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FACULTY OF MEDICINE
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DOCTORAL THESIS

CONTRIBUTIONS TO THE IDENTIFICATION OF NOVEL CARDIO- AND VASCULOPROTECTIVE PHARMACOLOGICAL APPROACHES IN AN ANIMAL MODEL AND IN PATIENTS UNDERGOING CARDIAC SURGERY

A B S T R A C T

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1. AIM AND OBJECTIVES OF THE RESEARCH

Oxidative stress is both a pathomechanism and therapeutic target in metabolic pathologies. However, the complexity of the sources and types of reactive oxygen species (ROS) and their contribution to different pathologies is far from being elucidated.

Monoamine oxidase (MAO) with 2 isoforms, MAO-A and MAO-B, is a flavin dehydrogenase located at the outer mitochondrial membrane that is involved in the catabolism of neurotransmitters and biogenic/dietary amines, a process in which hydrogen peroxide (H_2O_2) is constantly produced as the main by-product. MAO inhibitors have been introduced in the therapy of neuropsychiatric diseases back to the middle of the last century. In the past 2 decades, the role of increased MAO activity/expression and the related oxidative stress in the pathogenesis of cardio-metabolic disorders has been unveiled.

Metformin is the first-line drug for the treatment of type 2 diabetes mellitus (T2DM). The American Diabetes Association recommends metformin as preventive therapy in patients with obesity, prediabetes, and people < 60 years with multiple risk factors. The drug has pleiotropic effects, including antioxidant ones, which have been widely investigated; however, the underlying cellular and molecular mechanisms are far from being fully elucidated. *There are no data in the literature regarding a putative interaction between metformin and MAO-related cardiovascular oxidative stress in the setting of cardiometabolic pathologies, in animal models or humans.*

Research over the past decades has identified the peripheral blood cells, mainly platelets, as a source of functional mitochondria, which can be repeatedly isolated via minimally invasive procedures. Dynamic assessment of mitochondrial dys/function of circulating cells can mirror the bioenergetic dys/function at organ level with potential roles in diagnosis, prognosis and therapeutic response. Moreover, there is currently a growing interest for the development of pharmacological compounds that target mitochondria and support mitochondrial respiration in a variety of pathological entities. Such compound is the permeable succinate, NV118. *There are no data in the literature regarding the pharmacological support of platelet mitochondrial respiration in patients with cardiovascular pathologies and indication of open heart surgery and cardiopulmonary bypass, respectively*.

The aim of this PhD thesis was to evaluate the cardio- and vasculoprotective effects of two pharmacological agents, a classic one (metformin) and novel one (permeable succinate - NV118), in an experimental model of obesity and prediabetes induced by hypercaloric diet in rats and in vascular and blood samples taken from patients with cardiovascular pathology and indication for open heart surgery with cardiopulmonary bypass, respectively.

The specific objectives were achieved within 3 original studies**:**

I. **The first study**, conducted in cardiac samples (ventricular tissue) harvested from rats with obesity induced by hypercaloric ”cafeteria”-type diet, was aimed at assessing the effects of metformin on MAO expression and the related oxidative stress in order to contribute to the elucidation of the cardioprotective mechanisms.

II. **The second study**, conducted in vascular samples (internal mammary artery rings) isolated from patients with coronary heart disease undergoing coronary artery bypass grafting (CABG) surgery, was purported to assess the effects of metformin on the expression of nitric oxide (NO) synthases and angiotensin 2 (Ang2)-mediated oxidative stress in order to further dissect the mechanisms of vasculoprotection.

III. **The third study**, conducted in platelets isolated from peripheral blood of patients diagnosed with coronary artery disease and/or valvular pathologies undergoing open heart surgery, was designed to evaluate the effects of a novel compound, permeable succinate (NV118) on mitochondrial respiration, prior and after cardiopulmonary bypass, respectively.

The topic of the thesis, investigation of the mechanisms of cardiovascular protection in animal and human models, is in line with the research directions of the Centre for Translational Research and Systems Medicine at the Department of Pathophysiology - Functional Sciences (where I was affiliated as a PhD student), as well as those stipulated in the Research Strategy of "Victor Babeș" University of Medicine and Pharmacy of Timișoara.

