NOVEL MOLECULAR BIOLOGICAL MARKERS OF PREECLAMPSIA

PhD thesis book

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1 Introduction

Preeclampsia is a common and serious complication of pregnancy, affecting approximately 2-8% of all pregnancies worldwide. It is one of the main contributors to fetal and maternal morbidity and mortality. According to the guideline issued by the International Society for the Study of Hypertension in Pregnancy (ISSHP) this condition can be diagnosed after the 20th gestational week by the presence of hypertension accompanied by proteinuria and/or evidence of maternal end organ dysfunction (acute kidney injury, liver dysfunction, neurological features, hemolysis or thrombocytopenia) or fetal growth restriction.

Despite extensive research, the exact etiology of preeclampsia remains largely unknown. Preeclampsia can be classified into two subtypes: early onset and late onset. The boundary between early and late-onset type is commonly defined as the 34th gestational week.

The pathophysiology of preeclampsia is an ongoing topic of research and debate, as the processes underlying this disorder are complex and multifactorial, while novel technical advances are identifying additional mechanisms. In recent years, studies have focused on the role of angiogenic factors, immune dysregulation, and abnormal placental evolution in the development of preeclampsia, as well as the potential involvement of oxidative stress and extracellular vesicles.

The course of the disease can be explained by two stages. The first stage occurs in the first half of the pregnancy, when the disease is localized to the placenta and no clinical symptoms are present. The second stage of preeclampsia, the feto-maternal clinical syndrome can develop in the second half of the pregnancy. It seems that the key process of developing the clinical syndrome of preeclampsia is mainly the vascular endothelial dysfunction.
The resulting endothelial dysfunction can cause activation of the coagulation system and lead to a prothrombotic state. In addition, platelet activation and

aggregation are increased in preeclampsia, which further contributes to the hypercoagulable state. These changes can increase the risk of thrombotic complications, such as deep vein thrombosis, pulmonary embolism, stroke, and placental thrombosis.

Platelet activation also leads to increased production of platelet-derived extracellular vesicles (EVs). The elevated level of platelet derived EVs is generally considered as a marker of platelet activation. During pregnancy, the amount of circulating EVs significantly increases compared to the non-pregnant state. Notably, in preeclampsia, the count of EVs is further elevated, suggesting their potential involvement in the pathogenesis of preeclampsia.

The presence of oxidative stress has long been investigated in preeclampsia and it may play a central role in the development of the disease. It is a complex process that involves the generation of free radicals and other reactive oxygen species (ROS), which can cause cellular damage and dysfunction. In preeclampsia, oxidative stress is thought to be caused by a variety of factors, including placental ischemia and inflammation.

Several studies have investigated the relationship between oxidative stress and preeclampsia, and have demonstrated that oxidative stress markers are significantly elevated in women with the condition compared to healthy pregnant women. However, there is evidence of little or lacking effect of antioxidant therapy in preventing or treating preeclampsia

Peroxiredoxin-1 (PRDX1) and thioredoxin-1 (TRX1) are antioxidant enzymes that are involved in the regulation of oxidative stress. These coupled enzymes carry out the enzymatic reduction of peroxides thus protecting the organism against the toxic effects of peroxides. So far they have not been extensively studied in preeclampsia, and their potential role in this condition is not elucidated.

2 Objectives

We aimed to identify novel biomarkers of preeclampsia by analyzing platelet derived extracellular vesicles (P-EV) and by investigating the antioxidant systems of circulating lymphocytes and monocytes. Specifically, the objectives of our studies were:

1. To confirm increased platelet activation in preeclamptic women compared to healthy pregnant controls.
2. To determine the levels of platelet-derived extracellular vesicles (EVs) and tissue factor-bearing procoagulant EVs in the plasma of pregnant women with preeclampsia and to compare these levels to those of healthy pregnant women.
3. To confirm the presence of increased oxidative stress by measuring the level of the oxidative stress biomarker AOPP in pregnant women with preeclampsia and to compare its level to those of healthy pregnant women.
4. To investigate the exofacial expression of peroxiredoxin-1 (PRDX1) and thioredoxin-1 (TRX1) regulatory enzymes in lymphocytes and monocytes of pregnant women with preeclampsia and to compare these levels to those of healthy pregnant women.
5. To examine the relationship between oxidative stress, and adverse pregnancy outcomes, such as intrauterine growth restriction (IUGR).

