



Modeling type 1 cardiorenal syndrome in mice : Studies on risk factors and development of a therapeutic approach based on stimulation of NAD signaling

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Modélisation du syndrome cardiorénal de type 1 chez la souris : Etude des facteurs de risque et développement d'une approche thérapeutique basée sur la stimulation de la signalisation du NAD

Modeling type 1 cardiorenal syndrome in mice: Studies on risk factors and development of a therapeutic approach based on stimulation of NAD signaling

Thèse de doctorat de l'université Paris-Saclay

École doctorale n° 599, Innovation Thérapeutique : Du Fondamental À L'appliqué (ITFA)
Spécialité de doctorat : Physiologie et physiopathologie
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Titre : Modélisation du syndrome cardiorénal de type 1 chez la souris : Etude des facteurs de risque et développement d'une approche thérapeutique basée sur la stimulation de la signalisation du NAD

Mots clés : syndrome cardiorénal, infarctus du myocarde, modèle souris, signalisation, métabolisme

Résumé : L'infarctus aigu du myocarde (IM) est la principale cause de décès dans le monde. Plus de 20 % des patients hospitalisés avec un IM développent une insuffisance rénale aiguë (IRA), également connue sous le nom de syndrome cardiorénal de type I. Il a été démontré que plusieurs facteurs de risque sont impliqués dans l'exacerbation du SCR, notamment le tabagisme et le sexe masculin, entre autres. Les options thérapeutiques sont néanmoins limitées pour le traitement du SRC de type 1. Ces dernières années, plusieurs études ont mis en évidence les bénéfices d'une supplémentation en vitamine B3 pour activer la signalisation du nicotinamide adénine dinucléotide (NAD) dans différentes pathologies mais pas encore dans des modèles de SRC de type 1.

L'objectif de ma thèse était de caractériser un modèle de SCR de type I chez la souris et d'évaluer l'impact de la suppression des hormones sexuelles féminines

par l'ovariectomie et les effets du nicotinamide riboside (NR) en tant que thérapie prometteuse potentielle pour l'IRA suivant un IM expérimental. Nous avons constaté que l'ovariectomie aggrave les lésions rénales et l'inflammation à la suite d'un infarctus du myocarde et avons observé différentes perturbations dans la biosynthèse et la signalisation du NAD dans ce modèle. L'injection de NR n'améliore pas directement l'hémodynamique cardiaque dans la semaine suivant l'IM, mais fournit une protection robuste contre les lésions rénales, suggérant des mécanismes rénoprotecteurs directs médiés par la signalisation NAD. Ces résultats apportent une nouvelle compréhension de la pathogenèse du SRC de type 1 et ouvrent de nouvelles perspectives cliniques pour le traitement de l'IRA dans ce contexte..

Title : Modeling type 1 cardiorenal syndrome in mice: Studies on risk factors and development of a therapeutic approach based on stimulation of NAD signaling

Keywords : cardiorenal syndrome, myocardial infarction, mouse model, signaling, metabolism

Abstract : Acute myocardial infarction (MI) is the leading single cause of death worldwide. More than 20% of patients hospitalized with MI develop acute kidney injury (AKI), otherwise known as type I cardiorenal syndrome (CRS). Multiple risk factors have been shown to be implicated in exacerbating CRS-I including cigarette smoking (CS) and male sex among others. Therapeutic options are limited for the treatment of type 1 CRS. In the recent years, several studies have highlighted the benefits of vitamin B3 supplementation to activate nicotinamide adenine dinucleotide (NAD) signaling in different pathologies but not yet in models of type 1 CRS.

The objective of my thesis were to characterize a model of type 1 CRS in mice and assess the impact of female hormones suppression by ovariectomy and the effects of nicotinamide riboside (NR) as a potential promising therapy for AKI following experimental MI. We found that ovariectomy aggravates renal injury and inflammation following MI and observed different perturbation in NAD biosynthesis and signaling in this model. NR injection did not directly improve cardiac hemodynamics within one week after MI, yet, it provided a robust protection against renal injury suggesting direct renoprotective mechanisms mediated by NAD signaling.