The results of the studies included in the Special part are briefly presented below:

2. Contributions to the elucidation of the *cardioprotective* effect of metformin: interaction with MAO in an experimental model of diet-induced obesity

In the past decade, a plethora of experimental and clinical data supported the existence of "obesity cardiomyopathy", a pathological entity associated with obesity (and metabolic syndrome) which develops independently of the presence of cardiac diseases (hypertension, coronary artery disease) that induce heart disease. As such, there is a need to identify the mechanisms underlying its occurrence, including the role of mitochondrial dysfunction in the setting of metabolic stress, and also pharmacological approaches capable of mitigating it. Metformin is currently used as the main antidiabetic drug in the treatment of T2DM, with beneficial effects also in obese and overweight patients.

The present study aimed to investigate *a novel cardioprotective mechanism* of metformin, namely its *interaction with MAO* in the setting of experimentally-induced obesity.

The study group included male Wistar rats (purchased from Cantacuzino Institute, Bucharest, Romania) fed for 24 weeks, starting at 8 weeks of age (obese group, n = 8), hypercaloric food (5.2 kcal/g) on the top of standard chow. The control non-obese group (n = 8) received laboratory standard chow (2.5 kcal/g).

Blood glucose and body weight were monitored as markers of the metabolic disorder.

Rat ventricular samples harvested from obese and non-obese rats were incubated (12 h, 37 °C in endothelial cell growth basal medium supplemented with 0.1% BSA in the presence or absence of metformin (M, 10 μM) or MAO inhibitors: clorgyline (Clorg, the irreversible MAO-A inhibitor) and selegiline (Seleg, the irreversible MAO-B inhibitor, 10 μM). Subsequently, the tissue was used for ROS measurements (spectrophotometry and confocal microscopy) and MAO expression (gene and protein) assays (RT-PCR and immune fluorescence).

In the ventricular samples harvested from the control (CTL) group, MAO-A isoform was more abundant as compared to MAO-B. Twenty-four weeks of obesogenic diet elicited an increased expression of both MAO isoforms in the murine cardiac tissue (Fig. 1).

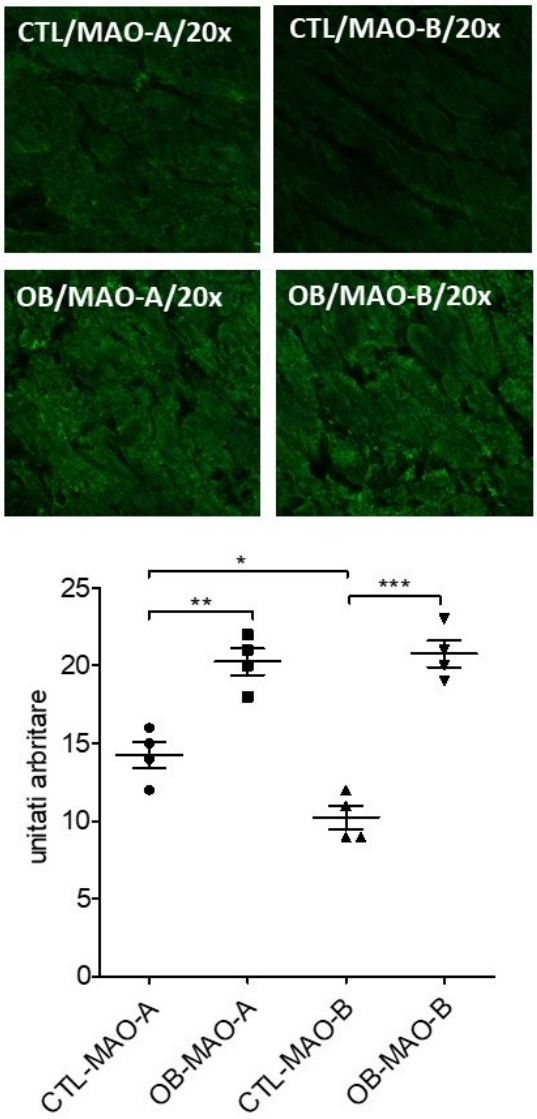


Figure 1. Immune fluorescence for MAO-A and MAO-B in hearts obtained from obese (OB) and control (CTL) rats, (CTL-MAO-A vs. OB-MAO-A, CTL-MAO-A vs. CTL- MAO-B, CTL-MAO-B vs. OB-MAO-B; *P≤0.05, **P≤0.01, ***P ≤ 0.001)

The obesogenic diet also induced the increase in H_2O_2 production as determined spectrophotometrically (FOX assay). This effect was attenuated by the *ex vivo* incubation of the ventricular samples with both MAO inhibitors A and B (clorgyline and selegiline, respectively, 10 μM, 12 h) - Fig. 2.

