# K113023 OTKA report

## Aim:

Discovery of new biomarkers for the diagnosis and prognosis of pregnancy-induced hypertension and preeclampsia (PE) by the investigation of circulating microvesicles and systems biology approaches.

## Hypothesis:

Extracellular vesicle (EV)-mediated intercellular communication has a very important role in the induction and maintenance of immune tolerance during pregnancy and also in the regulation of placental development. Trophoblast functional abnormalities and the altered immune homeostasis that are characteristic to PE may also have important roles in the regulation of EV production. Characterization of the circulating microvesicle (MV) pattern or the monitoring of MV production by placental and immune cells may be useful in the diagnosis and the prognosis of PE and/or other pregnancy complications.

The function of miRNAs in the placenta is poorly understood, but is clear that they take part in the regulation of placental development and are essential for normal physiology. Abnormally expressed miRNAs may contribute to complications of pregnancy by causing placental insufficiency. miRNAs are released from trophoblast cells to the maternal circulation via exosomes predominantly. In PE, impaired placental function with enhanced apoptosis and necrosis causes increased release of exosomes. The expression analysis of circulating exosomal miRNAs may facilitate the development of new biomarkers for hypertensive disorders of pregnancy.

## Questions to be answered:

1. Is there any difference between the circulating MV pattern of healthy pregnant women and patients with different pregnancy complications including chronic hypertension, pregnancy-induced hypertension or PE?
2. Can we use the circulating MV pattern in the prognosis of pregnancy complications including chronic hypertension, pregnancy-induced hypertension or PE?
3. Is there any difference between the biological effects of pregnancy–associated EVs isolated from healthy pregnant women and PE-associated EVs?
4. Through which mechanism could altered placental miRNA expression lead to the development of hypertensive disorders of pregnancy?
5. Is there a significant difference of the exosomal total-miRNA concentration in the circulation of pregnant women with gestational hypertension (GHT) or PE compared to healthy pregnant woman?
6. Is there any difference in the expression of key exosomal miRNAs in the circulation of pregnant women with GHT or PE compared to healthy pregnant woman?

## Methods:

1. Investigation of circulating MV pattern
    a. Detection of the circulating MV levels in healthy and pathological pregnant women by flow cytometry.
    b. Characterization of the circulating MV pattern by flow cytometric immunophenotyping.
    c. Comparison of the circulating MV patterns of the healthy and pathologic pregnant women.
    d. Characterization of circulating EVs by mass spectrometry

2. Investigation of circulating apoptotic bodies (ABs).
    a. Characterization of the circulating ABs by multicolor flow cytometry.
    b. Comparison the plasma levels of circulating ABs of the healthy and pathologic pregnant women.
3. Investigation of the target cells of circulating MVs isolated from the plasma of healthy and pathologic pregnant women by multicolor flow cytometry.
4. Investigation of the effects of MVs isolated from the plasma of healthy pregnant women and patients with pregnancy complications on the functionality of PBMC.
    a. Monitoring of the HLA-DR expression of PBMC after *in vitro* stimulation by EVs isolated from the plasma of healthy pregnant women and patients with pregnancy complications.
5. Investigation of the effects of pregnancy – associated EVs on THP1 human monocytic cell line (*in vitro* model system):
    a. Investigation of the effects of isolated pregnancy – associated EVs on phagocytic activity of THP1 monocyte model cells.
    b. Investigation of the effects of isolated pregnancy – associated EVs on cell adhesion of the THP1 monocyte model cells by xCELLigence SP System (Roche Applied Science, Indianapolis, IN, USA).
    c. Investigation of the effects of isolated pregnancy – associated EVs on the proliferative activity of PBMCs.
    d. Investigation of pregnancy – associated EVs induced cytokine production of PBMC by real time PCR, flow cytometry and protein array.
6. Characterization of the redox status of preeclamptic patients and healthy pregnant women at plasma, cellular and vesicular levels.
    a. Detection of the oxidative damaged proteins
        i. Detection of the total thiol levels in plasma by DTNB assay
        ii. Detection of the thiol content of membrane proteins both in circulating PBMCs and circulating MVs by the flow cytometric DyLight Maleimide staining
    b. Investigation of DNA oxidative damage by the quantitative flow cytometric analysis of oxoguanin8 in PBMC
    c. Characterization of the redox homeostasis by the detection of antioxidant enzymes like peroxiredoxin1 and thioredoxin both in healthy pregnant women and in patients with pregnancy complications
        i. Quantitative measurement of plasma peroxiredoxin1 levels by ELISA
        ii. Quantitative FACS measurements of exofacial thioredoxin and peroxiredoxin1 both in PBMC and circulating MVs
        iii. Quantitative FACS measurements of intracellular thioredoxin and peroxiredoxin1 in PBMC
    d. Flow cytometric analysis of the mitochondrial protein deacetylases, including SIRT3, SIRT4, SIRT5 in PBMC
7. miRNA-mRNA network analysis
    a. Creation of miRNA regulated interaction network by integration of publicly available miRNA and gene expression profiles in preeclamptic placenta samples
    b. Determination of key regulator miRNAs and their target genes in the network
    c. Investigation of the miRNA regulated pathways which could have a role in the pathogenesis of the disease
8. Total miRNA concentration measurement

