complex kit is designed for analysis of 30 blood serum samples in duplicates. The kit composition, its characteristics, operation principle and storage conditions are described in details in the Application Manual (**ANNEX 1**).

The ELI-P-Complex test is carried out using standard ELISA equipment. A blood sample is drawn from the patient to perform the test. Regulations for blood sampling and specimen preparation for examination are detailed in **ANNEX 2**.

The procedure is detailed in the Application Manual (**ANNEX 1**).

2.2 Evaluation of ELI-P-Complex test results

The results of the ELI-P-Complex test are processed by a special computer software (поставляется разработчиком is supplied by the developer). Clinician obtains the result in the form of a chart (**ANNEX 3**), where marker a-Abs profiles shifts (in arbitrary units), reflecting the alterations in different structures of reproductive system of investigated woman, are clearly seen.

1. The data obtained by the ELI-P-Complex Test represents a serum profile of the individual immune reactivity composed by 12 auto-Abs of IgG class with different antigen specificity, and is calculated in conditional units (CU; or percentages from an idealized level zero). In a healthy state, amplitude of each auto-Ab should be placed in a range from -20 to +10 CU (physiologic range). If amplitude of some auto-Abs is above +10 CU or below -20 CU, it may indicate pathology changes in certain cell populations in certain organs or systems. In the graphic form (**ANNEX 3**) the data is represented as bars in the histogram:
    - Green bars correspond to the norms of immune reactivity of respective auto-Abs;
    - Yellow parts of bars correspond to weak deviations from the norms of reactivity of respective auto-Abs;
    - Red parts of bars correspond to prominent deviations from the norms of reactivity of respective auto-Abs.
2. Besides the detailed profile of immune reactivity relating to the changes in production of auto-Abs to the selected antigens, the mean individual immune reactivity (MIR) of investigated women is also calculated and represented in graphic form (as X-axis). MIR implies an average deviation of the immune reactivity of investigated persons from the control data and is calculated in percentage from the reactions of the control sample with the same antigens. In a normal state, MIR should be situated in a range from -30% below to 0% to the control data.

The ELI-P-Complex test results histogram interpretation algorithm is presented in details in **ANNEX 4**.

Alterations to the serum content of specific auto-Abs reflect different pathologies in woman's reproductive system. The algorithm for interpreting the results of ELI-P-Complex test is given in **ANNEX 5**.

3. STUDY OBJECTIVES

3.1 Primary Objective

To demonstrate the efficacy of "Eli-P-Complex" testing in preconception care for increasing the frequency of healthy children birth in women with history of adverse pregnancy outcome.

3.2 Secondary Objective

To demonstrate the efficacy of "Eli-P-Complex" testing in preconception care for improving the pregnancy outcome in women with history of adverse pregnancy outcome.

3.3 Other Objectives

To demonstrate the efficacy of "Eli-P-Complex" testing in preconception care for reducing the complications and improving the pregnancy course in women with history of adverse pregnancy outcome.

4. STUDY DESIGN

The present study is designed as a multicenter, randomized, 2-arm parallel-group trial.

The estimated study duration that served as the assumption for sample size calculations is 75 months. All patients will be followed from randomization until the study end date, which will occur when the last patient has been followed for 15 months, based on 60-month recruitment period.

5. STUDY STEERING COMMITTEE

The Steering Committee of the study is composed of Investigator/Academic Members from participating sites and Sponsor Representatives. The Committee will provide scientific and strategic direction for the trial and will have overall responsibility for its execution. The Steering Committee is responsible for ensuring that study execution and management are of the highest quality. Detailed responsibilities and membership for the Committee and Sponsor are provided in **ANNEX 6.**

6. SELECTION OF PATIENTS

This clinical trial is conducted as collaboration between LLC MRC «IMMUNCULUS» (Moscow, Russia) and Obstetrics & Gynaecology Departments of 19 (nineteen) medical institutions (hospitals) of Russian Federation.

Based upon the anticipated event rates approximately 4,000 patients will be enrolled in the study, with approximately 2,000 patients in each arm (groups A and B).

6.1 Inclusion criteria

1. Written informed consent obtained
2. Women aged 17 to 45, **AND**
3. Presence of the history of adverse pregnancy outcome:
    a) Miscarriage (2 or more) **OR**
    b) Missed miscarriage **OR**
    c) Anembryonic pregnancy **(**blighted ovum) **OR**
    d) Foetal death (stillbirth) **OR**
    e) Intrauterine growth restriction **OR**
    f) Premature birth **OR**
    g) Foetal and Congenital malformations

6.2 Exclusion criteria

1. Women of childbearing age with no history of adverse pregnancy outcome.
2. Women diagnosed with infertility.
3. Presence of other condition (medical, psychological, social, or geographical) that would either interfere with participation in the trial or lead to inability to complete the trial.

7. STUDY PLAN

After randomization, 2 Groups are created: A and B.

- Group A women were tested with ELI-P Complex during the preconception preparation before pregnancy.
- In Group A women, the abnormalities, originally detected by ELI-P-Complex test, are confirmed by means of routine diagnostic methods. Individual treatment is conducted in accordance with the identified abnormalities. To assess the effectiveness of the treatment the ELI-P-Complex testing is performed one more time.
- Both groups (Group A and Group B) were tested with ELI-P-Complex during the first, second and third trimester.

In case of detecting abnormalities in serum content of auto-Abs in Group A women,

routine methods of laboratory and instrumental diagnostics are conducted during preconception preparation to confirm disorders identified by ELI-P-Complex test. The Unified Criteria for Patient Examination based on the alterations detected by means of ELI-P-Complex test is presented in **ANNEX 7**.

In each case, treatment should be prescribed individually and in accordance with generally accepted methods of treatment for female reproductive system diseases or diseases that affect reproductive function.

The control study is carried out by means of ELI-P-Complex test in 3-4 weeks after completion of treatment. For research purposes, the women in this study will be admitted to pregnancy planning, although the manufacturer recommends planning on the following criteria:

1. Normalization of serum content of embryotrophic auto-Abs
   OR
2. The decrease of the initially elevated level of immune reactivity of separate auto-Abs not less than by 50% of their original level
   OR
3. The increase in Median Individual Reactivity (MIR) level of at least 1.5 fold the initial value in women with immune suppression at baseline
   OR
4. The decrease in the Median Individual Reactivity (MIR) level to the normal values in women with initial immune activation.

Lack of such amendments in embryotrophic Abs indicates insufficiently effective therapy, as well as stability of a particular pathogenic reason that can lead to the recurrence of adverse pregnancy outcome.

8. ASSESSMENT OF INVESTIGATIONAL PRODUCT

8.1 Efficacy criteria

8.1.1 Primary criterion

Occurrence of one of the following Clinical Events:

- Newborns with Apgar score of 6 points or less
- Premature infant (Low birth weight)
- Intrauterine growth restriction
- Perinatal asphyxia
- Congenital malformations

8.1.2 Secondary criterion

Occurrence of one of the following Clinical Events:

- Miscarriage
- Foetal death (stillbirth)
- Missed miscarriage
- Anembryonic pregnancy **(**blighted ovum)
- Premature birth

8.1.3 Other criteria

Occurrence of one of the following Clinical Events:

- Threatened miscarriage
- Placental abruption
- Pre-eclampsia, eclampsia
- Intrauterine hypoxia
- Intrauterine growth restriction
- Foetal malformation
- Polyhydramnios
- Oligohydramnios

8.2 Definitions of components of the primary efficacy endpoint

8.2.1 Newborns with Apgar score of 6 points or less

Apgar score is a simple and replicable method to quickly and summarily assess the health of newborn children immediately after birth. The five criteria are summarized using words chosen to form a backronym (Appearance, Pulse, Grimace, Activity, Respiration). From each column in the table below, the infant is given a score of 0, 1 or 2. The scores are added up and the total sum is their Apgar score:

	Score of 0	**Score of 1**	**Score of 2**	**Component of Acronym**
Appearance/ Complexion	blue or pale all over	blue at extremities body pink (acrocyanosis)	no cyanosis body and extremities pink	**A**ppearance
Pulse rate	absent	<100	>100	**P**ulse
Reflex/ irritability	no response to stimulation	grimace/ feeble cry when stimulated	cry or pull away when stimulated	**G**rimace
Activity	none	some flexion	flexed arms and legs that resist extension	**A**ctivity
Respiratory Effort	absent	weak, irregular, gasping	strong, lusty cry	**R**espiration

The purpose of the Apgar test is to determine quickly whether a newborn needs immediate medical care. The test is generally done at one and five minutes after birth, and may be repeated later if the score is and remains low. Scores 7 and above are generally normal, 4 to 6 fairly low, and 3 and below are generally regarded as critically low. If the Apgar score remains below 3 at later times such as 10, 15, or 30 minutes, there is a risk that the child will suffer longer-term neurological damage.

8.2.2 Premature infant (Low birth weight)

Premature infant is defined as the newborn of less than 37 weeks gestational age with birth weight of 2500 gram or less. Preterm infants usually show physical signs of prematurity in reverse proportion to the gestational age.

8.2.3 Intrauterine growth restriction

Intrauterine growth restriction (IUGR) is defined as a poor growth of a baby while in the mother's womb during pregnancy. Intrauterine growth restriction refers to a condition in which a foetus is unable to achieve its genetically determined potential size. The most widely used definition of IUGR is a foetus whose estimated weight is below the 10th percentile for its gestational age. Growth percentiles for foetal weight versus gestational age are shown in **ANNEX 8**.

8.2.4 Perinatal asphyxia

Perinatal asphyxia (or birth asphyxia) is the medical condition resulting from deprivation of oxygen to a newborn infant that lasts long enough during the birth process to cause physical harm, usually to the brain.

Criteria of evaluation:

- Apgar score (0-3 for > 5 minutes)
- Umbilical cord arterial blood pH (pH < 7.0)
- Base deficit (> -12 -16 mmoll/l)
- Apnea
- Neurologic manifestations in the immediate neonatal period- seizure, hypotonia, drowsiness, coma, or hypoxic ischemic encephalopathy.
- Multi-organ system dysfunction in the immediate neonatal period - congestive cardiac failure and shock, persistent pulmonary hypertension (persistent foetal circulation) may by present. Respiratory distress syndrome, GI perforation, hematuria acute tubular necrosis or DIC

8.2.5 Congenital malformations

A congenital malformation, or congenital disease, is a condition existing at birth and often before birth, or that develops during the first month of life (neonatal disease), regardless of causation.

Primarily structural congenital disorders:

- A limb anomaly is called a dysmelia. These include all forms of limbs anomalies, such as amelia, ectrodactyly, phocomelia, polymelia, polydactyly, syndactyly, polysyndactyly, oligodactyly, brachydactyly, achondroplasia, congenital aplasia or hypoplasia, amniotic band syndrome, and cleidocranial dysostosis.
- Congenital anomalies of the heart include patent ductus arteriosus, atrial septal defect, ventricular septal defect, and tetralogy of fallot.
- Congenital anomalies of the nervous system include neural tube defects such as spina bifida, meningocele, meningomyelocele, encephalocele and anencephaly. Other congenital anomalies of the nervous system include the Arnold-Chiari malformation, the Dandy-Walker malformation, hydrocephalus, microencephaly, megencephaly, lissencephaly, polymicrogyria, holoprosencephaly, and agenesis of the corpus callosum.
- Congenital anomalies of the gastrointestinal system include numerous forms of stenosis and atresia, and imperforate.
- Congenital anomalies of the kidney and urinary tract (CAKUT) include renal parenchyma, kidneys, and urinary collecting system.

8.3 Definitions of secondary efficacy endpoint

8.3.1 Miscarriage

A miscarriage is the natural death of an embryo or foetus in the womb. Miscarriage is the loss of pregnancy before 20 weeks. The physical symptoms of a miscarriage are bleeding, low abdominal pain with or without cramping.

Miscarriage should be diagnosed only if any of the following criteria are met upon ultrasonography visualization [29]:

- Crown-rump length of at least 7 mm and no heartbeat.
- Mean gestational sac diameter of at least 25 mm and no embryo.
- Absence of embryo with heartbeat at least 2 weeks after an ultrasound scan that showed a gestational sac without a yolk sac.
- Absence of embryo with heartbeat at least 11 days after an ultrasound scan that showed a gestational sac with a yolk sac.

In addition, signs upon ultrasonography that are suggested to be suspicious for miscarriage, but not diagnostic of it, include [29]:

- Crown–rump length of less than 7 mm and no heartbeat.
- Mean gestational sac diameter of 16–24 mm and no embryo.
- Absence of embryo with heartbeat 7–13 days after an ultrasound scan that showed a gestational sac without a yolk sac.
- Absence of embryo with heartbeat 7–10 days after a scan that showed a gestational sac with a yolk sac.
- Absence of embryo at least 6 weeks after last menstrual period.
- Amniotic sac seen adjacent to yolk sac, and with no visible embryo.
- Yolk sac of more than 7 mm.
- Small gestational sac compared to embryo size (less than 5 mm difference between mean sac diameter and crown–rump length).

8.3.2 Foetal death (stillbirth)

Foetal death (stillbirth) occurs when a foetus dies in the uterus after 20 weeks of pregnancy. Once the foetus has died, the mother may or may not have contractions and undergo childbirth. So, foetal death can be divided into death prior to labour, antenatal (antepartum) death, and death during labour, intranatal (intrapartum) death.

A decrease or cessation in sensations of foetal activity may be an indication of foetal distress or death [30]. Foetal distress or death can be confirmed or ruled out via fetoscopy/doptone, ultrasound, and/or electronic foetal monitoring. If the foetus is alive but inactive, extra attention will be given to the placenta and umbilical cord during ultrasound examination to ensure that there is no compromise of oxygen and nutrient delivery.