3 Methods

3.1 Study group

Fifteen preeclamptic patients (PE) and fifteen third trimester healthy pregnant women (HP) were enrolled into the investigation of platelet markers. Third trimester healthy pregnant women (HP) were accepted as control group. Twelve patients with de novo preeclampsia (PE) and seven healthy pregnant controls (HP) were enrolled into the study analyzing the biomarkers of oxidative stress.

Preeclampsia was defined as a new onset high blood pressure (more than 140 mmHg systolic or 90 mmHg diastolic) in the presence of significant proteinuria according to the ISSHP guideline published in 2014.

Intrauterine growth restriction (IUGR) was defined as birth weight of the newborn being below the 10th percentile.

The studies were approved by the Ethic Committee of Medical Research Council of Hungary (ETT-TUKEB: 10147-4/2015/EKU (93/2014).

3.2 Plasma sample collection

The samples were obtained in the third trimester, but always before the onset of labor. Peripheral venous blood samples were collected from the median cubital vein. Samples were transferred at room temperature into the flow cytometric laboratory immediately after blood collection. Platelet count was measured by using an automated blood cell counter.

3.3 Measurement of platelet markers

3.3.1 Separation of platelet free plasma for EV measurements

Platelet-free plasma (PFP) was prepared using a 3-step centrifugation procedure: 1) 2000 rpm centrifugation for 5 minutes at room temperature for depletion of peripheral blood cells; 2) repeated centrifugation at 2500 g for 5 minutes at 20°C for preparation of PFP. Samples were stored at -80°C for future experiments.

3.3.2 Flow cytometric analysis of platelets and platelet derived circulating EVs

Unseparated plasma samples (1:500 dilution in sterile filtered phosphate buffered saline - PBS) were stained by phycoerythrin conjugated anti-CD42b (anti-von Willebrand factor receptor antibody) and fluorescein isothiocyanate (FITC) labelled Annexin V for the determination of resting and activated thrombocytes.

PBS-diluted PFP samples (1:500 dilution in sterile filtered PBS) were used for the identification of platelet derived EVs. Anti-CD41a-FITC (anti-

gpIIb/IIIa complex antibody, fluorescein isothiocyanate conjugated), anti-CD42b-Pe (anti-von Willebrand factor receptor antibody phycoerythrin conjugated) were used for the determination of platelet derived EVs. Activation state of circulating platelet derived EVs were analyzed by anti-CD62P APC (anti-P-selectin antibody, allophycocyanin conjugated), anti-CD142-Pe (anti-tissue factor antibody, phycoerythrin conjugated), anti-CD63-Pe (anti tetraspanin antibody, phycoerythrin conjugated) and Annexin V FITC staining.

The „Direct Immunofluorescence Staining of Cells Using a Lyse/No-Wash Procedure" protocol of BD Biosciences was adapted for the staining of exofacial molecules of EVs. The presence of EVs was confirmed by differential detergent lysis. Those events that did not disappear in the presence of 0.1% Triton-X 100 were rejected from analysis. Count Check Beads were used as an internal standard for the calculation of absolute number of circulating platelet-derived EVs.

Measurements were carried out by using a FACSCalibur flow cytometer on the day of the staining. Forward (FSC) and side scatter (SSC) parameters were set in log scale, and threshold was set at the SSC parameter. EV gating was accomplished by preliminary standardization experiments using Megamix-Plus SSC beads and was optimized with 1 μm Silica Beads Fluo-Green Green. CellQuestPro software was used for both the acquisition and analysis.

