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# Nicotinamide Riboside Supplementation Restores Myocardial Nicotinamide Adenine Dinucleotide Levels, Improves Survival, and Promotes Protective Environment Post Myocardial Infarction

Cynthia Tannous<sup>1,2,3</sup> Rana Ghali<sup>1,3</sup> Ahmed Karoui<sup>2</sup> Nada J. Habeichi<sup>1,2,3,4</sup> Ghadir Amin<sup>1,3,5</sup> George W. Booz<sup>5</sup> Mathias Mericskay<sup>2</sup> Marwan Refaat<sup>6</sup> · Fouad A. Zouein<sup>1,2,3,5</sup>

## Corresponding authors:

✉ Mathias Mericskay mathias.mericskay@inserm.fr

✉ Fouad A. Zouein fouadzouein@outlook.com

- 1 Department of Pharmacology and Toxicology, Faculty of Medicine, American University of Beirut Medical Center, Riad El-Solh, Beirut 1107 2020, Lebanon
- 2 Department of Signaling and Cardiovascular Pathophysiology, UMR-S 1180, Université Paris-Saclay, Inserm, 17 avenue des Sciences, 91 400 Orsay, France
- 3 The Cardiovascular, Renal and Metabolic Diseases Research Center of Excellence, American University of Beirut Medical Center, Riad El-Solh, Beirut, Lebanon
- 4 MatriceLab Innove Laboratory, Immeuble Les Gemeaux, 2Rue Antoine Etex, 94000 Creteil, France
- 5 Department of Pharmacology and Toxicology, School of Medicine, University of Mississippi Medical Center, Jackson, MS, USA
- 6 Department of Cardiovascular Medicine, Faculty of Medicine, American University of Beirut Medical Center, Beirut, Lebanon

## Abstract

**Aims** Myocardial infarction (MI) is a major cause of death. Nicotinamide adenine dinucleotide (NAD<sup>+</sup>) is a coenzyme in oxidative phosphorylation and substrate of sirtuins and poly-ADP ribose polymerases, enzymes critical for cardiac remodeling post-MI. Decreased NAD<sup>+</sup> is reported in several heart failure models with paradoxically an upregulation of nicotinamide riboside kinase 2, which uses nicotinamide riboside (NR) as substrate in an NAD<sup>+</sup> biosynthetic pathway. We hypothesized that stimulating nicotinamide riboside kinase 2 pathway by NR supplementation exerts cardioprotective effects.

**Methods and Results** MI was induced by LAD ligation in 2–3-month-old male mice. NR was administered daily (1 μmole/g body weight) over 7 days. RT-PCR showed a 60-fold increase in nicotinamide riboside kinase 2 expression 4 days post-MI with a 60% drop in myocardial NAD<sup>+</sup> and overall survival of 61%. NR restored NAD<sup>+</sup> levels and improved survival to 92%. Assessment of respiration in cardiac fibers revealed mitochondrial dysfunction post-MI, and NR improved complexes II and IV activities and citrate synthase activity, a measure of mitochondrial content. Additionally, NR reduced elevated PARP1 levels and activated a type 2 cytokine milieu in the damaged heart, consistent with reduced early inflammatory and pro-fibrotic response.

**Conclusion** Our data show that nicotinamide riboside could be useful for MI management.

**Keywords** Energy metabolism Cardiac remodeling Inflammation Cytokine

Mathias Mericskay and Marwan Refaat have equal contributions. Rana Ghali and Karoui Ahmed are co-second authors.

## Introduction

Despite significant improvement in mortality rates over the past two decades, coronary artery diseases, most commonly manifested as a myocardial infarction (MI), remain the leading cause of mortality and morbidity worldwide [1]. MI is caused by blood flow obstruction of one or multiple coronary arteries supplying the ventricles. Up until this day, a substantial proportion of MI patients are not reperfused for various reasons and suffer from permanent occlusion MIs with a high risk of heart failure development [2, 3].

The key components of the cardiac energetic system are downregulated in the failing heart. We focused our attention on pathways regulating nicotinamide adenine dinucleotide (NAD<sup>+</sup>) equilibrium in the failing heart. NAD<sup>+</sup> is emerging as a target for the development of metabolic therapy for heart failure, being a major coenzyme for glycolysis and mitochondrial oxidative phosphorylation [4]. Furthermore, NAD<sup>+</sup> is an essential substrate of crucial enzymes like sirtuin (SIRT) deacetylases and poly-ADP ribose polymerases (PARPs). SIRTs play a protective role in the heart by regulating nuclear gene expression and mitochondrial enzyme activities [5]. In the presence of acute cardiac injury, such as ischemic stress, PARP-1 activation depletes NAD stores [6], which reduces the cardioprotective effects of SIRTs 1 and 3 [6, 7].

In the normal physiological state, the consumption of NAD<sup>+</sup> by signaling pathways is counterbalanced by NAD<sup>+</sup> biosynthetic pathways relying on NAD<sup>+</sup> precursors (vitamin B3) and dietary tryptophan. However, levels of NAD<sup>+</sup> precursors are insufficient to sustain cardiac NAD<sup>+</sup> synthesis under pathological conditions, hence the necessity of NAD<sup>+</sup> precursor supplementation to correct this deficiency. Altered NAD homeostasis has been reported in several models of heart disease including ischemia reperfusion [8]. However, little is known about the regulation of NAD levels in the context of MI. Here, we show that NAD<sup>+</sup> equilibrium is altered at an early stage in the heart following MI with a striking upregulation as soon as day 4 following MI of the nicotinamide riboside kinase 2 (NMRK2) pathway for NAD<sup>+</sup> synthesis. NMRK2 kinase is involved in the NAD<sup>+</sup> salvage pathway from nicotinamide riboside (NR), which is a B3 vitamin, a trace nutrient in some food (e.g., milk) [9]. NR is safe for humans and increases levels of a metabolite that is critical for cellular energy production and protection against stress and DNA damage. NR is a NAD precursor with superior bioavailability in mice and humans without reported side effects in contrast to niacin [10]. Moreover, the alternative salvage pathway involving nicotinamide phosphoribosyl transferase (NAMPT) was reported to be repressed in the context of MI [11], reinforcing our interest in NR supplementation to correct myocardial NAD deficiency. Our aim in this study was to test the hypothesis that NR supplementation is cardioprotective post-MI.

