Laboratory Options for Risk Assessment of Pregnancy Pathologies

A. KESTLEROVÁ¹, L. KROFTA², A. ŽUFIĆ³, K. HAMPOLOVÁ BĚHÁVKOVÁ², J. RAČKO³, J. BENEŠ¹,⁴, J. FEYEREISL²

¹Institute of Biophysics and Informatics, First Faculty of Medicine, Charles University, Prague, Czech Republic, ²Institute for the Care of Mother and Child, Prague, Czech Republic, ³Faculty of Science, Charles University, Prague, Czech Republic, ⁴Institute of Biophysics, Second Faculty of Medicine, Charles University, Prague, Czech Republic

Received October 20, 2019
Accepted November 7, 2019

Summary
The most effective method of screening for chromosomal abnormalities and evaluating the risk of pregnancy pathologies in the first trimester is combined screening. The algorithm of screening is based on the combination of maternal age, measuring of the nuchal translucency and the fetal heart rate and analysis of the placental products of free ß-hCG and PAPP-A. For the screening of preeclampsia, placental growth factor (PlGF) is added. To distinguish between preeclampsia and other pathologies caused by placental dysfunction it is recommended to also extend the screening with selected immunological markers. We concluded that elevated biochemical and immunological markers can help to predict the threat of preeclampsia in the third trimester. Some markers can probably predict the development of particularly severe pathological conditions.

Key words
Pregnancy • Pathologies • Laboratory markers • Preeclampsia

Corresponding author
A. Kestlerova, Institute of Biophysics and Informatics, Salmovská 1, First Faculty of Medicine, Charles University, 120 00 Prague, Czech Republic. E-mail: 1andrea1@centrum.cz

Clarification of normal outcome of the pregnancy and pregnancy pathologies


Normal outcome
Normal outcome of the pregnancy involves delivery at the term (≥ 37 weeks) of a live baby with birth weight > 5 %-lower percentile and with none of the symptoms associated with pregnancy pathologies.

Chromosomal aneuploidies
Aneuploidy causes several human conditions and diseases including Down syndrome, mosaic variegated aneuploidy (MVA), and is associated with cancer. These disorders illustrate how aneuploidy can arise under different circumstances in an organism. Constitutional aneuploidy, prevalent in conditions such as Down syndrome, typically arises through fertilization of an aneuploid gamete generated through defective chromosome segregation during meiosis. Somatic aneuploidy is acquired through defective chromosome segregation in mitosis and is characteristic of individuals with MVA and is found in over 90 % of solid tumors.

Pre-eclampsia (PE)
PE have been considered to be a new onset hypertension after the 20th gestational week combined with proteinuria ≥ 300 mg per day. Heterogeneity of the disorder is more and more appreciated and therefore new diagnostic criteria have recently been introduced.
Proteinuria has been questioned as a sine qua non. According to the two new diagnostic criteria by The American College of Obstetricians and Gynecologists (ACOG) in 2013 and International Society for the Study of Hypertension in Pregnancy (ISSHP) in 2014, new onset hypertension in the absence of proteinuria but combined with hematological complications, renal insufficiency, impaired liver function, neurological symptoms, or uteroplacental dysfunction also fulfill diagnostic criteria for PE. This is to provide a broader definition of PE for clinical practice leaving proteinuria to ensure specificity of the diagnosis in scientific purposes. Affecting approximately 3-5% of pregnancies and causing as much as 10% of pregnancy related complications, better diagnostic criteria are needed to improve the recognition of PE and its diverse subtypes mild and severe.

**Eclampsia**
Eclampsia is a dangerous form of PE where blurred vision, apathy, nausea and dizziness turn into convulsions, indicating the severe disorder has affected the brain and the woman is experiencing a threat to her life.

**HELLP Syndrome**
HELLP Syndrome is a rapidly developing life threatening condition comprising hemolysis, elevated liver enzymes and low platelets (<100 000 cells per µl). HELLP Syndrome occurs in tandem with PE, but because HELLP Syndrome’s symptoms may appear before those characteristic of PE, they may be easily misdiagnosed.