Circulating platelets were defined on the basis of their CD42b expression, while activated and resting thrombocytes were distinguished by the exofacial presence or absence of phosphatidylserine (Annexin-V positivity).

The procoagulant activity of platelet derived EVs was examined by the presence of tissue factor (TF) on their surface. Circulating platelet derived EVs were demonstrated by exofacial labelling of gpIIb/IIIa complex (CD41a) and von Willebrand factor receptor (CD42b). Exofacial CD62P and CD63 were

used for the isolation of platelet derived EVs produced by activated thrombocytes.

3.4 Measurement of oxidative stress biomarkers

3.4.1 Isolation of lymphocytes and monocytes from the blood samples

Histopaque-1077 cell separation medium was used for the separation of peripheral blood mononuclear cells (lymphocytes and monocytes) under sterile conditions, as recommended by the supplier. Samples were frozen in the presence of 10% dimethyl sulfoxide containing fetal bovine serum at a final concentration of 2×10^6 cells/mL and were stored at -80°C degrees.

Plasma advanced oxidation protein products (AOPP) were determined by spectrophotometry. Chloramine-T solution which absorbs at 340 nm in the presence of potassium iodide was used for calibration.

3.4.2 Flow cytometry of the cells:

Measurements were carried out using a FACSCalibur flow cytometer on the day of the staining, collecting $3x10^4$ cells/tube. Lymphocytes and monocytes were defined on the basis of their size and granularity on forward scatter – side scatter (FSC/SSC) dot plots. CellQuestPro software was used for both the acquisition and analysis. Only those samples have been investigated, in which the viability of peripheral blood mononuclear cells exceeded 95%.

3.4.3 Peroxiredoxin-1 (PRDX1) and thioredoxin-1 (TRX1) in lymphocytes and monocytes

Exofacial presence of TRX1 and PRDX1 were detected by flow cytometry using fluorescein-isothiocyanate and phycoerythrine conjugated monoclonal antibodies. The „Direct Immunofluorescence Staining of Cells Using a Lyse/Wash Procedure" protocol of BD Biosciences was adapted for the staining of exofacial proteins on lymphocytes and monocytes. Cells were fixed with 4% paraformaldehyde solution for 15 min at room temperature. Cells were incubated with appropriate amounts of fluorochrome conjugated monoclonal antibodies for 15 minutes at room temperature. Unbound

antibodies were removed by washing. During the analysis the percentage of exofacial PRDX1 and TRX1 was calculated.

3.5 Statistical analysis

Statistical differences between the studied groups were tested using "GraphPad Prism 8" software. If datasets have passed the test of normality, statistical significances were analyzed by two-tailed Student's t-test, and descriptive statistics have been shown as mean ± standard error of mean (SEM). If datasets have not passed the test of normality, statistical significances were analyzed by the non-parametric Mann-Whitney test, and descriptive statistics have been shown as median and interquartile range (IQR). Regression analysis was performed and correlational coefficients were calculated to explore potential relationships between variables. Statistical significance was defined as $p < 0.05$.

4 Results

4.1 Measurement of platelet markers

4.1.1 Platelet count in normal pregnancy and preeclampsia

The mean value of platelet count was significantly lower in the preeclamptic group (168.9 G/l ± 19.08, mean ± SEM) compared to healthy pregnant group (242.1 G/l ± 9.90) ($p<0.05$, n=15 in both groups)

4.1.2 Flow cytometric analysis of activation state of circulating platelets

Significantly higher ratio of activated (CD42b+/AnnexinV+) platelets was detected in the plasma of preeclamptic patients (median: 4.05%, IQR: 1.72-6.48%, n=15) compared to third trimester healthy pregnant women (median: 15.23%, IQR: 2.50-21.19%, n=15)($p<0.05$). (Figure 1.)