## Materials and Methods

### Experimental Design

Animals were housed in sterilized cages in rooms maintained on a 12-h light–dark cycle, with access to autoclaved rodent chow and water ad libitum. Surgical procedures were performed in the animal care facility of the American University of Beirut Medical Center (AUBMC) and the University of Paris-Saclay. All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of AUB (IACUC # 18–03-470) and conducted according to the guidelines of the Declaration of Helsinki, conformed to the Directive 2010/63/EU of the European Parliament and approved by the National Ethics Committee #26 of the French Ministry of Research (agreement APAFIS #20327-2019062415368026v1). Adult wild-type (WT)

male C57/BL6J mice (3 months of age) were randomized into four different groups: SHAM + vehicle, SHAM + NR, MI + vehicle, and MI + NR. In the sham and MI groups treated with NR, a single daily injection of NR chloride (Chromadex, Irvine, CA, USA) (1  $\mu$ mole/g of body weight) was administered intraperitoneally (i.p.) right after sham operation or left anterior descending (LAD) coronary artery ligation and continued for 4 or 7 days thereafter. In the sham and MI groups treated with vehicle, a single daily injection of PBS (200  $\mu$ l/day) was administered i.p. right after sham operation or LAD coronary artery ligation and continued for 4 or 7 days thereafter. For experimental procedures, mice are anesthetized with 1.5–2% isoflurane. For MI, mice are administered 0.05 mg/kg tramadol for pre-emptive and post-operative (daily for 3 days) analgesia. For heart extractions, animals are anesthetized with 4% isoflurane and hearts rapidly excised. The animals do not regain consciousness. To ensure death before disposing the carcass, a cervical dislocation was performed.

### Myocardial Infarction

MI was induced by left anterior descending (LAD) coronary artery ligation. Heart rate, body temperature, and respiratory rate were monitored to avoid surgical complications. The mouse was placed on a heating pad to prevent anesthesia-induced hypothermia. Fifteen minutes prior to surgery, tramadol (0.05–0.1 mg/kg i.p.) and isoflurane (1.5–2% in oxygen)

inhalation were given to induce analgesia and general anesthesia, respectively. Orotracheal intubation was performed to maintain a normal respiratory rate by placing a needle into the trachea and connecting it to an automated ventilator (Harvard Apparatus). LAD coronary artery, left ventricle, and left atria were exposed by excising between the ribs of the left thorax. MI was induced by LAD coronary artery ligation with a 7-0 polypropylene suture at 1–3 mm underneath the left atrium appendage. Successful MI induction was confirmed by blanching of the tissue downstream of the ligation site, by ECG, and by echocardiography 24 h after surgery. Immediately following successful LAD coronary artery ligation, the chest was closed, and the mice were placed on a warm pad for recovery. The same procedure was applied to the SHAM group but without LAD coronary artery ligation. Once they were groomed freely, MI mice were caged back individually and monitored on a daily basis for full recovery.

## Echocardiography

Echocardiographic measurements were assessed using a Vevo 2100™ High-Resolution Imaging System (Visual Sonics, Toronto, Canada) according to the American Society of Echocardiography guidelines. Animals were anesthetized using isoflurane (1.5% in oxygen). Body temperature was maintained at 37 °C using a rectal thermostat and a heat pad. The transducer was placed on the left thorax and ultrasound beam directed at the mid-papillary muscle level in order to obtain M-mode and B-mode echocardiography images, in the parasternal long- and short-axis views. The mean calculation of cardiac parameters was measured at baseline, on day 1 following the MI induction surgery, and before sacrifice (on days 4 or 7 post-surgery).

## NAD Extraction and Quantification

We used the NAD cycling colorimetric assay which detects both NAD<sup>+</sup> and NADH, so that the total pool NADt is measured. Snap-frozen heart tissue (20 to 30 mg) was crushed in buffered ethanol (BE) (75% ethanol, 25% HEPES 10 mM pH 7.1) as previously described [12]. Extracts were cooled on ice for 20 min and centrifuged for 10 min at 13,000 g. Extracts were diluted 1:20. For each sample, 25 µL of NADt extract was quantified in duplicate by addition of 100 µL of reaction buffer (600 mM ethanol, 0.5 mM 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2 mM phenazine ethosulfate (PES), 120 mM Bicine (pH 7.8), yeast alcohol dehydrogenase (SIGMA A3263, > 300 u/mg) 0.05 mg/ml). Kinetics of the reaction (OD at 570 nm, every 30 s for 40 min) was followed on a Tristar Multimode Reader. NADt levels were quantified by comparison to a range of standard NAD concentrations (linear regression curve equation method). Data are expressed as pmole of NADt/mg of tissue.

## RNA Extraction and Quantification

cDNAs were reverse transcribed from 1.5 µg RNA extracted in TRIzol (ThermoFisher) from cardiac tissue using superscript II reverse transcriptase (Life Technologies). Quantitative PCR was carried out with a CFX-96 RT-PCR system (Bio-Rad) using Fast Start SYBR Green Master Mix (Roche Diagnostics). Quantification of gene expression was calculated as  $R = 2\%Ct$  (Ref Cp-target Cp), with HPRT used as a reference. Primers were designed using the NCBI Primer Blast Software and are shown in Table 1.

## Western Blot Analysis

Proteins were homogenized in a lysis buffer (RIPA buffer) containing protease inhibitors (ThermoFisher). Equal amounts of protein (30 µg) were separated on a 15% SDS polyacrylamide gel. Proteins were transferred onto a nitrocellulose membrane (Hybond C, Amersham Biosciences) and the blots blocked with TBS buffer (20 mM Tris, pH 7.5, 136.8 mM NaCl, and 0.1% (v/v) Tween 20) containing 5% (w/v) milk. Proteins were detected by overnight incubation at 4 °C with primary antibodies, followed by HRP-labeled secondary antibodies (1:10,000), and then exposed to ECL (Clarity Western ECL substrate, Bio-Rad) and scanned with the ChemiDoc MP imaging system. The antibodies used for Western Blot are listed in Table 2. Band densities of the target proteins were measured using ImageJ and normalized to the density of the total protein bands. As previously noted by us [13] and others [14], total protein is the most appropriate internal control for Western blot analysis with animal models of myocardial ischemic infarction due to the instability of housekeeping proteins such as p-actin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and p-tubulin.