These results bring a new understanding of type I CRS pathogenesis and open clinical perspectives for the treatment of AKI in this context.

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3 LIST OF ABBREVIATIONS

ACE: Angiotensin-converting enzyme	G3P: Glycerol 3 phosphate
ACMS: Amino-3-carboxymuconate semialdehyde	GECs: Glomerular endothelial cells
ADH: Antidiuretic hormone	GFB: Glomerular filtration barrier
ADHF: Acute decompensated heart failure	IDO-1: Indoleamine 2,3-dioxygenase 1
ADQI: Acute Dialysis Quality Initiative	IL: Interleukin
AHA: American Heart Association	LDL: Intermediate density lipoprotein
AKI: Acute kidney injury	KDIGO: Kidney Diseases Improving Global Outcomes
AKIN: Acute Kidney Injury Network	KIM-1: Kidney injury molecule-1
Ang-II: Angiotensin-II	LDL: low density lipoprotein
ANS: Autonomic nervous system	LV: Left ventricle
ATP: Adenosine triphosphate	LVH: Left ventricle hypertrophy
CKD: Chronic kidney disease	M-A: Malate-aspartate
CO: Cardiac output	MDA-LDL: Malondialdehyde-Modified Low-Density Lipoprotein
CRP: C-reactive protein	MI: Myocardial infarction
CRS: Cardio-renal syndrome	NA: Nicotinic acid
CS: Cigarette smoking	NAAD: NA dinucleotide
CVDs: Cardio vascular diseases	NAD: Nicotineamide dinucleotide
DAMPs: Dangerous associated molecule patterns	NAM: Nicotinamide
DM: Diabetes mellitus	NAMPT: Nicotinamide phosphoribosyl transferase
E2: 17- β Ethinyl Estradiol	NAPRT: NA phosphoribosyl transferase
ECM: Extracellular matrix	NAR: Nicotinic acid riboside
EF: Ejection fraction	NGAL: Neutrophil gelatinase-associated lipocalin
ETC: Electron transport chain	NMRK: Nicotinamide riboside kinase
FMN: Flavin mononucleotide	NOX: NADPH oxidases
	NPs: Natriuretic peptides

NR:	Nicotinamide riboside	STMI:	ST-elevated MI
PARP:	Poly-ADP ribosyl polymerases	TCA:	Tricarboxylic acid cycle
PNP:	Purine nucleoside phosphorylases	TDO:	Tryptophan 2,3-dioxygenase
PT:	Proximal tubules	TNF- α :	Tumour necrosis factor-alpha
RAAS:	Renin angiotensin aldosterone system	TP:	Tryptophane
ROS:	Reactive oxygen species	vLDL:	Very-low density lipoprotein
SIRT:	Sirtuins	WHO:	World Health Organization
SNS:	Sympathetic nervous system		

4 INTRODUCTION

4.1 STRUCTURE AND FUNCTION OF THE HEART AND KIDNEYS

The cardiovascular system and the kidneys are intimately correlated in order to keep cardio renal homeostasis. This bidirectional interrelationship is known as cardiorenal syndrome (**CRS**). CRS is the umbrella term used to describe a vast array of interrelated derangements in the clinical setting in which cardiac diseases and kidney injury coexist. A growing body of evidence has shed the light on CRS since this condition is highly correlated with substantial increase in morbidity and mortality rates. Kidney dysfunction is highly prevalent amongst patients with cardiovascular diseases (**CVDs**) and has been considered as a powerful and independent marker of adverse prognosis. Similarly, patients with kidney injury are considerably more likely to develop CVDs compared to matched subjects from the general population (Shah and Greaves 2010, Pan, Guo et al. 2014).

