

**„VICTOR BABEȘ” UNIVERSITY  
OF MEDICINE AND PHARMACY TIMIȘOARA**

**FACULTY OF MEDICINE  
Department of Functional Sciences**

**AVRAM VLAD-FLORIAN**



**ABSTRACT**

**MITOCHONDRIAL DYSFUNCTION ASSOCIATED WITH  
STATIN THERAPY: NOVEL PATHOGENIC INSIGHTS AND  
THERAPEUTIC TARGETS**

Scientific coordinator:

**PROF. MUNTEAN MIRELA-DANINA, MD, PhD**

Joint scientific coordinator:

**PROF. TIMAR ROMULUS-ZORIN, MD, PhD**

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**Key words:** mitochondrial dysfunction, platelets, HepG2 cell line, high resolution respirometry, statins, cell permeable succinate (NV118), diabetes mellitus.

## I. AIM AND OBJECTIVES OF THE RESEARCH

Diabetes mellitus represents a group of metabolic disease that has currently reached pandemic proportions. In 2019 the International Diabetes Federation estimated that approximately half a billion people suffered from this disease worldwide, with predictions of an increase by 50% in the following 25 years. It is important to note that all the predictions by the International Diabetes Federation regarding the future increase in diabetes prevalence, have been underestimated to date. The severity of this disease is closely tied to the doubling of patient cardiovascular risk as a consequence of the associated atherogenic dyslipidemia, characterized by the formation of small, dense LDL-cholesterol particles.

Statins represent the elective lipid-lowering medication and the current guidelines recommend in diabetic patients the treatment with potent statins in the highest recommended or tolerated dose. Statin adherence is lacking in some patients, due to the risk that in high doses side effects might appear; the most frequently reported side-effects are statin-associated muscle symptoms, and the most severe (but fortunately rare) is rhabdomyolysis.

Mitochondrial dysfunction is currently unanimously accepted as the central mechanism in the pathogenesis of diabetes mellitus (as insulin production in pancreatic  $\beta$ -cells is strictly dependent on mitochondrial function) and its complications and at the same time, is implicated in the generation of statin-induced side effects. One of the major mitochondrial functions which is impaired is mitochondrial respiration with the reduction of NADH-linked respiration (mitochondrial complex I dysfunction) being commonly incriminated.

Mitochondrial dysfunction of peripheral blood cells, mainly platelets, has become a veritable biomarker in the last decade, as mirror of organ-related mitochondrial dysfunction in various acute or chronic pathologies.

The aim of the current doctoral thesis was to assess the changes induced by statins on mitochondrial respiration, either in acute administration in platelets isolated from healthy volunteers, or in platelets harvested from patients with type 2 diabetes undergoing chronic statin therapy, using the high-resolution respirometry technique. In both cases, a cell permeable succinate (NV118) compound, generously provided by Prof. Eskil Elmer from Lund University, Sweden was used as innovative pharmacological approach to improve mitochondrial respiration.

**The research objectives** were as follows:

- 1. Characterization of the effects of acute administration of different statins on mitochondrial respiration of platelets harvested from healthy volunteers and in a cell line of hepatic origin.**
- 2. Evaluation of the cell permeable succinate potential to alleviate the acute mitochondrial dysfunction induced by the exposure to increasing concentrations of statins in human platelets.**
- 3. Characterization of the mitochondrial respiratory dysfunction in platelets isolated from patients with type 2 diabetes chronically-treated with statins.**
- 4. Evaluation of the capability of the cell permeable succinate to improve mitochondrial respiration in platelets isolated from patients with type 2 diabetes chronically treated with statins, as a novel potential method to alleviate the mitochondrial dysfunction in clinical setting.**

## II. Characterization of statin-induced acute mitochondrial dysfunction in human platelets

The effects of three different statins: simvastatin, atorvastatin and cerivastatin (the latter being withdrawn from the market due to myotoxicity/rhabdomyolysis) on mitochondrial respiration of platelets isolated from healthy volunteers were evaluated *in vitro* by means of high-resolution respirometry. At first, statins were titrated in increasing concentrations vs. the corresponding volume of solvent (DMSO) and by determining oxygen consumption one can determine that concentration-dependent alterations in mitochondrial respiration appear in the presence of statins (Fig 1A). To localize the defect caused by the three statins at the level of the electron transport system (ETS), in another set of experiments, platelets were permeabilized (with digitonin) and exposed to 3 different concentrations (40, 80 and 160  $\mu$ M) of statins (vs DMSO as control). All three statins decrease OXPHOS capacity (Fig.1B), ET capacity (ET - electron transport, Fig.1C), mainly through the reduction of NADH-linked respiration - complex I (CI) of the respiratory system (Fig.1D). Simvastatin (but not compared to atorvastatin and cerivastatin) further decreased succinate-dependent mitochondrial respiration, by inhibiting respiratory complex II (CII) (Fig.1E).

Apart from the direct inhibition of the electron transport system (of oxidative phosphorylation, noted OXPHOS), atorvastatin and cerivastatin (but not simvastatin) also interfered with ATP generation by increasing non-phosphorylating respiration (noted as LEAK, Fig.1F). In order to yet again confirm (indirectly) the interference in ATP production 2 ratios were calculated for the three statins: P-L control efficiency (Fig.1G) and E-L coupling efficiency (Fig.1H), both being significantly reduced in the presence of atorvastatin and cerivastatin even for the lowest concentration used (40  $\mu$ M).