    a. Isolation of exosomes using a precipitation method and miRNA extraction on columns from diluted exosomes; Measurement of total-miRNA concentration with Qubit fluorometer applying miRNA specific assay.
9. Circulating exosomal miRNA expression analysis
    a. Reverse transcription of miRNA samples and SYBR green-based real-time PCR with miRNA specific primer on LightCycler 2.0 instrument; Determination of expression by relative quantification method (ΔΔCT) and normalization to internal control miRNA.

**Patients and sample collection:**

Peripheral venous blood of healthy pregnant women and patients with pregnancy complications including chronic hypertension, gestational hypertension or PE was collected from the median cubital vein using the Vacutainer® Brand Plus ACD-A Tubes of Becton Dickinson (BD San Jose, California, USA). Samples were transferred at room temperature into the flow cytometric laboratory immediately after blood collection.

Pregnant women underwent routine obstetrical control at the 1st Department of Obstetrics and Gynecology of Semmelweis University Budapest, Hungary. The study was approved by the Ethical Community of the Semmelweis University and was also authorized by Ethic Committee of Scientific Research (ETT-TUKEB).

Number of the patients:

3rd trimester healthy pregnant women (K3): 34
2nd trimester healthy pregnant women (K2): 9
1st trimester healthy pregnant women (K1): 17
Chronic Hypertension (CHT): 23
Pregnancy induced hypertension (GHT): 31
Preeclampsia (PE): 40

**Sample preparation:**

Platelet free plasma (PFP) was prepared from anticoagulated blood by double centrifugation at 2500 × g for 15 min. The PFP was centrifuged at 12,500 × g for 20 min and washing by PBS and centrifuged at 12,500 g for 15 min. The supernatant PFP plasma was stored at − 80 °C for subsequent analysis.

PBMC were isolated by Histopaque-1077 (Sigma-Aldrich Co.) density gradient centrifugation. Isolated PBMC samples were frozen in DMSO/FCS containing freezing medium and was stored at − 80 °C for subsequent analysis.

Apoptotic bodies were analyzed in plasma samples which were diluted with sterile filtered PBS solution (1:100).

**Results:**

1. Investigation of circulating MV pattern
    a. Significantly higher amounts of AnnexinV+ MVs could be detected in the plasma of preeclamptic women and in patient with pregnancy induced hypertension compared to the K3 control group.
    b. The expression level of phosphatidylserine (AnnexinV fluorescence intensity) was significantly higher in K3 group then in patients with pregnancy complications.

