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OF MEDICINE AND PHARMACY TIMIŞOARA

FACULTY OF MEDICINE
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LELCU THEIA

ABSTRACT

CONSIDERATIONS REGARDING MITOCHONDRIAL DYSFUNCTION IN HEMATOLOGICAL MALIGNANCIES

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TABLE OF CONTENTS

Key words: hematological malignancies, children, adults, platelet mitochondrial respiration, high-resolution respirometry, cell-permeable succinate (NV118), eugenol, bromelain, human keratinocytes bioenergetics, extracellular flux analyzer.

AIM AND OBJECTIVES OF THE RESEARCH

Cancer incidence, including hematological malignancies, are increasing worldwide despite tremendous research efforts in the discovery of novel biomarkers, diagnostic methods and novel therapeutical agents. Leukemias are characterized by an abnormal proliferation of malignant precursors in the bone marrow and can be classified according to onset and proliferating cell type in acute and chronic lymphoid or myeloid types.

In the past decades, mitochondrial dysfunction has emerged as a central pathomechanism and therapeutic target in various chronic diseases, including all types of malignancies. Mitochondrial respiratory dysfunction is nowadays assessed by measuring oxygen consumption in tissue samples and cells with the aid of two state-of-art research equipments, the oxygraph-2K (Oroboros Instr.) and the extracellular flux analyzer (Seahorse Bioscience, Agilent), the latter allowing the measurement of both oxygen consumption and glycolysis.

In recent years, an increasing number of research groups were preoccupied by the assessment of peripheral blood cells bioenergetics, and in particular of platelet respiratory function, suggesting that alteration of mitochondrial function in circulating cells can serve as putative biomarker of mitochondrial dys/function in distant tissues/organs in various pathologies.

Literature data regarding platelet mitochondrial dysfunction in hematological malignancies is scarce. One study reported the impairment of mitochondrial function in platelets isolated from the peripheral blood of adults diagnosed with hematological malignancies undergoing treatment with standard chemotherapy. There are no data in the literature regarding platelet respiration in children with hematological malignancies.

Mitochondrial dysfunction in the setting of malignancies is further aggravated by side effects of classical anti-neoplastic drugs on both normal and cancerous cells. Recent years have witnessed the emergence of novel pharmacological compounds, called mitochondriotropic agents aimed at restoring mitochondrial function that has been impaired by genetical or acquired disease. Among these agents, the class of permeable succinate prodrugs, have been initially investigated as potential candidates for mitochondrial diseases with complex I defects. Recent studies reported that a cell permeable succinate prodrug, NV118, alleviated mitochondrial respiration in the setting of mitochondrial dysfunction induced by different drugs, such as paracetamol, metformin, statins and amiodarone.

Despite major achievements in improving the survival and prognosis of patients diagnosed with neoplastic diseases, including hematological malignancies, a significant percentage of patients have multi-organ involvement, including skin lesions. In the search of therapeutic alternatives, in the cancer literature a plethora of natural compounds and their effects on mitochondrial metabolism and respiration have been investigated.

Accordingly, phytochemicals have been widely used in association with classic systemic chemotherapy in various types of malignancies. Skin deposition of circulating tumor cells causes infiltrative lesions known as leukemia cutis, more frequently in acute myeloid leukemia. Several phytochemicals have been investigated, including at mitochondrial level, for their beneficial, anti-cancer effects on various malignant cell lines; however, whether they interfere

with the mitochondrial bioenergetic function of normal human keratinocytes cells when has been less investigated.

Bromelain (BR) derived from the pineapple stem, *Ananas comosus,* with several medical effects, including: antibacterial, antifungal, anti-aggregant, fibrinolytic, anti-inflammatory and anti-neoplastic effects.

Eugenol (EUG) or 4-allyl-2-methoxyphenol is commonly found in clove oil and presents several beneficial properties: anti-viral, anti-bacterial, anti-fungal, anti-helminthic, antioxidant, anti-inflammatory and anti-neoplastic. Eugenol displays a hormetic behavior, with lower doses being protective, while higher doses are toxic and induce cell death in malignant cells.