## III. Characterization of the alteration in NADH-linked respiration induced by acute statin exposure in human platelets

In permeabilized mitochondria (with alamethicin) we further analyzed the response of NADH-dehydrogenase in the presence of the three statins (in the concentration of 160  $\mu$ M) and in the enzyme's specific substrate (NADH), before and after statin addition. As presented in Fig. 2A, the addition of simvastatin and atorvastatin reduced oxygen consumption from  $18,7 \pm 1,1$  to  $10,9 \pm 3,3$  and from  $18,7 \pm 1,1$  to  $12,5 \pm 1,4$  respectively; surprisingly, the addition of cerivastatin slightly increased mitochondrial oxygen consumption as compared to the solvent ( $19,8 \pm 2,5$  vs.  $18,7 \pm 1,1$ ). To confirm this effect a ratio of the oxygen consumption after each of the two NADH additions was calculated, the result being lower for the first two statins and paradoxically higher for cerivastatin (Fig.2B).

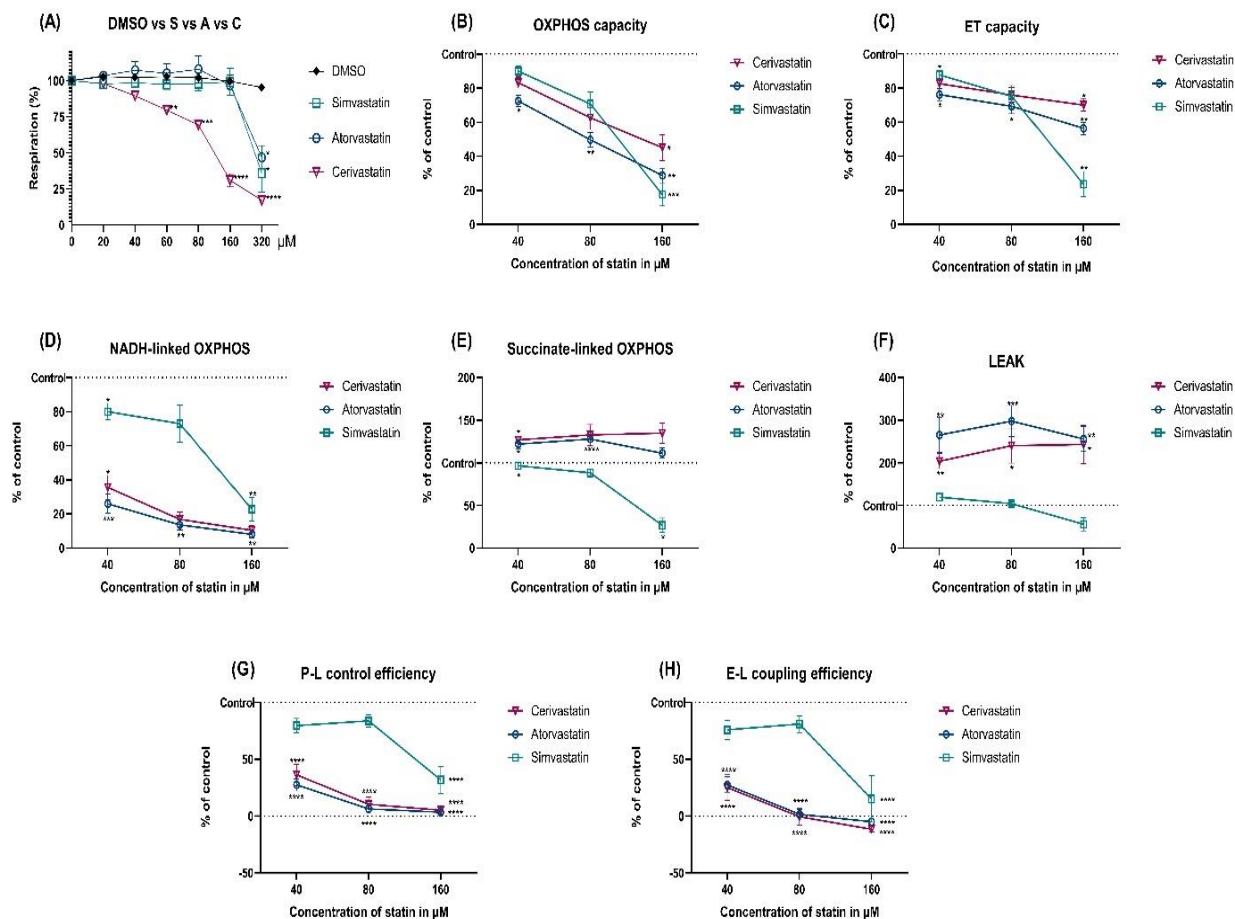


Figure 1. The acute effect of statins on the mitochondrial respiration of human platelets.

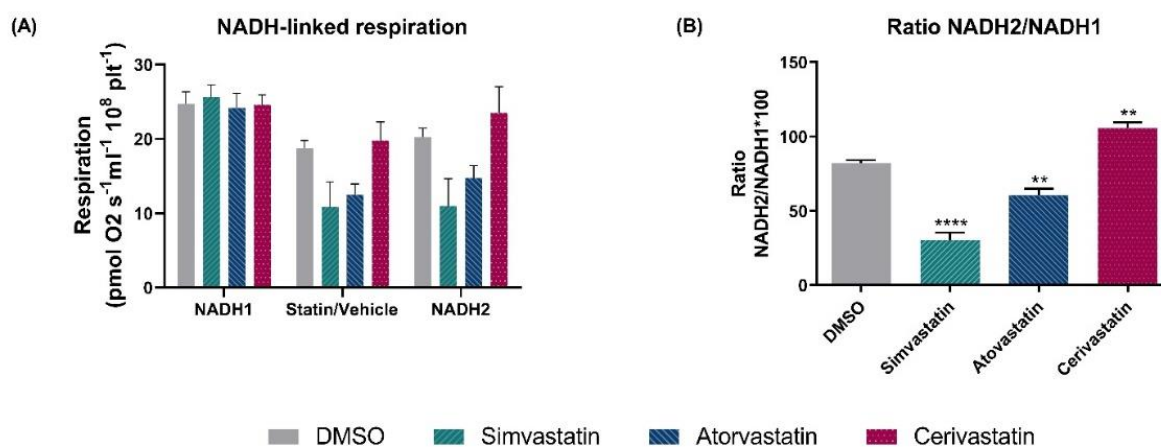


Figure 2. The acute effect of statins on NADH-linked mitochondrial respiration.

