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LUNGU ANCA-MIHAELA



DOCTORAL THESIS

**CONTRIBUTIONS TO THE ELUCIDATION OF THE ROLE OF
MITOCHONDRIAL DYSFUNCTION IN OBSTETRIC
PATHOLOGY**

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TABLE OF CONTENTS

List of publications.....	VI
List of abbreviations	VII
Figure index	X
Table index.....	XIV
Acknowledgments	XVI
INTRODUCTION.....	XVII

GENERAL PART – REVIEW OF THE LITERATURE

1. MITOCHONDRIAL FUNCTION AND DYSFUNCTION IN PREGNANCY	1
1.1. Mitochondrial general functions and adaptation during pregnancy	1
1.2. Mitochondrial dysfunction in pregnancy.....	7
2. OXIDATIVE STRESS IN PREGNANCY	9
2.1. Sources of reactive oxygen species in pregnancy.....	9
2.2. The role of oxidative stress during pregnancy	10
3. PREECLAMPSIA – OVERVIEW AND PATHOGENESIS	12
3.1. General pathogenesis of PE.....	13
3.2. Mitochondrial dysfunction in preeclampsia	16
3.2.1. Placental dysfunction in PE.....	16
3.2.2. Platelet dysfunction in PE.....	21
3.3. Oxidative stress in the pathogenesis of preeclampsia.....	23
3.3.1. Placenta as source of ROS in PE	23
3.3.2. Antioxidant defense in PE	26

SPECIAL PART – PERSONAL CONTRIBUTIONS

1. MOTIVATION AND AIMS.....	28
1.1. Research objectives	29
2. STUDY I – ASSESSMENT OF PLATELET MITOCHONDRIAL RESPIRATION IN PREECLAMPSIA	30
2.1. Aim of the study	30
2.2. Materials and methods	30

2.2.1. Study population.....	30
2.2.2. Platelet isolation	31
2.2.3. High-resolution respirometry of isolated platelets	33
2.2.4. Data analysis	34
2.2.5. Chemicals.....	34
2.3. Results: Contributions regarding platelet mitochondrial dysfunction in preeclamptic pregnancies	35
2.4. Discussions regarding platelet mitochondrial dysfunction in the setting of preeclampsia.....	47
3. STUDY II - ASSESSMENT OF PLACENTA MITOCHONDRIAL RESPIRATION IN PREECLAMPSIA	51
3.1. Aim of the study	51
3.2. Materials and methods	51
3.2.1. Study population.....	51
3.2.2. Placental mitochondria isolation	52
3.2.3. Assessment of placental mitochondria respiration	53
3.3. Results: Contributions regarding complex I-linked placental mitochondrial dysfunction in preeclamptic pregnancies	55
3.4. Results: Contributions regarding complex II-linked placental mitochondrial dysfunction in preeclamptic pregnancies.....	61
3.5. Discussions regarding placental mitochondrial dysfunction in the setting of preeclampsia	68
4. STUDY III: ASSESSMENT OF LOCAL OXIDATIVE STRESS IN PREECLAMPSIA	72
4.1. Aim of the study	72
4.2 .Materials and methods	72
4.2.1. Study population.....	72
4.2.2. Assessment of placental oxidative stress.....	73
4.2.3. Assessment of MAO gene transcript by RT-PCR	74
4.2.4. Assessment of MAO protein expression by immunohistochemistry	74
4.3. Results: Characterization of local oxidative stress in preeclampsia	74
4.3.1. Contributions regarding placental reactive oxygen species in preeclampsia.....	74

4.3.2. Contributions regarding placental monoamine oxidase in preeclampsia.....	77
4.4. Discussions regarding placental oxidative stress and the role of MAO in preeclampsia	80
5. STUDY IV- ASSESSMENT OF SYSTEMIC OXIDATIVE STRESS IN PREECLAMPSIA	84
5.1. Aim of the study	84
5.2. Materials and methods	84
5.2.1. Study population.....	84
5.2.2. Assessment of systemic oxidative stress in preeclampsia: reactive oxygen metabolites and plasma antioxidant capacity	85
5.3. Results: Contributions to the assessment of systemic oxidative stress in preeclampsia	86
5.4. Discussions regarding the systemic oxidative stress in the pathomechanism of preeclampsia	88
GENERAL DISCUSSIONS	91
CONCLUSIONS	93
REFERENCES.....	96

Key words: *preeclampsia, fetal growth restriction, mitochondrial dysfunction, placenta, platelets, high-resolution respirometry, monoamine oxidase, local and systemic oxidative stress.*

I. AIM AND OBJECTIVES OF THE RESEARCH

Preeclampsia is defined by a spectrum of clinical and laboratory features that develop after 20 weeks of gestation: increased systolic (≥ 140 mm Hg) and/or diastolic (≥ 90 mm Hg) blood pressure values in a previously normotensive patient, associated with at least one of the following new-onset dysfunctions: proteinuria (>300 mg/24h) or increased protein/creatinine ratio (≥ 30 mg/mmol), acute kidney injury, thrombocytopenia, disseminated intravascular coagulation, hemolysis, liver impairment, neurological complications, and utero-placental dysfunction.