Partial HELLP is the term applied when a woman meets one or two of the criteria for HELLP but not all three.

**Pregnancy induced hypertension (PIH) or gestational hypertension (GH)**
PIH is defined with blood pressure of >140/90 mm Hg measured normally 4-6 hours apart in previously normotensive women.

**Intrauterine growth restriction (IUGR)**
IUGR is the failure of the fetus to realize its growth potential as a result of genetic environmental factors. Approximately 30% of IUGR cases are associated with chromosomal aberrations. A number of PE cases are associated with the delivery of IUGR babies.

**Small for gestational age (SGA)**
Small for gestational age babies are those with birth weight below the 10th percentile for their gestational age.

**Preterm delivery (PTD)**
Preterm delivery applies to pregnancy outcome when delivery is before 37 complete weeks of gestation.

**Selection markers**

**Alpha-fetoprotein (AFP)**
In the mid-1970s, raised levels of maternal serum alpha fetoprotein (AFP) was shown to be a useful second-trimester screening test for open spina bifida and anencephaly (Brock and Sutcliffe 1972). In the early 1980s Merkatz and colleagues investigated the possibility that low maternal serum alpha-fetoprotein (AFP), obtained from maternal blood in the second trimester of pregnancy could be associated with chromosomal abnormalities in the fetus. Their retrospective case-control study showed a statistically significant relationship between fetal trisomy, such as Down’s syndrome, and lowered maternal serum AFP (Merkatz et al. 1984). These results were explored by Cuckle and colleagues in a larger retrospective trial using data collected as part of a neural tube defect (NTD) screening project (Cuckle et al. 1984). When used unconjugated oestriol (uE3) in combination with AFP and maternal age, it appeared to identify more pregnancies affected by Down’s syndrome than AFP and age alone (Canick et al. 1988). Further work suggested that all three serum markers (AFP, hCG and uE3) showed even higher detection rates when combined with maternal age (Wald et al. 1988) and appeared to be a cost-effective screening strategy (Wald et al. 1992).

AFP had been tested for a long time only in the second trimester of pregnancy, then researchers began performing AFP tests in the first trimester of pregnancy. The study containing cases of exomphalos, anencephaly and two cases of open spina bifida also confirmed that first-trimester maternal serum AFP was increased in cases with anencephaly and exomphalos but not in the two cases with open spina bifida (Sebire et al. 1997). A recent first trimester study of cases with anencephaly confirmed the elevation of maternal serum AFP, however, this same study also found elevation of maternal serum AFP in cases with open spina bifida (Bredaki et al. 2012). A further study of 44 cases of open spina bifida found a small but significant increase in maternal serum AFP in
addition to a small but significant decrease in free β-hCG (Bernard et al. 2013). On the basis of the study of Bernard and the results of study of Spencer (Spencer et al. 2014) in conjunction with studies published in 1993 (Aitken et al. 1993), one cannot confirm the value of first-trimester maternal serum AFP as a possible screening marker for open spina bifida in the first trimester. Spencer thinks, that one possible explanation for the differences between the few studies may be how cases identified by second trimester ultrasound are accurately classified as open spina bifida as opposed to closed lesions. Because most cases of closed spina bifida are not identified until term unless a detailed fetal examination is undertaken on the fetus, a clear diagnosis may not be possible. This in itself could lead to some heterogeneity amongst the cases. In the more recent publications (Bredaki et al. 2012, Bernard et al. 2013) there was no indication that diagnosis was confirmed by fetal exam, only that diagnosis occurred as a result of second trimester ultrasonography (Spencer et al. 2014).

**Fee beta HCG and PAPPa**

Human chorionic gonadotropin (hCG) free β-subunit measurement, together with pregnancy associated protein A (PAPP-A) is used as a screening test for Down syndrome during the first trimester of pregnancy. Combination of both parameters has higher distinctive value; however, it also brings some problems. Difficulties arise mainly from low stability of free β-hCG subunit. In collected blood, free β-subunit does not dissociate but it is subjected to nicking and other forms of degradation even in properly separated serum (Springer et al. 2008). Nicked free β-hCG lacks peptide linkages between either β-subunit residues 44 and 45 or β-subunit residues 47 and 48. The percentage of nicked free β-hCG increases after the second month of pregnancy (Cole and Kardana 1993, Cole and Kardana 1997).