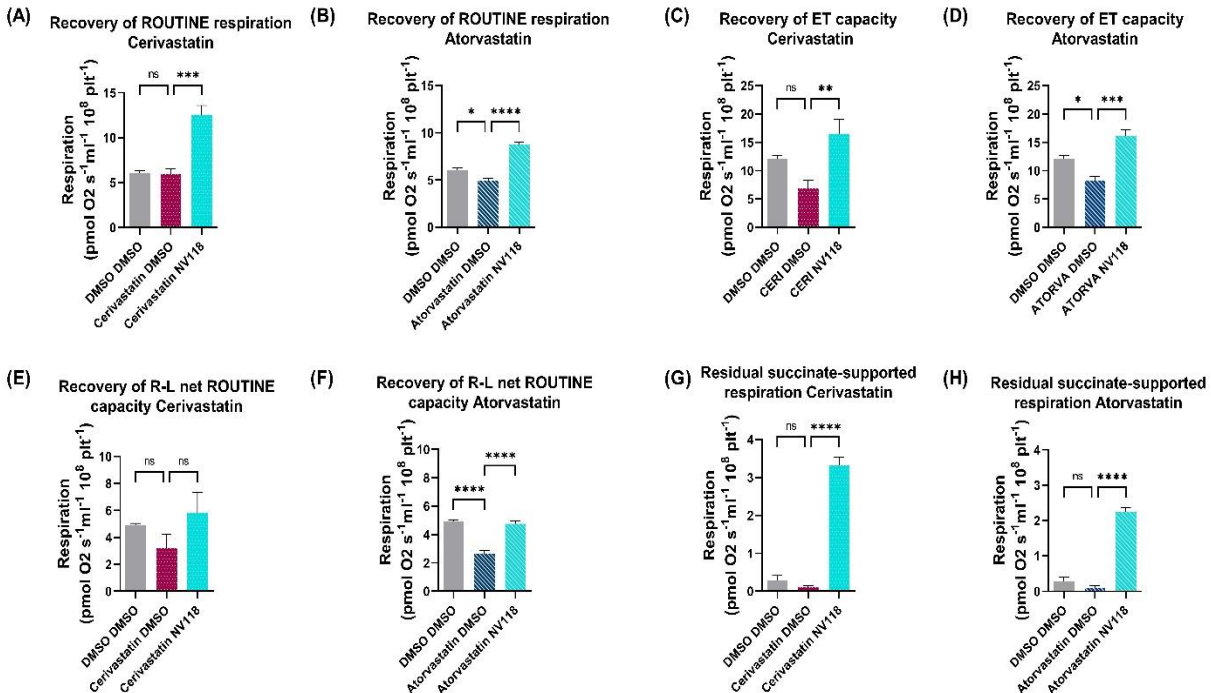
#### IV. A cell permeable succinate (NV118) corrected the statin-induced acute mitochondrial dysfunction in human platelets

Since two of the statins (atorvastatin and cerivastatin) determined mitochondrial dysfunction by significantly reducing CI-dependent respiration, we hypothesized that a rational therapeutic approach would be to apply a compound that would sustain the CII-supported respiration. The substrate for CII is succinate, which under normal circumstances cannot travel through intact cell membranes; for this reason we used a prodrug known as a cell permeable succinate (NV118), produced by a research team in Lund.

To test the effects of NV118, platelets were firstly exposed to a toxic concentration (80  $\mu$ M) of cerivastatin and atorvastatin, respectively; afterwards, oxygen consumption was measured in the presence of NV118 or its solvent (DMSO); as an extra measure of control, platelets were exposed only to DMSO (instead of the statin).

NV118 increased ROUTINE oxygen consumption (Fig.3A și B) for both statins. It also increased ET capacity (Fig.3C și D) for both cerivastatin and atorvastatin. In order to determine if this increase in oxygen consumption is indeed efficient, net ROUTINE respiration was calculated (by subtracting non-phosphorylating respiration); NV118 increased ATP generating respiration for platelets exposed to cerivastatin ( $3,21 \pm 1,02$  vs.  $5,82 \pm 1,51$ ) and for those exposed to atorvastatin ( $2,62 \pm 0,25$  vs.  $4,76 \pm 0,19$ ,  $p < 0,0001$ ) - Fig.3E and F.

To prove that NV118 functions by increasing succinate-supported respiration, oxygen consumption was measured after the inhibition of complex I with rotenone. In the case of cerivastatin exposure (Fig.3G), NV118 increased oxygen consumption after complex I inhibition from  $0,1 \pm 0,04$  to  $3,31 \pm 0,21$  ( $p < 0,0001$ ), and in the case of atorvastatin inhibition (Fig.3H), from  $0,1 \pm 0,06$  to  $2,25 \pm 0,12$  ( $p < 0,0001$ ).