According to the onset, PE is classified in two phenotypes with different pathomechanisms, clinical features, biochemical markers and prognosis: early-onset PE (with symptoms and delivery occur <34 weeks of gestation being linked to abnormal placentation) and late-onset PE (with symptoms and delivery occur >34 weeks of gestation being linked to maternal chronic inflammatory syndrome).

PE has a complex, partially elucidated pathophysiology with genetic susceptibility, placental dysfunction, oxidative stress, and altered immune system being the major contributors. An increasing body of evidence suggests a multi-step model of PE progression, firstly, the abnormal placental perfusion and secondly, the maternal endothelial dysfunction. Despite the fact that oxidative stress is widely accepted to contribute to the pathophysiology of the disease throughout its development, the sources of ROS are far from being elucidated.

Placental mitochondrial dysfunction has been previously described in numerous animal and more recently, human studies as a central pathomechanism of PE, but whether it plays a causal role or occurs with the progression of the disease has not been established so far. Important, its relationship with the fetal growth restriction has been scarcely addressed in the literature. In PE, the alteration of the placental mitochondrial population reduces the energy production (ATP) and increases oxidative stress beyond the antioxidant defense capacity. However, the sources of reactive oxygen species (ROS) are far from being fully characterized.

The past decades witnessed an increasing interest for the assessment of mitochondrial function in circulating blood cells, platelets have particularly emerged as potential biomarkers of impaired organ bioenergetics in various pathologies. *There are no data available in the literature regarding platelet respiratory function in patients with pre-eclampsia.*

The research carried out in this thesis was aimed **to characterize the mitochondrial respiratory function in both peripheral platelets and placenta** as well as the **systemic and local oxidative stress in the setting of PE**. With respect to the latter, **the presence and contribution of monoamine oxidase (MAO), a mitochondrial enzyme, to placental oxidative stress** was assessed in national *premiere*.

The research objectives were as follows:

1. **Assessment of mitochondrial respiration in *peripheral platelets*** isolated from preeclamptic pregnancies as compared to healthy pregnancies and age-matched non-pregnant women.

2. **Assessment of mitochondrial respiration in *placental mitochondria*** isolated from preeclamptic pregnancies with or without fetal growth restrictions as compared to healthy pregnancies.
3. **Characterization of *local* oxidative stress in preeclampsia** by assessing: i) **MAO expression**, and ii) **ROS production**, in central vs peripheral placental tissue.
4. **Characterization of *systemic* oxidative stress in preeclampsia** by measuring the plasma level of reactive oxygen metabolites and the antioxidant capacity, respectively.

These objectives are in line with the research directions of the Center for Translational Research and Systems Medicine at the Discipline of Pathophysiology within the Department of Functional Sciences, Faculty of Medicine, "Victor Babeș" University of Medicine and Pharmacy from Timișoara and also, with the research strategy of our university.

The studies included in the Special Part of the thesis are briefly presented below:

II. Contributions regarding platelet mitochondrial dysfunction in preeclamptic pregnancies

In the past decades, the impairment of mitochondrial respiration in various tissues and solid organs has emerged as a disease biomarker and/or a potential prognostic tool, yet tissue biopsy is more difficult accepted by patients.

Circulating blood cells, in particular platelets, have been increasingly used in translational research as a reliable proxy for the tissue defects in cellular respiration, with higher chances for clinical applicability.

Accordingly, in the first study, platelet mitochondrial respiration was evaluated through HRR in pregnancy, healthy or complicated by preeclampsia (PE), as compared to controls, non-pregnant age-matched women. Participants (n=33) were randomized into 3 groups: PE pregnancies (n=14, gestational age - GA 35.31 ± 0.84 weeks), non-PE pregnancies (n=10, GA - 33.71 ± 0.92 weeks), and controls (n=9).

Our results showed that ROUTINE respiration (Fig. 1A) was significantly increased in healthy pregnancies as compared to healthy non-pregnant women, and this increase disappeared in the presence of preeclampsia. Furthermore, platelet active respiration shows a significant decrease (mainly for CI-supported OXPHOS (Fig.1B), but also for CI and CII-supported OXPHOS, Fig. 1C) in healthy pregnancies and this decrease was further aggravated in the presence of PE, particularly for complex I-supported respiration, strongly suggesting an early impairment of CI in the setting of PE. The same pattern was further noticed in non-phosphorylating (LEAK) respiration (Fig. 1D) and maximal uncoupled respiration (ET capacity, Fig. 1E), yet for the latter mainly for complex II (Fig. 1F), in pregnancies complicated by preeclampsia.