Ultrasound evaluation:

- No heartbeat on ultrasound

8.3.3 Missed miscarriage

A missed miscarriage (delayed miscarriage, silent miscarriage) is when the embryo or foetus has died, but a miscarriage has not yet occurred.

Ultrasound evaluation [31]:

- A Crown-rump length (CRL) of ≥ 7mm without a heartbeat on a transvaginal ultrasound.
- Additional clues are presence of abnormal hyperechoic material within the uterine cavity and an irregular gestational sac.
- If there is an absence of heart beat in a foetus that is less than 7mm, the diagnosis of miscarriage cannot be made with certainty. This scenario is termed "pregnancy of uncertain viability (PUV)", and

follow up with ultrasound (generally in 7-10 days) and serial bHCG recommended.

8.3.4 Anembryonic pregnancy (blighted ovum)

An anembryonic pregnancy is characterized by a normal-appearing gestational sac, but the absence of an embryo. It likely occurs as a result of early embryonic death with continued development of the trophoblast [32].

Ultrasound evaluation:

An anembryonic pregnancy may be diagnosed when there is no fetal pole identified on trans-vaginal scanning, and:

- the size of the gestational sac is such that a foetal pole should be seen : Mean sac diameter (MSD) ≥ 25 mm (by RCOG criteria), **OR**
- there is little or no growth of the gestational sac between interval scans (normally the MSD should increase by 1 mm per day)

Transabdominal imaging without transvaginal scanning may be sufficient for diagnosing early pregnancy failure when an embryo whose crown–rump length is 15 mm or more has no visible cardiac activity.

8.3.5 Premature birth

Premature birth is the birth of a baby of less than 37 weeks gestational age and defined as the birth of a baby before the developing organs are mature enough to allow normal postnatal survival [33].

8.4 Definition of other efficacy endpoint

8.4.1 Threatened miscarriage

Threatened miscarriage is a loose term referring to pain, bleeding or contractions during the first 20 weeks of gestation, with a closed cervical os [34]. Threatened miscarriage is a common complication of early pregnancy and does not mean that a miscarriage is inevitable. When a miscarriage is inevitable, the cervical os is open (dilated).

Symptoms of threatened miscarriage:

- Bleeding
- Low abdominal pain (or no pain)
- Cramping (or without cramping)
- At abdominal or vaginal ultrasound: foetal heart sounds present and foetal movements may be present. So the foetus remains viable and the pregnancy continues.

The ultrasound signs:

- CRL of 7mm and no heart beat

- Mean gestational sac diameter of 25mm and no foetal pole
- Persistent absence of a foetal pole with cardiac activity, 2 weeks post a scan demonstrating a gestational sac with a yolk sac
- Yolk sac of more than 7 mm.
- Small gestational sac compared to foetal pole
- Foetal bradycardia: <80-90 bpm
- Small or irregular gestational sac: MSD-CRL <5 mm
- Large subchorionic haemorrhage

8.4.2 Placental abruption

Placental abruption refers to a premature separation of the normally implanted placenta after the 20th week of gestation and before the 3rd stage of labour. It is a potentially fatal complication of pregnancy and is a significant cause of third-trimester bleeding.

Clinical symptoms of placental abruption:

- Contractions that don't stop (and may follow one another so rapidly as to seem continuous)
- Pain in the uterus
- Tenderness in the abdomen
- Vaginal bleeding (sometimes)
- Uterus may be disproportionately enlarged
- Pallor

The sonographic signs of placental abruption include [35]:

- Retroplacental clot (often poorly echogenic)
- Intraplacental anechoic areas
- Separation and rounding of the placental edge
- Thickening of the placenta: often to over 5.5 cm
- Thickening of the retroplacental myometrium: usually should be 1-2 mm unless there is a focal myometrial contraction
- Disruption in retroplacental circulation
- Intra-amniotic clot

8.4.3 Pre-eclampsia, eclampsia

Pre-eclampsia is diagnosed when a pregnant woman develops [36]:

- Blood pressure ≥ 140 mm Hg systolic or ≥ 90 mm Hg diastolic on two separate readings taken at least four to six hours apart after 20 weeks gestation in an individual with previously normal blood pressure.
- Proteinuria ≥ 0.3 grams (300 mg) or more of protein in a 24-hour urine sample or a SPOT urinary protein to creatinine ratio ≥ 0.3.

In the absence of proteinuria, the presence of new-onset hypertension and the new onset of one or more of the following is suggestive of the diagnosis of preeclampsia:

- Evidence of kidney dysfunction (oliguria, elevated creatinine levels)
- Impaired liver function (impaired liver function tests)
- Thrombocytopenia (platelet count <100,000/microliter)
- Pulmonary oedema
- Ankle oedema pitting type
- Cerebral or visual disturbances
- Epigastric/right upper quadrant abdominal pain, headaches, and vomiting during severe preeclampsia

Eclampsia is a serious complication of preeclampsia. Warning symptoms for eclampsia in an individual with current preeclampsia may include headaches, visual disturbances and right upper quadrant or epigastric abdominal pain. Eclampsia characterized by the appearance of tonic–clonic seizures and coma that happen during pregnancy but are not due to pre-existing or organic brain disorders [37]. Placental bleeding and placental abruption may also occur.

8.4.4 Intrauterine hypoxia

Intrauterine hypoxia occurs when the foetus is deprived of an adequate supply of oxygen. It may be due to a variety of reasons such as prolapse or occlusion of the umbilical cord, placental infarction, intrauterine growth restriction (IUGR) and maternal smoking.

Doppler ultrasound evaluation

The severity of foetal hypoxia can be assessed with several Doppler parameters which include:

1. Umbilical artery Doppler assessment

The umbilical arterial waveform usually has a "saw tooth" type pattern with flow always in the forward direction. In growth-retarded foetuses and foetuses developing intrauterine distress, the umbilical artery blood velocity waveform usually changes in a progressive manner as below:

- Reduction in end diastolic flow: increasing RI values , PI values and S:D ratios
- Absent end diastolic flow (AEDF): RI = 1
- Reversal of end diastolic flow (REDF)

For technique and measurement see **ANNEX 9.**

2. Umbilical vein Doppler assessment

   - Can become pulsatile

   For technique and measurement see **ANNEX 9.**

3. Fetal middle cerebral artery (MCA) Doppler assessment

   - In the **normal** situation the foetal MCA has a **high** resistance flow which means there is minimal antegrade flow in foetal diastole.
   - In **pathological states** this can turn into a **low** resistance flow mainly as a result of the foetal head sparing theory.
   - Paradoxically in some situations such as with severe cerebral oedema, the flow can revert back to a **high** resistance pattern when the pathology hasn't yet resolved. This is a very poor prognostic sign.
   - Cerebroplacental ratio > 1:1 is normal , <1:1 is abnormal

   For technique and measurement see **ANNEX 9.**

4. Ductus venosus Doppler assessment

   1. Reduction flow in ductus venosus A wave
   2. Absent flow in ductus venosus A wave
   3. Reversal of flow in ductus venosus A wave
   4. Abnormal indices:

      - Abnormal pulsatility index (PI)
      - Abnormal S wave to A wave ratio (S:A)
      - Abnormal peak velocity index

   For technique and measurement see **ANNEX 9.**

8.4.5 Intrauterine growth restriction

The ultrasound scanning is performed three times during pregnancy: in the first trimester (ideally at 10 to 13 weeks of pregnancy), in the second trimester at 20 to 22 weeks of pregnancy and in the third trimester at 32 - 34 weeks.

Ultrasound biometry of the foetus is now the gold standard for assessing foetal growth. The measurements most commonly used are the biparietal diameter, head circumference, abdominal circumference and femur length. Percentiles have been established for each of these parameters, and foetal weight can be calculated (**ANNEX 10**).

Also useful is the ratio of the head circumference to the abdominal

circumference (HC/AC). Between 20 and 36 weeks of gestation, the HC/AC ratio normally drops almost linearly from 1.2 to 1.0. The ratio is normal in the foetus with symmetric growth restriction and elevated in the infant with asymmetric growth restriction.

Another important use of ultrasound is estimating the amount of amniotic fluid. A decreased volume of amniotic fluid is closely associated with IUGR. Percentiles for the amniotic fluid index at each gestational age are shown in **ANNEX 10**.

8.4.6 Foetal malformation

The introduction of high-frequency vaginal probes has enabled early diagnosis of certain foetal abnormalities from the 12th to 14th week of pregnancy.

Anomalies of the central nervous system (see **ANNEX 11**):

- Anencephaly
- Microcephaly
- Meningocele and Encephalocele
- Hydrocephaly
- Choroid Plexus Cyst
- The Posterior Fossa
- Spina Bifida

Externally visible body defects and skeletal abnormalities

- Skeletal Dysplasia (In most skeletal dysplasias the first noticeable feature is short limbs)

8.4.7 Polyhydramnios

Polyhydramnios is a medical condition describing an excess of amniotic fluid in the amniotic sac. For ultrasound technique and measurement see **ANNEX 10.**

8.4.8 Oligohydramnios

Oligohydramnios is a condition in pregnancy characterized by a deficiency of amniotic fluid. For ultrasound technique and measurement see **ANNEX 10.**

9. STATISTICAL ANALYSES

Analysis of the frequency of healthy children birth, pregnancy outcome and the amount of pregnancy complications comparing 2 groups with significance level 0.05

will perform using the SPSS 21.0.0 for Windows (IBM, NY, USA) and STATA/SE 13.0 software (STATA Corp., Texas, USA). Indicators (positive predictive value (PPV), sensitivity, specificity) of ELI-P-Complex test will calculate for pregnancy complications. The relative risk of adverse pregnancy outcome and of an unhealthy child birth will be calculated by groups A and B comparison.

10. ETHICAL ISSUES

The study protocol will be submitted for approval to the Institutional Ethical Committee of hospitals of the each study site, and the clinical trial will be conducted in accordance with the principles laid down by the 18th World Medical Assembly (Helsinki, 1964) and all applicable amendments laid down by the World Medical Assemblies, and the ICH guidelines for Good Clinical Practice (GCP).

The study design and its execution do not involve any significant ethical issues except those in other clinical studies of similar type. The only additional operation will be carried out to the patients is the blood sampling. The maximum amount of venous blood taken is 10 ml.

All patients must sign the Informed Consent Form for their participation. The copy of the Informed Consent Form is shown in **ANNEX 12**. When the results of the ELI-P-Complex examination are available, the patients will be informed about the results including an explanation of the meaning of these test results, and the appropriate measures for further conduct.

11. STUDY MONITORING

The Investigators undertake to perform the Clinical Trial in accordance with this Clinical Trial Protocol, ICH guidelines for Good Clinical Practice and the applicable regulatory requirements. The Investigators are required to ensure compliance with all procedures required by the Clinical Trial Protocol. They agree to provide reliable data and all information requested by the Clinical Trial Protocol with the help of the Case Report Form (CRF) in an accurate and legible manner and to ensure direct access to source documents to Sponsor.

The Sponsor of this Clinical Trial is responsible to Health Authorities for taking all reasonable steps to ensure the proper conduct of the Clinical Trial Protocol as regards ethics, Clinical Trial Protocol compliance, integrity and validity of the data recorded on the Case Report Forms.

During the Clinical Trial, each study site will be contacted, through monitoring visits, letters, e-mail messages or telephone calls, by a representative of the Monitoring Team to review study progress, Investigator and patient compliance with Clinical

Trial Protocol requirements, and any emergent problems. According to the ICH guidelines for Good Clinical Practice, the Monitoring Team must check the Case Report Form entries against the source documents. The Informed Consent Form will include a statement by which the patient allows the Sponsor's duly authorized personnel, the Ethical Committee, and the regulatory authorities to have direct access to source data which supports the data on the Case Report Forms (e.g., patient's medical file, appointment books, original laboratory records, etc.).

12. PROPERTY RIGHTS

All information, documents and Investigational Product provided by the Sponsor or its designee are and remain the sole property of the Sponsor. All the results, data, documents and inventions, which arise directly or indirectly from the Clinical Trial in any form, shall be the immediate and exclusive property of the Sponsor. The Sponsor may use or exploit all the results at its own discretion, without any limitation to its property right (territory, field, continuance). The Sponsor shall be under no obligation to patent, develop, market or otherwise use the results of the Clinical Trial.

13. DATA PROTECTION

The patient's personal data and Investigator's personal data which may be included in the Sponsor database shall be treated in compliance with all applicable laws and regulations. When archiving or processing personal data pertaining to the Investigator and/or to the patients, the Sponsor shall take all appropriate measures to safeguard and prevent access to this data by any unauthorized third party.

14. SPONSOR AUDITS AND INSPECTIONS BY REGULATORY AGENCIES

For the purpose of ensuring compliance with the Clinical Trial Protocol, Good Clinical Practice and applicable regulatory requirements, the Investigator should permit auditing by or on the behalf of the Sponsor and inspection by applicable regulatory authorities. The Investigator agrees to allow the auditors/inspectors to have direct access to his/her study records for review, being understood that this personnel is bound by professional secrecy, and as such will not disclose any personal identity or personal medical information. The Investigator will make every effort to help with the performance of the audits and inspections, giving access to all necessary facilities, data, and documents.

15. CLINICAL TRIAL RESULTS

The Sponsor will be responsible for preparing a Clinical Study Report. When the data from all investigational sites have been fully analyzed by the Sponsor, the latter will communicate the results of the Clinical Trial to the Investigator(s).