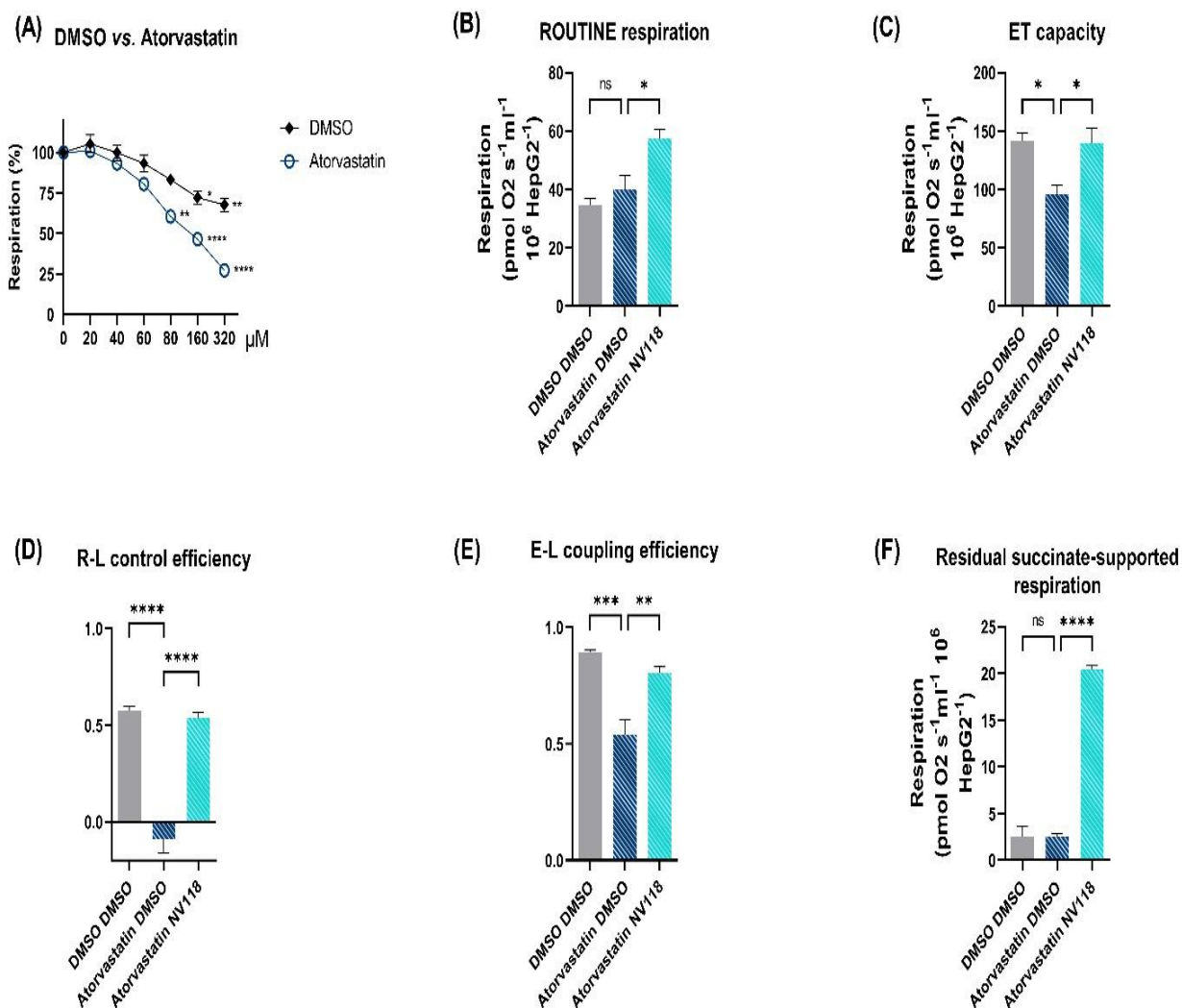


**Figure 3. Cell permeable succinate corrects mitochondrial dysfunction induced by acute statin exposure in intact human platelets.**

## V. A cell permeable succinate (NV118) corrected the statin-induced acute mitochondrial dysfunction in HepG2 cells

In order to investigate the possibility to recapitulate the effects of NV118 in other cells, the experiments carried out on human platelets were repeated in HepG2 cells (a human malignant hepatocyte cell line) using atorvastatin.

In Fig.4A we see the same type of concentration-dependent inhibition of mitochondrial respiration. Similarly to the results obtained in human platelets, NV118 improved ROUTINE respiration (Fig.4B), ET capacity (Fig.4C), in the context of a more efficiently coupled electron transport system (Fig.4D) by increasing succinate-supported respiration (Fig.4F) in the HepG2 cell line.



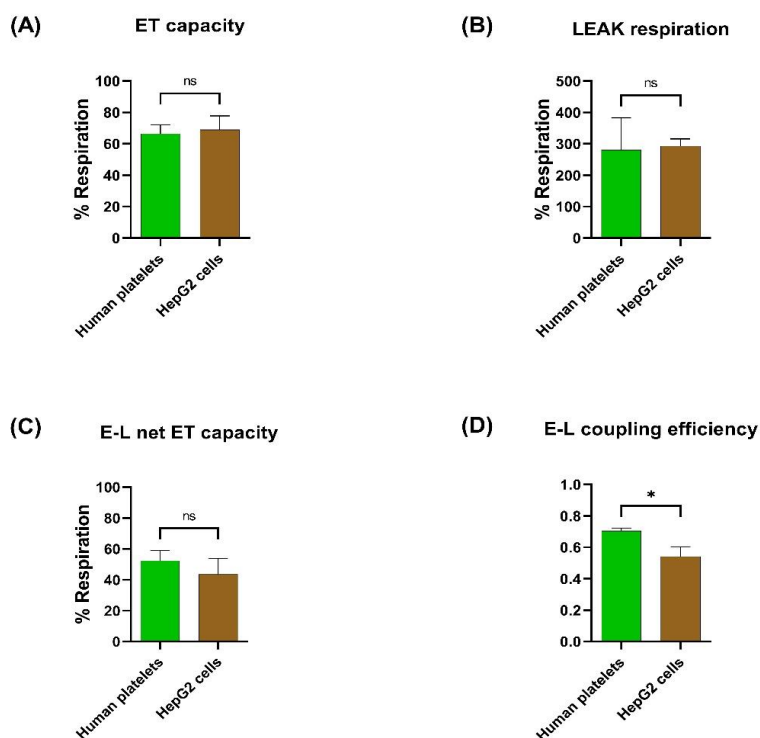
**Figure 4. Cell permeable succinate corrects mitochondrial dysfunction induced by acute statin exposure in HepG2 cells.**