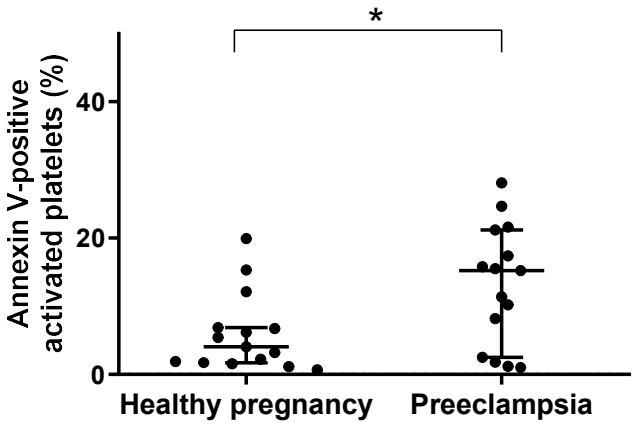

Figure 1. Activation state of circulating platelets in preeclamptic patients (n=15) and healthy controls (n=15) (median and IQR) * $p<0.05$

4.2 Flow cytometric analysis of platelet-derived extracellular vesicles (P-EVs)

4.2.1 Plasma level of tissue factor (TF, CD142) positive P-EVs

Significantly higher amount of procoagulant TF bearing (CD142+) circulating P-EVs was detected in the plasma of preeclamptic patients (20638 events/μL ±7143, mean ± SEM, n=10) than in healthy pregnancy (5283 events/μL ± 788, mean ± SEM, n=13) ($p < 0.03$). (Figure 2.)

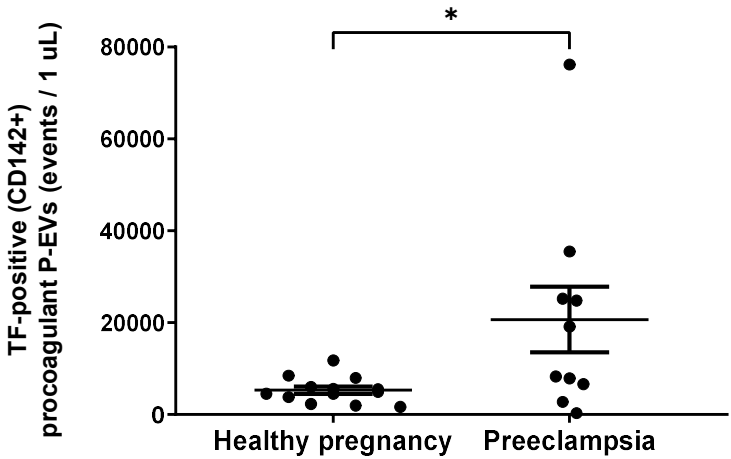

Figure 2. Tissue factor (TF) bearing (CD142+) circulating procoagulant EVs in preeclamptic patients (n=10) and healthy controls (n=13) (mean ± SEM). * $p<0.05$

4.2.2 Plasma level of GpIIb/IIIa complex (CD41a) positive P-EVs

The median value of circulating CD41a+ P-EVs produced by activated thrombocytes was significantly lower in the plasma of preeclamptic patients (median: 11903 events/µL IQR: 8041-19533, n=15) compared to third trimester healthy women (median: 54297 events/µL, IQR: 30395-116438, n=15) ($p<0.05$). (Figure 3.)