Pregnancy associated plasma protein-A (PAPP-A) is a high-molecular-weight zinc-binding metalloproteinase belonging to metzincin superfamily of metalloproteinases and was originally identified in the plasma of pregnant women (Lin et al. 1974, Zakiyanov et al. 2013).

PAPP-A belongs to a group of biomarkers that predict later preeclampsia development, primarily early onset preeclampsia; however, it should be combined with a Doppler ultrasonography of the uterine artery (pulsatile index) and other biochemical and maternal factors to achieve a higher detection rate with an acceptable false positivity rate (Kalousová et al. 2014).

**Placental growth factor (PIGF)**

Placental growth factor (PIGF), which is a member of the vascular endothelial growth factor (VEGF), stimulates angiogenesis and growth of collateral vessels in ischemic tissues via VEGF receptor-1 (Flt1) (Autiero et al. 2003, Luttun et al. 2002). PIGF is upregulated in atheromatic lesions, and antiFlt1 suppresses atherosclerotic process and plaque vulnerability (Luttun et al. 2002). First trimester combined screening can be concentrated on risk detection of preeclampsia. The best results are reached with combination of mean arterial pressure, uterine artery pulsatility index and maternal serum level of PlGF. In pregnancies that develop PE, compared to those without PE, MoM values of serum PAPP-A and PIGF are decreased, and the deviation from normal was greater for early than late PE (Tan et al. 2018). Combined screening by maternal factors, mean arterial pressure, uterine artery pulsatility index and PIGF predicted 90 % of early PE, 75 % of preterm PE and 41 % of term PE (Tan 2018).

**Heat shock proteins (Hsps)**

Heat shock proteins (Hsps) are ubiquitously distributed phylogenetically conserved molecules present in the cells of all living organisms. Under physiological conditions, the stress proteins are expressed in low concentrations as the constitutive proteins, regulating cellular homeostasis and maintaining the integrity and function of other cellular proteins (Nover 1984, Morimotto et al. 1990).

Human Hsps are categorized under distinct families based on their functions in the cells, their homologies in the primary structures and their approximate molecular weight, measured in kDa. These families are as follows: a family of small Hsps, Hsp40, Hsp60, Hsp70, Hsp90 and Hsp110 (Kampinga et al. 2009).

Maternal circulation can reflect both maternal and placental pathologic conditions through the mediation of diverse Hsp gene expression profiles. Since Hsp60 and HspBP1 mRNA are not detectable in maternal plasma samples and Hsp27 and Hsp90 mRNA show comparable levels regardless of the course of gestation, Hsp70 represents the sole plasmatic marker. (Hromadníková et al. 2013). Elevated circulating Hsp70 concentrations reflecting systemic inflammation, oxidative stress and hepatocellular injury were found to

Fetal DNA

Circulating nucleic acids such as DNA, mRNA and microRNAs present in maternal plasma or serum samples are increasingly being used as biomarkers for monitoring of pregnancy-related complications. Extracellular nucleic acids present in maternal circulation are predominantly haematopoietic in origin (Zheng et al. 2012). Extracellular vesicles (EVS)

In biological systems, extracellular vesicles (EVs) represent a heterogeneous population of microvesicles (MVs) with a diameter below 1000 nm and exosomes with a diameter mostly below 100 nm (in some reports up to 250 nm). (Sittar et al. 2015, Lo Cicero et al. 2015, Varga et al. 2014)

Syncytial surface of the human placenta is known to shed EVs, commonly called syncytiotrophoblast microparticles (STBMs) directly to maternal blood, as a result of blebbing and programmed cell death (Roos et al. 2010, Huppertz et al. 1998, Knight et al. 1998).

STBEVs are recognised as a form of intercellular communication important for interactions between the fetus and the mother, allowing a continual adaptation of the maternal immune system throughout normal pregnancy (Messerli et al. 2010, Mincheva – Nilsson et al. 2010).