16. PUBLICATIONS

It is the policy of the Sponsor to encourage the presentation and/or publication of the results of their studies, using only clean, checked and validated data in order to ensure the accuracy of the results. All study participants give full authority to the Steering Committee for primary presentation and/or primary publication of the results in the name of wholehearted collaborators. No other publication is allowed before the primary publication.

17. FINANCING

The Clinical Trial is being sponsored by LLC MRC «IMMUNCULUS» (Moscow, Russia).

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19. ANNEXES

ANNEX 1. ELI-P-COMPLEX KIT. APPLICATION MANUAL

INTENDED USE

ELI-P-Complex Kit is intended for measuring of profiles of specific serum immune reactivity (IR) determined by twelve autoantibodies (a-Abs) of IgG class interacting with Chorionic Gonadotropin (hCG), dsDNA, β2-Glycoprotein I, Fc-fragment of IgG (rheumatoid factor), collagen II, insulin, thyroglobulin, S100 proteins, Spr-06 (specfic antigen of sperm membranes), TrM-03 (platelet membranes antigen), ANCA, KiM-05 (antigen of kidney cell membranes). Abnormal changes in serum content of specified a-Abs reflecting by changes in profiles of serum IR, may influence prominently upon pregnancy development and fetus formation and can be cause of infertility, habitual miscarriages, stillbirth, or fetal malformations.

The following antigens used in the Kit:

Antigen	Clinical consequences of changes in profiles related to according natural autoantibodies
Human Chorionic Gonadotropin (hCG)	Excess of a-Abs to the AG lead to infertility, placental malformation, and placental insufficiency, and indicate to endocrine dysfunction. Most cases elevated production of such autoantibodies (prolonged) related to pre-IVF treatment by Pregnyl or Choragon. Besides excess of autoantibodies against this AG can be marker sign of premature ovarian insufficiency. Rarely indicate for malignancy (tumor of pituary gland).
ds-DNA	Excess of a-Abs to the AG is marker sign for activation of apoptosis (virus-induced mainly). Rarely may indicate for systemic autoimmune disease or malignancy. Excess of such autoantibodies may reveal embryotoxic effects.
B2-Glycoprotein I	Excess of a-Abs to the AG is marker sign of anti-phospholipid syndrome which may be the cause of a small vessels thrombosis (in placenta and other organs) and leads to placental insufficiency. Rarely may indicate for systemic autoimmune disease or malignancy. Excess of such autoantibodies may reveal embryotoxic effects.
Collagen II	Excess of a-Abs to the AG is marker sign for adhesions and scars formation. Besides may be sign of pathological changes in a connective tissue. Excess of such autoantibodies may reveal embryotoxic effect.
Fc-fragments of IgG	Excess of a-Abs to the AG (Rheumatoid factor) is marker sign of inflammatory processes of any localization as well as of elevated production of proinflammatory cytokines. Excess of such autoantibodies may reveal embryotoxic effects.
Insulin	Excess of a-Abs to the AG may be pathogenic factor for diabetic fetopathy. Besides it can be marker sign of forming gestational diabetes or insulin-dependent diabetes type I, the endothelial disfunction and vasculopathy,
Thyroglobulin	Excess of a-Abs to the AG is marker sign of the existing or forming disturbances (chronic stress-related mostly) in the thyroid gland. Excess of such autoantibodies may reveal embryotoxic effects.
S100	Excess of a-Abs to the AG is human papilloma virus infection (by mechanism of molecular mimicry). Excess of a-Abs may lead to deviations in morphogenesis and cell differentiation in embryo, and be cause for stillbirth at any term of pregnancy. Besides it may be reason of malformation of the nervous system because S100 is involved in differentiation and migration of neuroblasts of primordial nervous system.

Spr-06	Excess of a-Abs to the AG is marker sign of endometritis (in the most cases) and/or inflammatory processes in other pelvic organs (rarely). Excess of autoantibodies against Spr-06 may be cause of declining fertility in women and men.
ANCA	Excess of ANCA-specific autoantibodies is typical sign of small vessel vasculites and vasculopathy. The last may be reason for insufficiency of placental blood flow and other malfunction related to the problems with circulation.
TrM-03	Excess of a-Abs to the AG is marker sign of trombocytopathy and may lead to the following deviations: 1) Elevated lysis of platelets and consequent pathological bleeding; 2) excessive aggregation of platelets and trombotic events.
KiM-05	Excess of a-Abs to the AG is marker sign of inflammatory process in the renal tissue.

CLINICAL SIGNIFICANCE

The surplus rise in a-Abs to the used antigens may induce embryotoxic effect, being one of the most common causes of miscarriage and/or pregnancy abnormalities. Harmful environmental factors, infectious diseases, or metabolic changes can affect the serum level of examined a-Abs. Changes in a-Abs does mirrored most sensitive by profiles of blood serum immune reactivity changes. The evaluation of a-Abs panel in blood serum using ELI-P-Complex Kit can be instructive for obstetrician who observe woman before planned pregnancy and during pregnancy development.

ELI-P-Complex Kit is designed for clinical use in obstetrics, gynecology and immunology as an additional method of pregnancy supervision, and namely for the examination of:

a. women planning pregnancy, first of all, those of risk groups, to establish the state of immune chain in the regulation of embryogenesis, 1-2 months prior to planned conception;

b. women at early stages of pregnancy (first trimester) to identify patients with high risk of pregnancy abnormalities;

c. women with infertility of unknown genesis (to identify possible immune causes of situation).

MATERIALS PROVIDED

- 96-well polystyrene plates with adsorbed antigens – 10 pieces;
- Standard sample - control serum sample (examined for the lack of HIV and hepatitis B and C viruses) with normalized content of a-Abs to the examined antigens (close to the population average of the healthy persons) – 1 ml;
- Conjugate of horseradish peroxidase (HRP) with antibodies specific for human IgG (100X concentrate) – 2 ml;
- Buffer for diluting samples (10X concentrate) – 50 ml;
- Buffer for washing plates (10X concentrate) – 250 ml;
- Buffer for developing reaction (10X concentrate) – 20 ml;
- Stop-solution (1M sulfuric acid) – 50 ml;
- Solution of tetramethylbenzidine – 2 ml;
- Sticky tape – 20 sheets;
- Application instruction – 1 copy.

MATERIALS AND DEVICES REQUIRED BUT NOT PROVIDED

- ELISA photometer with wave length 450 nm;
- Thermostats maintaining temperature $+37 \pm 1^{O}C$ and $+56 \pm 1^{O}C$;
- ELISA plate washer;

- Semi-automatic one-channel pipettes with disposable tips, for 10 µL, 20 µL, 200 µL;
- Semi-automatic 8-channel pipette with disposable tips for 25 µL, 100 µL;
- Measuring cylinders (glasses) for preparing working buffer solutions 5-10 ml;
- Distilled or deionized water;
- Plastic reservoirs for working with multi-channel pipettes
- Reservoirs (10mL) for patient's sera dilution

NOTES

- For in vitro diagnostic use.
- IVD (in vitro diagnostic medical device).
- For professional use only.

PRINCIPLE OF PROCEDURE

ELI-P-Complex Kit is designed for evaluation of serum immune reactivity profiles determined by specific a-Abs of IgG class to adsorbed antigens. Each Kit is intended for screening of 30 examined samples of blood serum in two replications and Standardized template-sample added to the wells of each one immunoplate. After incubation period, a balance is established between bound and free a-Abs. After that wells are washed and filled with the solution of conjugate of horseradish peroxidase (HRP) with antibodies specific for human IgG, binding of conjugate in quantity proportional to the quantity of the bound a-Abs occurs. The excess of free conjugate is washed and enzyme reaction of peroxydase in the presence of hydrogen peroxide and chromogen (tetramethylbenzidine – TMB) takes place for measuring activity of HRP. The intensity of chromogen's color is proportional to the concentration of a-Abs in the examined sample. After the HRP reaction is stopped by a stop-reagent, the evaluation of results is done by photometric analysis of optical density (OD) at the wave's length 450 nm. Calculation of the relative immune reactivity (IR) of the analyzed a-Abs in the sample, expressed as the percentage of the immune reactivity of the control serum sample (in Conventional Units), as well as calculation of normalized relative immune reactivity of 12 analyzed a-Abs compared to individual average level of immune reactivity is done.

PRECAUTIONS

- Do not use the Kit if the packaging of any of its components is damaged.
- While working with components of the Kit personnel should be informed: TMB is toxic if inhaled or swallowed. Sulfuric acid can induce irritation and burns if allowed on skin or mucosa and, should that happen, is to be washed off immediately by large amounts of running water.
- While working with the Kit, one should wear expendable rubber or plastic gloves, as the Kit includes components of human blood, which should be considered as potentially infectious.
- Used materials contained blood or serum, should be handled as potentially infectious. All liquid remained should be disinfected before being disposed, by 30%-solution of chloramines, or by 15 minute autoclaving under +120^{o}C. Dishes used for the storage of examined samples also have to be disinfected.

STORAGE CONDITIONS AND APPLICATION CONDITIONS

During the whole period of operability (6 months), ELI-P-Complex Kit should be stored under the following conditions:

- Conjugate of HRP with antibodies specific for human IgG and control serum must be kept at -20^{o}C;
- Sample buffer, washing buffer, substrate buffer, stop-solution, TMB solution and immunoplates at +2...8^{o} C.
- Store working sample buffer and working washing buffer at +2...8^{o} C no longer than 7 days.

- Analysis should not be conducted on blood serum samples containing sodium azide, as well as on opaque and highly hemolyzed samples.
- Reliable results can be received only under the condition of strict compliance with the application instructions.

ASSAY PROCEDURE

Preparing reagents for analysis (volumes are given for **one** immunoplate):

1. Keep the Kit's components under the room's temperature for no less than 60 min.
2. **Sample buffer** is made immediately before use. For that, add **27** ml of distilled water to **3** ml of 10X concentrated buffer for diluting samples, and stir thoroughly. The prepared dilution can be stored at +2...8°C no longer than 48 hours.
3. **Preparation of dilutions of examined blood serum samples:** Serum samples can be stored at +2...8°C for no longer than 3 days. Prior to the analysis, blood serum samples should be warmed at +56°C for 30 min. For diluting samples, add **3000** µL of working sample buffer solution to **15** µL of each of the sample, and stir. The dilutions of examined serum samples should be prepared immediately before use, they cannot be stored.
4. **Preparation of dilutions of standard sample:** Standard serum sample should not be warmed! Standard serum dilutions should be prepared immediately before use, and cannot be stored. For preparation of standard serum dilution add indicated (Table 1) volume of sample buffer to indicated volume of standard serum and mix thoroughly.

TABLE 1.

Dilution *	Standard serum, µL	Sample buffer, µL	Used for antigens (number of strip
1/50	20	1000	2, 6
1/100	10	1000	5, 8, 9
1/200	10	2000	1, 4, 10, 11, 12
1/300	10	3000	7
1/400	5	2000	3

* (**Important note:** specific dilutions of standard serum sample and placements may be changed from lot to lot. Please, refer to printed product information and manual included with kit)

5. **Working washing buffer dilution** for washing plates should be prepared immediately before use. Before the dilution, warm 10X concentrate of washing buffer at 30–40°C till the residual matter is fully dissolved. Add 225 ml of distilled water to 25 ml of 10X concentrated buffer stock solution, and stir. Solution can be stored at +2...8°C for no longer than 7 days.
6. **Preparation of working conjugate dilution:** Add 10 ml of working sample buffer solution to 100 µL of conjugate, and stir. Conjugate dilution should be prepared immediately before use and cannot be stored.
7. **Preparation of chromogene dilution:** Working substrate buffer solution should be prepared immediately before use. For that, add 9,0 ml of distilled water and 100 µL of concentrated TMB to 1 ml of 10X concentrate of substrate buffer and mix. Chromogene dilution should be prepared immediately before use and cannot be stored.
8. Add 100 µL of the respective standard dilutions and examined sera samples, both diluted, in each of the immunoplate's wells according to the scheme (see Fig.1). Note that standards and samples are used in duplicates.
9. Seal the plate with adhesive film and incubate overnight (15-20 hours) at +2...8°C.

10. Empty the wells and wash 3 times with working dilution of washing buffer by filling each well with 300 µL of buffer, and emptying by aspiration each time. Procedure should not last more than 15 min.
11. Immediately after washing, fill each of the wells with 100 µL of working dilution of conjugate; procedure should not last more than 5 min, then incubate for 1,5 hours at +37°C.
12. Empty the wells and wash 4 times according point 10 of this Assay Procedure.
13. Immediately after washing, fill each of the wells with 100 µL of working dilution of buffer for developing reaction. This procedure should not last more than 2 min.
14. Incubate the plate in the dark at +18...25°C during 15-20 min, then stop the reaction by filling each well with 25 µL of stop-solution using multi-channel pipette (after wells with standard sera turn blue). Procedure should not last more than 1 min.
15. Immediately after the reaction is stopped, measure optical density in each well on ELISA photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length).

	hCG	**DNA**	**b2-GP**	**Col**	**Fc**	**Ins**	**Tg**	**S100**	**Spr-06**	**TrM-03**	**ANCA**	**KiM-05**
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	1/200	1/50	1/400	1/200	1/100	1/50	1/300	1/50	1/200	1/200	1/200	1/200
B	1/200	1/50	1/400	1/200	1/100	1/50	1/300	1/50	1/200	1/200	1/200	1/200
C	S1	S1	S1	S1	S1	S1	S1	S1	S1	S1	S1	S1
D	S1	S1	S1	S1	S1	S1	S1	S1	S1	S1	S1	S1
E	S2	S2	S2	S2	S2	S2	S2	S2	S2	S2	S2	S2
F	S2	S2	S2	S2	S2	S2	S2	S2	S2	S2	S2	S2
G	S3	S3	S3	S3	S3	S3	S3	S3	S3	S3	S3	S3
H	S3	S3	S3	S3	S3	S3	S3	S3	S3	S3	S3	S3

Fig 1. Immunoplate scheme with the order of adsorbed antigens; The order of the standard serum dilutions (1/50, 1/100, 1/200, 1/300, 1/400) placement and three samples of examined sera (S1, S2, S3) are indicated. Abbreviations used for antigens are following: **hCG** – human chorionic gonadotropin; **DNA** – dsDNA; **b2-GP** – Beta2-Glycoprotein I; **Col** – collagen; **Fc** – Fc-fragments of immunoglobulins; **Ins** – Insulin; **Tg** – Thyroglobulin; **S100** – protein S100; **Spr-06** – antigen of spermatozoids' membranes; **TrM-03** – antigen of thrombocytes' membranes; **ANCA** – antigen of vessel's endothelium; **KiM-05** – antigen of kidneys.