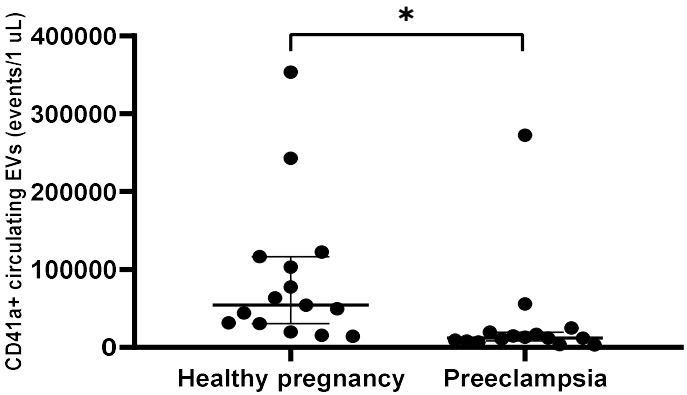

Figure 3. Circulating CD41a+ platelet derived EVs

Significantly lower amount of CD41a+ platelet derived EVs could be detected in the plasma of preeclamptic patients (n=15) compared to third trimester pregnant-matched group (n=15). (median and IQR; *$p<0.05$)

4.2.3 Plasma level of P-Selectin (CD62) positive P-EVs

The absolute amount of circulating CD62P/CD41a double positive P-EVs produced by activated thrombocytes was significantly lower in the plasma of preeclamptic patients (median: 191 events/µL IQR: 62-677, n=15) compared to third trimester healthy women (median: 2717 events/µL, IQR: 997-5445, n=14) ($p<0.05$). (Figure 4.)

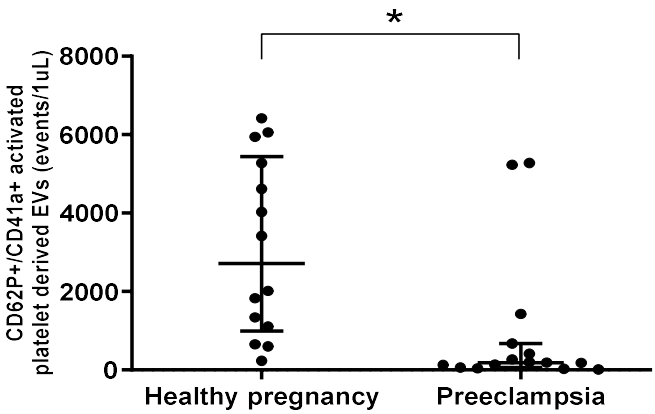

Figure 4. Circulating CD62P+/CD41a+ platelet derived EVs

Significantly lower amount of CD62P+/CD41a+ activated platelet derived EVs could be detected in the plasma of preeclamptic patients (n=15) compared to third trimester pregnant-matched group (n=14). (median and IQR; * $p<0.05$)

4.2.4 Plasma level of tetraspanin (CD63) positive P-EVs

We could observe higher amount of circulating tetraspanin positive (CD63+) P-EVs in preeclamptic patients (median = 4107 events/µL IQR: 2095-19247, n=13) compared to third trimester healthy pregnant women (median: 3352 events/µL, IQR: 1110-5716, n=13), but the difference was not statistically significant p=0.15). (Figure 5.)

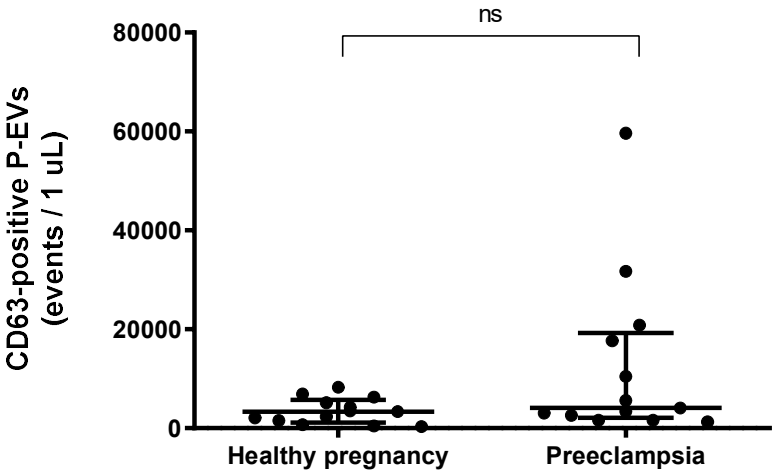

Figure 5. The median amount of CD63 (tetraspanin) positive activated platelet derived extracellular vesicles in preeclampsia (n=13) and healthy controls (n=13). (median and IQR, p=0.15). ns = not significant