The research objectives were as follows:

1. Characterization of the age-dependency of mitochondrial respiration in platelets isolated from healthy children.

2. Characterization of platelet mitochondrial respiration at the onset of pediatric acute lymphoblastic leukemia (ALL).

3. Assessment of the effect of a novel permeable succinate compound on platelet mitochondrial respiration in the remission phase of pediatric ALL.

4. Characterization of mitochondrial dysfunction in platelets sampled from adult patients newly diagnosed with acute myeloid leukemia (AML) and chronic lymphoid leukemia (CLL).

5. Assessment of the effects of two natural compounds, eugenol and bromelain, on bioenergetics of HaCaT cells.

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 " , where the experiments were carried out, and are also in line with the research strategy of our university.

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

I. Characterization of the age-dependency of mitochondrial respiration in platelets isolated from children with no hematological malignancies.

The objective of the first study of the present PhD thesis was to assess the age dependency of mitochondrial respiration in platelets isolated from healthy children. The study group consisted of pediatric patients aged between 10-17 years, in whom pathologies that may influence mitochondrial respiration, namely infectious, inflammatory and oncological pathologies, were excluded. The study group was further divided into 3 study groups consisting of children aged 10-11 years, 13-14 years and 16-17 years respectively. The results of this pilot study revealed the presence of an age related decrease in platelet mitochondrial respiration. Regarding basal mitochondrial respiration, a significant increase was reported in children aged between 10-11 years old when compared to the older pediatric groups. (Figure 1A, 6 $p<0.0001$). However, no significant differences regarding basal respiration were detected between the 13-14 year old and 16-17 year old groups. In case of active respiration dependent on complex I, no significant differences were noted between the three pediatric groups (Figure 1B). However, the maximal active respiration driven by both complexes exhibited a significant decrease with age when comparing the younger group and the two older groups (Figure 1C, $p<0.01$). No significant differences were noted in the maximal active respiration of both complexes in case of the two older groups. As per the non-phosphorylating respiration dependent on both complexes, a significant decrease with age was observed (Figure 1D, $p<0.01$). Likewise, in the case of maximum non-coupled respiration dependent on complex I and II, a significant age-dependent decrease was observed, with significant differences being observed between the 10-11 year old group and the other two groups, 13-14 and 16-17 years old, respectively (Figure 1E, $p<0.01$). As for complex II-dependent maximum non-coupled respiration, a significant decrease with age was also noted (Figure 1F, $p<0.05$).

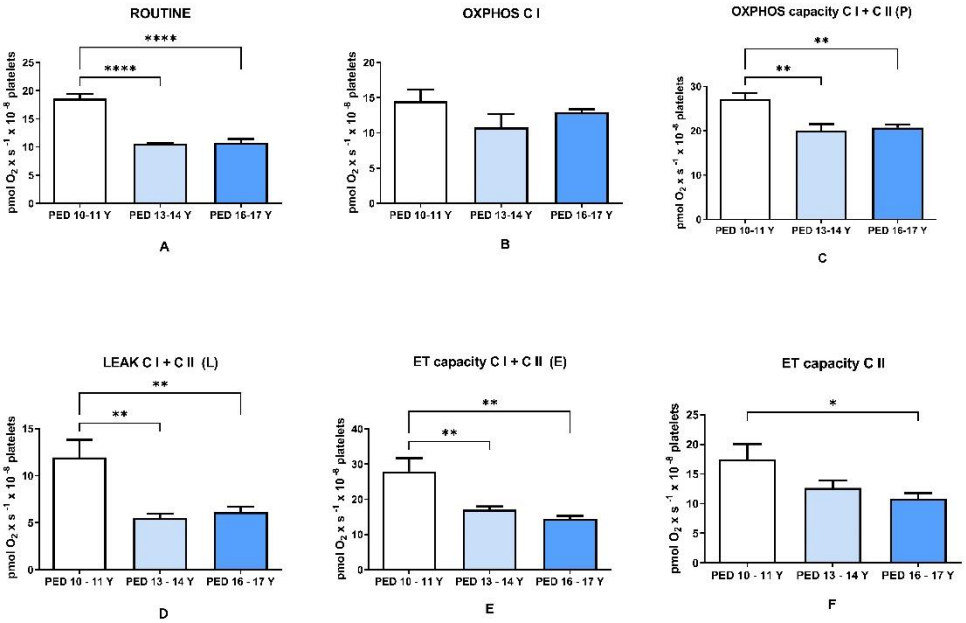

Figure 1. Age dependency of platelet mitochondrial respiration in healthy children. Data are presented as pmol O2 x s -1 x 10-8/platelets.