    c. Significantly lower amounts of CD42b+/CD62P+ activated platelet derived MVs could be detected in the plasma of preeclamptic women compared to the K3 control group.
    d. Funrich analysis of MV fraction of healthy (K3) and preeclamptic (PE) women showed different protein distributions. The majority of the preeclamptic MV proteins are involved in the complement activation and inflammatory responses, meanwhile the protein content of MVs isolated from healthy pregnants involved in blood coagulation and platelet-associated biological processes.
2. Investigation of circulating apoptotic bodies (ABs)
    a. Circulating apoptotic bodies could be detected in the plasma samples of both healthy pregnant women and preeclamptic patients by AnnexinV and propidium iodide (PI) stainings.
    b. Significantly higher amounts of PI+ (propidium iodide) and AnnexinV+ ABs could be detected in the plasma of preeclamptic women compared to the K3 control group.
3. Investigation of the target cells of circulating MVs isolated from the plasma of healthy and pathologic pregnant women by multicolor flow cytometry.
    a. EVs bind to T lymphocytes in all groups, but their binding ability to T cell subsets differs: a) EVs isolated from healthy pregnant women or from the plasma of women in the chronic hypertension group, show more pronounced binding to Th than to Tc cells. b) EVs isolated from preeclamptic patients have preferential binding to Tc cells c) equal binding to Tc and Th cells could be detected in the case of gestational hypertension.
    b. EVs bind to B cells in each group, but the most characteristic binding can be observed in PE.
4. Investigation of the effects of MVs isolated from the plasma of healthy pregnant women and patients with pregnancy complications on the functionality of PBMCs.
    a. HLA-DR expression
        i. MVs isolated from either healthy pregnants or patients with pregnancy complications did not alter the expression level of HLA-DR on the surface of monocytes.
        ii. MVs isolated from healthy pregnants (K3) or patients with pregnancy complications increased the expression level of HLA-DR on the surface of CD20+ B lymphocytes. There was no difference between the clinically different groups (healthy pregnants, chronic hypertension, gestational hypertension or PE).
        iii. MVs had no effects on the expression levels of HLA-DR on CD3+ T cells.
5. Investigation of the effects of pregnancy-associated EVs on THP1 human monocytic cell line.
    a. Phagocytosis of PE–associated EVs by THP1 cells was less effective due to their lower “eat-me” signal expression.
    b. PE-associated-EVs induced chemoattractant effect, adhesion molecule expression, migratory activity and adhesiveness of THP1 cells were reduced.
    c. The effects of circulating MVs of preeclamptic or healthy pregnants on proliferation did not differ.
    d. PE associated EVs induced proinflammatory cytokine expression in THP1 cells.
6. Characterization of the redox status of preeclamptic patients and healthy pregnant women.
    a. Detection of the oxidative damage of proteins

i. Significantly higher total thiol levels could be detected in hypertonic patients, both in the chronic hypertonic and the gestational hypertonic groups, compared to the healthy pregnant women (control group).
ii. The exofacial thiol content of circulating PBMCs was significantly higher in pregnancy complications (both in PE and gestational hypertension) compared to control group.
iii. Lower exofacial thiol level of circulating EVs could be detected in the healthy pregnant group compared hypertensive pregnancy complications. This difference was statistically significant only between the healthy pregnant women and the gestational hypertension group.
iv. Significantly higher plasma advanced oxidation protein products (AOPP) level could be detected in preeclamptic patients compared to healthy pregnant women.

b. We could not detect any differences in the oxoguanin8 expression of PBMC between the investigated groups.
c. Characterization of the redox homeostasis by the detection of antioxidant enzymes like peroxiredoxin1 and thioredoxin both in healthy pregnant women and in patients with pregnancy complications
    i. We could not detect any alterations in the plasma soluble peroxiredoxin1 levels.
    ii. Significantly higher expression levels of peroxiredoxin1 and thioredoxin could be detected on the surface of lymphocytes and monocytes in preeclamptic patients.
    iii. We demonstrated the presence of exofacial PRDX1 expressing circulating EVs in all patient groups. Neither the amounts of PRDX1+ EVs, nor the exofacial expression levels of PRDX1 on EVs did not differ in the investigated groups.
    iv. Significantly higher intracellular levels of TRX1 were found both in lymphocytes and in monocytes in preeclamptic patients.
    v. The presence of exofacial TRX1 expressing circulating EVs could be detected in all patient groups. Neither the amounts of PRDX1+ EVs nor the exofacial expression levels of TRX1 on EVs did not differ in the investigated groups.
d. Analysis of mitochondrial regulatory proteins.