4.3 Measurement of oxidative stress biomarkers

4.3.1 Plasma advanced oxidation protein products (AOPP) level

A significantly higher plasma AOPP level was detected in preeclamptic patients compared to healthy pregnant women (mean ± SEM: PE group = 27.97 μM chloramine T equivalents ± 3.6; HP group = 11.75 μM chloramine T equivalents ± 3.7, $p<0.05$).

4.3.2 The ratio of peroxiredoxin-1 positive lymphocytes and monocytes

The ratio of PRDX1 positive circulating lymphocytes in the third trimester was significantly higher in patients with preeclampsia (mean ± SEM: 73.64% ± 4.9) compared to healthy pregnant women (mean ± SEM: 29.97% ± 6.1, $p<0.05$). (
Figure 6/A.)

Similarly to lymphocytes, a significantly higher ratio of circulating monocytes expressed cell surface PRDX1 in patients with preeclampsia (mean ± SEM: 81.69% ± 5.0) compared to the control group (mean ± SEM: 36.81% ± 8.0, $p<0.05$). (
Figure 6/B.)

4.3.3 The ratio of thioredoxin-1 positive lymphocytes and monocytes

The ratio of circulating lymphocytes that carried cell surface TRX1 was significantly higher in preeclamptic patients (mean ± SEM: 80.72% ± 3.9) compared to the third trimester healthy pregnant women (mean ± SEM: 16.67% ± 4.0, $p<0.05$). (Figure 6./C.)

We have also found that a significantly higher ratio of circulating monocytes carried cell surface TRX1 in preeclamptic patients (mean ± SEM: 89.10% ± 2.8) compared to the control group (mean ± SEM: 31.51% ± 7.0, $p<0.05$). (Figure 6./D.)