## VI. Statin-induced mitochondrial dysfunction is comparable in human platelets vs. HepG2 cells

Since the experimental protocol for the evaluation of NV118 in the presence of atorvastatin (80 $\mu$ M) was done in both platelets and HepG2 cells, we compared the effects of atorvastatin on mitochondrial oxygen consumption in the two cell types and the results were expressed as a percentage of control.

In Fig. 5 we can observe that atorvastatin elicited a comparable reduction of ET capacity (Fig.5A), net ET capacity (Fig.5C), as well as relatively similar effect on non-phosphorylating respiration (Fig.5B) in both cell types. In Fig.5D atorvastatin is shown to determine a decrease in E-L coupling efficiency which is more pronounced in HepG2 cells than it is in platelets ( $p < 0,05$ ).

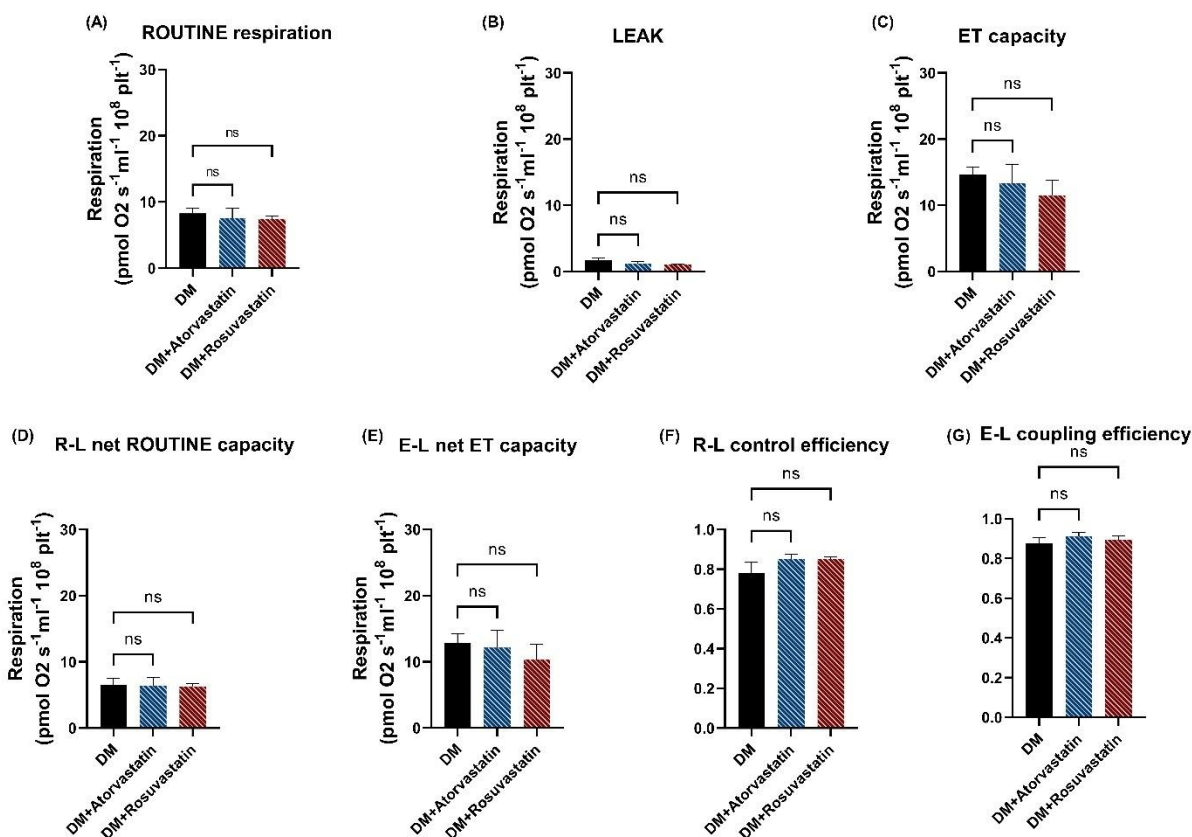


**Figura 5. Comparison of statin-induced mitochondrial dysfunction between human platelets vs. HepG2 cells.**

## VII. Characterization of the effects of chronic statin treatment on mitochondrial respiration of *intact* platelets isolated from patients with type 2 diabetes

Using the results of the study of the effects of acute statin exposure on platelets isolated from healthy volunteers as starting point, in the second study we evaluated the effects of chronic statin treatment on platelet mitochondrial respiration. To this aim patients with type 2 diabetes were divided into three subgroups: treated with atorvastatin (DM+Atorvastatin), treated with rosuvastatin (DM+Rosuvastatin) and with no statin treatment (DM). Mitochondrial respiration parameters were