ASSAY QUALITY CONTROL

- Standardized template-sample must be run along with analyzed samples in each immunoplate in order for results to be valid.
- Optical density (OD) of the wells with Standardized template-sample is in the range from 0,15 - 0,4 units of optical density (OD), except wells with adsorbed hCG where OD range is 0,1 - 0,2.

CALCULATION OF RESULTS

1. The method ELI-P-Complex and specialized kit was registered by the Russian Ministry of Health for diagnostic and prognostic investigation of planning pregnancy or already pregnant women. Serum samples processing, based on conventional ELISA, was executed in accordance to instruction to the Kit. Intensity of reaction of the Standardized template-sample with each of antigens (Optical density; OD) was considered as 100%; Intensity of reaction of investigated serum samples with the same antigens was calculated in per cents to OD of the Standardized template-sample**.** Mediane of immune reactivity each of investigated serum sample calculated

in accordance to the equations:

$$\text{MIR} = \left(\frac{R(ag1)\cdot 100}{R(k1)} - 100 + \frac{R(ag2)\cdot 100}{R(k2)} - 100 + \ldots \frac{R(ag12)\cdot 100}{R(k12)} - 100\right) : 12,$$

Symbols: MIR — mediane of individual immune reactivity of investigated serum sample calculated as per cents to immune reactivity of the Standardized template-sample. The normal (physiological) level of MIR should been restricted by ranges from -30%… to 0% from MIR of the Standardized template-sample.
*R(ag*1), *R(ag*2), ... *R(ag*12) — optical density (OD) of reaction of investigated sample with each of 12 used antigens.
*R(k*1), *R(k*2), ... *R(k*12) — OD of reaction of the Standardized template-sample with each of the same 12 used antigens.

2. Profiles of immune reactivity of investigated sample was presented as combination of reactions with each one used antigens (ag1, ag2, … ag12) impressed in per cents to MIR of given sample. The next equations were used:

$$R(nrm)ag1 = \left(\frac{OD(ag1)\cdot 100}{OD(k1)}\right) - 100 - \text{MIR}; \qquad R(nrm)ag2 = \left(\frac{OD(ag2)\cdot 100}{OD(k2)}\right) - 100 - \text{MIR};$$

$$\ldots\ldots\ldots\ldots\ldots \qquad R(nrm)ag12 = \left(\frac{OD(ag12)\cdot 100}{OD(k12)}\right) - 100 - \text{MIR};$$

Symbols: *R (nrm) ag*1, *R (nrm) ag*2, ... *R (nrm) ag*12 — deviation of the reaction of investigated sample with each one used antigens (ag1, ag2, … ag12) expressed in per cents from MIR of given individual sample.

OD (*ag*1), *OD(ag*2), ... *OD* (*ag*12) — optical density of reaction of investigated sample with each of used antigens.

OD(*k*1), *OD*(*k*2) ... OD(k12) — OD of reaction of the Standardized template-sample with each of the same 12 used antigens.
MIR —mediane of individual immune reactivity of investigated serum sample calculated as per cents to immune reactivity of the Standardized template-sample.

3. The normal (physiological) level of each one "peak" in profile, corresponding the reaction R(nrm) of a-Ab any one antigen specificity should been restricted by the ranges from -20% to 10% from MIR of given individual sample.
4. Specialized software (included in each Kit) was used for calculations.

CLINICAL EXPLANATION OF RESULTS

1. Optimal level of mean individual serum median immune reactivity (MIR) should be in range between -30%... 0% from the Standardized template-sample MIR. Women with mean individual immune reactivity level fits to optimal level range, and without prominent deviations in one or few immune reactivity related to any of specific a-Abs above 10% from individually normalized individual serum median immune reactivity (MIR) level the risk of pregnancy pathology is no more 4-5%.
2. Abnormally elevated level of individual serum median immune reactivity (above 1% from the Standardized template-sample MIR) indicates polyclonal immune activation. This is typical for acute infection diseases, inflammatory processes, system autoimmune diseases in stage of exacerbation. This situation may negatively influence pregnancy development, and may be related to additional risk for miscarriage, stillbirth, etc.

3. Abnormally low level of individual serum median immune reactivity (below -30% from the Standardized template-sample MIR) indicates polyclonal immune supression (immune deficiency). This state is typical for chronic intracellular infections (viral and bacterial, such as herpetic infection, chlamydiosis, mycoplasmosis, etc.) and/or chronic intoxications. This situation may influence negative upon the pregnancy development, and be related to additional risk for miscarriage, pre-eclampsia, stillbirth, etc.
4. Profiles of individual immune reactivity (elevation in serum content/affinity of a-Abs with different antigen specificity) are represented by histogram: each bar of the histogram reflects the shift in serum content/affinity (immune reactivity) of a-Abs with defined antigenic specificity expressed as a per cents from individually normalized median level of the immune reactivity (MIR). Any single "peaks" in profiles above +10% or belov -20% from individual MIR level should be considered as indicator for pathological processes (forming or present now) in respective systems, organs or metabolic links.

Important: Permanent rise in the production of specific a-Abs, reflecting the activation of apoptosis, is the earliest sign of pathological changes, which shall become detectable by usual biochemical tests usually in a few months or even years later. Clinical symptoms will occur still later. *NB:* Biochemical (earlier) and clinical (later) signs reflect already a serious functional deficiency of an organ, while the changes in the a-Abs content/affinity reflect a pathological intensification of the apoptosis of an organ's specified cells, which is the first step in the development of a disease, long before any signs of organ's deficiency occur.

LIMITATIONS AND SOURCES OF ERRORS

Poor test reproducibility may result from:

- Inconsistent delivery of reagents;
- Improper storage of reagents;
- Improper reconstitution of reagents;
- Incomplete and non uniform washing of microwells;
- Substrate reagents old or exposed to light;
- Unstable/defective spectrophotometer;
- Unstable/defective washing device;
- Poor operating of multi-channel auto-pipettes
- Error in following the assay procedure.

SYMBOLS

	Storage Temperature	CC REP	Authorized Representativ
LOT	Lot Code		Caution, see instructions
	Expiration Date	IVD	For in vitro diagnostic use
	Manufacturer	REF	Catalog No.

MANUFACTURER
Medical Research Center "Immunculus", Ltd.
Okruzhnoy projezd, 30a
105187, Moscow, Russia
Tel/Fax: +7(495)780-72-87
website:www.immunculus.ru

ANNEX 2. REGULATIONS FOR BLOOD SAMPLING AND SPECIMEN PREPARATION FOR EXAMINATION

Blood serum used for examination is obtained by the standard preparation method that includes the following steps:

1. Venous or capillary blood is drawn into a dry test tube or into a vacuum system for drawing blood **with coagulation activator or gel (red or yellow cap)**. Attention: do NOT use plasma with heparin, EDTA or sodium citrate! The minimum amount of blood serum for a single examination is at least 2 ml.
2. Centrifugation at 3000 rpm during 5 minutes.
3. Serum separation into type Eppendorf micro test tube.

Serum samples storage conditions.

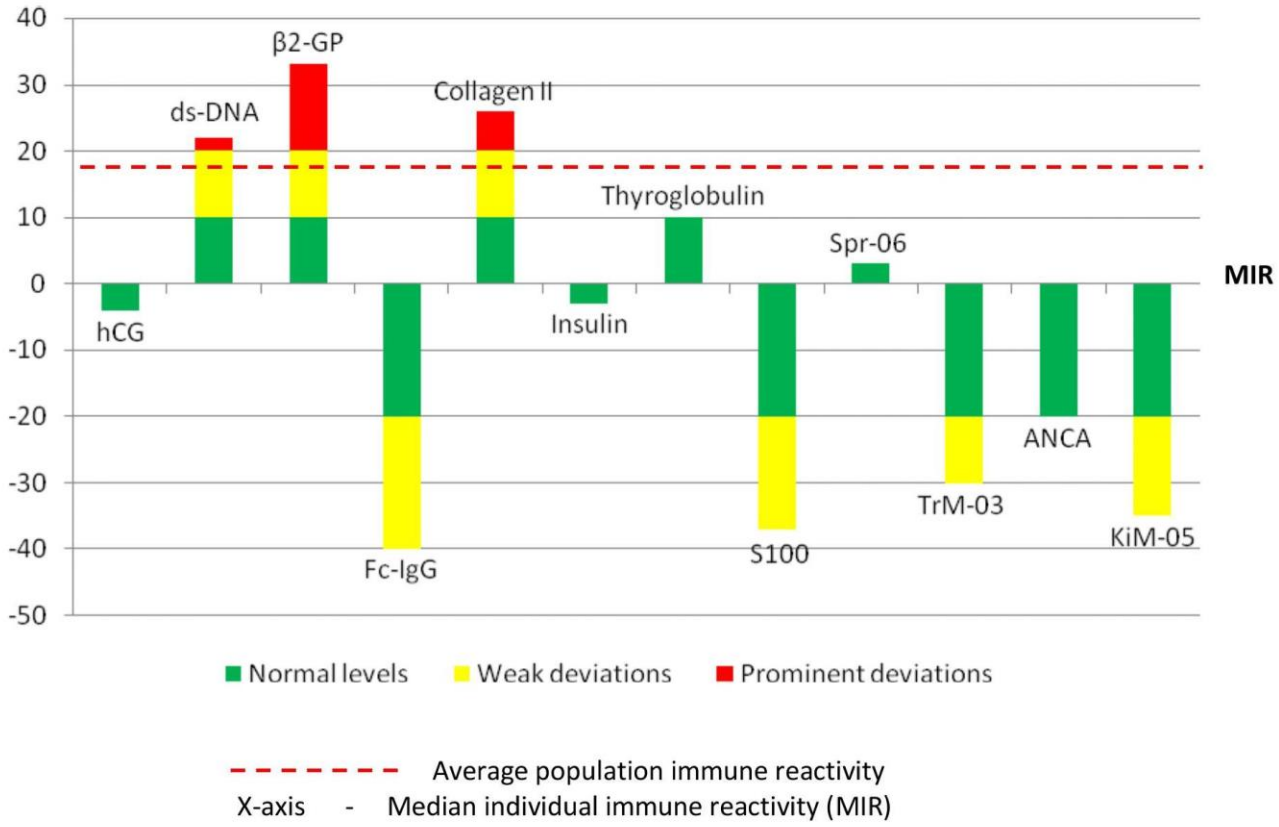
1. 24 hours at room temperature
2. 1 week at +2…+8˚C
3. 1 year at -20˚C
4. up to 10 years at -70˚C

Samples re-thawing/re-freezing is not permitted.

Examination contraindications - presence in medical history within the last 3-4 weeks of one of the following events:

1. Acute infectious or inflammatory disease.
2. Treatment with immune modulators (immune suppressants and immune stimulants).
3. Course of antibacterial treatment or chemotherapy.
4. Preventive vaccination.
5. Course of radiotherapy.

ANNEX 3. ELI-P-COMPLEX TEST CHART



ANNEX 4. INTERPRETATION ALGORITHM OF THE ELI-P-COMPLEX TEST RESULTS HISTOGRAM (AUTOIMMUNOGRAM)

1. Immune system general condition (total activity) assessment

a. First, it is recommended to pay attention to the quantitative comparison of Median Individual Reactivity (MIR) of the examined serum (matches the X-axis) and an average population norm, indicated by the red dotted line (see **ANNEX 3**). The latter may coincide with the immunoreactivity of the examined serum, may be above it (in case of immunosuppression) or below it (in case of immunoactivation). Normal fluctuation of the Median Individual Reactivity is to be in the range of -30 to 0 conventional units. The values of the Median Individual Reactivity below -30 CU. indicate immunosuppressive condition, while the excess of zero value indicates polyclonal activation of the immune system.

b. Durational general immunosuppression is accompanied by clearance disruption, i.e. reduced effectiveness of removal of apoptosis products and other antigens that are to be disposed from the body and leads to chronic autointoxication by products of metabolism. This increases the risk of miscarriage, causes chronic fatigue syndrome and is a risk factor for developing cancer.

c. General immunoactivity, that is typical for purulent-septic and system autoimmune processes, as well as for initial stages of malignant neoplastic processes, is accompanied by disturbances of the regulation of various components of molecular and cellular homeostasis. In case of general immunoactivity immune stimulators or preventive vaccination are not advisable.