II. Characterization of platelet mitochondrial respiration at the onset of pediatric ALL.

The second study aimed to characterize changes in platelet mitochondrial respiration in a pilot group of pediatric patients newly diagnosed with acute lymphoblastic leukemia (ALL) prior to chemotherapy initiation, compared to age matched healthy children.

A significant decrease in basal respiration was observed in children newly diagnosed with ALL when compared to age-matched healthy children (Figure 2A, $p<0.0001$).

Complex I-dependent active respiration was significantly increased at ALL onset (Figure 2B, $p<0.05$). However, in terms of active respiration dependent on both complexes, no significant differences between the two pediatric groups were observed (Figure 2C).

At the onset of pediatric ALL, a significant decrease in maximum non-coupled respiration driven by complex I and II was detected (Figure 2D, $p<0.05$).

Furthermore, in pediatric patients newly diagnosed with ALL, a significant decrease of complex II supported respiration was noted (Figure 2E, $p<0.05$).

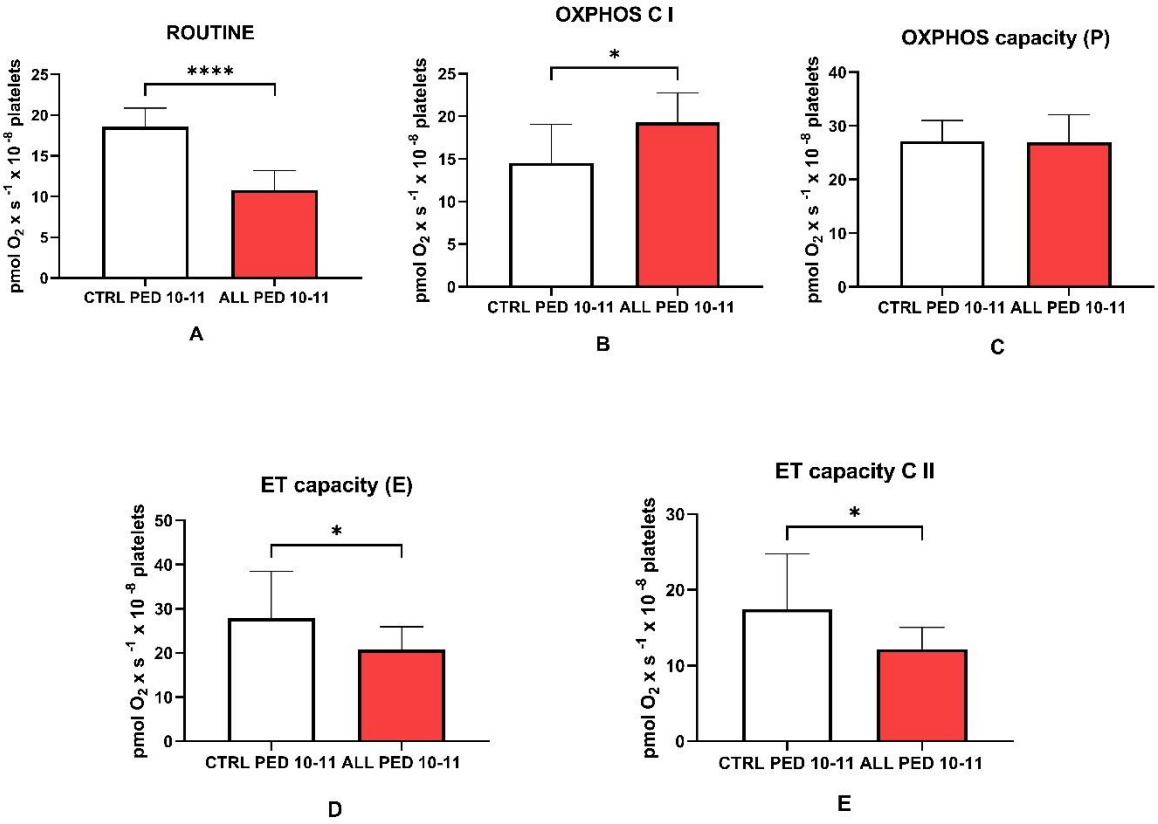

Figure 2. Platelet mitochondrial respiration in pediatric patients newly diagnosed with ALL. Data are presented as pmol O2 x s -1 x 10-8/platelets.