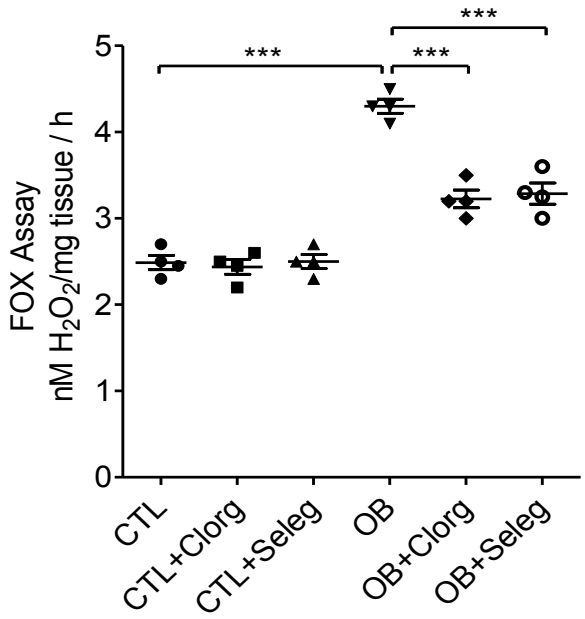
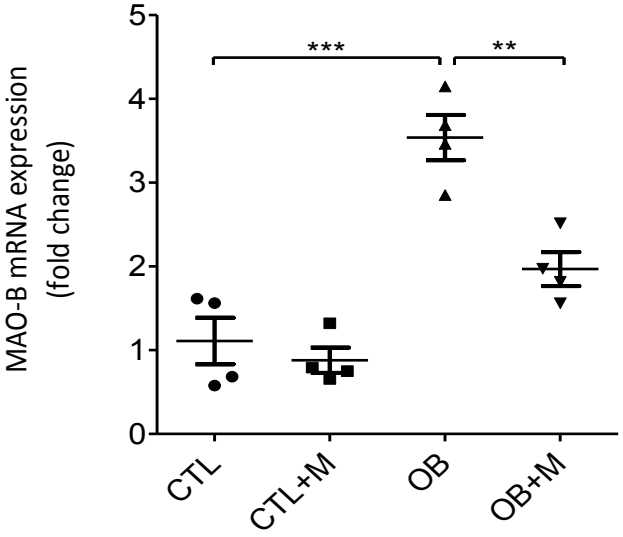


Figure 2. H_2O_2 measurements (spectrophotometry) in the presence or absence of MAO inhibitors (Clorg clorgyline, Seleg selegiline, 10 μM) (CTL vs. OB, OB vs. OB+Clorg, OB vs. OB+Seleg; ***P ≤ 0.001)

In vitro exposure to metformin was able to reduce MAO A and B gene expression in heart samples isolated from obese rats with no effect in control samples (Fig. 3).



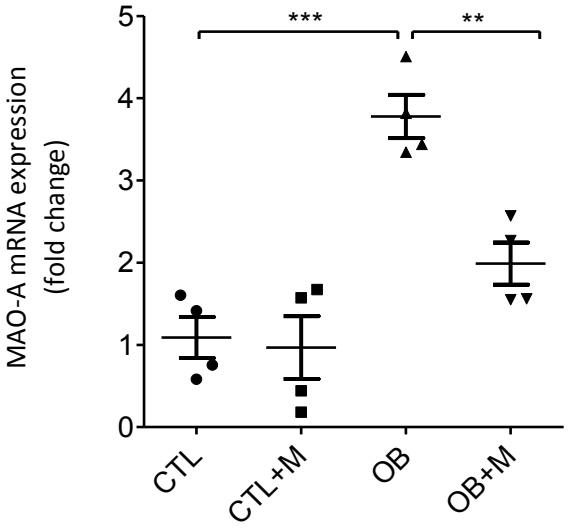


Figure 3. Metformin reduced MAO-A and MAO-B mRNA expression in heart samples isolated from obese rats. CTL control, OB obese, M metformin (CTL vs. OB, OB vs. OB+Clorg, OB vs. OB+Seleg; **P ≤ 0.01, ***P ≤ 0.001)

Also, metformin was able to mitigate the degree of oxidative stress assessed by both methods, spectrophotometry (FOX assay, Fig. 4) and confocal microscopy (DHE staining, Fig. 5).