## Oxygen Consumption Rate Assay in Permeabilized Cardiac Fibers

Cardiac muscle fibers from the remote myocardium were isolated and permeabilized using saponin. Mitochondrial respiration was assessed using a Clarke electrode as previously described [15]. Respiration solution was made from CaK<sub>2</sub>EGTA (100 mM) diluted to 2.77 mM, K<sub>2</sub>EGTA (100 mM) diluted to 7.23 mM, and 6.56 mM MgCl<sub>2</sub> to obtain 100 nM free Ca<sup>2+</sup> and 1 mM free Mg<sup>2+</sup> with 20 mM taurine, 0.5 mM DTT, 50 mM potassium methanesulfonate (160 mM ionic strength), 20 mM imidazole (pH 7.1) at 23 °C. The protocol was started by the addition of fibers to respiration solution containing glutamate-malate (10 mM and 4 mM, respectively) and ADP (0.1 mM), followed by sequential addition of ADP (2 mM), pyruvate (10 mM), succinate (15 mM), amytal (inhibitor of complex I; 1 mM), and tetramethyl-p-phenylenediamine (TMPD, 0.5 mM) to activate complex IV respiration. Ascorbate (2 mM) was added to avoid auto-oxidation of TMPD. The rates of respiration are given as nmoles O<sub>2</sub>/min/mg of cardiac tissue dry weight.

**Table 1 List of primers used for RT-qPCR analysis**

Primer	Forward	Reverse
<i>Hprt</i>	GTTGGGCTTACCTCACTGCT	TAATCACGACGCTGGGACTG
<i>Nampt</i>	ACCAGCGGGGAACCTTTGTTA	ACATAACAACCCGGCCACAT
<i>Nmrk2</i>	TACAGCCAACGCTACTTCCT	GGGACTTCATGCCATCTAAA
<i>Parp1</i>	ACACCACAAAACCTCAGCCA	ACAAACCACAAAACCCGGC

**Table 2 Antibodies used for Western Blot analysis**

Target protein	Antibody supplier, catalog #	Dilution
Sirtuin 3 (SIRT3)	Abcam, ab86671	1:1000
Interleukin-4	Abcam, ab9728,	1:1000
Citrate Synthase	Abcam, ab96600	1:1000
OxPhos Complex IV subunit (COX IV)	Mitosciences, MS604	1:1000

## Enzymatic Activities

Frozen tissue samples were weighed and homogenized with Precellys 24 (Bertin Instruments) in ice-cold buffer (50 mg/ml) containing 5 mM HEPES (pH 8.7), 1 mM EGTA, 1 mM DTT, and 0.1% Triton X-100. Citrate synthase (CS) and cytochrome oxidase (COX) activities were determined in homogenized ventricles as previously described [16]. The activities of enzymes were determined by spectrophotometric assays. For citrate synthase (CS), approximately 0.5 µg of protein was added to 1 mL Buffer M (100 mM Trizma (pH 8), 0.1 mM 5,5'-dithiobis-(2-nitrobenzoic acid), 0.3 mM acetyl-CoA, 0.5 mM oxaloacetic acid), and the absorbance at 412 nm was measured for 3 min. Cytochrome oxidase (COX) activity was determined by the addition of approximately 0.25 µg of protein in 1 mL of phosphate buffer (50 mM K<sub>2</sub>HPO<sub>4</sub> (pH 7.4)) containing 50 µM cytochrome c (previously reduced 90% with sodium dithionite). Loss of reduced cytochrome c was followed by measuring the absorbance at 550 nm over 3 min using fully oxidized (by the addition of excess potassium ferricyanide) cytochrome c as reference.

## Enzyme-Linked Immunosorbent Assay (ELISA)

The concentrations of IL-4 (STEMCELL technologies, Cat#02038A) were determined in plasma using ELISA kits according to the manufacturer's instructions. All samples were analyzed in duplicate.

## Picrosirius Red Staining

At the time of collection, hearts were perfusion-fixed with 4% paraformaldehyde (PFA). Fixed hearts were embedded in paraffin blocks, then cut into 5-µm-thick sections and placed on slides. Heart sections were deparaffinized and hydrated through a graded ethanol series (100, 95, 75, and 50%), then stained with Picrosirius Red to visualize fibrosis. Images were

acquired on an Olympus CX41 microscope (total scan of a transversal cardiac section of  $n=3$  per experimental group). For myocardial fibrosis quantification, the red color represents the fibrotic area, and the results are presented as the ratio of the fibrotic area to the total area of the myocardium.

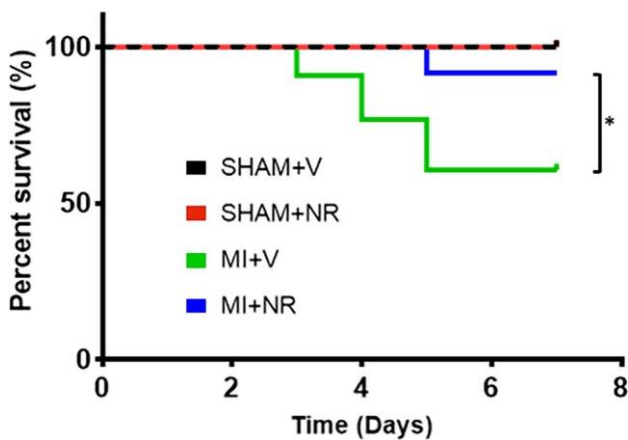
## Statistics

We performed statistical analyses using Prism 8 software (<https://www.graphpad.com>). For survival curve analysis, we used the Gehan–Breslow–Wilcoxon test that is adapted to short-term survival studies with early mortality. We used an unpaired two-tailed Student's *t*-test for the comparison of two groups or a one-way analysis of variance (ANOVA) for the comparison of four groups (SHAM and MI) with different treatments, followed by Tukey's post hoc test. For repeated measurements (echocardiography follow-up), we used two-way repeated measure ANOVA for independent samples followed by Šidak's multiple comparison tests for group comparisons. Statistical significance was considered to be  $P<0.05$ .

## Results

### NR Treatment Decreases Mortality Rate Post-MI

Kaplan–Meier survival curve analysis showed that the NR-treated MI group had a marked survival advantage over the vehicle-treated MI group. The survival rate of the vehicle-treated MI group exhibited a decline starting on D3 post-MI, eventually reaching a survival percentage of 61% by D7. However, the survival rate of the NR-treated MI group did not drop before D5 post-MI to reach a survival percentage of 92% at D7. Mice died from cardiac rupture. None of the mice died from sham operation in both groups (Fig. 1).