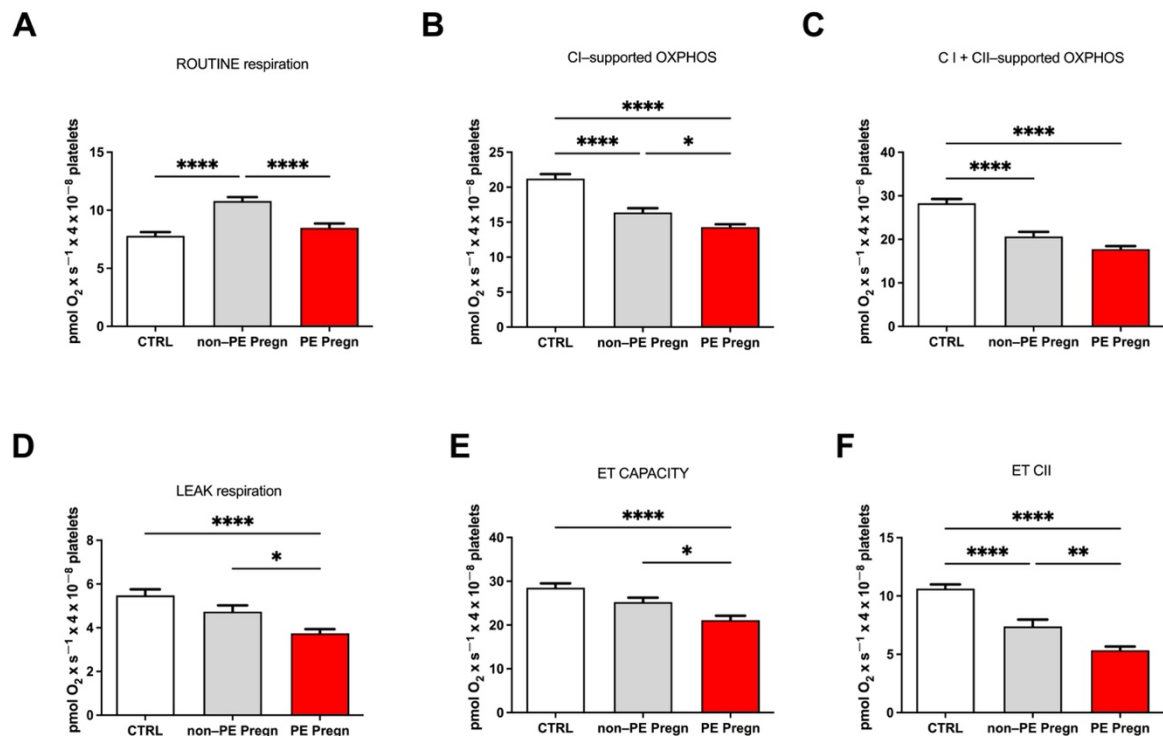


Figure 1. Mitochondrial respiratory parameters in permeabilized platelets. A: ROUTINE respiration, B: CI-supported OXPHOS, C: CI+CII-supported OXPHOS, D: LEAK respiration, E: ET capacity for CI + CII, F: ET capacity for CII. OXPHOS = oxidative phosphorylation; LEAK= non-phosphorylating respiration; ET capacity = electron transport system capacity. Data is presented as mean±SEM.

III. Contributions regarding complex I-linked placental mitochondrial dysfunction in preeclamptic pregnancies

In the second study, placenta mitochondrial dysfunction was evaluated through HRR in preeclampsia, in relation with the presence vs the absence of fetal growth restriction. Complex I and II-dependent respiration was separately analyzed in chamber A and B.

The participants (n=24) were randomized into 3 groups: non-PE pregnancies (n=14, GA=38 ± 0.31) and PE pregnancies (n=10, GA=35 ± 1.2, p<0.05), with (n=4) or without (n=6) fetal growth restriction.

Different results observed in placental mitochondria study in PE are intriguing, normal weight fetuses reflected a significant enhance of both mitochondrial complexes-supported maximal coupled and uncoupled respiration as compared to PE with FGR or healthy pregnancies. Particularly for complex I-supported respiration, the parameters were more than double in the group of PE with no FGR as compared to the ones with FGR.