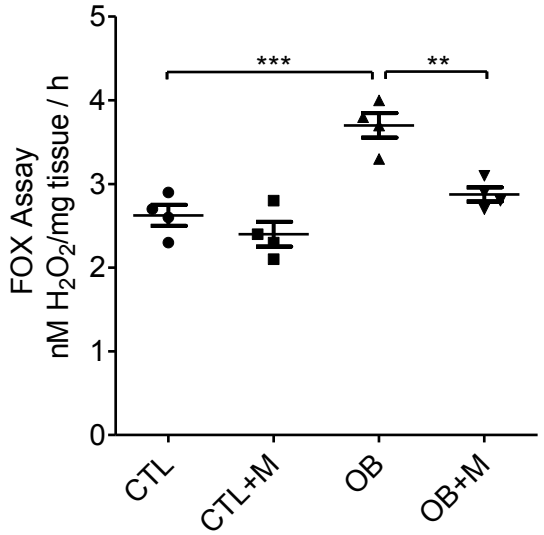


Figure 4. Metformin reduced oxidative stress in heart samples isolated from obese rats (FOX Assay); CTL control, OB obese, M metformin (CTL vs. OB, OB vs. OB + M; **P ≤ 0.01, ***P ≤ 0.001)

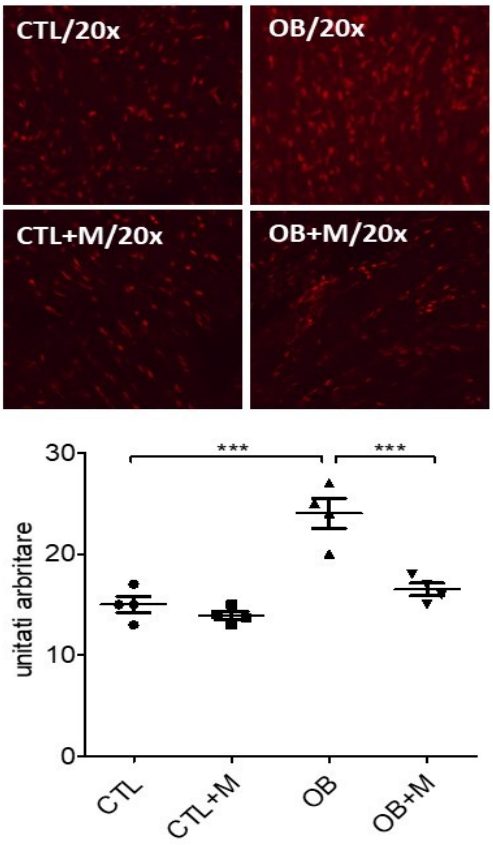


Figure 5. Metformin reduced oxidative stress in heart samples isolated from obese rats (DHE staining); CTL control, OB obese, M metformin, (CTL vs. OB, OB vs. OB + M; ***P ≤ 0.001)

3. Contributions to elucidating the vasculoprotective effect of metformin: interaction with nitric oxide synthases in the internal mammary artery

The present study aimed to investigate a *novel vasculoprotective mechanism of metformin in humans*, namely the modulation of nitric oxide synthases expression, in the presence vs absence of acute stimulation with angiotensin 2 (Ang2) of human internal mammary arterial samples harvested from patients with coronary heart disease subjected to (CABG).

The pilot study included patients hospitalized at the Institute of Cardiovascular Diseases from Timisoara (n = 7) with indication for revascularization, with primary diagnostics: stable angina pectoris, unstable angina pectoris, myocardial infarction, trivascular coronary artery disease (and secondary diagnostics: carotid artery atherosclerosis, mitral/tricuspid regurgitation, hypertension, heart failure NYHA II, hypercholesterolemia).

Ang2 induced vascular dysfunction by increasing contractility and reducing relaxation, and metformin was able to attenuate these effects by reducing contractility (Fig. 6) and increasing relaxation, respectively (Fig. 7).