**Fig. 1** Effect of NR administration on survival post-MI. Kaplan–Meier survival curve analysis of mice with myocardial infarction treated with vehicle or with nicotinamide riboside (NR). NR was administered using intraperitoneal injection during 7 days after LAD ligation. ( $n = 9$  in the vehicle-treated sham-operated group,  $n = 5$  in the NR-treated sham-operated group,  $n = 31$  in the vehicle-treated LAD-ligated group (includes historical data), and  $n = 12$  in the NR-treated LAD-ligated group)

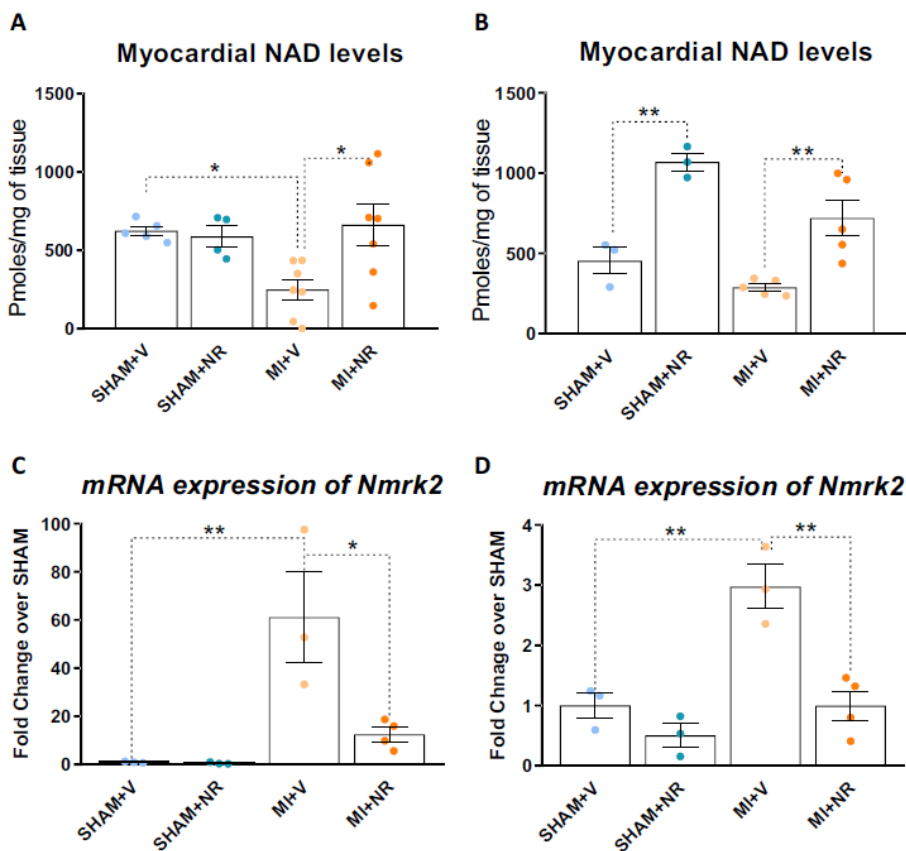
### NAD Levels and NMRK2 Pathway are Affected in the Failing Heart of MI Mice

We assessed the consequence of MI on the NAD pathway at both 4 and 7 days post-MI. Myocardial NAD pools significantly dropped by 60% in the vehicle-treated MI group, compared to vehicle-treated SHAM 4 days following surgery (Fig. 2A). In parallel, the mRNA expression level of the *Nmrk2* gene, involved in the NAD salvage pathway and which uses NR as substrate to synthesize NAD, was strikingly upregulated (61-fold) in the MI group treated with vehicle compared to the SHAM group (Fig. 2C). This gene expression pattern suggested a MI-induced compensatory mechanism to engage the NR pathway but may lack immediately bioavailable NR reserves. We tested this hypothesis by giving mice i.p. injections of NR following LAD ligation and for 4 days thereafter. Consistent with our hypothesis, we found that myocardial NAD levels were preserved by NR injection in the MI group, with NAD levels comparable to those in the SHAM group (Fig. 2A). The increase in NAD levels in Sham + NR mice between days 4 and 7 might be explained as a gradual buildup in NAD in the absence of any change in *Nmrk2* levels. Intraperitoneal injection of NR right after LAD coronary artery ligation and for 7 days thereafter, resulted in increased myocardial NAD levels compared to the vehicle-treated MI group (Fig. 2B). The mRNA expression levels of *Nmrk2* were significantly increased by threefold in the MI group treated with vehicle compared to the SHAM group (Fig. 2D), although this was

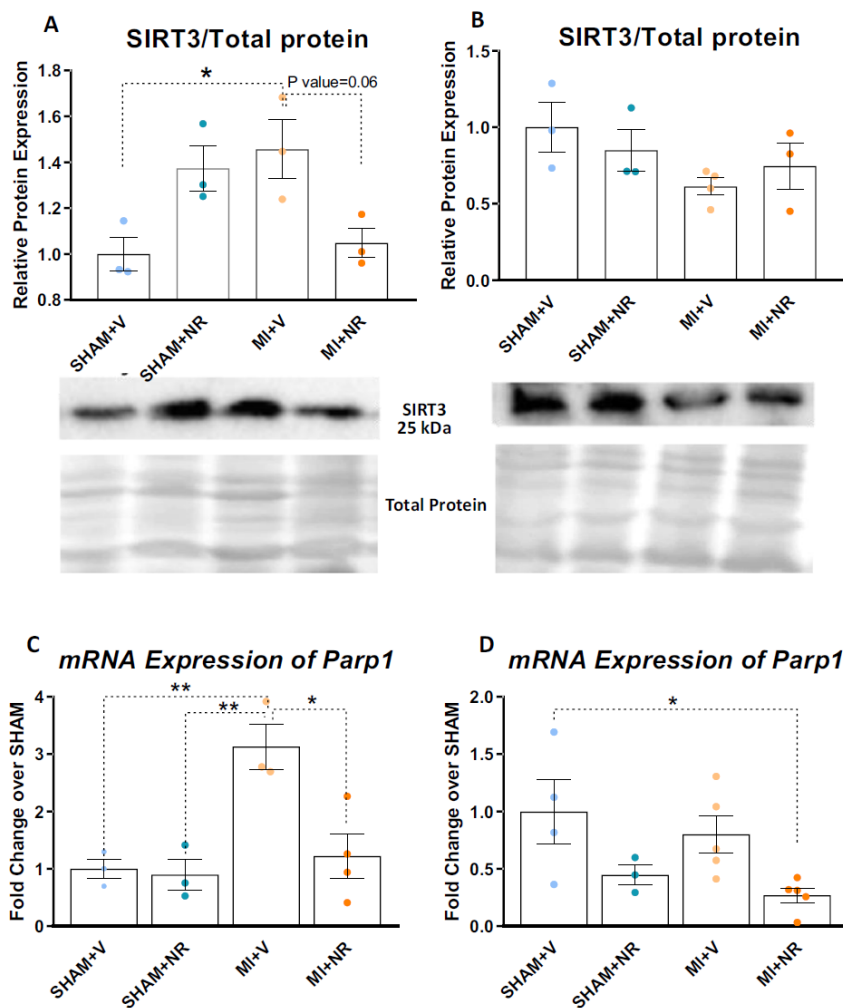
significantly less than at day 4. However, the expression level of NAMPT, the enzyme converting nicotinamide (NAM) to nicotinamide mononucleotide (NMN), a direct pre-cursor of NAD, was reduced after MI as reported, although this did not reach statistical significance (data not shown).