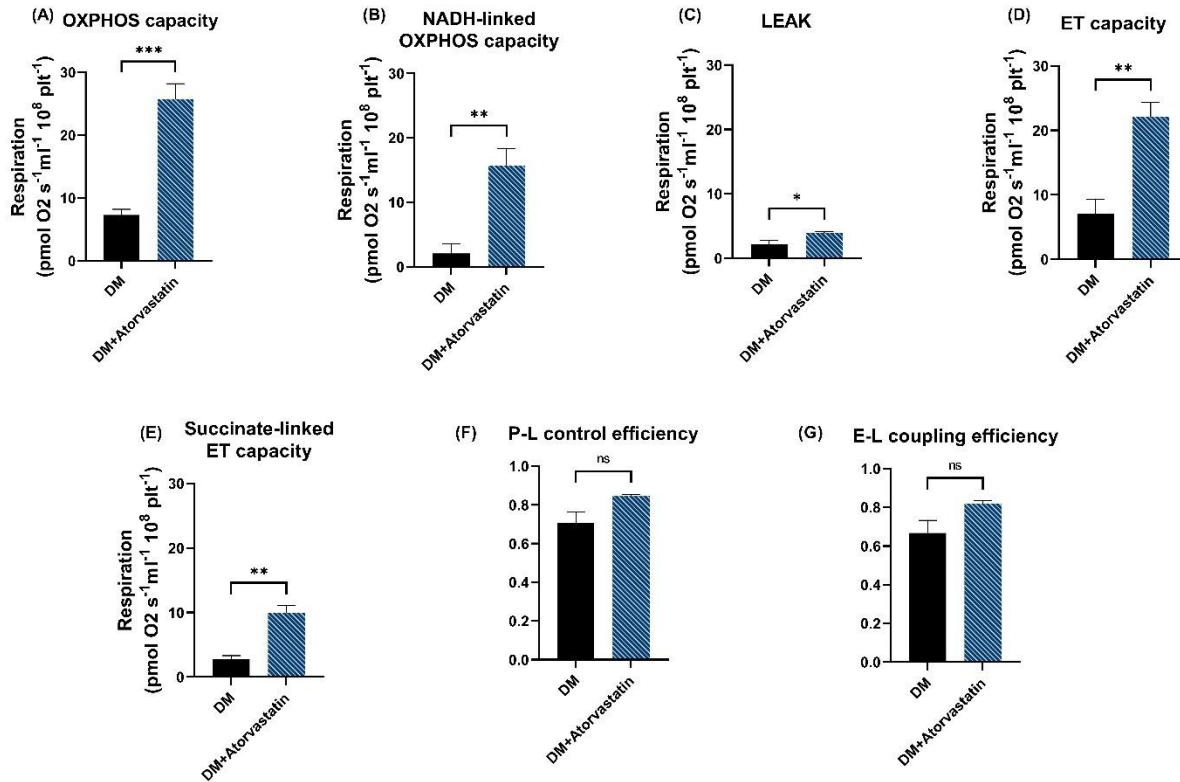
evaluated for each of these patients in intact platelets. Chronic treatment with either atorvastatin or rosuvastatin did not modify these parameters in intact platelets (Fig. 6).



**Figure 6. The effects of chronic statin treatment on the mitochondrial respiration of intact platelets isolated from patients with type 2 diabetes.**

### VIII. Characterization of the effects of chronic atorvastatin treatment on the mitochondrial respiration of *permeabilized* platelets isolated from patients with type 2 diabetes

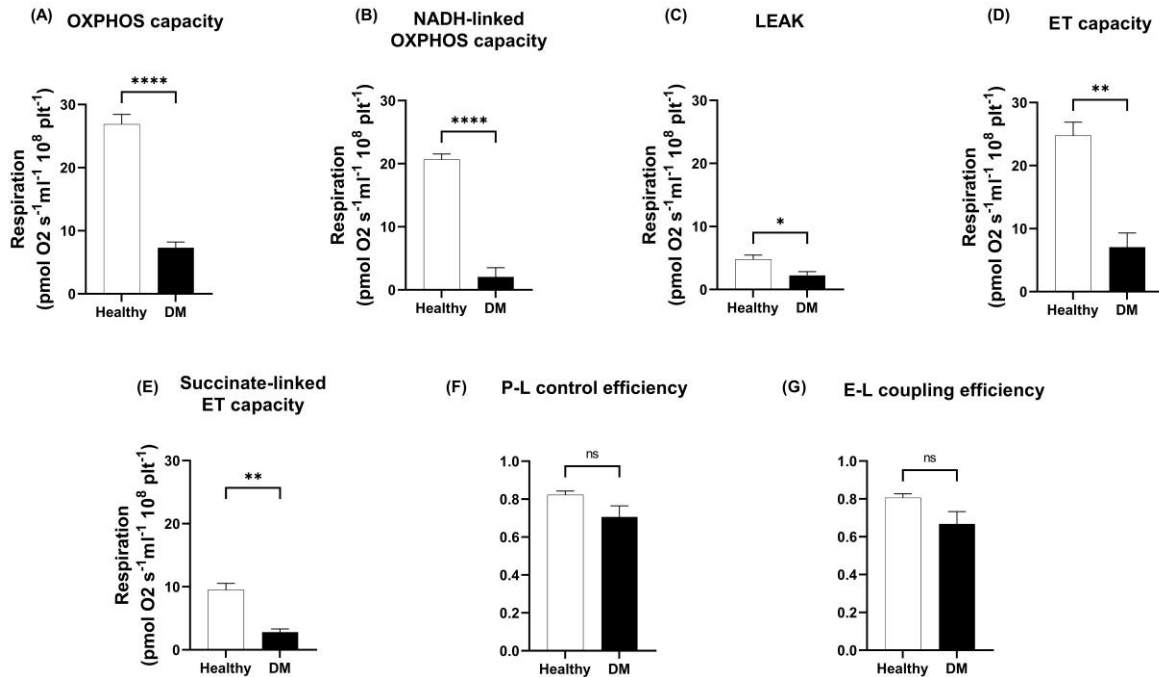
In order to identify any potential modifications of mitochondrial respiration, if statins were to have free access to the mitochondria (possible only for lipophilic statins such as atorvastatin in chronic administration) the same respiratory parameters were measured in permeabilized platelets harvested from patients in the DM and DM+Atorvastatin groups (Fig.7). Interestingly, patients treated with atorvastatin presented an increase in OXPHOS capacity (Fig.7A,  $p<0,001$ ), in NADH-linked OXPHOS capacity (Fig.7B,  $p<0,01$ ), in ET capacity (Fig.7D,  $p<0,01$ ) and in succinate-linked ET capacity (Fig.7E,  $p<0,01$ ). Although it must be mentioned that there was also an increase in LEAK respiration (Fig. 7C,  $p<0,05$ ), however this does not appear to affect the production of ATP as the efficiency of the electron transport system as unaffected (Fig.7F și G).