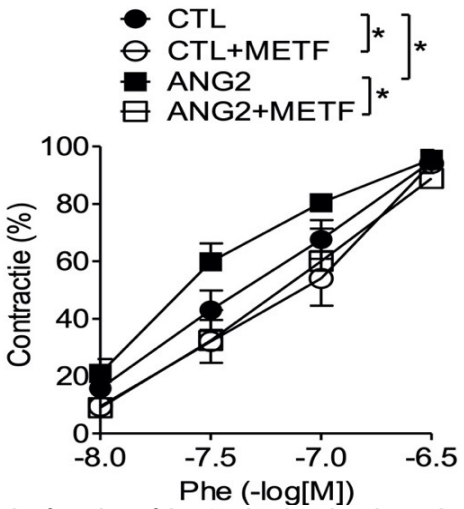


Figure 6. Metformin improves vascular function of Ang2-stimulated and non-Ang2-stimulated mammary artery rings by reducing phenylephrine (Phe)-induced contraction (n=7, *p<0.05).

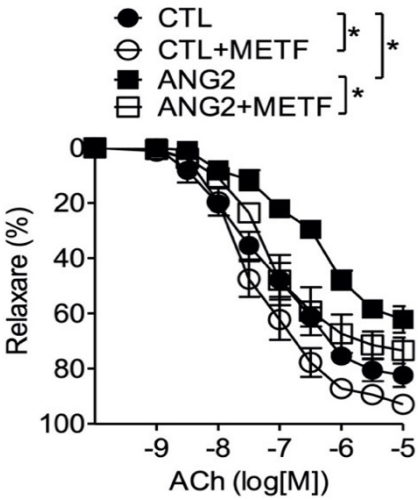


Figure 7. Metformin improves vascular function of Ang2- and non-Ang2-stimulated mammary artery rings by increasing the endothelium-dependent relaxation induced by acetylcholine (Ach) (n=7, *p<0.05).

The beneficial vasomotor effects of Metf were observed in all vascular rings (stimulated or not with Ang2), demonstrating the activation of the local renin-angiotensin system in the mammary arteries harvested from patients with coronary heart disease. Nevertheless, the magnitude of response was higher in the Ang2-stimulated group. In the presence of L-NAME (the eNOS inhibitor), acute incubation with Ang2 augmented the contractile response but metformin addition was able to attenuate this effect, suggesting its interference with the intracellular signaling pathways responsible for NO generation as a possible vasculoprotective mechanism provided by the main antidiabetic drug used in type 2 diabetic patients (Fig. 8).

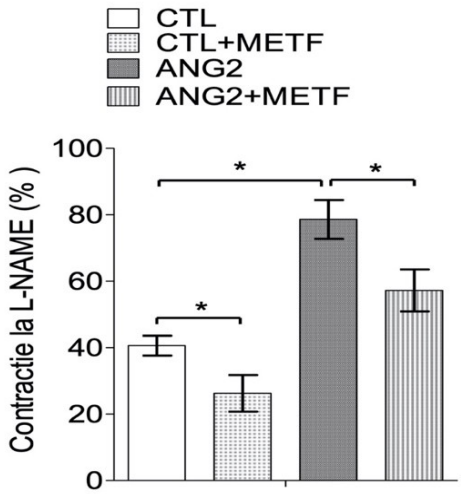


Figure 8. Metformin improves vascular function of Ang2-stimulated and non-Ang2-stimulated mammary artery rings by reducing Ang2-induced contraction in the presence of L-NAME (n=7, *p<0.05).

Incubation of vascular segments with Ang2 significantly decreased eNOS (Fig. 9) and nNOS (Fig. 10) expression and increased iNOS expression (Fig. 11), respectively. Metformin was able to reverse all of these effects.

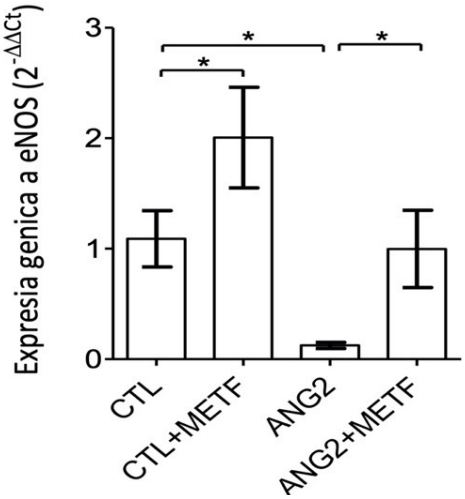


Figure 9. Metformin modulates NOS isoform expression in Ang2-stimulated and non-Ang2-stimulated internal mammary artery rings: increased eNOS expression decreased by Ang2 on RT-PCR (n=7, *P<0.05).