### NAD Consuming Enzymes are Affected Post-MI

The role of NAD is established as a main substrate of sirtuins (SIRT3) and poly-ADP ribose polymerases (PARPs), enzymes involved in regulating metabolism and maintaining cellular homeostasis [17]. SIRT3 protein levels tended to increase at 4 days post-MI (Fig. 3A). Interestingly, while NR increased SIRT3 levels in the SHAM animals, it prevented the increase in the MI group. However, at day 7, SIRT3 protein levels did not show significant changes among groups (Fig. 3B). Our data demonstrate a substantial increase (threefold) in the myocardial mRNA expression level of *Parp1* in the vehicle-treated MI group at 4 days post-surgery (Fig. 3C), which correlates with the drop in NAD levels at this stage. In the NR-treated MI group, *Parp1* expression levels were similar to the ones in the SHAM groups (Fig. 3C). On the other hand, the myocardial mRNA expression level of *Parp1* was not increased in the vehicle-treated MI group at 7 days post-surgery. In the NR-treated MI group, *Parp1* expression levels significantly dropped compared to the vehicle-treated SHAM group (Fig. 3D).



**Fig. 2** Quantification of NAD content in the heart of SHAM and MI mice and expression level of genes involved in NAD salvage and bio-synthesis pathways. **A, B** Total NAD content in the myocardium of SHAM and MI mice at 4 days and at 7 days following vehicle or NR treatment was determined by a spectrophotometric assay. ( $n = 4-5$  in sham-operated groups,  $n = 5-7$  in LAD-ligated groups). **C, D** RT- qPCR analysis of cardiac mRNA from SHAM and MI mice with vehicle or NR treatment. Relative expression of *Nmrk2* gene involved in NAD salvage pathway ( $n = 3-4$  in sham-operated groups,  $n = 4-5$  in LAD-ligated groups). Data normalized to *hprt* reference mRNA level and expressed as fold change over the mean value of vehicle- treated SHAM group. Data are presented as means  $\pm$  SEM. Statistical analysis: one-way ANOVA for independent samples; Tukey's multiple comparisons test: \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ .



**Fig. 3 Quantification of NAD consumers in the heart of SHAM and MI mice.** A, B Western Blot analysis of SIRT3 protein involved in NAD signaling pathway. C, D RT-qPCR analysis of cardiac mRNA from SHAM and MI mice with vehicle or NR treatment. Relative expression of *Parp1* gene involved in NAD consuming pathway ( $n = 3-4$  in sham-operated groups,  $n = 4-5$  in LAD-ligated groups). Data were normalized to *hprt* reference mRNA level and expressed as fold change over the mean value of the vehicle-treated SHAM group

### NR Improves Mitochondrial Respiration Rate in Isolated Cardiac Fibers When Given for 4 Days Post-MI

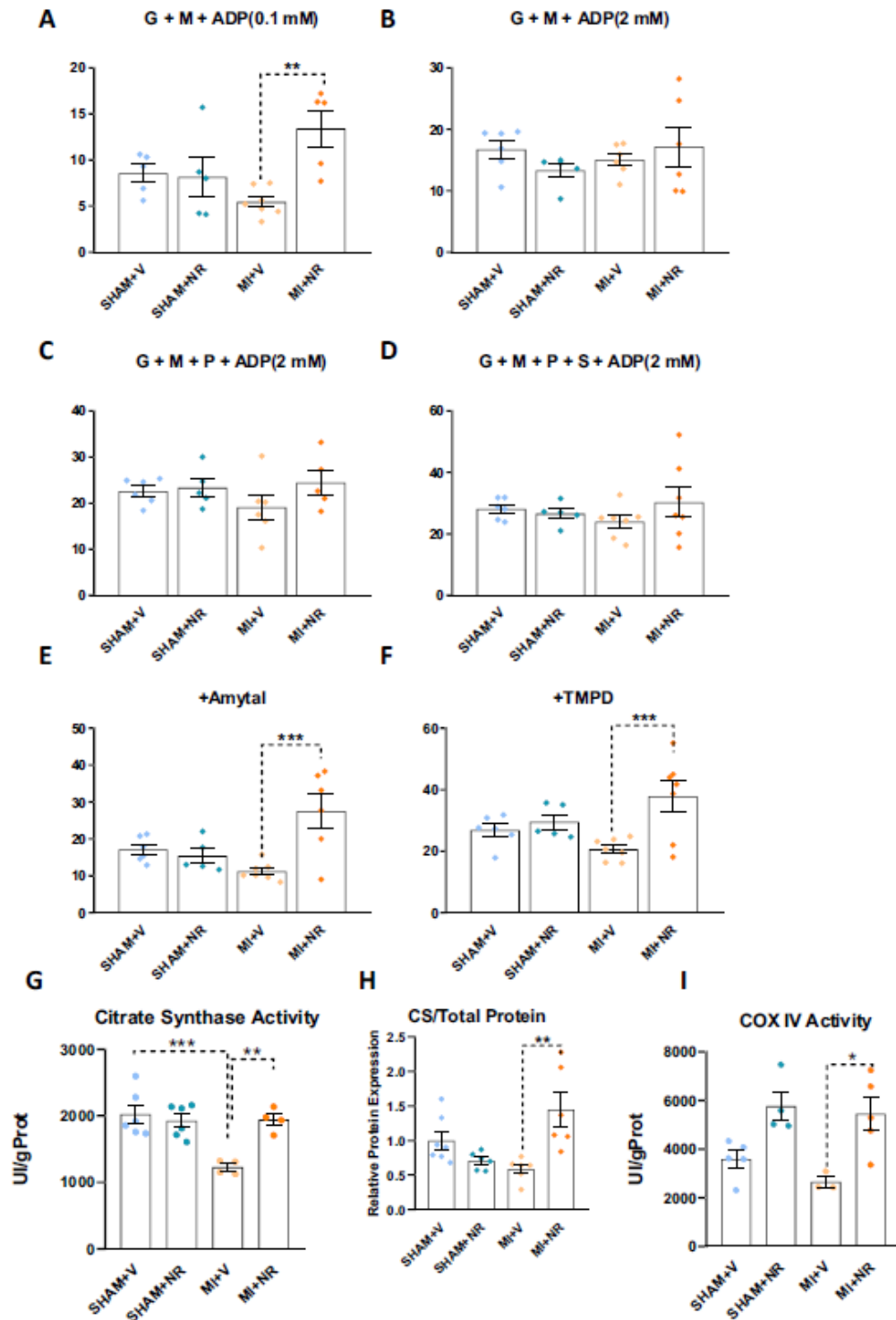
In order to know if the deregulation in NAD homeostasis observed in the MI group is associated with alterations of mitochondrial function, mitochondrial oxidative capacities were measured in permeabilized cardiac fibers from the left ventricle (Fig. 4). We harvested tissue from the remote LV posterior wall to analyze mitochondrial function in cardiomyocytes outside of the necrotic area. In the presence of glutamate and malate stimulating the malate-aspartate shuttle (complex I driven respiration), the respiration rate measured in the presence of limiting ADP concentration (0.1 mM) was lower in the vehicle-treated MI group than the SHAM group, and NR administration restored this rate (Fig. 4A). However, when increasing ADP to saturating levels (2 mM), respiration rates were not different between groups (Fig. 4B). Successive addition of pyruvate (more production of NADH and acetyl CoA and activation of complex I) and then succinate (activation of complex II on top of complex I), increased respiration rates, but did not show changes between groups (Fig. 4B-D). However, the subsequent addition of amytal to inhibit complex I revealed a decrease in complex II dependent-respiration rate in the MI vehicle group, and NR administration significantly restored this rate (Fig. 4E). TMPD was added to measure maximal complex IV-dependent respiration, which was reduced in the vehicle-treated MI group compared to the sham group and was increased in the NR-treated MI group (Fig. 4F).