Type I CRS is defined as an acute cardiac disease that leads to acute kidney injury (**AKI**). Type I CRS has gained a great attention since it occurs in 24-45% patients hospitalized with CVDs such as acute myocardial infarction (**MI**) (Pan, Guo et al. 2014, Atici, Emet et al. 2018). Multiple risk factors are implicated in the pathogenesis of AKI including cigarette smoking (**CS**) and gender among others. Of note, kidney metabolic impairment has been well-documented in the setting of AKI, however, to date there is no metabolic drug that can lessen AKI progression.

In order to address these issues, my thesis research aimed at assessing the impact of MI on AKI and studying the impact of risk factors such as CS exposure and the reduction in female sex hormones protection in a mouse model of this disease. Additionally, following previous observations obtained during my Master thesis with

Dr. Fouad Zouein and results from Dr. Mericskay's group, I aimed at evaluating a new metabolic therapy approach by using nicotinamide riboside **(NR)**, a precursor implicated in the generation of NAD⁺ coenzyme on MI-induced AKI.

4.1.1 Cardiac anatomy and physiology

The cardiovascular system is made up of the heart and the circulation system, together. The heart has been over decades the center of the cardiovascular research due to its important role in maintaining homeostasis, though delivering oxygen and nutrients to all vital organs in order to perform their function, in addition to the fact that cardiac diseases are the leading cause of death worldwide. The role of the heart is to pump blood to the whole body in order to supply different organs with sufficient amount of oxygen and nutrients. Therefore, any disruption of the orchestrated function and the interaction between the heart components leads to initiate cardiac pathogenesis and eventually multi organ damage.

The heart is located in the chest cavity and surrounded by an outer protective covering called the pericardium. Three layers made up the heart wall: The endocardium; the inner layer, the myocardium, known as the muscular middle layer, and the epicardium; the outer layer. The heart is also composed of 4 chambers: Two lower chambers called ventricles and two upper chambers known as atria **(Figure 1)**. The right atria receives the deoxygenated blood from the body, then blood passes through the tricuspid valve to the right ventricle, moves afterward through the pulmonic valve to the lung for re-oxygenation. Afterward, the oxygenated blood enters the left atrium and passes to the left ventricle through the mitral valves then to be ejected through the aortic valve to supply all organs and tissues (Mohrman and Heller 2006, Katz 2010).

The cardiac cycle consists of two phases: systole and diastole. During the systole the heart contracts in order to pump blood to the aorta and subsequently to other arteries. Of note, the coronary arteries are the first vessels that branch from the aorta

to perfuse the cardiac wall in order to supply it with sufficient amount of the oxygenated blood. During diastole, the ventricles are relaxed and the heart fills with blood (Guyton and Hall 2011).

There are two major coronary arteries: The right and left coronary arteries. The right branches into the right marginal artery that provides blood to both surfaces of the right ventricles and the posterior descending artery that supplies blood to the posterior third of the inter ventricular septum. The left coronary artery, on the other hand, is divided into the left descending coronary artery that supplies the anteroseptal wall of the heart and the circumflex coronary artery that supplies the posterior and lateral portions. Since supplying the heart with a sufficient amount of blood is crucial for maintaining normal cardiac cycle and healthy cardiac tissue, any decrease in blood flow to the heart may result in coronary heart diseases otherwise known as ischemic heart diseases (**IHD**) (Phibbs 2007, Katz 2010)

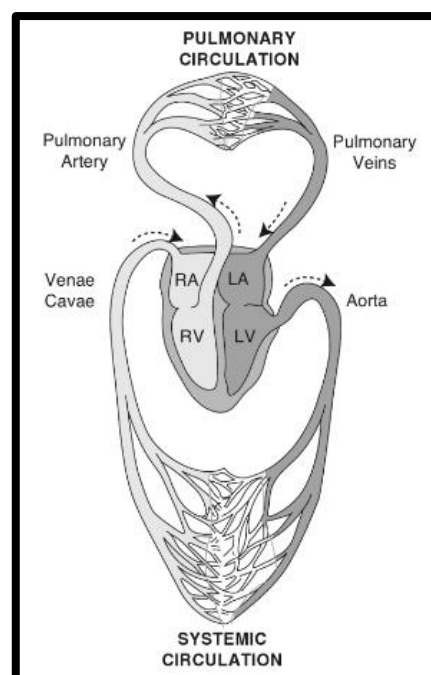


Figure 1: Circulation of the blood

Light shedding: deoxygenated blood, dark shedding: oxygenated blood. RA: right atrium, LA: left atrium, RV: right ventricle, LV: left ventricle.