2. The rise in relative content of specific auto-Abs (columns of the histogram are directed upwards from the X axis). Possible interpretations:

a. **Normal condition**
Immunogram columns, painted in green, not beyond the norm limits, i.e. beyond the values of +10 to -20% of the Median Individual Reactivity (from X-axis), reflect normal levels of serum content of the relevant auto-Abs markers.

b. **Weak deviations from the norm**
Important prognostic and/or clinical significance belongs to the histogram (immunogram) columns beyond the norm (within the "yellow" and especially within the "red" area of the deflection range) indicating abnormalities in the content of any marker auto-Abs.

Histogram columns, facing the positive direction from the X-axis (from the level of Median Individual Reactivity) and reaching the "yellow" zone (+11...+20% of Median Individual Reactivity, i.e. from the X-axis) indicate the existence of an active and relatively "fresh" (less than 6 months) pathological process in the

organ or system, with which antigens corresponding auto-Abs interact.

c. **Prominent deviations from the norm**
Evident increase in relative levels of auto-Abs of the appropriate specificity (columns reach the "red zone" above +20% of the Median Individual Reactivity, i.e. from the X-axis) indicates an active pathological process of increased intensity and/or duration.

3. The decrease in relative content of specific auto-Abs (the histogram columns are directed downwards from the X axis). Possible interpretations:

Autoimmunogram columns if directed downward from the Median Individual Reactivity, i.e. from X-axis, and reaching the "yellow" (-20...-30% from X-axis) and especially the "red" zone (below -30% from X-axis) typically indicate a long-lasting (many months or years) pathological process, the intensity of which gradually decreases.

4. Immunogram variations with time (dynamic observation).

Changes in the intensity and severity of pathological process, as well as the size of lesion focus, are naturally reflected by dynamic alterations in relative levels of auto-Abs of the appropriate specificity.

ANNEX 5. INTERPRETATION OF ELI-P-COMPLEX DATA (GENERAL ALGORITHMS)

Used antigens:	Steady state elevation of auto-Abs in blood serum may indicate for:
Human Chorionic Gonadotropin (hCG)	• Anti-hCG syndrome • Premature ovarian failure • General endocrine dysfunction
dsDNA	• Active acute infection process (mostly viral) • Systemic autoimmune disease (rarely) • Variant of paraneoplastic reaction at malignancy (rarely)
β-2 Glycoprotein 1	• Antiphospholipide syndrome (APS) • Systemic autoimmune disease (rarely) • Variant of paraneoplastic reaction at malignancy (rarely)
Collagen	• Scars formation and other changes in interstitial tissue as a result of inflammatory processes • Systemic autoimmune disease (rarely)
Fc-fragment of IgG	• Chronic inflammation of any localization
Insulin	• Chronic pancreatitis with activation of apoptosis of insulin-secreting islet cells (risk for developmeny of diabetes mellitus type I and gestational diabetes)
Thyroglobulin	• Thyroiditis (expected hypofunction of thyroid gland later)
S100 Protein	• Papilloma virus infection • Elevated risk for miscarriage and stillbirth • Variant of paraneoplastic reaction at malignancy (rarely) • Neural changes (emotional disturbances, depression, phobias, aggressiveness)
Spermal-Prostatic antigen Spr-06	• Endometritis, or other inflammatory processes in pelvic organs(often induced by *Chlamidia trachomatis*) • Many sexual partners (5 or more) • May be ground for decreased fertility or (rarely) infertility
Platelet membrane antigen TrM-03	• Changed platelet's functions (may be accomplished by elevation as well as decreasing of blood coagulation
Antigen ANCA	• System or local vasculites and vasculopathies
Kidney antigen KiM-05	• Changes in kidney's tissue as a result of acute or chronic inflammation, urolithiasis, malignancy, or other reasons

ANNEX 6. THE STEERING COMMITTEE

Membership:

Name	Function	Expertise	Member
Svetlana Tsakhilova	Chairman/ Principal Investigator	Obstetrics&Gynecology	Expert member
Rosa Zamaleeva	Principal Investigator	Obstetrics&Gynecology	Expert member
Natalia Cherepanova	Principal Investigator	Obstetrics&Gynecology	Expert member
Inna Tutereva	Principal Investigator	Obstetrics&Gynecology	Expert member
Elena Koshel	Principal Investigator	Obstetrics&Gynecology	Expert member
Alexander Poletaev			Company's member
Alexander Pukhalenko			Company's member

Steering committee responsibilities:

- Coordination, organization of meetings, distribution of documents and minutes
- Coordination of protocol and CRF review
- Development of Clinical Events review forms and instructions
- Review of monitoring visit reports
- Direct communication with the sites regarding the trial and any relevant issues
- Re-training of sites staff in case of turnover
- Distribution of newsletters
- Maintenance of a hot-line service for medical questions
- Reviewers contracts and payments
- Prepare final study manuscript for publication

Sponsor responsibilities:

- ELI-P-Complex kits Supplies
- Funding
- Study Monitoring
- Data Management
- Study Archives
- Study Reporting

ANNEX 7. THE UNIFIED CRITERIA FOR PATIENT EXAMINATION RECOMMENDED AFTER ELI-P-COMPLEX TEST

Abnormal changes in serum content of autoantibodies against:	**Recommended examinations**
Human Chorionic Gonadotropin (hCG)	• **Strictly recommended laboratory examinations:** 1. Measuring the blood content of the following hormones: LH, FSH, progesteron, testosteron, prolactin, estradiol, DHEA-S, cortisol, TSH 2. a-Abs against hCG. • **Strictly recommended special instrumental examinations:** US-examination of Pelvis Additional examination aimed at revealing the possible endocrine disturbances or malignancy can be recommended.
ds-DNA	• **Strictly recommended laboratory examinations:** 1. Full blood count 2. Urine examination 3. Blood and urine culture 4. Bacteriological, serological or PCR examination of blood and cervical smears for infection detection including chlamydia, mycoplasma, ureaplasma, trichomonas vaginalis, gardnerella, HIV, HPV, cytomegalovirus, herpes simplex virus, syphilis, candida, etc. 5. Broad hemostasiogram: fibrinogen, prothrombin, PT, APTT, INR, thrombin time (TT), lupus anticoagulant, D-dimer, antithrombin III. • **Strictly recommended special instrumental examinations:** 1. US-examination of organs of *Pelvis minor* 2. X-ray of lungs Additional examination aimed at revealing the possible autoimmune disorders or malignancy can be recommended.
β2-Glycoprotein I	• **Strictly recommended laboratory examinations:** 1. Full blood count 2. Broad hemostasiogram: fibrinogen, prothrombin, PT, APTT, INR, thrombin time (TT), lupus anticoagulant, D-dimer, antithrombin III. Additional examination aimed at revealing the possible autoimmune disorders or malignancy can be recommended.
Collagen II	• **Strictly recommended laboratory examinations:** 1. Full blood count 2. Erythrocyte sedimentation rate (ESR) 3. C-reactive protein of the blood 4. Blood and urine culture 5. Bacteriological, serological or PCR examination of blood and

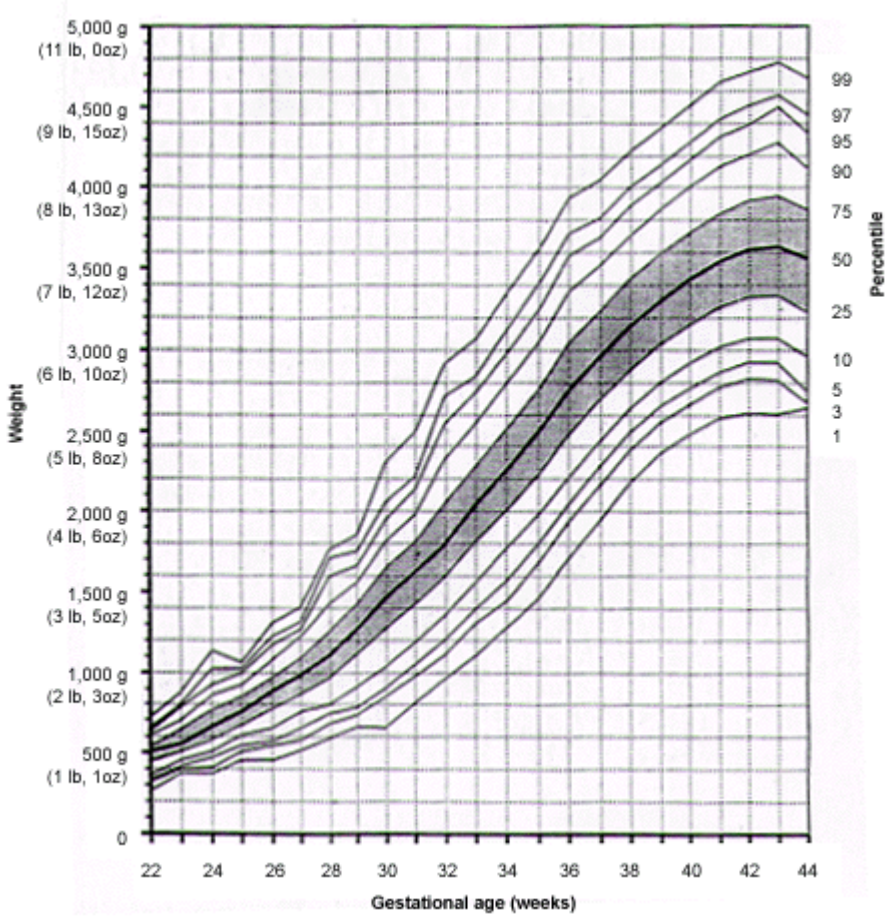
	cervical smears for infection detection including chlamydia, mycoplasma, ureaplasma, trichomonas vaginalis, gardnerella, HIV, HPV, cytomegalovirus, herpes simplex virus, syphilis, candida, etc. • **Strictly recommended special instrumental examinations:** US-examination of *Pelvis minor* for signs of adhesion process. Additional examination aimed at revealing the possible adhesion process in pelvis minor or autoimmune disorders can be recommended.
Fc-fragment of IgG	• **Strictly recommended laboratory examinations:** 1. Full blood count 2. Erythrocyte sedimentation rate (ESR) 3. C-reactive protein of the blood 4. Blood and urine culture 5. Bacteriological, serological or PCR examination of blood and cervical smears for infection detection including chlamydia, mycoplasma, ureaplasma, trichomonas vaginalis, gardnerella, HIV, HPV, cytomegalovirus, herpes simplex virus, syphilis, candida, etc. • **Strictly recommended special instrumental examinations:** 1. US-examination of abdominal organs and organs of the *Pelvis minor*. 2. X-ray of lungs Additional examination aimed at revealing the possible chronic inflammatory process of any localization can be recommended.
Insulin	• **Strictly recommended laboratory examinations:** 1. Fasting plasma glucose (FPG) 2. Testing with a 2-hour plasma glucose (2hPG) in a 75 g oral glucose tolerance test (OGTT) if FPG> 5,5mmol/l • **Strictly recommended special instrumental examinations:** US-examination of pancreas Additional examination aimed at revealing the possible risk of development of diabetes mellitus, pancreatitis, and endothelial dysfunction can be recommended.
Thyroglobulin	• **Strictly recommended laboratory examinations:** FT_3, FT_4, TSH • **Strictly recommended special instrumental examinations:** US-examination of thyroid gland Additional examination aimed at revealing the possible risk of thyroid dysfunction can be recommended.
S100	• **Visual examinations for marker signs of HPV infection (condylomas and warts any localization)** • **Strictly recommended laboratory examinations:** PCR study of blood and cervical smears for different types of HPV • **Strictly recommended special instrumental examinations:** US-examination of *Pelvis minor*

	Additional examination aimed at revealing the possible malignancy can be recommended.
Spr-06	• **Strictly recommended laboratory examinations:** 1. Full blood count 2. C-reactive protein 3. Bacterial inoculation of cervical smears • **Strictly recommended special instrumental examinations:** US-examination of *Pelvis minor* Additional examination aimed at revealing of possible endometritis, and other inflammatory processes in organs of *Pelvis minor* including bacteriological, serological or PCR examination of blood and cervical smears for different infection agents can be recommended.
TrM-03	• **Strictly recommended laboratory examinations:** Broad hemostasiogram: fibrinogen, prothrombin, PT, APTT, INR, thrombin time (TT), lupus anticoagulant, D-dimer, antithrombin III. Additional examination aimed at revealing of possible risk of thrombophilia including antithrombin, factor V Leiden, factor II (prothrombin) gene mutation, MTHFR gene mutation, protein S, protein C, hyperhomocysteinemia can be recommended.
ANCA	• **Strictly recommended laboratory examinations:** 1. Full blood count 2. Erythrocyte sedimentation rate (ESR) 3. C-reactive protein 4. Broad hemostasiogram: fibrinogen, prothrombin, PT, APTT, INR, thrombin time (TT), lupus anticoagulant, D-dimer, antithrombin III. Additional examination aimed at revealing the possible risk of vasculites and vasculopathies (including liver function tests, urine examination, serum creatinine, interleukin 6, serological examination of antinuclear antibodies and antibodies against neutrophil cytoplasmic antigens) can be recommended.
KiM-05	• **Strictly recommended laboratory examinations:** 1. Full blood count 2. Urine examination 3. Bacterial inoculation of urine 4. C-reactive protein of the blood • **Strictly recommended special instrumental examinations:** Kidney US-examination Additional examination aimed at revealing the possible risk of renal dysfunction can be recommended.
General immunosuppression	• **Strictly recommended laboratory examinations:** 1. Full blood count 2. C-reactive protein of the blood An additional examination aimed at revealing the possible cause of immune suppression can be recommended.