### NR Improves Citrate Metabolism in MI Fibers When Given for 4 Days Post-MI

The activity of citrate synthase (CS) is commonly used as a marker of mitochondrial mass [18]. CS enzymatic activity was



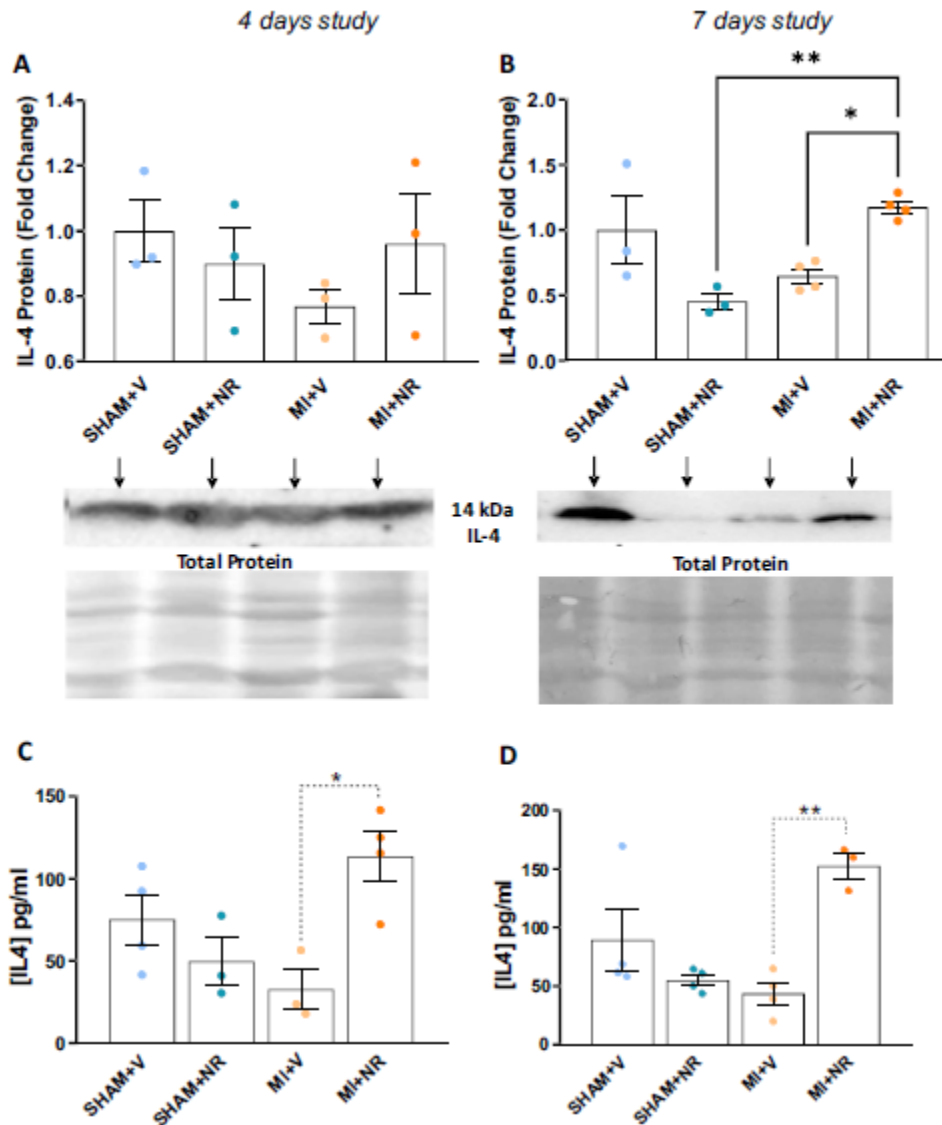
reduced by 39.35% in the vehicle-treated MI group. NR administration protected against this decline of CS activity in the MI group (Fig. 4G). In addition, the CS protein level showed a similar pattern as activity (Fig. 4H). The cytochrome oxidase (COX) activity tended to decrease in the vehicle-treated MI group. Interestingly, 4 days of NR administration prevented the drop in COX activity in the MI group (Fig. 4I).