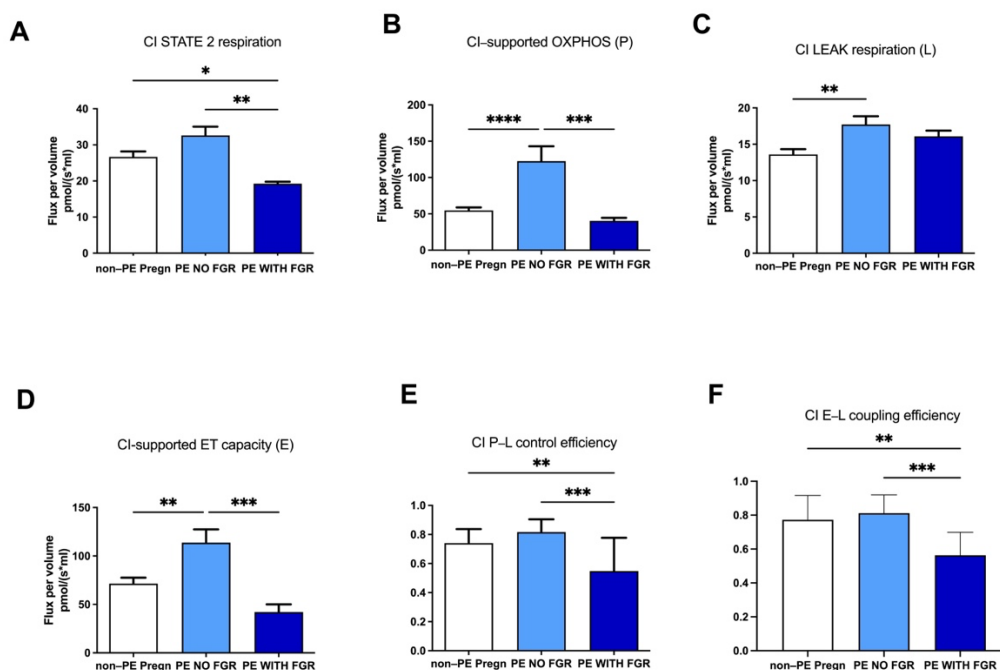


Figure 2. Mitochondrial respiratory parameters for complex I (CI) in isolated placental mitochondria. A: State 2 respiration, B: OXPHOS, C: LEAK respiration, D: ET capacity, E: P-L control efficiency, F: E-L coupling efficiency. OXPHOS (P) = oxidative phosphorylation; LEAK (E) = non-phosphorylating respiration; ET (E) capacity = electron transport capacity. P-L control efficiency was calculated as $1 - \text{LEAK}/\text{P}$; E-L coupling efficiency was calculated as $1 - \text{LEAK}/\text{ET capacity}$. Data is presented as mean \pm SEM.

In isolated placental mitochondria, the basal respiration is represented as the STATE 2 respiration (Fig. 2A), as compared to healthy placentas, the mitochondrial oxygen consumption was increased in preeclampsia without FGR, and significantly decreased in the PE with FGR.

ADP addition in the presence of mitochondrial complex I substrates (glutamate, malate) induce oxidative phosphorylation (Fig. 2B), a significant increase of 123.57% appears in PE without FGR compared to healthy pregnancies, while in the presence of FGR the OXPHOS was reduced to half of the one measured in the non-PE pregnancies.

Oligomycin, as ATP synthase inhibitor, induces the LEAK respiration or non-phosphorylating resting state, as compared to the non-PE group, all preeclamptic placental mitochondria showed a higher non-phosphorylating respiration with 30.36% in non-FGR and 18.3% in FGR (Fig. 2C).

Complex I-supported ET capacity or the maximal uncoupled respiration in the presence of CCCP titration showed as compared to healthy pregnancies an 59.18% increase in PE with no FGR and a 41% decrease in PE with FGR (Fig. 2D).

IV. Contributions regarding complex II-linked placental mitochondrial dysfunction in preeclamptic pregnancies

Complex II-supported basal respiration was assessed in the presence of succinate, after rotenone was injected in order to provide complex I inhibition. No changes in STATE 2

respiration were found in PE vs healthy pregnancies regardless the presence or absence of FGR, albeit a tendency of decrease was observed for the former one (Fig. 3A).

The ADP addition in the presence of mitochondrial complex II substrate (succinate) induce oxidative phosphorylation (Fig. 3B), a significant increase of 42.7% appears in PE without FGR compared to healthy pregnancies, while in the presence of FGR a significant decrease (46.5%) was reported, the changes are similar to the ones previously described for CI-dependent respiration.

The non-phosphorylating respiration or LEAK respiration (Fig. 3C) presented no differences in mitochondrial oxygen consumption among the three investigated groups, similarly to the results for STATE 2.