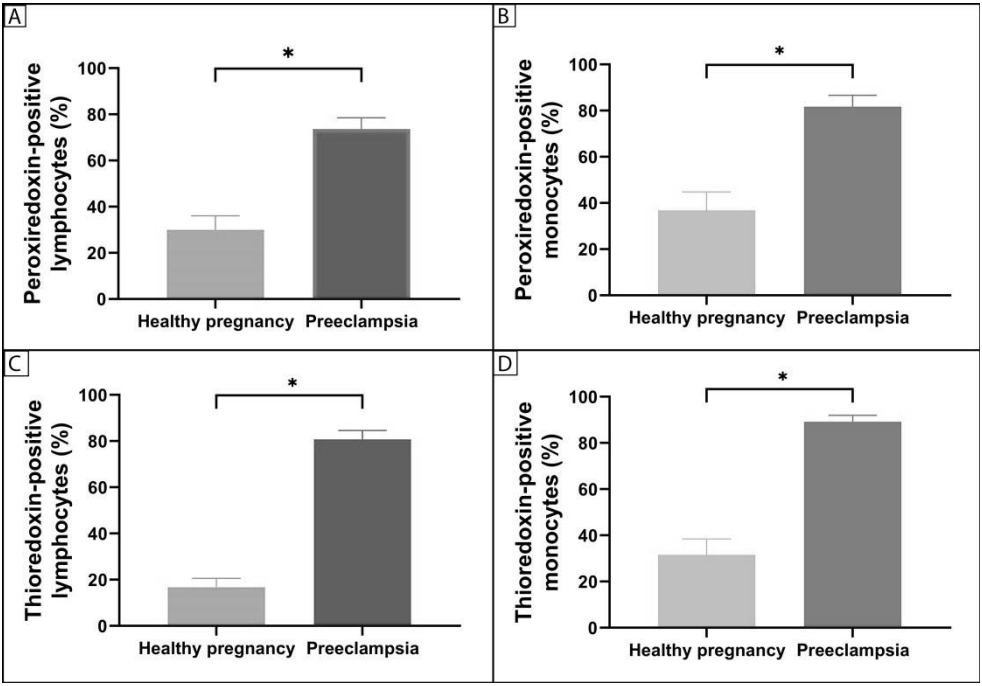

Figure 6. The ratio of PRDX1-positive lymphocytes [A] and monocytes [B] in the blood samples of healthy (n=7) and preeclamptic (n=12) pregnant women. The ratio of TRX1-positive lymphocytes [C] and monocytes [D] in the blood samples of healthy (n=7) and preeclamptic (n=12) pregnant women. * $p<0.05$

4.3.4 Correlation of the ratio of peroxiredoxin-1 positive lymphocytes and monocytes with the birth weight of newborns

The ratio of PRDX1-positive lymphocytes showed a significant inverse correlation with the birth weight of newborns. (r: -0.88, $p<0.05$) (Figure 7./A.)

Similarly, a significant inverse correlation was observed between the ratio of peroxiredoxin-positive monocytes and the birth weight of newborns. (r: -0.86, $p<0.05$) (Figure 7./B.)

4.3.5 Correlation of the ratio of thioredoxin-1 positive lymphocytes and monocytes with the birth weight of newborns

We have found that the ratio of TRX1-positive lymphocytes had a significant inverse correlation with the birth weight of newborns. (r: -0.80, $p<0.05$) (Figure 7./C.)

In concert with the above findings, the ratio of TRX1-positive monocytes has shown a significant inverse correlation with the birth weight of newborns. (r: -0.75, $p<0.05$) (Figure 7./D.)

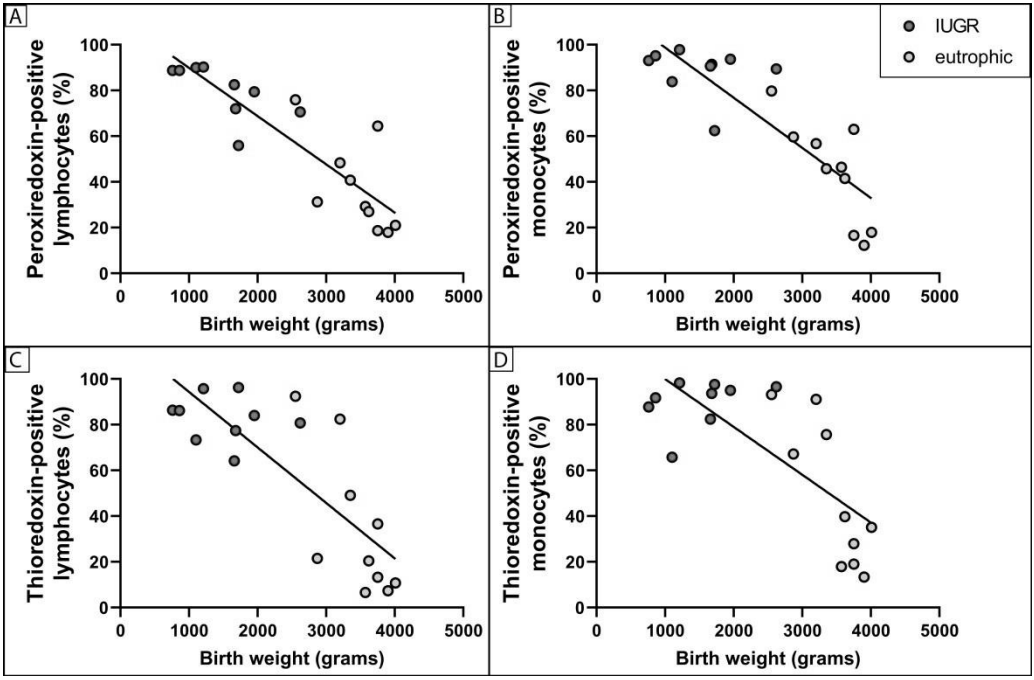

Figure 7.