III. Assessment of the effect of a novel permeable succinate compound on platelet mitochondrial respiration in the remission phase of pediatric ALL.

The third study aimed to evaluate platelet mitochondrial respiration during the remission phase of pediatric ALL and the effect of a novel cell-permeable succinate compound, NV118 (diacetoxymethyl succinate), on platelet mitochondrial respiration in a pediatric patient in whom chemotherapy was concluded and disease remission confirmed.

Comparative analysis of platelet mitochondrial respiration rates detected in the same patient at disease onset and in remission was performed and in the presence of NV118 (500μM) and DMSO (control) during ALL remission.

During the remission phase of pediatric ALL, an improvement in the active complex I and II dependent respiration was observed. Furthermore, an increase in maximal non-coupled complex I and II supported respiration was also detected.

Regarding the effect of the permeable succinate compound, NV118, on platelet mitochondrial respiration in the remission phase of ALL, a major improvement in all the studied respiratory rates was reported (Figure 3). Mainly, NV118 elicited a 75.96% increase in basal respiration. Moreover, in the presence of this cell permeable succinate prodrug, an increase of 96.11% was noted regarding the non-phosphorylating respiration. As per the maximal non-coupled respiration, NV118 induced an increase of 187%. Lastly, NV118 determined an increase of 242% of the maximal non-coupled respiration driven by complex II.

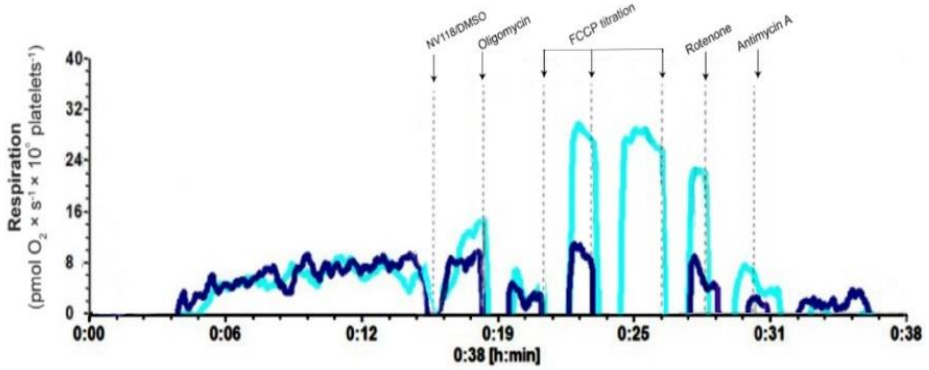

Figure 3. An overlay of the HRR tracings in the presence of DMSO (dark blue) vs NV118 (light blue).

IV. Characterization of mitochondrial dysfunction in platelets sampled from adult patients newly diagnosed with AML and CLL.

This study aimed to characterize mitochondrial respiration in platelets isolated from two adult patients newly diagnosed with HM prior to treatment initiation, and to compare platelet mitochondrial respiratory rates of these patients to those of a healthy adult volunteer.

In the newly diagnosed adult patient with AML, basal mitochondrial respiration was increased when compared to the basal respiration detected in case of the adult volunteer.

No significant differences were noted regarding the maximal active respiration driven by complex I of the AML patient and the healthy control.

However, the maximal active respiration dependent on both complexes, exhibited a decrease in case of the AML patient.

An increase in the non-phosphorylating complex I and II dependent respiration was also observed in the setting of AML.

Furthermore, a decrease in the maximal non-coupled complex I and II supported respiration was observed in the case of the AML patient. Lastly, the same decreasing trend was revealed in the case of the maximal non-coupled complex II supported respiration. As for coupling efficiency, a clear decrease was noted in the mitochondria of the AML patient.