Both the ratio of SIRT3+ cells and the expression levels of SIRT3 were significantly higher in preeclamptic patient compared to the control group.

7. miRNA-mRNA network analysis
    a. We created an interaction network, which consists of the differently expressed miRNAs and their predicted target genes detected in two microarray studies.
    b. In the group of upregulated miRNAs, hsa-mir-210 was unique regarding the number of its targeted genes. It is one of the so-called “hypoximirs”, which are upregulated in hypoxic conditions. Hypoxia is one of the main characteristics of PE and it modulates miRNA expression in trophoblast cells.
    c. We identified several miRNA-mRNA regulatory mechanism which may contribute to the pathogenesis of PE.
8. Total miRNA concentration measurement
    a. The mean total-miRNA concentration was significantly higher in the disease groups. There was a difference between the two disease subgroups, which can

be the consequence of the enhanced release of exosomes – therefore miRNAs – to the maternal circulation in the case of PE.

9. Circulating exosomal miRNA expression analysis
    a. We found that exosomal hsa-miR-210 was significantly upregulated in the circulation of pregnant women with hypertension compared to the normotensive pregnant women: in GHT more than 3-fold and in PE more than 4-fold compared to controls. This observation can be explained by the increased level of hypoxia in hypertension disorders of pregnancy.

**Summary:**

Successful pregnancy is based on the perpetual immunological communication between the fetus and the mother which is mediated by direct cell-cell interactions, extracellular vesicles and soluble regulators. Although the regulatory role of trophoblasts has been established, there remain many open questions in the field of microvesicle-mediated regulatory pathways.

We could certify that circulating MV pattern including circulating apoptotic bodies and activated platelet-derived MVs differ in healthy and pathological pregnancies.

We proved that both T and B lymphocytes are the target cells of circulating MVs, and circulating MVs isolated from the plasma of pregnant women have an effect on the functionality of PBMC.

We demonstrated in *in vitro* cell culture system, that circulating PE-associated EVs may have a pathogenic role by modifying the Mo-Mph system.

We showed that the redox status of pregnant women depends on the pathophysiology of pregnancy.

We applied bioinformatics approaches to integrate high-throughput experimental data on miRNA and mRNA expression profiles in PE. Several miRNA-mRNA regulatory mechanism were identified which may contribute to the pathogenesis of the disease.

Circulating exosomal hsa-miR-210 expression was assessed and compared between different forms of hypertensive disorders of pregnancy. We found that the total-miRNA concentration was significantly higher in the circulation of pregnant women with hypertension, and it was correlated to the severity of the disease. Similarly, hsa-miR-210 was significantly overexpressed in the hypertensive groups compared to control group, which underlies the importance of hypoxia in the pathogenesis of the disease.

According to our preliminary results, hsa-miR-210 is a potential biomarker for hypertension disorders of pregnancy. Further measurements are needed to decide whether this miRNA could predict the onset of hypertension in pregnancy, or the combination with other placenta-specific miRNAs would improve the prognostic value of the expression analysis.

**Publications:**

Orsolya Biró, Bálint Nagy, János Rigó Jr. (2016): Identifying miRNA regulatory mechanisms in preeclampsia by systems biology approaches, Hypertension in Pregnancy, DOI: 10.1080/10641955.2016.1239736

**Some research projects supported by the K113023 Hungarian Scientific Research Fund have not been finished yet. Further publications are expected, therefore we would like to ask for the reevaluation of the final report in two years.**