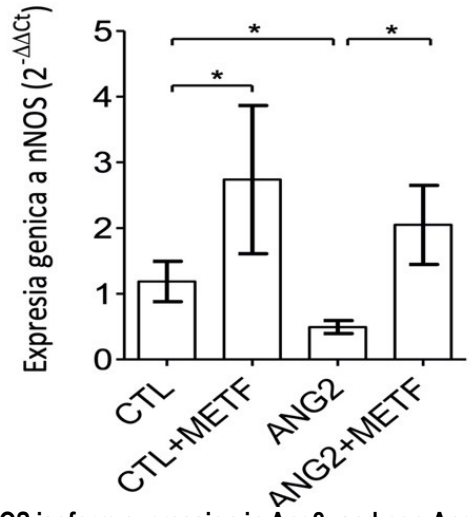


Figure 10. Metformin modulates NOS isoform expression in Ang2- and non-Ang2-stimulated internal mammary artery rings: increased nNOS expression, decreased by Ang2 at RT-PCR (n=7, *P<0.05).

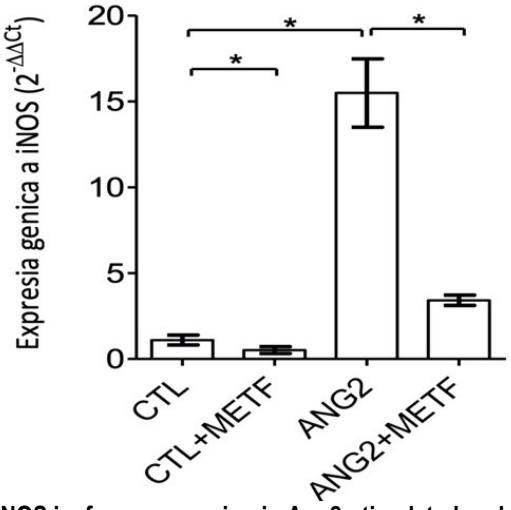


Figure 11. Metformin modulates NOS isoform expression in Ang2-stimulated and non-Ang2-stimulated internal mammary artery rings: decreased iNOS expression, increased by Ang2 at RT-PCR (n=7, *P<0.05).

4. Contributions to the elucidation of the protective effect of nv118 permeable succinate on platelet respiration isolated from patients undergoing cardiopulmonary bypass

The present study aimed to evaluate *the effects of NV118 (diacetoxymethyl succinate) on platelets bioenergetics* collected from a group of patients undergoing open heart surgery and cardiopulmonary bypass, respectively.

This pilot study included 13 patients (4 M, 9F) hospitalized at the Institute of Cardiovascular Diseases from Timisoara, diagnosed with multivessel coronary artery disease, valvular pathology or both, with indication for cardiac surgery / cardiopulmonary bypass (CPB).

Platelets were isolated form the venous blood collected before (prior to heparin administration) and after the CPB (within 10 minutes after protamine sulphate administration). Mitochondrial respiration was assessed by high-resolution respirometry.

Cell permeable succinate, NV118 elicited a global improvement of the respiratory function in intact platelets isolated both prior to (Fig. 12) and after (Fig. 13) cardiopulmonary bypass.