In case of the adult patient newly diagnosed with CLL, active respiration driven by both mitochondrial complexes, exhibited a clear decrease in platelets sampled from the CLL patient when compared to the healthy control. A major descending trend was observed regarding the maximum non-coupled respiration of both complexes as well as for the maximum non-coupled respiration of complex II of platelets isolated from the CLL patient.

V. Assessment of the effects of two natural compounds, eugenol and bromelain, on bioenergetics of HaCaT cells.

In this last study we aimed at characterizing the effects of two phytochemicals with known anticancer effects on mitochondrial bioenergetics of human immortalized keratinocytes (HaCaT) cells, by evaluating two parameters, oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) respectively.

The time-dependent effects of eugenol (EUG, 50 μM) on the bioenergetic profile of HaCaT cells was assessed at 24, 48 and 72 hours. The HaCaT cells for this experimental protocol were divided into 3 groups:

• Group 1 (CTRL, untreated cells);

• Group 2 (cells treated with DMSO 50 μM, which was used to prepare the stock solution of EUG);

• Group 3 (cells treated with 50 μM EUG).

At 24 hours of treatment with eugenol (EUG), no significant difference was found regarding both the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). At 48 hours of treatment with eugenol (EUG), a significant decrease of both OCR (Figure 4A) and ECAR (Figure 4B) was detected, $p<0.0001$. Furthermore, the 72-hour treatment with eugenol of HaCaT cells led to the lowest values of OCR (Figure 4C) and ECAR (Figure 4D), respectively, $p<0.0001$, aspect suggestive of a toxic effect on the normal skin cells.

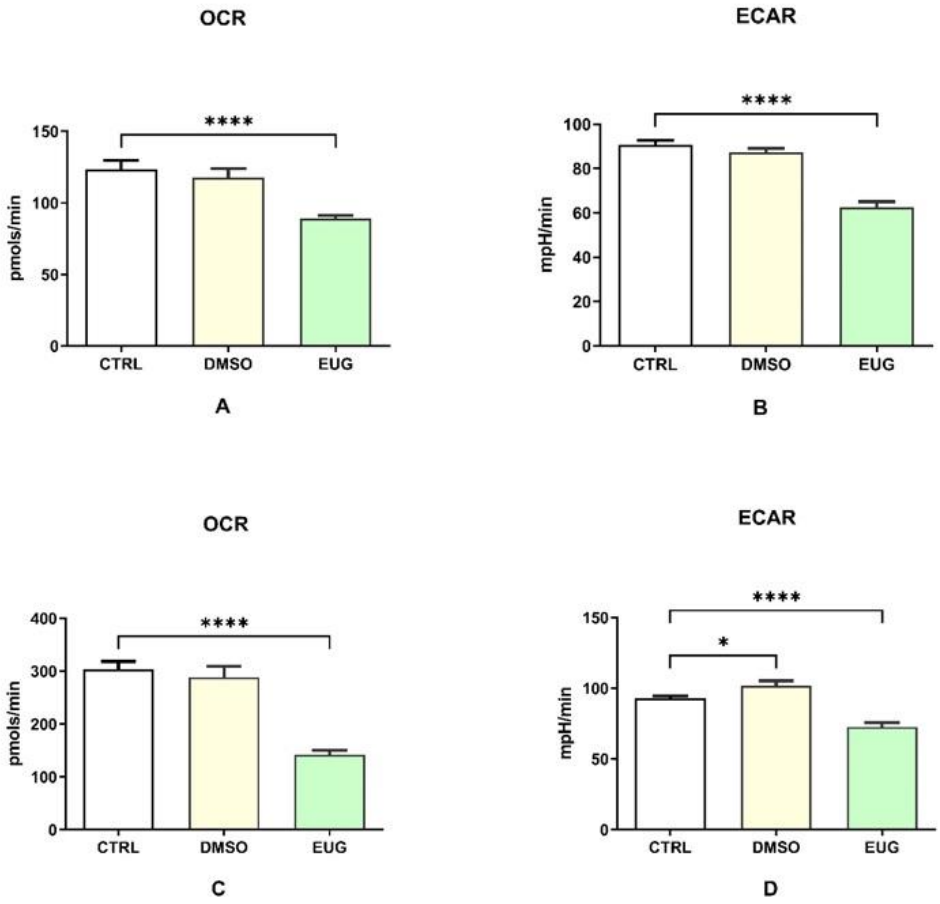

Figure 4. The time-dependent effects of eugenol (EUG, 50 µM) on the bioenergetic profile of HaCaT cells 24, 48 and 72 hours. Data are presented as mean ± SD.