Notes:

1. Detection of alterations by means of the above recommended examinations indicates decompensation process and requires appropriate therapeutic measures prescription.
2. Detection of no alterations by means of the above recommended examinations indicates compensated process and requires a period of 1-3 months waiting, after which control ELI-P-Complex testing is advisable.
3. Alterations stability detected by means of the ELI-P-Complex Test in 1-3 months indicates the absence of body reparative capacity and requires additional diagnostic measures and appropriate treatment prescription.
4. The control ELI-P-Complex testing is to be conducted in 3-4 weeks after completion of treatment.

ANNEX 8. FETAL WEIGHT PERCENTILES THROUGHOUT GESTATION



ANNEX 9. DOPPLER ULTRASOUND EVALUATION

1. Umbilical artery Doppler assessment

Doppler waveforms of the umbilical arteries can be obtained from any segment along the umbilical cord. Waveforms obtained from the placental end of the cord show more end-diastolic flow, thus lower ratio values (RI, S/D) than waveforms obtained from the abdominal cord insertion. Differences in Doppler indices of arterial waveforms obtained from different anatomic locations of the same umbilical cord are generally minor and have no significance on clinical practice.

TABLE 1. Resistance Index (RI) of the Umbilical Artery Between 20 and 40 Weeks of Gestation [38].

Gestation (wks)	5th Percentile	50th Percentile	95th Percentile
20	0.567	0.690	0.802
21	0.557	0.680	0.793
22	0.548	0.671	0.784
23	0.539	0.663	0.776
24	0.530	0.655	0.768
25	0.522	0.646	0.760
26	0.514	0.639	0.752
27	0.506	0.631	0.745
28	0.498	0.623	0.737
29	0.490	0.615	0.730
30	0.482	0.608	0.723
31	0.474	0.600	0.715
32	0.465	0.592	0.707
33	0.457	0.584	0.700
34	0.449	0.576	0.692
35	0.440	0.567	0.684
36	0.431	0.559	0.675
37	0.422	0.550	0.667
38	0.412	0.540	0.657
39	0.402	0.530	0.648
40	0.390	0.519	0.637

TABLE 2. Pulsatility Index (PI) of the Umbilical Artery Between 20 and 40 Weeks of Gestation [38].

Gestation (wks)	5th Percentile	50th Percentile	95th Percentile
20	0.940	1.216	1.505
21	0.913	1.189	1.476
22	0.890	1.165	1.450

Gestation (wks)	5th Percentile	50th Percentile	95th Percentile
23	0.869	1.142	1.427
24	0.849	1.122	1.405
25	0.831	1.102	1.385
26	0.813	1.084	1.365
27	0.798	1.065	1.346
28	0.780	1.048	1.327
29	0.764	1.031	1.308
30	0.748	1.014	1.290
31	0.732	0.997	1.272
32	0.716	0.980	1.254
33	0.700	0.963	1.236
34	0.684	0.946	1.218
35	0.668	0.928	1.199
36	0.651	0.910	1.180
37	0.634	0.891	1.160
38	0.615	0.872	1.139
39	0.595	0.851	1.117
40	0.573	0.828	1.093

TABLE 3. Umbilical Artery Systolic: Diastolic Ratio (S:D ratio) [39].

	Percentile								
Gestation (wk)	2.5th	5th	10th	25th	50th	75th	90th	95th	97.5th
19	2.73	2.93	3.19	3.67	4.28	5.00	5.75	6.26	6.73
20	2.63	2.83	3.07	3.53	4.11	4.80	5.51	5.99	4.43
21	2.51	2.70	2.93	3.36	3.91	4.55	5.22	5.67	6.09
22	2.43	2.60	2.83	3.24	3.77	4.38	5.03	5.45	5.85
23	2.34	2.51	2.72	3.11	3.62	4.21	4.82	5.22	5.61
24	2.25	2.41	2.62	2.99	3.48	4.04	4.63	5.02	5.38
25	2.17	2.33	2.52	2.88	3.35	3.89	4.45	4.83	5.18
26	2.09	2.24	2.43	2.78	3.23	3.75	4.30	4.66	5.00
27	2.02	2.17	2.35	2.69	3.12	3.63	4.15	4.50	4.83
28	1.95	2.09	2.27	2.60	3.02	3.51	4.02	4.36	4.67
29	1.89	2.03	2.20	2.52	2.92	3.40	3.89	4.22	4.53
30	1.83	1.96	2.13	2.44	2.83	3.30	3.78	4.10	4.40
31	1.77	1.90	2.06	2.36	2.75	3.20	3.67	3.98	4.27
32	1.71	1.84	2.00	2.29	2.67	3.11	3.57	3.87	4.16
33	1.66	1.79	1.94	2.23	2.60	3.03	3.48	3.77	4.06
34	1.61	1.73	1.88	2.16	2.53	2.95	3.39	3.68	3.96
35	1.57	1.68	1.83	2.11	2.46	2.87	3.30	3.59	3.86
36	1.52	1.64	1.78	2.05	2.40	2.80	3.23	3.51	3.78
37	1.48	1.59	1.73	2.00	2.34	2.74	3.15	3.43	3.69
38	1.44	1.55	1.69	1.95	2.28	2.67	3.08	3.36	3.62

	Percentile								
Gestation (wk)	2.5th	5th	10th	25th	50th	75th	90th	95th	97.5th
39	1.40	1.51	1.64	1.90	2.23	2.61	3.02	3.29	3.54
40	1.36	1.47	1.60	1.85	2.18	2.56	1.96	3.22	3.48
41	1.33	1.43	1.56	1.81	2.13	2.50	2.90	3.16	3.41

2. Umbilical vein Doppler assessment

Umbilical venous flow in the physiological situation comprises of a monophasic non pulsatile flow pattern with a mean velocity of ~10-15 cm/s. The presence of pulsatility implies a pathological state unless in the following situations:

1. Early in pregnancy: up to ~13 weeks gestation
    - The presence of pulsatility may be higher in chromosomally abnormal foetuses even in early pregnancy [40]
2. When confounded by other movement variables such as
    - Foetal breathing movement
    - Foetal hiccups [41]

3. Foetal middle cerebral artery (MCA) Doppler assessment

For an accurate measurement, the foetal head should be in the transverse plane. The MCA vessels are often found with colour or power Doppler ultrasound overlying the anterior wing of the sphenoid bone near the base of the skull. An angle of insonation of < 15^{o} should be used; typically, an angle that approximates zero degrees can be achieved by moving the transducer on the maternal abdomen.

Parameters used include:

1. Foetal MCA pulsatility index (PI).
2. Foetal MCA peak systolic velocity (PSV): the highest velocity should be recorded.
3. Foetal MCA systolic/diastolic (S/D) ratio: a normal foetal MCA S/D ratio should always be **higher** than the umbilical arterial S/D ratio.
4. Cerebroplacental ratio (CPR): ratio of pulsatility index of MCA and umbilical artery.

TABLE 4. Foetal MCA pulsatility index (PI) [42].

Gestation (wks)	5th Percentile	50th Percentile
24	1.38	1.86

Gestation (wks)	5th Percentile	50th Percentile
25	1.44	1.94
26	1.50	2.01
27	1.55	2.06
28	1.58	2.11
29	1.61	2.15
30	1.62	2.16
31	1.62	2.16
32	1.61	2.14
33	1.58	2.10
34	1.53	2.04
35	1.47	1.96
36	1.39	1.86
37	1.30	1.75
38	1.2	1.63
39	1.1	1.49
40	1.02	1.29
<5th percentile is abnormal		

TABLE 5. Foetal MCA peak systolic velocity (PSV) [43].

Gestation (wks)	5th Percentile	95th Percentile
20	18.1	26.0
21	19.5	29.0
22	20.8	32.0
23	22.2	35.0
24	23.6	38.1
25	24.9	41.1
26	26.3	44.1
27	27.7	47.1
28	29.0	50.1
29	30.4	53.1
30	31.8	56.1
31	33.1	59.1
32	34.5	62.1
33	35.9	65.1
34	37.2	68.2
35	38.6	71.2
36	40.0	74.2
37	41.3	77.2
38	42.7	80.2
39	44.1	83.2

Gestation (wks)	5th Percentile	95th Percentile
40	45.4	86.2

TABLE 6. Foetal MCA systolic/diastolic (S/D) ratio [44].

Gestation (wks)	S/D ratio
20	5.34 ± 1.55
21	5.81 ± 1.9
22	5.91 ± 1.79
23	6.21 ± 1.61
24	6.31 ± 1.97
25	6.36 ± 1.47
26	6.38 ± 1.59
27	6.69 ± 1.37
28	6.87 ± 1.46
29	6.95 ± 1.64
30	7.13 ± 1.35
31	7.09 ± 2.21
32	6.60 ± 1.66
33	5.84 ± 2.0
34	5.33 ± 1.84
35	5.10 ± 1.18
36	4.31 ± 1.15
37	4.12 ± 1.31
38	3.22 ± 0.67
39	3.22 ± 0.46
40	3.16 ± 0.44

TABLE 7. Cerebroplacental ratio (CPR): ratio of pulsatility index of MCA and umbilical artery (CPR = MCA PI/UA PI) [42].

Gestation (wks)	5th Percentile	50th Percentile
24	1.16	1.74
25	1.24	1.85
26	1.32	1.95
27	1.40	2.05
28	1.47	2.14
29	1.53	2.21
30	1.58	2.28
31	1.62	2.32
32	1.64	2.35
33	1.65	2.36
34	1.63	2.35
35	1.60	2.32

Gestation (wks)	5th Percentile	50th Percentile
36	1.55	2.27
37	1.48	2.19
38	1.40	2.09
39	1.29	1.97
40	1.24	1.8
<5th percentile is abnormal		

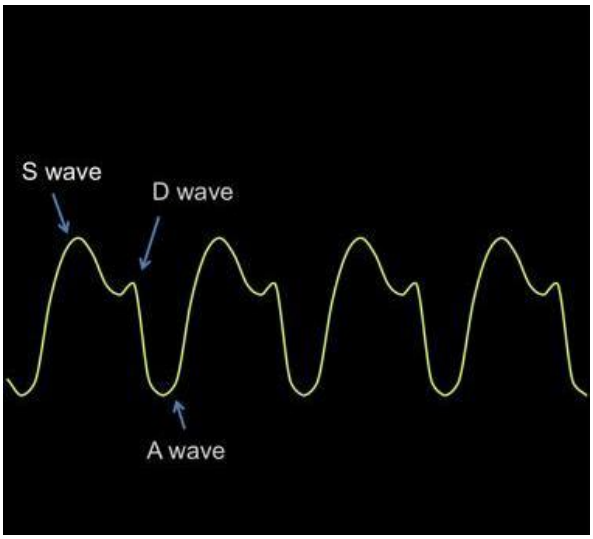
4. Ductus venosus Doppler assessment

Technique

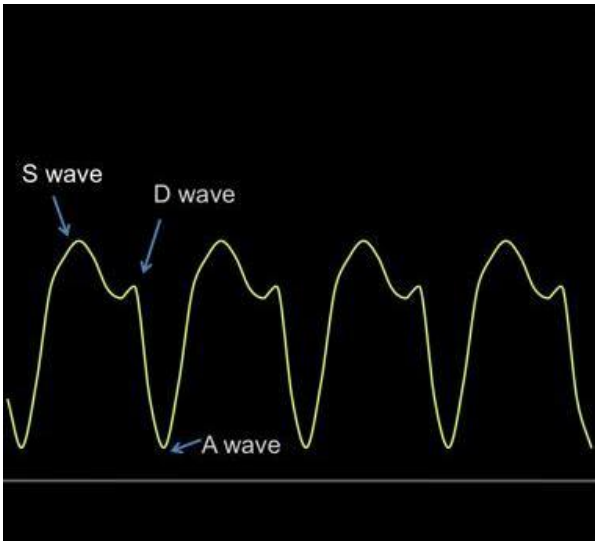
- The probe is ideally focused so sampling is done where the umbilical vein joins the ductus venosus.
- The probe is ideally angled to allow a mid sagittal plane or a transverse oblique plane through the foetal abdomen.
- Do not contaminate the ductus venosus from flow from the foetal inferior vena cava.
- The foetus should be as still as possible.

On Doppler ultrasound the flow in the ductus venosus has a characteristic **triphasic** waveform where in a **normal physiological situation** flow should always be in the forward direction [7] (i.e. towards the foetal heart).

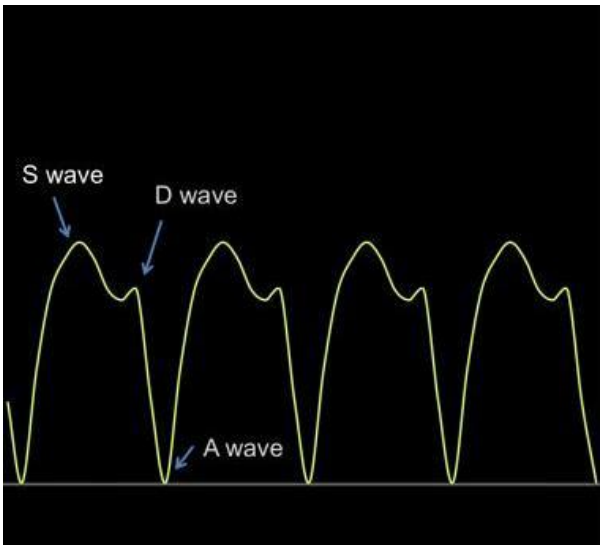
Normal flow in ductus venosus



Reduction flow in ductus venosus



Absent flow in ductus venosus



Reversal of flow in ductus venosus

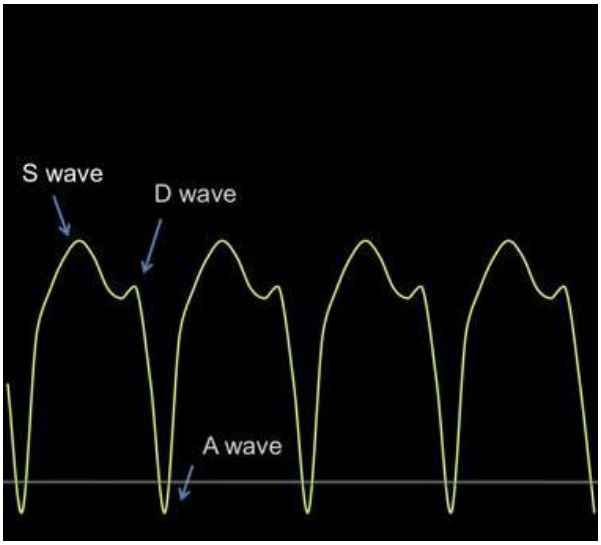


TABLE 8. Ductus Venosus Pulsatility Index (S-a)/Tamx [45].