Significantly elevated amounts of STBEVs have been found in the circulation of women with preeclampsia (Redman et al. 2008, Redman et al. 2005), indicating a possible causative role in a number of pathophysiological states such as systemic inflammation, endothelial damage and organ failure (Redman et al. 2012). It is still under discussion if elevated STBEV concentrations in preeclampsia are cause or consequence of this disease. STBEVs may be involved in the development of the associated hypertension (Tesse et al. 2007).

A few studies have analyzed quantities of EVs from different cell types in severe preeclampsia and have found increased amounts of EVs derived from T cells, monocytes, and granulocytes (VanWijk et al. 2002, Lok et al. 2009). Women with preeclampsia have an increased number of monocyte-, lymphocyte- and platelet-derived EVs compared with healthy pregnant women (Meziani et al. 2006). Furthermore, elevated levels of endothelial cell-derived EVs have been described in preeclampsia (Gonzalez-Quintero et al. 2004). STBEVs can alter the fibrinolytic and angiogenic balance at the maternal-fetal interface, which may be involved in the pathophysiology of preeclampsia (Guller et al. 2011). The pathomechanisms leading to preeclampsia are still not completely understood, but it may be expected that EVs are involved (Foster et al. 2016).

Anti-phospholipid antibodies (APA)

Long-lasting primary infertility or repeated pregnancy losses after spontaneous and/or artificial fertilization should be examined along with antiphospholipid abnormalities. Present levels of APA potentially improve the chance of female conception through appropriately directed treatment. Today, we have a panel of eight different phospholipids for the diagnosis of antiphospholipid syndrome. Concerning pregnancy loss, there is general agreement that IgG antibodies against CL, against ph-serine, ph-ethanolamine, beta-2-GPI, ph-inositol at moderate or high titer and/or LA, identify the patients with a greater risk of future obstetric complications than low-titer IgG or IgM of the same phospholipid factors. (Ulcova-Gallova et al. 2014)

Prevalence of anti-phospholipid antibodies in preeclampsia patients is twice that in healthy pregnant women. Multipositive aPL test, IgM-anti-cardiolipin and IgM-anti-ß(2)glycoprotein-I isotypes showed an association with severe and early-onset preeclampsia (Ferrer-Oliveras et al. 2012). Other authors indicate that the risk of preeclampsia is markedly increased in women with anti-phospholipid syndrome (relative risk 9.72) (Duckitt and Harrington 2005), but there are no data concerning other pregnancy pathologies.

Anti-cardiolipin autoantibodies form one part of a complex phospholipid antibodies and may therefore be one of the possible indicators of pregnancy complications such as gestational hypertension or preeclampsia. The familiar relationship between elevated levels of antiphospholipid antibodies and type I diabetes (Wangel et al. 1992) might play an important role e.g. the development of gestational diabetes mellitus.

In our previous study we have shown that the
immunological factors, i.e., increased predisposition to the autoimmune reaction, and elevated sensitivity of cell-mediated immunity to the antigens of trophoblast origin, could represent a signal leading to the onset of preeclampsia (Kestlerova et al. 2012). There was a dilemma whether the elevation of anti-cardiolipin autoantibodies (ACLA) is a cause of PE or a consequence of the general inflammation accompanying PE. We proposed that the presence of anti-phospholipid autoantibodies in pregnant women was rather the cause, representing a significant risk factor for a development of PE. In order to verify this opinion, we decided to analyze the presence of ACLA as early as in the first trimester in women suffering from preeclampsia in the third trimester of gravidity. We also tried to prove whether the presence of ACLA is an exclusive marker for future preeclampsia or if it could signal other common pregnancy pathologies.