**Fig. 4** Effect of NR treatment on the mitochondrial oxygen consumption rate ( $VO_2$ ) and mitochondrial function in cardiac fibers of SHAM and MI mice following vehicle or NR treatment. Glutamate and malate (A), ADP (B), pyruvate(C), succinate (D), amytal inhibitor of complex I (E) and TMPD, and ascorbate (F) were successively added to permeabilized myocardial fibers from the left ventricle ( $n = 7$  per group). The oxygen consumption rate is normalized to myocardial fiber dry weight ( $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}$  of dry fibers). Each value represents the mean  $\pm$  SEM. Groups were compared using one-way ANOVA and Tukey post hoc. *TMPD*, tetramethyl-p-phenylenediamine; *VO<sub>2</sub>*, oxygen consumption. citrate synthase activity (G), citrate synthase protein expression level (H), and COX activity (I) in the left ventricle. Data are presented as means  $\pm$  SEM. Statistical analysis: one-way ANOVA for independent samples; Tukey's multiple comparisons test: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

## NR Treatment Affects Plasma and Myocardial Level of IL-4 Cytokine Post-MI

Western blot analysis of myocardial levels of IL-4 showed a nonsignificant increase at day 4 of the NR-treated MI group that was significantly amplified at day 7 post-MI (Fig. 5A, B). In the plasma, a similar pattern was observed with NR administration robustly increasing IL-4 levels in the MI group at days 4 and 7 post-MI (Fig. 5C-D).

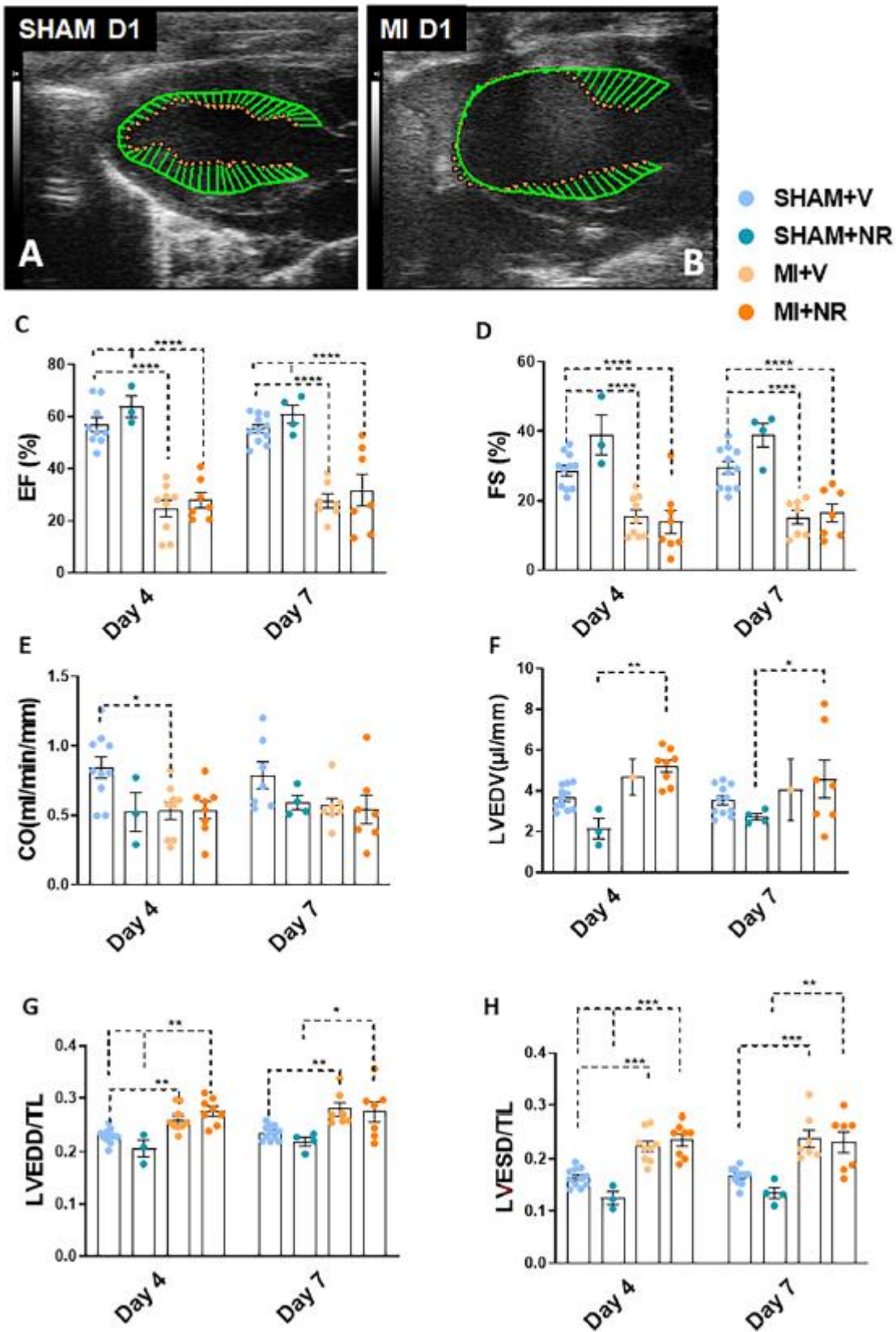


**Fig. 5 Plasma and myocardial levels of IL-4 post-MI.** A, B Western Blot analysis of IL-4 in cardiac tissue ( $n = 4$  per group). Quantitative ELISA: Plasma protein concentrations of (C, D) IL-4 of SHAM and MI groups at 4 and 7 days post-following NR treatment ( $n = 4-5$  per group). Data are presented as means  $\pm$  SEM. Statistical analysis: one-way ANOVA for independent samples; Tukey's multiple comparisons test: \* $p \leq 0.05$ , \*\* $p \leq 0.01$

## Cardiac Dysfunction and LV Remodeling Following MI Induction

To assess the effect of NR on cardiac parameters, we performed 2D echocardiography in SHAM and MI mice. Twenty-four hours of permanent LAD coronary artery ligation caused severe LV enlargement compared to the SHAM group (Fig. 6A, B), indicating successful ligation of the LAD coronary artery. Moreover, the induction of MI caused severe cardiac dysfunction as evidenced by the decrease in ejection fraction (EF), fractional shortening (FS), and cardiac output (CO) in the vehicle-treated MI group at days 4 and 7 following LAD (Fig. 6C-E). A similar decrease in all hemodynamic parameters (EF, FS, and CO) was observed in both the vehicle-treated MI group and NR-treated MI group at days 4 and 7 post-MI. LV end-systolic and diastolic volumes and diameters (LVESV, LVEDV, LVESD, and LVEDD) (normalized to tibia length) increased in both vehicle-treated MI group and NR-treated MI group at days 4 and 7 following LAD coronary artery ligation compared to SHAM group, and NR had no effect on these parameters (Fig. 6F-H). NR treatment had no

effect on cardiac fibrosis post-MI (Supplementary Figure I).