[A] The correlation of the ratio of PRDX1-positive lymphocytes and the birth weight of eutrophic newborns (n=10) and newborns with IUGR (n=9). $p<0.05$, $r=-0.88$

[B] The correlation of the ratio of PRDX1-positive monocytes and the birth weight of eutrophic newborns (n=10) and newborns with IUGR (n=9). $p<0.05$, $r=-0.86$

[C] The correlation of the ratio of TRX-positive lymphocytes and the birth weight of eutrophic newborns (n=10) and newborns with IUGR (n=9). $p<0.05$, $r=-0.80$

[D] The correlation of the ratio of TRX-positive monocytes and the birth weight of eutrophic newborns (n=10) and newborns with IUGR (n=9). $p<0.05$, $r=-0.75$

5 Conclusions

The study analyzing platelet biomarkers in preeclamptic and healthy pregnant women confirmed a reduction in platelet count, and the higher ratio of activated (phosphatidylserine-expressing) platelets within the preeclamptic group. The findings indicate that pregnant women with preeclampsia have a significantly higher quantity of tissue factor-positive, thrombogenic platelet-derived extracellular vesicles in their plasma when compared to healthy pregnant women. Preeclampsia is associated with a shift towards a procoagulant state, which can lead to thromboembolic complications, and is particularly prominent during the postpartum period. The higher ratio of activated platelets and tissue factor-expressing platelet-derived extracellular vesicles in the plasma of preeclamptic women may explain the hypercoagulable state observed in preeclampsia.

Furthermore, the study found that the amount of CD41a and CD62P-positive platelet-derived extracellular vesicles was lower in preeclamptic patients than healthy pregnant women, which may indicate increased aggregate formation and highlight differences in the exofacial expression patterns between platelets and platelet-derived extracellular vesicles. Finally, the study found a higher expression of CD63 in preeclamptic patients' platelet-derived extracellular vesicles, although not significantly elevated.

These findings provide a better understanding of the pathophysiology of preeclampsia and can be utilized to develop diagnostic and therapeutic interventions to prevent maternal and fetal morbidity and mortality associated with this disease.

The study analyzing oxidative stress biomarkers aimed to investigate the involvement of the peroxiredoxin-thioredoxin system in the development of preeclampsia, a pregnancy-related condition associated with oxidative stress and inflammation. The study confirmed the elevated levels of oxidative stress in preeclamptic women and proposed that the upregulation of the

peroxiredoxin-thioredoxin system in lymphocytes and monocytes may play an important protective role against oxidative stress in preeclampsia.

The study found an increased ratio of exofacial PRDX1 and TRX1 expressing lymphocytes and monocytes in preeclamptic women, assuming their upregulation by micro-environmental factors including enhanced ROS production and inflammatory mediators.

The study also found a significant negative correlation between the percentage of lymphocytes and monocytes expressing PRDX1 and TRX1 and the birth weight of newborns across all the examined groups, suggesting a higher burden of oxidative stress in pregnancies complicated with intrauterine growth restriction (IUGR) and a higher compensatory effect of these antioxidant regulatory enzymes. The study suggests that a ratio of PRDX1 expressing lymphocytes below 50% could potentially exclude IUGR.

6 Bibliography of the candidate's publications

6.1 Journal articles related to the dissertation

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- **Alasztics B**, Kovács ÁF, Molvarec A, Koller Á, Szabó G, Fekete N, Buzás EI, Pállinger É, Rigó J. (2021) Platelet-derived extracellular vesicles may contribute to the hypercoagulable state in preeclampsia. J Reprod Immunol, 148: 103380. IF: 3.993

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ΣIF: 20.438