Natural killers (NK), Interleukins (IL)

Natural killer (NK) T or NKT cells are recently discovered cells. They are a small subset of TcR+ T lymphocytes which also express natural killer cell surface receptors (see under cells). They have a very restricted TcR repertoire. They can secrete several cytokines, especially IL-4 and IFN-γ, shortly after stimulation and kill target cells, mostly via cell/target interaction of Fas molecule with Fas L (Fas ligand). The cytokines they secrete endow them with potential immunoregulatory functions, which are exerted through their ability to induce apoptosis and to modulate the development of Th1 or Th2 cells. Finally, there exist T cells capable of down-regulating or ‘suppressing’ T cell effector functions. T regulatory cells (Tregs) have recently been described (Shao et al. 2005, Chauuat et al. 2007) and might be involved in the success of tolerance to the fetus (see below). In the same vein, transforming growth factor β (TGFβ) secreting Th cells (named Th0) have been described, and identified at the materno-fetal interface. Since TGFβ is both a growth factor for the embryonic derived fibroblast component of the placenta and a very potent immune suppressor, these cells have equally been implicated in the non-rejection of the fetus. (Chauuat et al. 2007)

Placental dysfunction increases syncytiotrophoblast microvesicle production leading to exaggerated systemic inflammation (Redman and Sargent 2005). In fact, treatment of peripheral blood mononuclear cells by first trimester microvesicles has been shown to increase IL1β secretion (Holder et al. 2012). Thus, increased systemic IL1β in the first trimester may indicate an immunological preeclampsia subtype perhaps mediated by abnormal placentation and increased placental microvesicles. Maternal character-ristics may impact systemic immune markers (Curry et al. 2008). Thus, second trimester controls may have been more likely to have elevated immune markers via unmeasured maternal factors biasing results towards the null. Lastly, it is also possible that a decrease in IL1β in the second trimester is capturing a different preeclampsia subtype (Leavey et al. 2016). Exploratory analyses of Brandies team did show that in the second trimester IL1β was significantly decreased in preeclampsia without SGA but displayed trends towards increased levels in preeclampsia with SGA (Brandie et al. 2016).

Initial analyses of human epithelial and Jurkat T cell cultures revealed a high concentration of IL-36Ra in supernatants and, to a lesser extent, in the cytoplasm, indicating the secretion of this cytokine (Towne et al. 2011). While cleavage of IL-1β and IL-18 by caspase-1 promotes their activation and secretion (Barton et al. 2000, Debets 2001), the 17–20 kDa protein encoded for IL-36Ra lacks N-glycosylation at Asn91, a caspase cutting site and conventional leading peptide sequence (Muler et al. 1999).

IL-18 has been described as a paracrine regulator of endometrial function. Its elevated levels have been associated with implantation failure (Ledee-Bataille et al. 2003, Ledee-Bataille et al. 2004). Human decidual and glandular cells express IL-18 whereas trophoblast cells express IL-18R, indicating its role in maternofetal communication. Moreover, IL-18 stimulates the cytotoxic capacity of uterine and peripheral blood NK cells (Ledee-Bataille et al. 2004, Tokmadzic et al. 2002). During normal human pregnancy, IL-18 expression is relatively high in the first and second trimester. At the labor stage, its levels are even more elevated. Furthermore, during several pregnancy disorders IL-18 concentrations are exacerbated, suggesting the participation of this cytokine in pathogenic processes (Ida et al. 2000).

IL-37 is the antagonist member of the IL-18 subfamily. An active form of 22 kDa is generated by cleavage through caspase-1 and binds to IL-18Ra, leading to inhibition of this receptor (Kumar et al. 2002). The participation of IL-37 in pregnancy related processes remains unknown.

Expression of IL-36 cytokines has also been described in human uteri (Busfield et al. 2000, Murrita-Coxca et al. 2019). All IL-36 agonists are also expressed
in the human placenta (Smith et al. 2000, Murrieta-Coxca et al. 2019).