4.1.2 Kidney anatomy and physiology

The kidneys are complex organs that exert a crucial role in maintaining normal range of homeostasis (Wallace 1998). Their main function is to regulate acid-base and electrolyte balances and fluid homeostasis in order to sustain a stable environment for cell metabolism. The kidneys filter the blood, balance solute and water transport, contributing to blood pressure control and excrete metabolic waste products (Wallace 1998). Kidneys also contribute to many other functions such as synthesis of vitamin D that is needed for bone mineralization, or erythrocyte development by generating erythropoietin (Scott and Quaggin 2015).

The kidney parenchyma is divided into two regions, the cortex or and the medulla (**Figure 2A**). The functional unit of the kidneys that are responsible for kidney functions is the nephron (Miyoshi, Hiratsuka et al. 2020). Importantly, an adult human kidney has been shown to contain around 1 million nephrons, which in turn consists of more than twenty cell types (Yousef Yengej, Jansen et al. 2020). A nephron can be functionally subdivided into a filtration and reabsorption units. The filtration unit is known as the glomerulus, whereas the reabsorption unit is the segmented tubular reabsorption compartment, which can also be subdivided into four different segments: 1) proximal tubule (**PT**), 2) loop of Henle, 3) distal tubule, and 4) collecting duct (**Figure 2B**) (Scott and Quaggin 2015). The glomerulus is a highly specialized cell type located at the proximal part of the nephron and is responsible for filtrating incoming blood by excreting excess metabolic waste and water in order to generate pro-urine (Greka and Mundel 2012). Of note, the glomerulus consists of four cell types: glomerular endothelial cells (**GECs**), podocytes, parietal epithelial cells of Bowman's capsule, and mesangial cells (Scott and Quaggin 2015) (**Figure 2C**). The internal layer of GECs otherwise known as the glomerular filtration barrier (**GFB**) faces the capillary lumen,

whereas the podocytes are located on the external layer facing the urinary capsule (Wang, Tao et al. 2017). Noteworthy, GFB is a size dependent selective molecular that prevents the filtering of large molecules and allows the passage of small molecules and water on the basis of charge and size (Wang, Tao et al. 2017). Consequently, circulating cells such as erythrocytes and high molecular weight proteins including albumin are retained in the blood stream, while small molecules including glucose and urea among others and water are filtrated through this specialized cell (Scott and Quaggin 2015). These filtrated products then pass through the barrier into the Bowman's capsule area and they move to the renal tubular compartments (Wang, Tao et al. 2017).