Gestation (wks)	5th Percentile	50th Percentile	95th Percentile
20	0.410	0.643	0.875

Gestation (wks)	5th Percentile	50th Percentile	95th Percentile
21	0.409	0.642	0.874
22	0.408	0.641	0.873
23	0.407	0.640	0.872
24	0.406	0.639	0.871
25	0.405	0.638	0.870
26	0.404	0.637	0.869
27	0.403	0.636	0.868
28	0.402	0.635	0.867
29	0.401	0.634	0.866
30	0.400	0.633	0.865
31	0.399	0.632	0.864
32	0.398	0.631	0.863
33	0.397	0.630	0.862
34	0.396	0.629	0.861
35	0.395	0.628	0.860
36	0.394	0.627	0.859
37	0.393	0.626	0.858
38	0.392	0.625	0.857
39	0.391	0.624	0.856
40	0.390	0.623	0.855

TABLE 9. Ductus Venosus S/A ratio [45].

Gestation (wks)	5th Percentile	50th Percentile	95th Percentile
20	1.331	2.161	2.991
21	1.329	2.159	2.989
22	1.327	2.157	2.987
23	1.324	2.154	2.984
24	1.322	2.152	2.982
25	1.320	2.150	2.980
26	1.318	2.148	2.978
27	1.315	2.145	2.975
28	1.313	2.143	2.973
29	1.311	2.141	2.971
30	1.308	2.138	2.968
31	1.306	2.136	2.966
32	1.304	2.134	2.964
33	1.301	2.131	2.961
34	1.299	2.129	2.959
35	1.297	2.127	2.957
36	1.295	2.125	2.955
37	1.292	2.122	2.952
38	1.290	2.120	2.950
39	1.288	2.118	2.948

Gestation (wks)	5th Percentile	50th Percentile	95th Percentile
40	1.285	2.115	2.945

TABLE 10. Ductus Venosus Peak Velocity Index (S-a)/D [45].

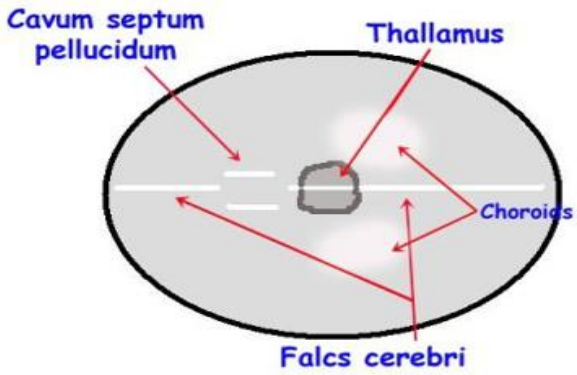
Gestation (wks)	5th Percentile	50th Percentile	95th Percentile
20	0.381	0.580	0.779
21	0.380	0.579	0.779
22	0.380	0.579	0.778
23	0.379	0.578	0.777
24	0.678	0.578	0.777
25	0.378	0.577	0.776
26	0.377	0.576	0.776
27	0.377	0.576	0.775
28	0.376	0.575	0.774
29	0.375	0.575	0.774
30	0.375	0.574	0.773
31	0.374	0.573	0.773
32	0.374	0.573	0.772
33	0.373	0.572	0.771
34	0.372	0.572	0.771
35	0.372	0.571	0.770
36	0.371	0.570	0.770
37	0.371	0.570	0.769
38	0.370	0.569	0.768
39	0.369	0.569	0.768
40	0.369	0.568	0.767

ANNEX 10. ULTRASOUND BIOMETRY OF THE FOETUS

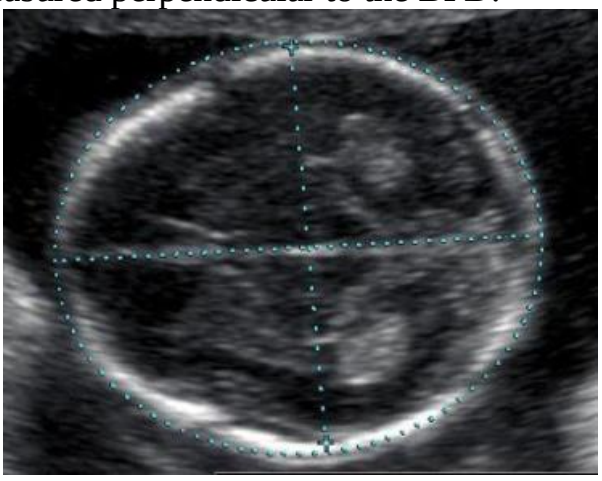
The ultrasound scanning is performed three times during pregnancy: in the first trimester (ideally at 12 to 14 weeks of pregnancy), in the second trimester at 20 to 22 weeks of pregnancy and in the third trimester at 32 - 34 weeks.

BIPARIETAL DIAMETER AND HEAD CIRCUMFERENCE

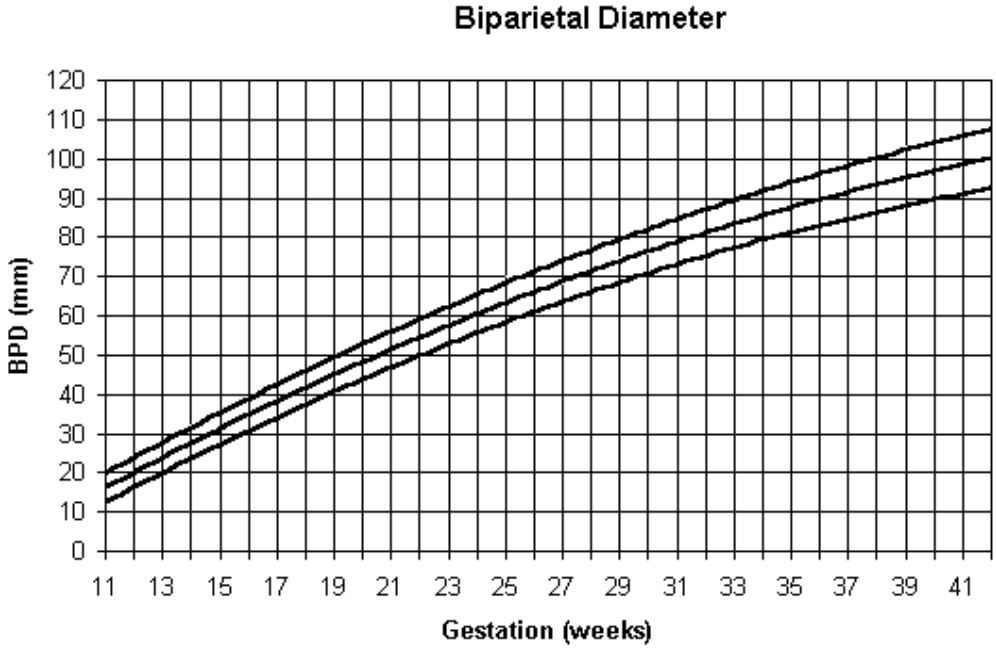
The BPD (Biparietal Diameter) and OFD (Occipital Frontal Diameter) are measured on a transverse axial section of the foetal head which includes the falx cerebri anteriorly and posteriorly, the cavum septum pellucidum anteriorly in the midline and the thalami. The BPD is measured from the outer edge of the nearer parietal bone to the inner edge of the more distant parietal bone. The OFD is measured perpendicular to the BPD.

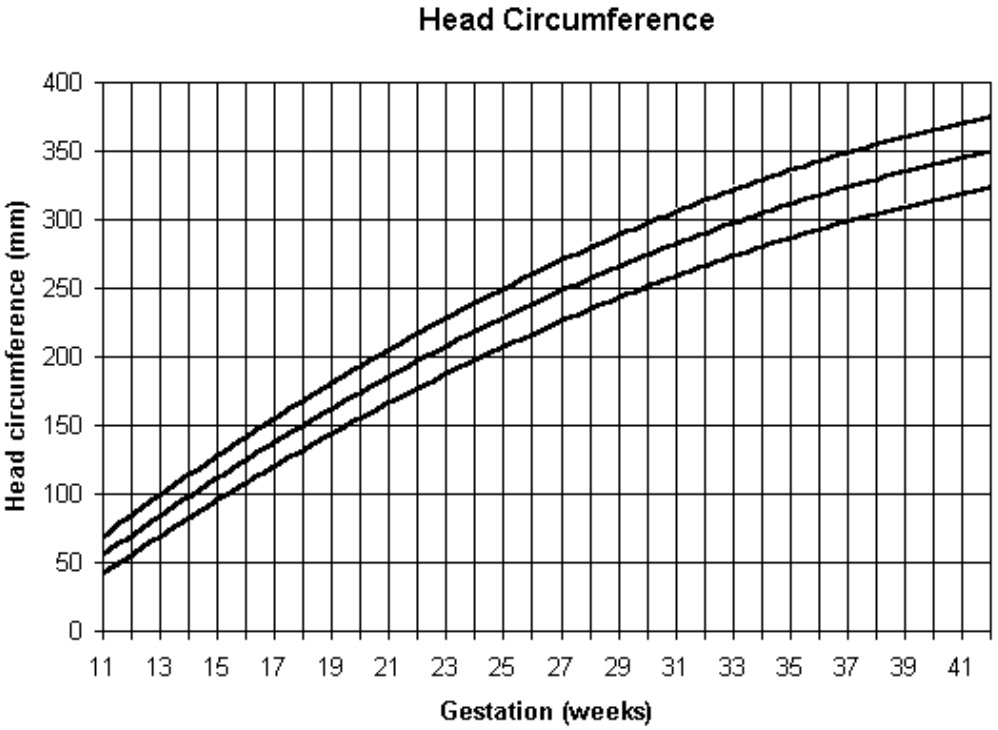


The correct plane for the measurement of the head circumference (HC) and bi-parietal diameter (BPD)must include the cavum septum pellucidum, thallamus and choroid



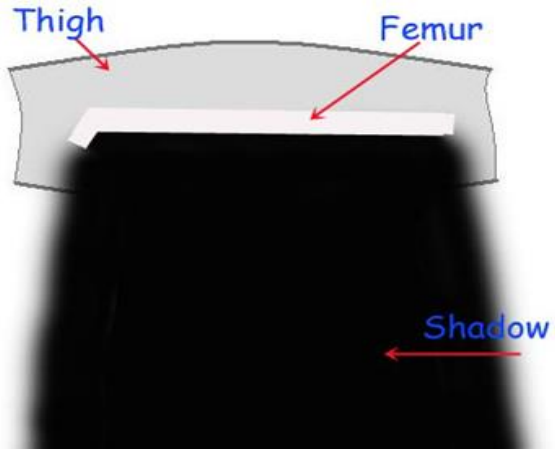
BPD: Measure outer table of the skull to the inner table.
HC: Measure around the outer table of the skull.



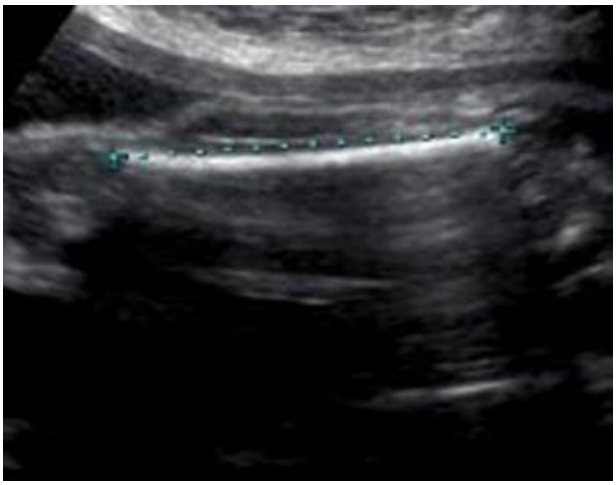


FEMUR LENGTH

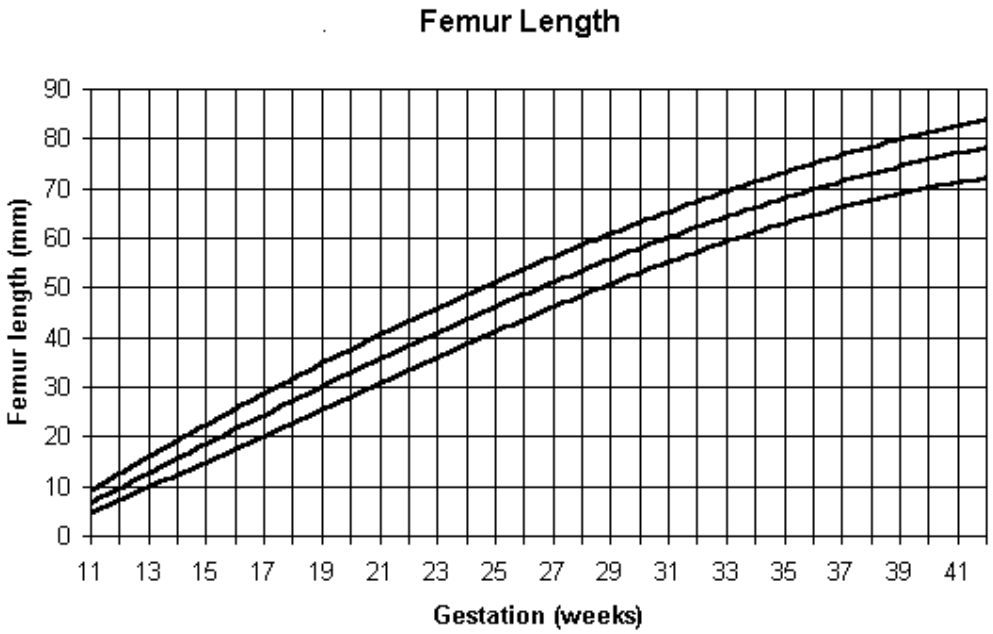
The long bones are measured with the bone across the beam axis. The strong acoustic shadow behind the femoral shaft and the visualisation of both cartilaginous ends indicates that the image plane is on the longest axis and is the optimal measurement plane. The calipers are placed along the diaphyseal shaft excluding the epiphysis.