A recent study reported the expression of IL-36 (α, β, γ) and IL-36Ra in the placenta of women with normal pregnancy and preeclampsia. Although the concentrations of IL-36 (α, β, γ) in serum do not change, IL-36Ra is expressed at lower levels in patients with preeclampsia who underwent emergency cesarean at 27-39 weeks of gestation compared with patients with normal pregnancy under elective cesarean at term (>38 weeks). Histologically, IL-36Ra is located around fetal blood vessels of the placental villi. Serum IL-36Ra is significantly higher in later stages of normal pregnancy compared to non-pregnant women. IL-38 seems to be also decreased in pre-eclamptic placentas (Southcombe et al. 2015). IL-36Ra and IL-38 are also relevant players in this scenario; reduction of their levels is associated with the development of inflammatory processes and contributes to dysregulated pro-inflammatory IL-36 (α, β, γ) activity (Marrakchi et al. 2011, Onoufriadis et al. 2011, Lea et al. 2011, Kim et al. 2008, Murrieta-Coxca et al. 2019).

IL-6 and TNFβ were associated with term preeclampsia (Tayolor 2016). The conflicting results may be due to differences in the study populations and IL6 was associated with preeclampsia but most often in white women.

IL-1β was significantly associated with preterm preeclampsia in the first trimester and the elevation IL-1β in early pregnancy may indicate a subtype of preeclampsia. However, these associations were not observed in the second trimester. Longitudinal changes in IL1β in relation to preeclampsia subtypes may be warranted. Overall, no single immune biomarker is likely a strong predictor for preeclampsia or preeclampsia subtypes, particularly in the second trimester. Investigations which combine several immune markers, biomarkers from pathways which may induce inflammation and clinical data may be useful to define an immunological subtype of preeclampsia (Brandie 2016).

C3, C4 complements

The role of the complement system is fundamental in protecting the fetal–maternal interface against invading pathogens and in promoting the removal of immune complexes and apoptotic cells. In addition, the presence of complement inhibitors is crucial in protecting the fetus from attack by the maternal immune system. However, excessive complement activation or the lack of complement inhibition can determine bad pregnancy outcomes in murine models. These observations were also confirmed in humans, increased circulating levels of complement proteins and their activation fragments being a common finding in patients with pre-eclampsia, recurrent miscarriage and intrauterine growth restriction (Girardi et al. 2011).

C3 complement component was without significant changes, but C4 complement component was reduced by woman with preeclampsia (Kestlerová et al. 2012). Measurement of serum C3 and C4 can help differentiate between SLE activity and pre-eclampsia, since both C3 and C4 are significantly lower in women with SLE than women with pre-eclampsia, and serum C3 and C4 concentrations rise during uncomplicated or pre-eclamptic pregnancy in women with SLE (Buyon et al. 1986).

Imunoglobulins Ig

B cells express IgD and IgM as cell surface receptors and secrete IgM, IgG, IgE and IgA antibodies. IgM, and IgG1, IgG2, and IgG3 in human are complement fixing antibodies and are thus (most of the time) cytotoxic. However, complement functions at the materno−fetal interface are blocked by specific regulatory molecules, MCP (membrane cofactor protein) and DAF (decay accelerating factor) in humans, which are expressed on germ cells, the fertilized egg and early embryo, and later on by trophoblasts. These antibodies can also act as immobilizing antibodies (especially IgM and IgA), and indeed there are many cases of infertility linked to IgM or IgG antisperm agglutinating or cytotoxic antibodies; these are a concern in IVF (Kamada et al. 1985, Chaouat et al. 2007).

In our study of pregnancy pathologies in the third trimester were concentrations of serum immunoglobulins of the IgA, IgG and IgM classes elevated in gestational hypertension or in preeclampsia, when compared with normal pregnancies. While in the IgA class the differences are not statistically significant, serum levels of IgG were markedly higher in the preeclampsia group and slightly higher also in women with gestational hypertension. IgM-class immunoglobulins were also elevated in the preeclampsia group (Kestlerová et al. 2012).

Conflict of Interest

There is no conflict of interest.
Acknowledgements
I would like to thank prof. MUDr. Tomáš Zima, DrSc., MBA for his consultations and his expert advice.

References


BARTON JL, HERBST R, BOSISIO D, HIGGINS L, NICKLIN MJ: A tissue specific IL-1 receptor antagonist homolog from the IL-1 cluster lacks IL-1, IL-1ra, IL-18 and IL-18 antagonist activities. Eur J Immunol 30: 3299-3308, 2000.