The maximal uncoupled respiration for mitochondrial complex II showed only a significant reduction of 45.84% in the presence of FGR as compared to healthy pregnancies (Fig. 3D).

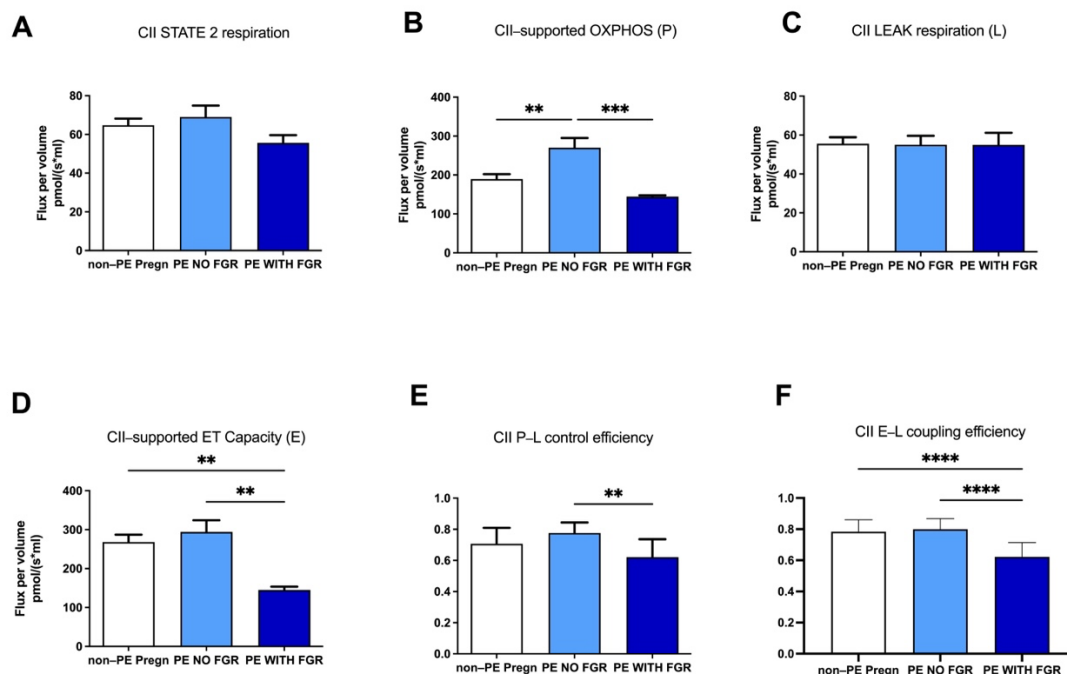


Figure 3. Mitochondrial respiratory parameters for complex II (CII) in isolated placental mitochondria. A: State 2 respiration, B: OXPHOS, C: LEAK respiration, D: ET capacity, E: P-L control efficiency, F: E-L coupling efficiency. OXPHOS (P) = oxidative phosphorylation; LEAK (L) = non-phosphorylating respiration; ET capacity (E) = electron transport capacity. P-L control efficiency was calculated as $1 - \text{LEAK}/\text{P}$; E-L coupling efficiency was calculated as $1 - \text{LEAK}/\text{ET}$ capacity. Data is presented as mean \pm SEM.

V. Contributions regarding placental reactive oxygen species in preeclampsia

Placental samples, collected immediately after delivery from central and peripheral areas were incubated with DHE following a slide examination in confocal microscopy in order to evaluate the ROS production. The preeclamptic group (n=11) was additionally divided upon the severity into mild forms (n=4) and severe forms (n=7) vs controls, healthy pregnancies (n=8).

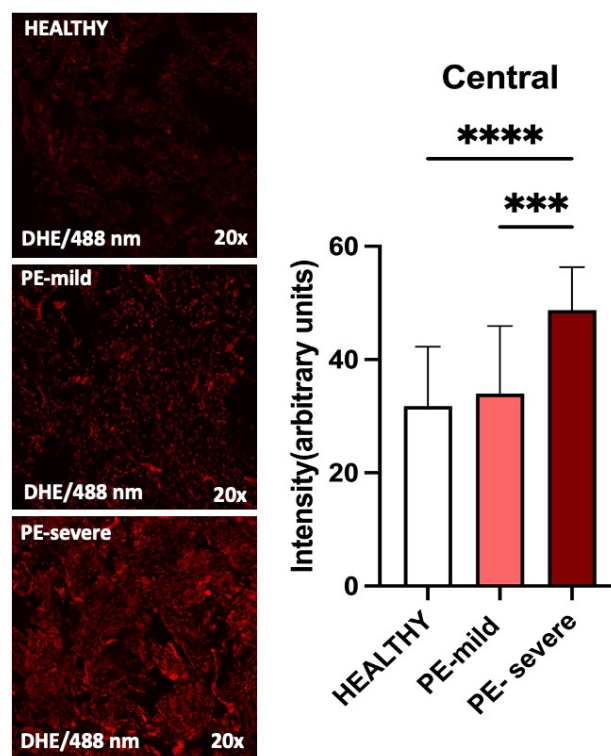


Figure 4. Assessment of placental oxidative stress (DHE staining) in central samples, healthy vs mild and severe PE pregnancies. Data is presented as mean \pm SEM.