Femur Length

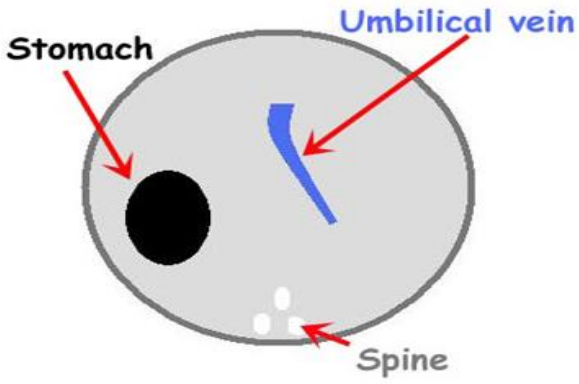


The Femur length should only be measured when the femur is horizontal (beam is perpendicular) and shadows evenly- at least from both ends.

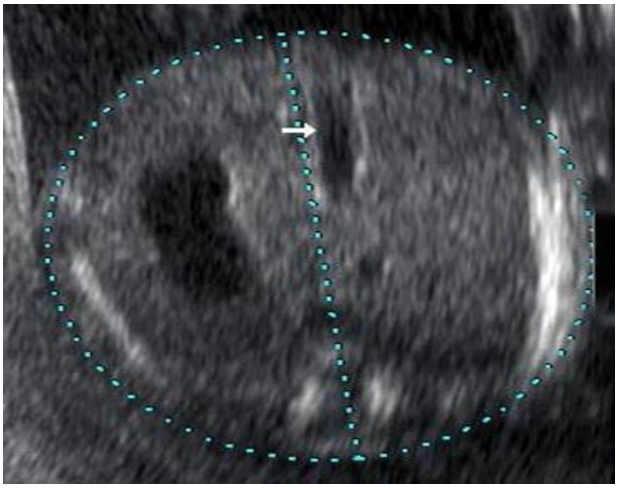


ABDOMINAL CIRCUMFERENCE

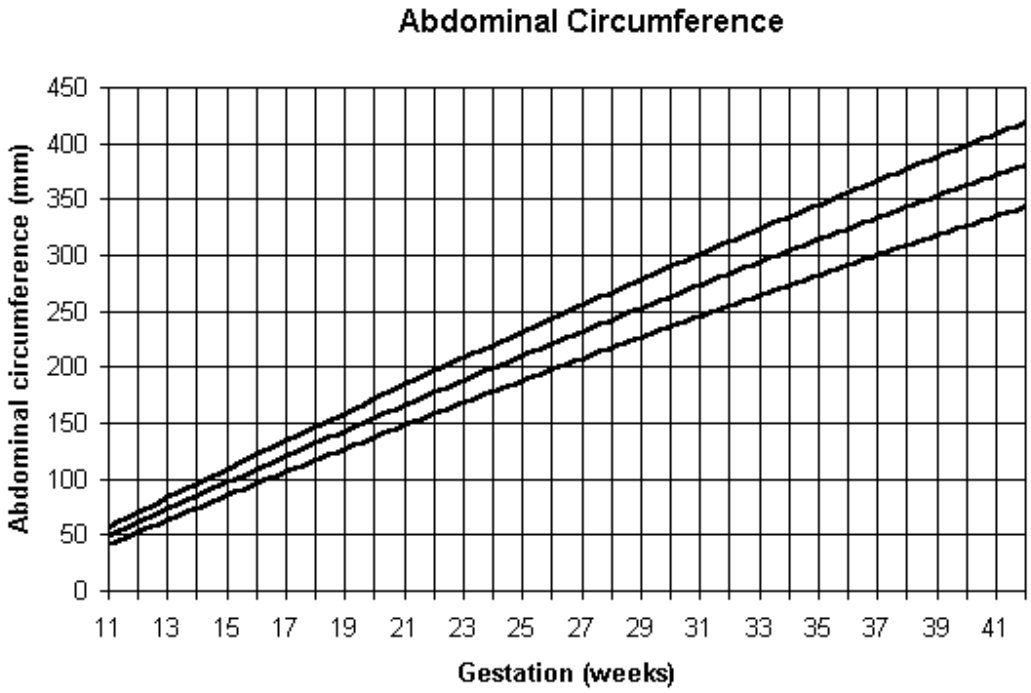
These measurements are more appropriately used in the assessment of foetal growth, particularly in the second half of the pregnancy, than in the assessment of gestational age. It is, however, an appropriate measurement in the second trimester to demonstrate normal foetal proportions. The abdominal circumference is measured at the level of the liver and stomach, including the left portal vein at the umbilical region.



Abdominal Circumference



The abdominal circumference is taken with a transverse image to include the stomach, portal vein and the spine in a true transverse plane.

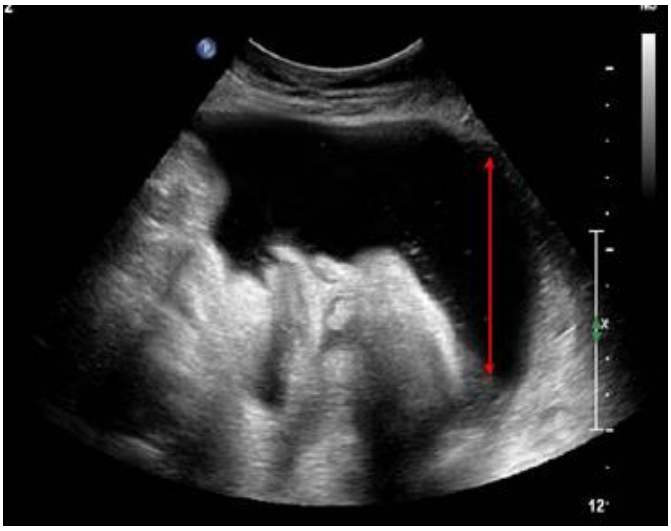


AMNIOTIC FLUID

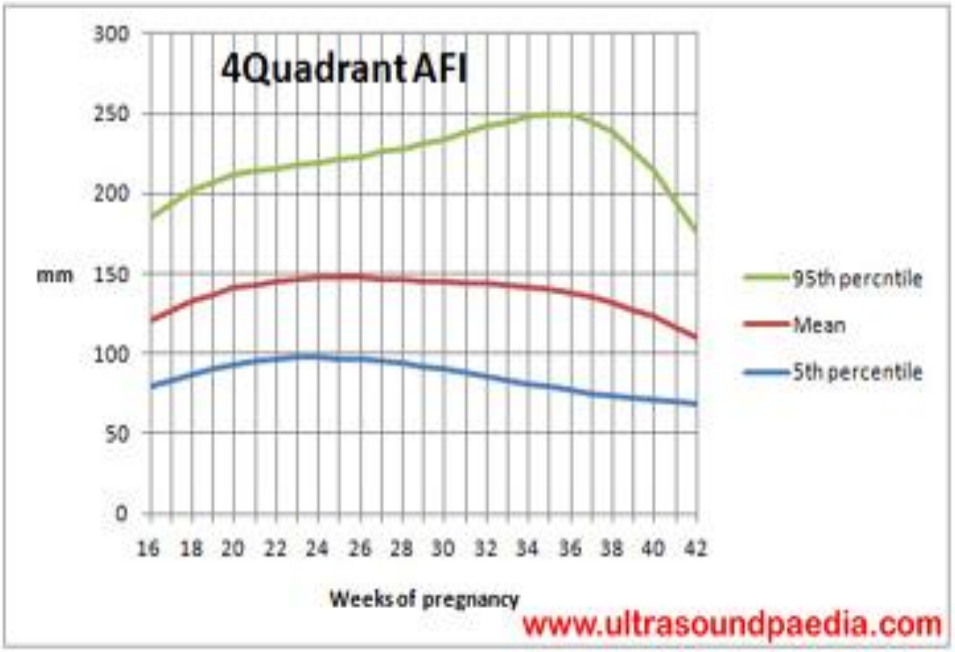
A decreased volume of amniotic fluid is closely associated with IUGR. Significant morbidity has been found to exist in pregnancies with an amniotic fluid index value of less than 5 cm. The combination of oligohydramnios and IUGR portends a less favourable outcome, and early delivery should be considered. Generally, if the pregnancy is at 36 weeks or more, the high risk of intrauterine loss may mandate delivery.

Amniotic Fluid Index (AFI) technique [46]:

- Position patient supine.
- A linear, curvilinear, or sector transducer can be used.
- Divide the uterus into four quadrants using the maternal sagittal midline vertically, and arbitrary transverse line approximately halfway between the symphysis pubis and the upper edge of the uterine fundus.
- The transducer must be kept parallel to the maternal sagittal plane and perpendicular to the maternal coronal plane throughout.
- The deepest unobstructed and clear pocket of amniotic fluid is visualised, and the image frozen. The ultrasound calipers are manipulated to measure the pocket in a strictly vertical direction.
- The process is repeated in each of four quadrants and the pocket measurements summed = AFI.
- If the AFI is <8 cm, perform the four quadrant evaluation three times and average the values.



Amniotic Fluid index (AFI)
Amniotic fluid index values are obtained by measuring the vertical depth of the largest clear amniotic fluid pocket in each of four equal uterine quadrants. The values from each quadrant are measured in millimetres and added together.



REF: Moore TR and Cayle JE. The amniotic fluid index in normal human pregnancy. Am J Obstet Gynecol 1990;162:1168-1173

Greater than the 95th centile = polyhydramnios.
Less than the 5th centile = oligohydramnios.

ANNEX 11. ULTRASOUND DIAGNOSIS OF FOETAL ANOMALIES [47]

Anencephaly

In anencephaly, the cerebral hemispheres and overlying skull and scalp are absent. In exencephalus, the cranial bones are also absent, but a large amount of disorganized brain tissue is present.

Microcephaly

Small Head Perimeter Measurements (mm). 2-and 3-SD mean for foetal age in weeks of value in diagnosis of microcephaly [48].

Age (wk)	-2 SD	-3 SD	-4 SD	-5SD
20	145	131	116	101
21	157	143	128	113
22	169	154	140	125
23	180	166	151	136
24	191	177	162	147
25	202	188	173	158
26	213	198	183	169
27	223	208	194	179
28	233	218	203	189
29	242	227	213	198
30	251	236	222	207
31	260	245	230	216
32	268	253	239	224
33	276	261	246	232
34	283	268	253	239
35	289	275	260	245
36	295	281	266	251
37	301	286	272	257
38	306	291	276	262
39	310	295	281	266
40	314	299	284	270

Meningocele and Encephalocele

In cranial meningocele, the meninges protrude through a bony defect in the skull. Occipital presentation of meningocele is most common, occurring in 75% of cases.

Hydrocephaly

Enlargement of the cerebral ventricular system.

Choroid Plexus Cyst

A choroid plexus cyst is visualized in approximately 1% of foetuses scanned at 16 to 22 weeks' gestation.

Posterior Fossa The posterior fossa shows the cerebellum and the cisterna magna. Normally, the cisterna magna measures 1 to 10 mm.

Spina Bifida In spina bifida, splaying of the spine is noted in the longitudinal plane. However, small spinae bifida located in the lumbosacral area are best visualized by careful cross-sectional imaging. The size of spinae bifida varies from a tiny bone defect to one involving many segments of the spine (myeloschisis).

ANNEX 12. CLINICAL TRIAL CONCENT FORM

Centre Number:
Study Number:
Patient Identification Number for this trial:

CONSENT FORM FOR CLINICAL TRIAL

Title of Project: Evaluation of pregnancy course and outcome by means of the diagnostic «ELI-P-Complex» test for preconception care in women with history of adverse pregnancy outcome.

Name of Researcher:

	Please tick to confirm
• **I confirm that I have read and understand the information sheet dated ..... (version .....) for the above study.**	•
• **I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.**	•
• **I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.**	•
• **I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by responsible individuals from [……], from regulatory authorities, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.**	•
I agree to my Obs. & Gyn. being informed of my participation in the study.	•
I agree to take part in the above research study.	•

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent (if different from researcher)	Date	Signature
_____	_____	_____
Researcher	Date	Signature

When complete, 1 copy for patient: 1 copy for researcher site file: 1 (original) to be kept in medical notes.