In addition, the dose-dependent effects of bromelain (BR) in increasing concentrations (0.25 µg/mL, 2.5 µg/mL and 25 µg/mL) on bioenergetics and glycolysis on HaCaT cells at 24 hours and the effects were compared to the ones of methylene blue (MB, 0.1 µM).

The HaCaT keratinocytes from this experimental protocol were divided into 5 groups:

• Group 1: control group (untreatead cells);

• Group 2: cells treated with bromelain in a concentration of 0.25 µg/mL (BR 0.25);

• Group 3: cells treated with bromelain in a concentration of 2.5 µg/mL (BR 2.5);

• Group 4: cells treated with bromelain in a concentration of 25 µg/mL (BR 25);

• Group 5: cells treated with methylene blue in a concentration of 0.1 µM (MB).

Bromelain elicited a determined a steady increase of the OCR, mainly the highest dose of bromelain induced the highest OCR (Figure 5A).

In accordance, dose titration of bromelain determined a steady decrease in ECAR, similarly, the highest dose of bromelain induced the lowest ECAR (Figure 5B). As for MB (0.1 µM), no effect on of OCR and a decrease in ECAR, without any statistical significance were found.

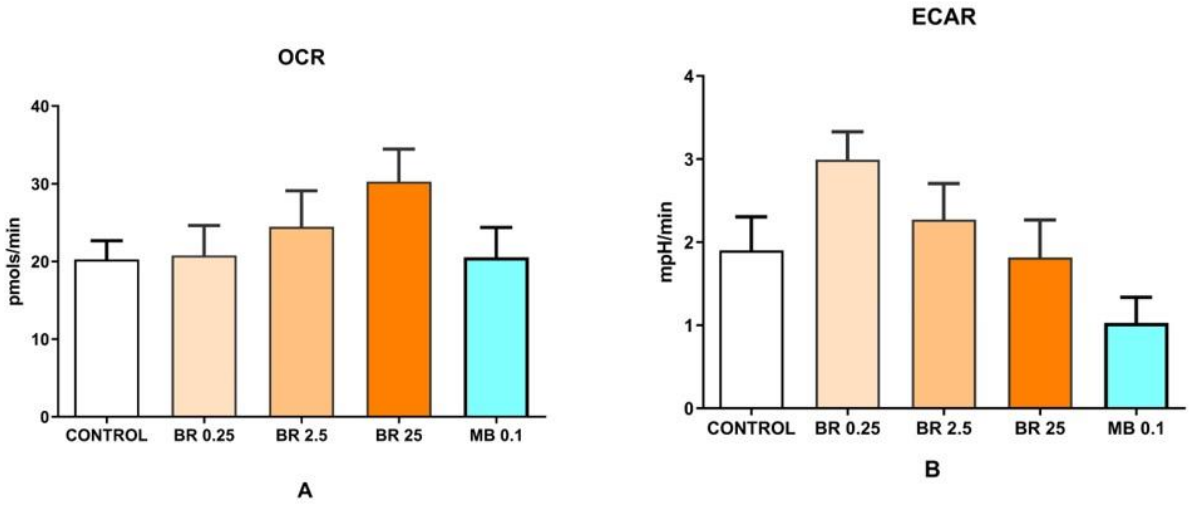

Figure 5. The dose-dependent effects of bromelain on the bioenergetic profile of HaCaT cells at 24 hours. Data are presented as mean ± SD.

VI. CONCLUSIONS (summary)

1. Age-dependent changes of platelet mitochondrial respiration occur in healthy children.

2. The changes consisted in a significant decrease in complex I and II supported active and maximal non-coupled respiration with the age.

3. Changes in complex II-supported respiration appear to be age-dependent since no significant changes were found for CI-related respiratory parameters.

4. Platelet mitochondrial dysfunction is present at the onset of pediatric acute lymphoblastic leukemia, prior to treatment initiation.