**Figure 7. The effects of chronic atorvastatin treatment on the mitochondrial respiration of permeabilized platelets isolated from patients with type 2 diabetes**

## IX. Characterizing platelet mitochondrial dysfunction in patients with type 2 diabetes mellitus

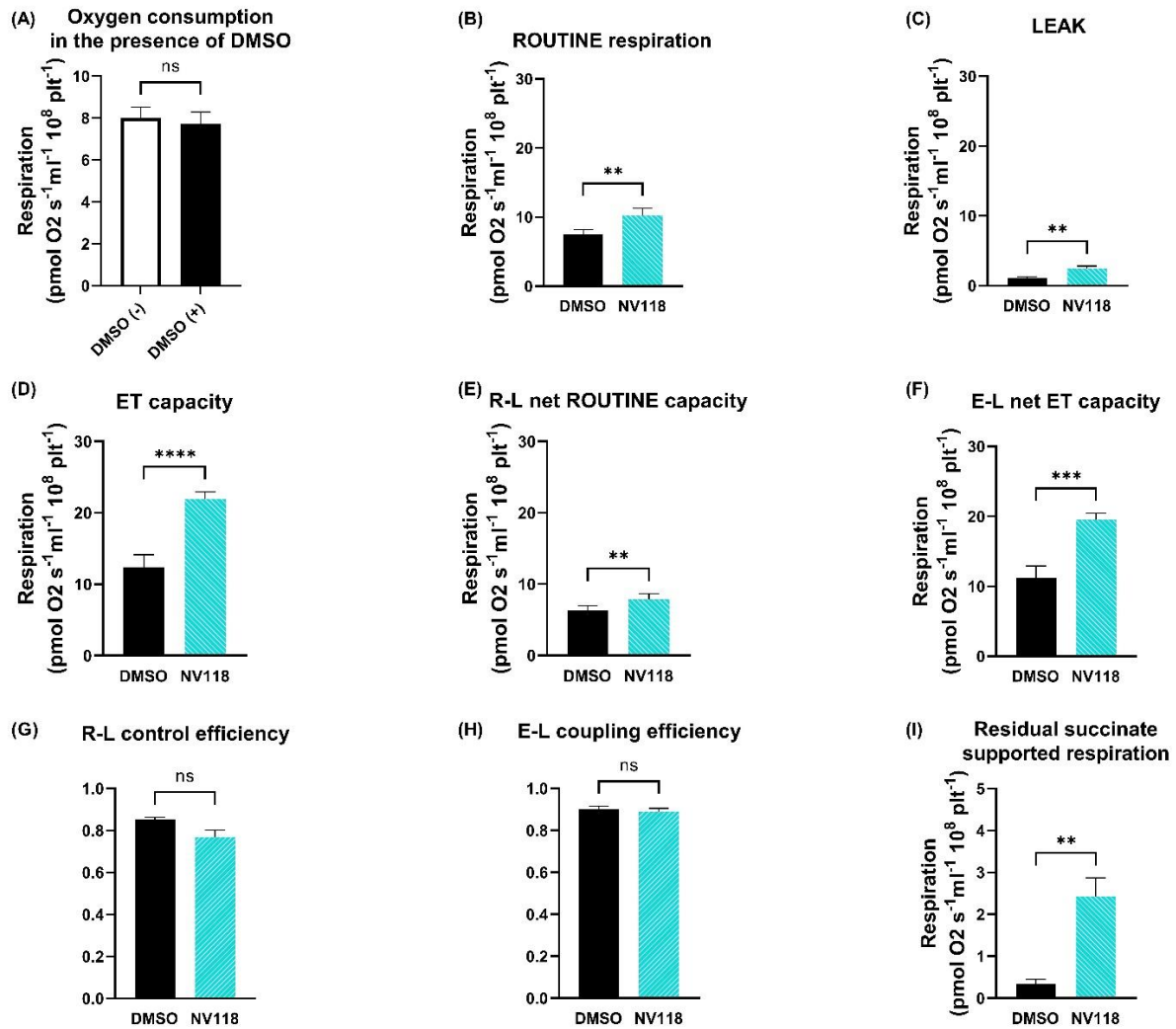
In a supplementary set of experiments we compared platelets mitochondrial respiration parameters in the DM group to those of a group of healthy volunteers. As can be seen in Fig. 8, patients with diabetes present with a global dysfunction of mitochondrial respiration characterized by the reduction of all mitochondrial respiration parameters: OXPHOS capacity ( $p < 0.0001$ ), NADH-linked OXPHOS capacity ( $p < 0.0001$ ), LEAK respiration ( $p < 0.05$ ), ET capacity ( $p < 0.01$ ) and succinate-linked ET capacity ( $p < 0.01$ ), with a tendency of reduction in P-L control efficiency (Fig.8F) and in E-L coupling efficiency, respectively (Fig.8G).