**Fig. 6** Quantification of cardiac function using B-mode and M-mode echocardiography of SHAM and MI mice, 4 and 7 days after surgery. **A, B** Representative echocardiogram images (B-Mode, parasternal Long Axis), 1 day after surgery (SHAM and MI). Cardiac dysfunction and left ventricle dilation take place following LAD ligation. Ejection fraction (EF) (**C**), fractional shortening (FS) (**D**), cardiac output (CO) (**E**), LV end-diastolic volume (LVEDV) (**F**), left ventricular end-diastolic diameter (LVEDD) (**G**), left ventricular end-systolic diameter (LVESD) (**H**). SHAM and MI ( $n = 7-10$ ) (8-9

weeks of age). Data are presented as means  $\pm$  SEM. Statistical analysis: two-way ANOVA for independent samples; Tukey's multiple comparisons test: \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001.

## Supplementary Material

The supplementary figures cited in the results section of this manuscript are provided in a separate supplementary file as follows: (1) Picrosirius red staining findings for fibrosis, 4- and 7-days post-MI; (2) Western blots.

## Discussion

Several studies have shown the key roles of NAD and NAD-dependent enzymes in protecting against oxidative stress-induced injuries, improving metabolism, protecting mitochondrial function, inhibiting cell death, decreasing DNA damage, and inhibiting inflammation [19–23]. In this study, we report that depressed NAD homeostasis and activation of NMRK2 kinase are key events in a mouse model of large MI and that administration of NR, the most energetically favorable NAD<sup>+</sup> precursor and substrate of NMRK2, preserves myocardial NAD levels and mitochondrial respiration, and significantly increases survival after a large MI.

The alteration of NAD homeostasis has been reported in several models of cardiac diseases including DCM [12, 24], pressure overload [25], and hypertension [26]. In our study, we found a marked upregulation of *Nmrk2* expression in the heart as early as 4 days following LAD coronary artery ligation. The synthesis of NMN from NR by NMRK enzymes requires only one ATP, whereas synthesis from NAM by NAMPT requires >3 ATP equivalents: one for the autophosphorylation of the enzyme, and 2 (plus a carbohydrate) for the formation of phosphoribosyl pyrophosphate. Thus, the shift from the NAMPT to the NMRK2 routes for NAD<sup>+</sup> synthesis could be considered as an energy-sparing mechanism that may be favored in cardiac diseases where energy homeostasis is altered.

Our findings also show that PARP1 is highly expressed as early as day 4 following LAD coronary artery ligation. This is a pivotal feature of MI which leads to NAD depletion, causing the slowing of the rate of glycolysis, electron transport, and ATP production, and eventually leading to activation of inflammatory pathways [27], cell death, and functional impairment. While the NMRK2 pathway is activated following MI, myocardial NAD levels are depressed, suggesting that circulating bioavailable NR is not sufficient to sustain cardiac NAD biosynthesis in the context of acute cardiac injury. NR supplementation reversed this imbalance by both reducing PARP-1 expression and increasing NAD levels at days 4 and 7 post-MI. A limitation of our study is that we did not look at PARP-1 protein levels, although MI-associated PARP-1 cleavage would likely have complicated the analysis.

Mitochondrial dysfunction is a key feature following cardiac ischemic lesions [28]. Our present work shows alterations in the respiration capacity of the respiratory chain complexes after MI. We observed that NR administration in vivo rescued mitochondrial citrate synthase activity and respiration capacities of complexes I (using NADH coenzyme) and IV. Overall, these data indicate that the decrease in myocardial mitochondrial function and mass in mice subjected to MI is blunted by NR injection.

The roles of NAD<sup>+</sup> extend beyond that of a coenzyme. NAD<sup>+</sup> links cellular metabolism status to inflammation and immune response [22]. In this study, we provided evidence that the effect of NR exceeds its action on NAD levels and mitochondrial function by targeting the inflammatory status following MI. The inflammatory response has been shown to play a major role in the evolution of the fibrotic scar following MI [29]. The utility of our approach is supported by a recent study, which showed that NR supplementation in heart failure patients boosted NAD levels of peripheral blood mononuclear cells (PBMCs), thereby suppressing their inflammatory activation [30]. Our findings show that NR administration increased plasma and myocardial levels of IL-4 after MI. IL-4 is a Th2 cytokine with several biological functions including a main anti-inflammatory effect, and elevated IL-4 plasma levels have been associated with reduced risk of cardiovascular diseases [31].

## Perspectives and Significance

This is the first study performed in the context of a murine model of acute MI to investigate whether boosting NAD levels with NR administration exerts cardioprotective effects. Although human efficacy data are lacking, the effects of NR therapy on preserving cardiac NAD levels, improving cardiac function and cardiac mitochondrial respiration, modulating systemic and myocardial inflammation, and improving overall survival, constitute a promising therapy to curb the detrimental acute effects of a large permanent MI. The present study has a few limitations. First, the severity of the experimental MI surgery (large MI)

could have affected the analysis post-MI especially that a large number of vehicle-treated MI animals died before day 7, which reduced statistical power in terms of functional analysis. Second, the cardioprotective effects of NR at early stages of MI are not translated into a strong and visible phenotype at the functional and structural levels. One possible explanation is the involvement of NR in modulating cardiac electrophysiology and limiting the occurrence of reentry tachyarrhythmias that can rupture the heart. Hence, a thorough investigation of calcium handling and delayed-after-depolarization-triggered reentry tachyarrhythmias is warranted.

In conclusion, our work shows that NR supplementation is a powerful approach to preserving cellular homeostasis by correcting NAD levels following MI, restoring mitochondrial function, and improving overall survival. Our working hypothesis is that NR supplementation protects the heart by bolstering the periinfarct region of the heart and limiting infarct expansion, as well as by limiting wall thinning by tempering the inflammatory response post-MI. Future studies are warranted in order to discern the underlying mechanisms behind these interesting findings.

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**Data Availability** The authors will make all original data available upon reasonable request.

## Declarations

**Ethics Approval** All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of AUB (IACUC # 18–03–470) and conducted according to the guidelines of the Declaration of Helsinki, conformed to the Directive 2010/63/EU of the European Parliament and approved by the National Ethics Committee #26 of the French Ministry of Research (agreement APAFIS #20327-2019062415368026v1).

**Consent to Participate** Not applicable.

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**Conflict of Interest** The authors declare no competing interests.

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