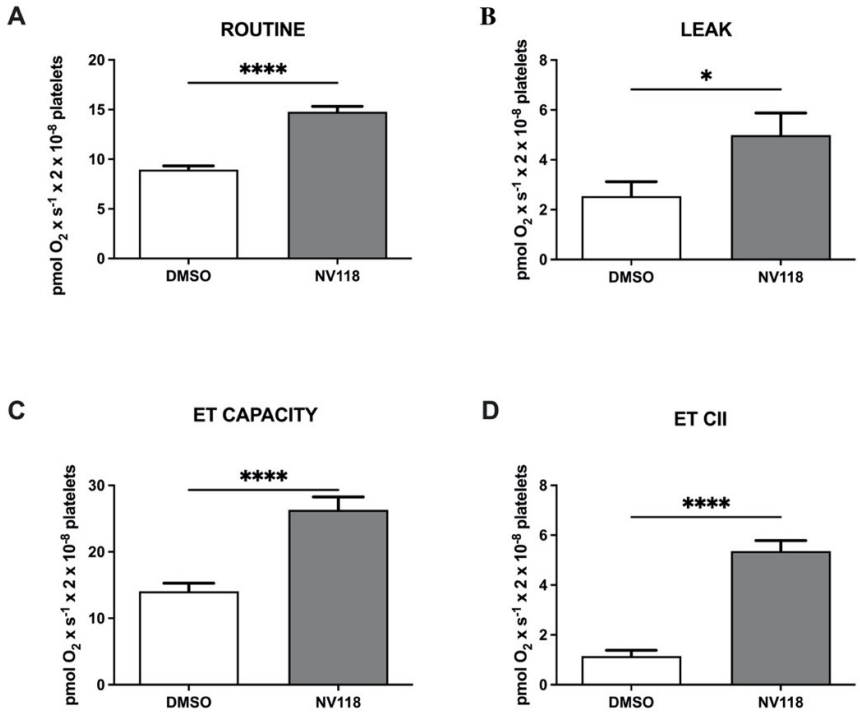


Figure 12. Platelet respiratory parameters prior to CBP in the absence vs. the presence of cell-permeable succinate NV118. (A) ROUTINE respiration, (B) LEAK respiration, (C) ET CAPACITY, (D) ETII CAPACITY. Data are expressed as the mean ± SEM of ROX-corrected respiration. * p < 0.5; **** p < 0.0001. ET capacity: electron transport capacity.

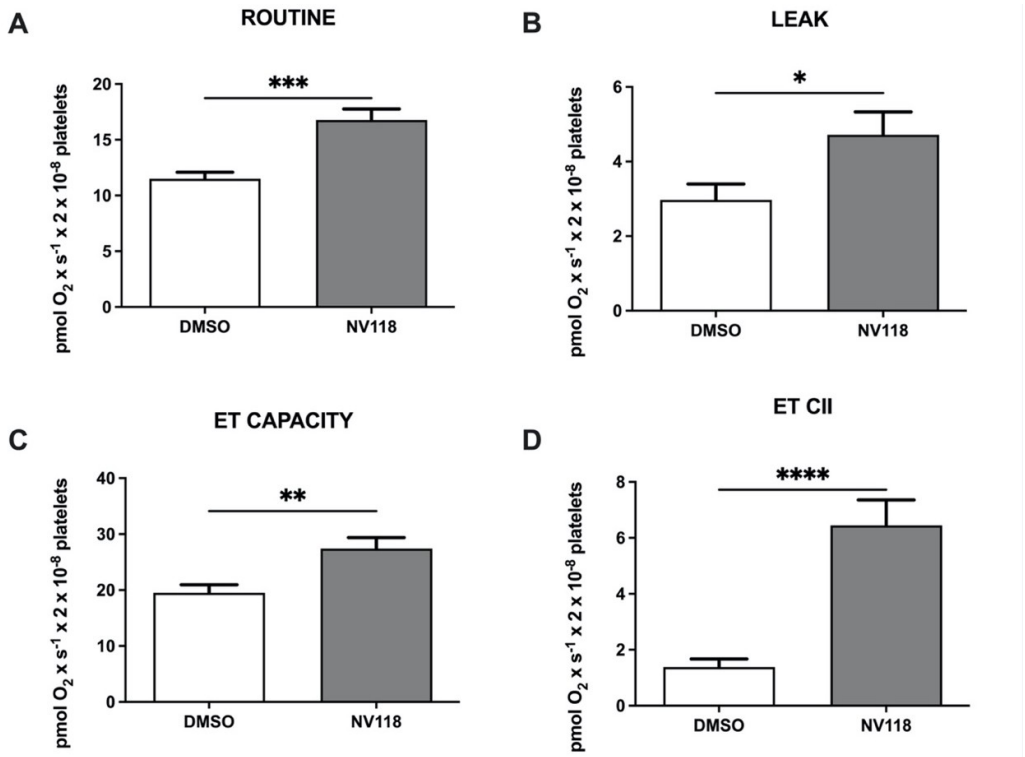


Figure 13. Platelet respiratory parameters after CPB in the absence vs. the presence the cell-permeable succinate NV118. (A) ROUTINE respiration, (B) LEAK respiration, (C) ET CAPACITY, (D) ETII CAPACITY. Data are expressed as the mean ± SEM. * p < 0.5; ** p < 0.01; *** p < 0.001; **** p < 0.0001. ET capacity: electron transport capacity.

CONCLUSIONS

The aim of the present PhD thesis was to evaluate the cardio- and vasculoprotective effects of two pharmacological agents in an animal model and in humans. The research objectives were achieved by conducting the 3 original studies described in the Special Part of the thesis.