Confocal images were analyzed using the Icy Bioimage analysis software. The main result of this experiment demonstrates higher levels of oxidative stress in severe forms of PE as compared to both healthy pregnancies or mild PE, regarding the placental region (Fig. 4 and Fig. 5).

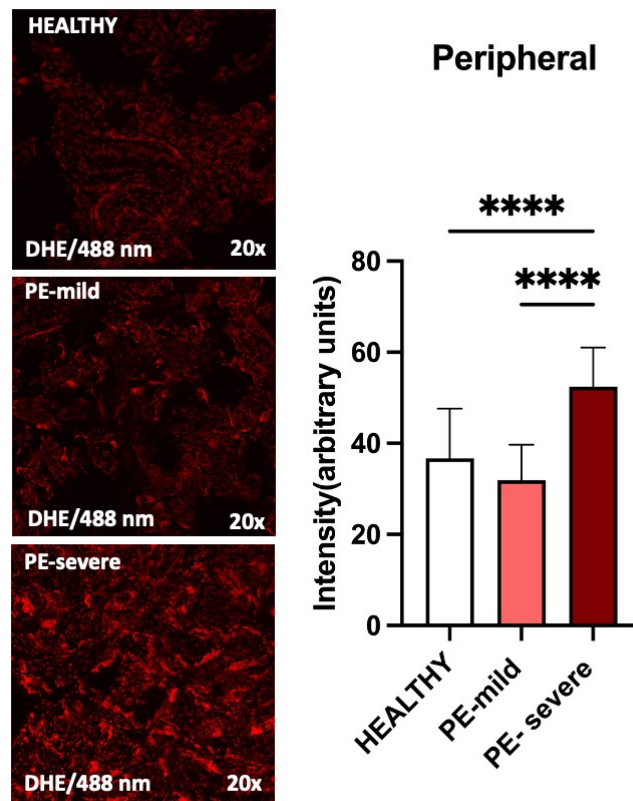


Figure 5. Assessment of placental oxidative stress (DHE staining) in peripheral samples, healthy vs mild and severe PE pregnancies. Data is presented as mean±SEM.

VI. Contributions regarding placental monoamine oxidase in preeclampsia

MAO presence was evidenced in frozen sections as mRNA transcript in placental samples from healthy pregnancies (n=3), and severe PE (n=3). Both monoamine oxidase isoforms, MAO A (Fig. 6) and B (Fig. 7), were detected in placenta, being significantly upregulated in severe PE.

No difference in MAO gene expression was found in central and peripheral placental areas in both healthy and preeclamptic pregnancies (Fig. 6 and Fig. 7).

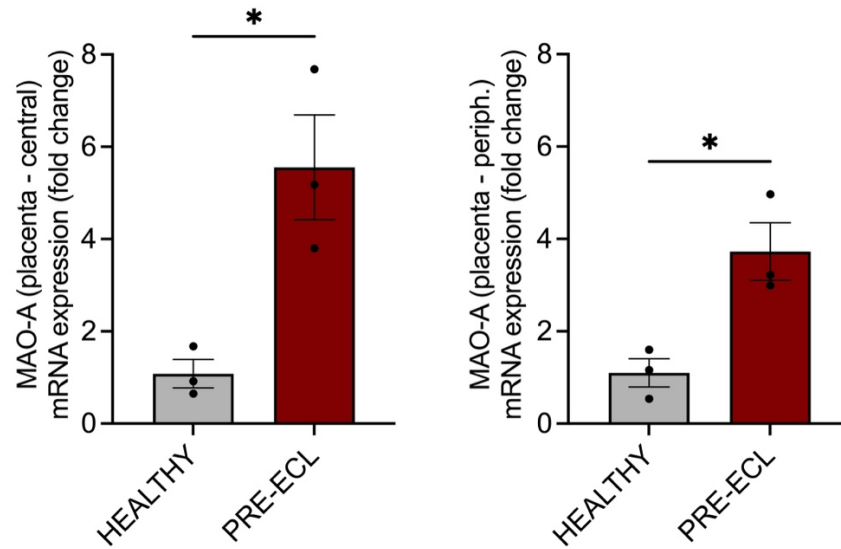


Figure 6. Assessment of placental expression of and MAO-A isoform in central and peripheral samples from healthy vs severe PE pregnancies. Data is presented as mean±SEM