5. The impairment of platelet respiration in children with ALL was characterized by an increase in the active CI-supported respiration while a significant decrease in the maximal non-coupled respiration for both complexes occurred.

6. Platelet mitochondrial respiration is improved in the remission phase of pediatric ALL, in particular with respect of maximal non-coupled respiration CI and II-supported.

7. NV118, a permeable succinate compound, was able to significantly improve all respiratory parameters in the remission phase of pediatric acute lymphoblastic leukemia.

8. Platelet respiratory dysfunction was also found in adults newly diagnosed with an acute and a chronic hematological malignancy, before therapeutic intervention.

9. The impairment of platelet respiration in adults with acute myeloid leukemia and chronic lymphoid leukemia were comparable to the ones found in children, with a significant decrease in oxidative capacity and the maximal non-coupled respiration for both complexes.

10. Eugenol elicited a time-dependent decrease of the bioenergetic markers, the oxidative phophosphorylation and anaerobic glycolysis, with a maximum effect at 72 hours of treatment.

11. Bromelain elicited a dose-dependent change in the bioenergetical profile of human keratinocytes, with an increase in oxidative phophosphorylation and a corresponding decrease in anaerobic glycolysis.

12. Methylene blue elicited a comparable, yet milder response of the HaCaT cells as bromelain with respect to the decrease in anaerobic glycolysis.

VII. ORIGINAL CONTRIBUTIONS

• *In premiere* characterization of the age-dependency of mitochondrial respiration in platelets isolated from healthy children.

• *In premiere* characterization of platelet mitochondrial respiration at the onset of pediatric ALL.

• Assessment of the effect of a novel permeable succinate compound on platelet mitochondrial respiration in the remission phase of pediatric ALL.

• Characterization of mitochondrial respiratory dysfunction in platelets sampled from adult patients newly diagnosed with AML and CLL.

• Assessment of the effects of two natural compounds, eugenol and bromelain, on bioenergetics of HaCaT cells.

VIII. FUTURE RESEARCH DIRECTIONS

❖ Investigation of the occurence of age-dependent changes of mitochondrial respiration in peripheral blood cells (platelets and PBMCs) sampled from a significant pediatric population.

❖ Characterization of the effects of permeable succinate on bioenergetic profile of several malignant cell lines.

❖ Assessment of the effects of bromelain on the bioenergetic profile of malignant skin cells alone and in association with the conventional drugs prescribed in the therapy of leukemia cutis view possible interferences.

❖ Assessment of the bioenergetical effect of phytochemical-based nano-formulations/nanostructures that target mitochondria developed by the collaborators from the Faculty of Pharmacy and/or from abroad.

❖ Assessment of the age-dependency of ROS production/antioxidant defense in pediatric population with and without HM.

IX. SCIENTIFIC PUBLICATIONS

1. Theia Lelcu, Anca M. Bîna, Maria D. Dănilă, Calin M. Popoiu, Oana M. Aburel, Smaranda T Arghirescu, Claudia Borza, Danina M. Muntean, *Assessment of Platelet Mitochondrial Respiration in a Pediatric Population: A Pilot Study in Children With Acute Lymphoblastic Leukemia*, **Children** 2021; https://www.mdpi.com/2227-9067/8/12/1196 **ISI journal IF 2.863**

2. Oana M. Aburel, Ioana Z. Pavel, Maria D. Dănilă, **Theia Lelcu**, Alexandra Roi, Rodica Lighezan, Danina M. Muntean, Laura C. Rusu, *Pleiotropic Effects of Eugenol: The Good, the Bad, and the Unknown*, **Oxidative Medicine and Cellular Longevity** 2021, Article ID 3165159, https://www.hindawi.com/journals/omcl/2021/3165159/ **ISI journal IF 6.543**

3. Theia Lelcu, Anca-Mihaela Bînă, Vlad-Florian Avram , Smaranda-Teodora Arghirescu, Claudia Borza, Mirela-Danina Muntean, *Permeable Succinate Improved Platelet Mitochondrial Respiration in Pediatric Acute Lymphoblastic Leukemia in Remission – Case Report***, Scripta Medica** 2022; DOI:10.5937/scriptamed53-37038, **BDI journal**