**Citable conference abstracts:**

**ISSHP (International Society for the Study of Hypertension in Pregnancy) Conference 2015**: 2 lectures and 2 poster

Hajnalka Héjja, Nóra Fekete, Bálint Alasztics, József Gábor Joó, Attila Molvarec, Katalin Szabó-Taylor, Edit Buzás, János Rigó Jr., Éva Pállinger (2015). O18. Oxidative stress in preeclampsia. Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health, 5(3), 214. doi:10.1016/j.preghy.2015.07.017

Éva Pállinger, Nóra Fekete, Anikó Árokszállási, József Gábor Joó, Edit Buzás, János Rigó Jr. (2015). O72. Monocyte–macrophage system in pregnancy complications from the prospective of extracellular vesicles. Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health, 5(3), 227. doi:10.1016/j.preghy.2015.07.046

Orsolya Biró, Bálint Nagy, János Rigó Jr. (2015). P39. Connection between placenta specific miRNA clusters and preeclampsia: a hypothetical miRNA–mRNA interaction network. Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health, 5(3), 244. doi:10.1016/j.preghy.2015.07.092

Kovács, Á. F., Fekete, N., Alasztics, B., Joó, J. G., Prosszer, M., Buzás, E., Rigó J., Pállinger, É. (2015). P45. Target cells of pregnancy-associated extracellular vesicles. Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health, 5(3), 247. doi:10.1016/j.preghy.2015.07.098

**ISEV (International Society for Extracellular Vesicles) 2016**: 1 poster

Árpád Ferenc Kovács, Orsolya Láng, László Kőhidai, János Rigó, Nóra Fekete, Edit Buzás and Éva Pállinger (2016). PF3.09. Impact of pre-eclampsia–associated extracellular vesicles on the monocyte–macrophage system: an in vitro analysis. J Extracell Vesicles. 2016 May 30;5:31552. p. 149-150. doi: 10.3402/jev.v5.31552

**ESHG (European Society of Human Genetics) Conference, Barcelona, Spain, 2016**: 1 poster

Orsolya Biró, Bálint Nagy, János Rigó Jr. (2016). P01.054 Integrated analysis of miRNA-and mRNA expression profiles in preeclampsia. European Journal of Human Genetics. 2016 Volume 24 E-Supplement 1. p. 63.

**4th CEE-NIPD (Central Eastern European Symposium on Free Nucleic Acids in Non-Invasive Prenatal Diagnosis) Symposium, Split, Croatia, 2016**: 1 lecture

Orsolya Biró, Bálint Nagy, János Rigó Jr. (2016) O8. Identifying potential miRNA biomarkers for preeclampsia by systems biology approaches. PAEDIATR CROAT. 2016;60 (SUPPL 2):30.

**ISSHP Conference, Sao Paolo, Brazil, 2016**: 1 poster

János Rigó Jr, Bálint Alasztics, Attila Molvarec, Bálint Nagy, Orsolya Biró (2016). Expression analysis of circulating exosomal hsa-miR-210 in hypertensive disorders of pregnancy. Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health, Vol. 6, Issue 3, p183. doi:10.1016/j.preghy.2016.08.093

**Other conference participations:**

**Magyar Immunológiai Társaság 45. Vándorgyűlésére, Velence, 2016**. 1 lecture
Láng Orsolya, Kőhidai László, Rigó János Jr., Fekete Nóra, Buzás Edit Irén, Pállinger Éva. Potential Role of Preeclampsia-Associated Extracellular Vesicles as Immune Regulators of Monocyte-Macrophage System.

**XV. Magyar Genetikusok Egyesülete Minikonferencia, Szeged 2016**. 1 lecture
Kovács Árpád Ferenc, Pap Erna, Fekete Nóra, Rigó János, Buzás Edit, Pállinger Éva. Preeclampsia asszociált extracelluláris vezikulák monocita génexpressziós mintázatra kifejtett hatásai.

**XI. MHGT (Magyar Humángenetikai Társaság) Kongresszus, Pécs, 2016**: 1 lecture
Biró Orsolya, Alasztics Bálint, Molvarec Attila, Nagy Bálint, Rigó János Jr. A keringő exoszómális hsa-miR-210 miRNS expressziós profiljának vizsgálata magasvérnyomással szövődött terhességben.