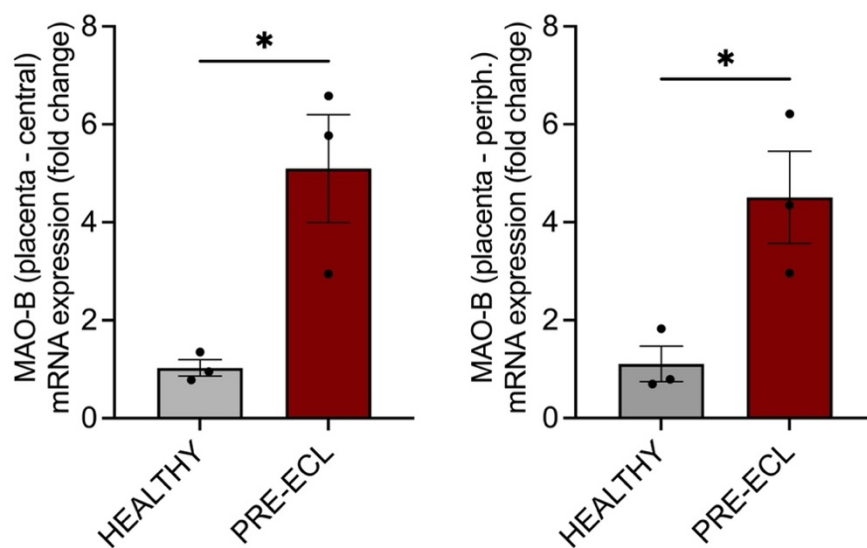


Figure 7. Assessment of placental expression of and MAO-B isoform in central and peripheral samples from healthy vs severe PE pregnancies. Data is presented as mean±SEM

VII. Contributions to the assessment of systemic oxidative stress in preeclampsia

The plasma oxidative stress was evaluated in preeclampsia (mild, n=8 and severe, n=2 forms) vs healthy pregnancies (n=10) by measuring the plasma derivatives of reactive

oxygen metabolites (dROM) and the antioxidant status (BAP test) using the Free Radical Analytical System 4 equipment (Diacron, Grosseto, Italy).

All the pregnant women showed higher levels of oxidative stress, the values being almost double in the healthy ones, specifically, dROMs (Fig. 8A) values were 1012 ± 103.3 CARR U in the control group as compared to 546.8 ± 32.22 CARR U in the PE group.

At variance, the antioxidant capacity of the plasma (BAP test, Fig. 8B) was decreased in the control group (1666 ± 151.3 $\mu\text{mol/L}$) and significantly increased in preeclamptic pregnancies (4651 ± 479.9 $\mu\text{mol/L}$). Interestingly, both dROMs and BAP test values were similar in mild and severe forms of preeclampsia.

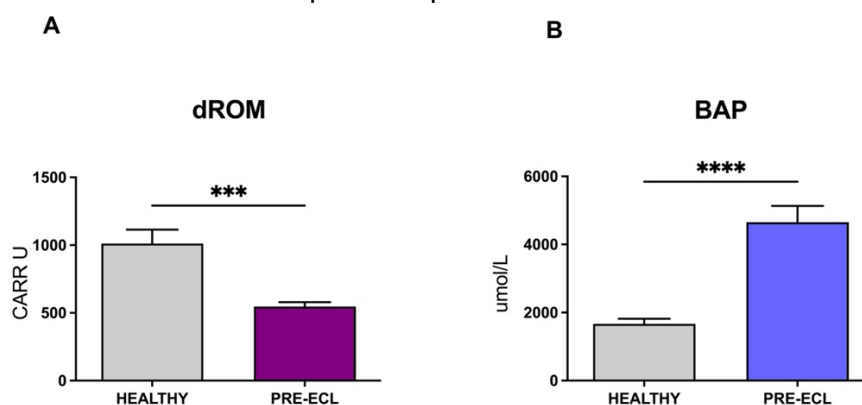


Figure 8. A) Concentrations of derivatives of reactive oxygen metabolites (d-ROMs) and (B) biological antioxidant potential (BAP) in plasma collected from healthy pregnancies and pregnancies complicated by preeclampsia. Data is presented as mean \pm SEM.

VIII. CONCLUSIONS

The results reported in this doctoral thesis present original information that contribute to the further elucidation of the complex pathophysiology of mitochondrial dysfunction and oxidative stress in preeclampsia.

The research was successful in achieving all the aims set out at the beginning of the doctoral studies.