The completion of the PhD research allows the following **general conclusions** to be drawn:

1. Gene expression of MAO-A and MAO-B is increased at ventricular level and contribute to cardiac oxidative stress in rats with hypercaloric diet-induced obesity and prediabetes, an experimental model that recapitulates the main cause of obesity in both children and adult population.
2. In ventricular samples from the control group of non-obese rats, MAO-A isoform was more abundant than MAO-B.
3. The obesogenic diet also induced increased H_2O_2 production, which was mitigated by acute *ex vivo* incubation of the ventricular samples with the MAO-A inhibitor, clorgyline and MAO-B inhibitor, selegiline, respectively.
4. These compounds had no effect in the control group demonstrating that under physiological conditions the enzyme activity does not cause oxidative stress.
5. Protein expression of both MAO isoforms is increased in the heart of obese and prediabetic rats.
6. Metformin reduced both ventricular gene and protein expression of MAO isoforms in obese and prediabetic rats and had no effect in control animals.
7. Metformin reduced oxidative stress in the heart of obese rats assessed by both spectrophotometry and confocal microscopy.
8. The mitigation of oxidative stress is secondary to the decrease in MAO expression, as metformin does not exhibit a ROS scavenger effect (as demonstrated for catalase).
9. Angiotensin 2 (Ang2) induced vascular dysfunction demonstrated by an increased contractility and a reduced endothelium-dependent relaxation in internal mammary artery samples harvested from diagnosed patients with coronary artery disease undergoing CABG.
10. Acute *in vitro* incubation with metformin improved the vasomotor function of internal mammary artery rings by decreasing contractility and increasing relaxation.
11. The beneficial effect of metformin was also present in control (non-Ang2-stimulated) mammary artery samples, suggesting the activation of the local, vascular renin-angiotensin system.
12. The contractile response to Ang2 was amplified in the presence of L-NAME, the endothelial nitric oxide synthase inhibitor and incubation with metformin attenuated this effect, suggesting the interference with the intracellular cascade of NO generation as a mechanism of protection.
13. Ang2 induced oxidative stress, as demonstrated by increased hydrogen peroxide generation in human internal mammary arteries.

14. Metformin reduced Ang2-induced oxidative stress in internal mammary artery rings.
15. Metformin also reduced hydrogen peroxide generation in control (non-Ang2-stimulated) vascular rings, demonstrating that oxidative stress is present, together with the endothelial dysfunction, in mammary arteries used for revascularization.
16. The cardio- and vasculoprotective effects of metformin, i.e. alleviation of endothelial dysfunction and reduction of ventricular and arterial oxidative stress in both animal and human models were observed at a clinically relevant concentration, 10 μM being the minimal value of the therapeutic plasma window.
17. Metformin modulated gene expression of nitric oxide synthases in internal mammary artery rings, inducing:
    - increased expression of constitutive endothelial (eNOS) and neuronal (nNOS) nitric oxide synthases decreased by Ang2
    - decreased expression of inducible nitric oxide synthase (iNOS), increased by Ang
18. NV118, a first-generation cell-permeable succinate compound, improved respiratory mitochondrial function of isolated platelets from patients with valvular and/or coronary heart disease (CHD) undergoing open heart surgery.
19. The effect of significantly increasing all respiratory parameters of platelets in the presence of NV118 was observed regardless of the time of their sampling, prior and after the cardiopulmonary bypass lasting 1.5 hours.

The **original contributions** are represented by the first-time evaluation of the:

- Metformin-monoamine oxidase interaction at cardiac level on a relevant animal model of obesity and prediabetes induced in rats, which have a comparable expression of MAO isoforms in the cardiovascular system to the humans.
- Interaction of metformin-nitric oxide synthases under vascular angiotensin-2-mediated oxidative stress in internal mammary arteries from patients with CHD undergoing revascularization.
- Effects of permeable succinate on mitochondrial respiration of peripheral platelets isolated from patients subjected to cardiac surgery for valvular diseases and/or CHD, prior and after cardiopulmonary bypass.

The potential **future research directions** are as follows:

- Investigation of the intracellular signaling pathways by which metformin attenuates the MAO-related cardiac oxidative stress in the setting of obesity.
- Investigation of the mechanisms/signaling pathways underlying metformin-nitric oxide synthases interaction.
- Assessment of the ability of serum harvested from patients, pre- and post-cardiopulmonary bypass, to induce mitochondrial dysfunction in platelets isolated from healthy volunteers.
- Investigation the effects of permeable succinate on atrial mitochondrial dys/function in samples collected from patients undergoing cardiac surgery.