**Figure 8. Characterization of the mitochondrial dysfunction in permeabilized platelets isolated from patients with type 2 diabetes mellitus.**

## **X. Improvement of platelet mitochondrial respiratory parameters in patients with type 2 diabetes under chronic statin therapy using a cell permeable succinate (NV118)**

To the aim of evaluating the cell permeable succinate as a means to treat the mitochondrial dysfunction of patients with type 2 diabetes, treated with statins, the oxygen consumption of intact platelets, harvested from these patients, was measured in the presence of NV118 or that of its solvent (DMSO). In Fig. 9A DMSO, the solvent used as a control in the experiments, did not affect platelet mitochondrial respiration. NV118 increased ROUTINE respiration (Fig.9B,  $p < 0.01$ ), net ROUTINE respiration (Fig.9E,  $p < 0.01$ ), ET capacity (Fig.9D,  $p < 0.0001$ ) and net ET capacity (Fig.9F,  $p < 0.001$ ). Although an increase in LEAK respiration we seen in the presence of NV118 (Fig.9C,  $p < 0.01$ ), the efficiency of the electron transport system was unaffected. (Fig.9G and H), suggesting that overall, NV118 increases ATP generating mitochondrial respiration.



**Figure 9. Cell permeable succinate (NV118) improves mitochondrial respiration parameters in intact platelets isolated from patients with type 2 diabetes mellitus, under chronic statin treatment.**

## **XI. CONCLUSIONS (summary)**

1. Simvastatin, atorvastatin and cerivastatin, during acute *in vitro* administration, determine a reduction of mitochondrial complex I-supported respiration in platelets isolated from healthy volunteers.
2. Simvastatin also determines a reduction of mitochondrial complex II supported respiration in platelets isolated from healthy volunteers.
3. All three statins, during acute *in vitro* administration, determine a concentration-dependent (in micromolar doses) reduction of total electron transport capacity and of the efficiency of the phosphorylation process.
4. Statins alter mitochondrial bioenergetics through two distinct mechanisms: the inhibition of electron transport along the electron transport chain and the uncoupling of the phosphorylation process (the increase of non-phosphorylating respiration).
5. At a variance from atorvastatin and simvastatin, cerivastatin (withdrawn from the market), does not exert a direct inhibition of the respiratory complex I (NADH-dehydrogenase).
6. Atorvastatin induces a similar profile of inhibition of mitochondrial respiration in both intact platelets and in a malignant human hepatocyte cell line, HepG2.
7. A cell permeable succinate prodrug (NV118), may be used to compensate the reduction of complex I-dependent mitochondrial respiration induced by statins.
8. Chronic treatment with atorvastatin or rosuvastatin does not affect mitochondrial respiration in intact platelets isolated from patients with type 2 diabetes mellitus.
9. Chronic treatment with atorvastatin increases mitochondrial respiration in permeabilized platelets isolated from patients with type 2 diabetes mellitus.
10. Patients with type 2 diabetes mellitus present mitochondrial dysfunction in platelets through the reduction of both complex I and complex II-dependent respiration.
11. Novel cell permeable succinate compounds may be used to improve mitochondrial bioenergetics in patients with diabetes mellitus through the increase in complex II- supported mitochondrial respiration.

## **XII. ORIGINAL CONTRIBUTIONS**

- Characterization of the dose-dependent mitochondrial dysfunction induced by simvastatin-, cerivastatin and atorvastatin in human platelets.
- Characterization of atorvastatin-induced mitochondrial dysfunction in HepG2 cells.
- Characterization of NADH-linked mitochondrial respiration of permeabilized platelet mitochondrial in the presence of simvastatin, atorvastatin and cerivastatin.
- Bypassing statin-induced mitochondrial dysfunction using a cell-permeable succinate in both human platelets and HepG2 cells.
- Assessment of the effects on mitochondrial respiration of chronic treatment with atorvastatin and rosuvastatin in human platelets from patients with type 2 diabetes.
- Characterization of mitochondrial respiration of platelets isolated from patients with type 2 diabetes.
- Improving the bioenergetics of platelet mitochondria in a group of patients with type 2 diabetes under statin therapy using a cell-permeable succinate.

## **XIII. FUTURE RESEARCH DIRECTIONS**

- Characterization the effects on mitochondrial respiration of acute exposure to statins in platelets harvested from patients with other pathologies that implicate mitochondrial dysfunction.
- Characterize the effects on mitochondrial respiration of acute exposure to statins in combination with other drugs reported to induce mitochondrial dysfunction.
- Evaluate the potential of the cell-permeable succinate to improve mitochondrial bioenergetics in other chronic diseases.
- Evaluate the effects of other cell-permeable succinates compounds.
- Evaluate other novel molecules that could improve mitochondrial bioenergetics in type 2 diabetes.

#### XIV. SCIENTIFIC PUBLICATIONS

1. **Avram V.F.**, Chamkha I., Åsander-Frostner E., Ehinger J.K., Timar R. Z., Hansson M.J., Muntean D.M., Elmér E. *Cell-Permeable Succinate Rescues Mitochondrial Respiration in Cellular Models of Statin Toxicity*. **International Journal of Molecular Science** 2021, 22(1), 424. **FI = 4.556**
2. **Avram V.F.**, Bîna A.M., Sima A., Aburel O.M. Sturza A., Burlacu O., Timar R.Z, Muntean D.M., Elmér E., Crețu O.M. *Improvement of Platelet Respiration by Cell-Permeable Succinate in Diabetic Patients Treated with Statins*. **Life** 2021, 11(4), 288. **FI = 2.991**