The final conclusions are as follows:

1. Preeclampsia is associated with platelet mitochondrial dysfunction evidenced as a decrease in all the respiratory parameters: basal, maximal active and maximal uncoupled respiration.
2. The decrease in maximal active respiration mainly affected complex I of the respiratory chain, suggesting its early impairment in the setting of preeclampsia.
3. Monitoring oxygen consumption in circulating blood cells by the high-resolution respirometry allows an accurate assessment of respiratory mitochondrial function and may represent a surrogate peripheral biomarker of bioenergetic organ dysfunction in pathological conditions.
4. Platelet respiratory mitochondrial dysfunction is a potential biomarker of placental mitochondrial stress in the setting of preeclampsia.

5. Preeclampsia associated with fetal growth restriction elicited placental mitochondrial respiratory dysfunction characterized by a global decrease in complex I-supported respiration and a partial decrease in complex II-supported respiration.
6. Preeclamptic pregnancies associated with normal weight fetuses showed an increase placental mitochondria respiration supported by both complexes I and II of the electron transport system.
7. Placental respiratory mitochondrial dysfunction appears to be a feature of preeclampsia associated with fetal growth restriction, whereas in preeclampsia without growth restriction, an adaptation of respiratory function apparently occurs, observations that require further investigation to elucidate the pathomechanisms.
8. Placental oxidative stress is present only in severe forms of preeclampsia.
9. No regional differences, in central and peripheral placental areas, were noticed in either healthy or preeclamptic pregnancies.
10. Monoamine oxidase, an enzyme with 2 isoforms, MAO-A and B, is upregulated both at gene and protein level in placental samples, harvested from severe preeclampsia.
11. Healthy pregnancies, but not the mild forms of preeclampsia, were associated with an increase in systemic oxidative stress assessed by the quantification of the derivatives of the reactive oxygen metabolites.
12. The antioxidant capacity of plasma was higher in preeclamptic pregnancies as compared to healthy pregnancies.

Original contributions:

- Investigation in international premiere of platelet mitochondrial respiratory function by high-resolution respirometry in preeclampsia vs healthy pregnancies.
- Investigation in national *premiere* of placental mitochondrial respiratory function in preeclampsia and the demonstration of its impairment in relation to the fetal growth restriction.
- Investigation of placental ROS generation in central and peripheral samples in confocal microscopy (DHE stain) in preeclampsia.
- Investigation in national *premiere* of MAO contribution to placental oxidative stress in severe preeclampsia

Future research directions:

- Evaluation of placental and platelet respiratory mitochondrial dysfunction in patients with preeclampsia correlated to the presence or absence of the fetal distress.
- Investigation of the mechanisms underlying the increase in placental respiratory function in preeclampsia with normal weight fetuses.
- Evaluation of temporal platelet mitochondrial respiratory function in the evolution of preeclampsia.
- Since complex I appears to be primarily affected in preeclampsia, investigation of molecules capable to sustain mitochondrial respiratory function starting from complex II is warranted.
- Evaluation of placental and platelet respiratory mitochondrial dysfunction in other gestational disorders such as diabetes and obesity.
- Assessment of monoamine oxidase-contribution to placental oxidative stress in a larger cohort of patients with mild and severe preeclampsia.

IX. LIST OF PUBLICATIONS

1. **Bîcă AM**, Aburel OM, Avram VF, Lelcu T, Lința AV, Chiriac DV, Mocanu AG, Bernad E, Borza C, Craina ML, Popa ZL, Muntean DM, Crețu OM. *Impairment of mitochondrial respiration in platelets and placentas: a pilot study in preeclamptic pregnancies*. **Mol Cell Biochem**. 2022 Apr 7. doi: 10.1007/s11010-022-04415-2. **ISI journal IF= 3.396**
2. **Bîcă AM**, Sturza A, Iancu I, Mocanu AG, Bernad E, Chiriac DV, Borza C, Craina ML, Popa ZL, Muntean DM, Crețu OM. *Placental oxidative stress and monoamine oxidase expression are increased in severe preeclampsia: a pilot study*. **Mol Cell Biochem**. 2022 June 01. doi: 10.1007/s11010-022-04499. **ISI journal IF= 3.396**
3. **Bîcă, AM**, Anechitei AI, Lelcu T, Lința AV, Chiriac DV, Mocanu AG, Bernad E, Popa ZL, Craina ML, Muntean DM, Borza C, Crețu OM. *Assessment of the systemic oxidative stress in preeclampsia*. **Serbian Journal of Experimental and Clinical Research**, vol.23, no.1, 2022, pp.45-50. <https://doi.org/10.2478/sjecr-2022-0010> **BDI journal**