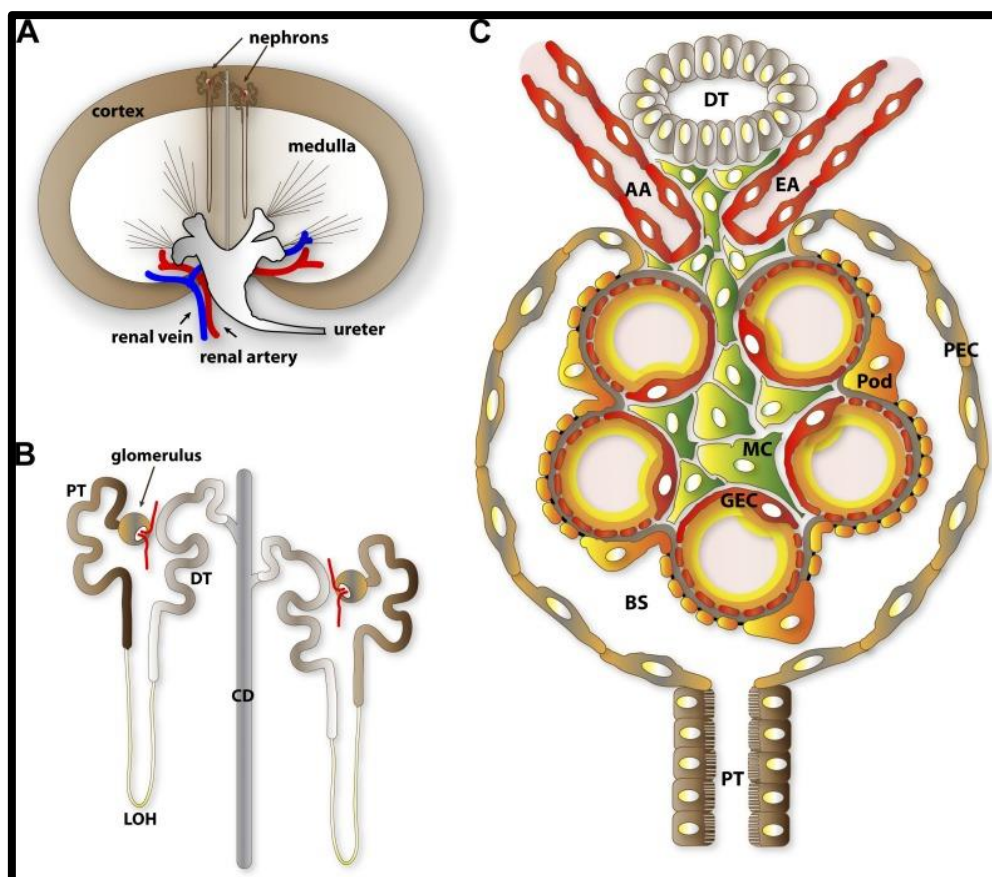


Figure 2: Anatomical overview of renal filtration

(A) Diagrammatic representation of nephron distribution in the kidney. Glomeruli, the filtration compartments of nephrons, are found within the kidney cortex. (B) Segmental structure of nephrons. The vascularized glomerulus is found at the proximal end and is connected through a series of renal tubules where urinary filtrate composition is refined through resorption and secretion. (C) Cellular

organization of the glomeruli. GEC, glomerular endothelial cell; AA, afferent arteriole; EA, efferent arteriole; Pod, podocyte; MC, mesangial cell; PEC, parietal epithelial cell; PT, proximal tubule; DT, distal tubule; LOH, loop of Henle; CD, collecting duct; BS, Bowman's space (Scott and Quaggin 2015).

With respect to the segmented tubular reabsorption compartment, the first segment is the PT. It is built up of a leaky epithelium that contains specialized transporters that regulate the reabsorption of different compounds, such as bicarbonate, electrolytes and glucose. Under physiological conditions, plasma glucose is filtered by glomeruli and almost completely reabsorbed in the PT. Glucose reabsorption is mainly mediated by two main sodium-glucose cotransporters (**SGLT**): SGLT-1 and SGLT2. It has been shown that SGLT2 is located in the early segment of the PT (S1-S2) and responsible for 80-90% of glucose reabsorption, whereas SGLT1 is located in the distal part of the PT and absorbs around 10-20% of glucose (Lu, Griffen et al. 2014). The second segment is known as the loop of Henle that is a long U-shaped tubular section, stretching down into the renal medulla and then ascending back into the renal cortex. The descending limb of the loop of Henle contributes to water reabsorption, while the ascending limb and the following segment, the distal tubule, control electrolyte reabsorption. The terminal tubular compartment is the collecting duct that contains two types of cells: principal cells and intercalated cells. Principal cells are the most abundant type and they reabsorb water and sodium, in exchange with potassium, under the control of aldosterone and vasopressin. Intercalated cells, on the other hand, maintain acid-base homeostasis by regulating proton and bicarbonate excretion (Roy, Al-bataineh et al. 2015, Scott and Quaggin 2015, Yousef Yengej, Jansen et al. 2020). The collecting ducts ultimately converge into a single ureter that acts as

one-way outlet for the urine formed in the nephron

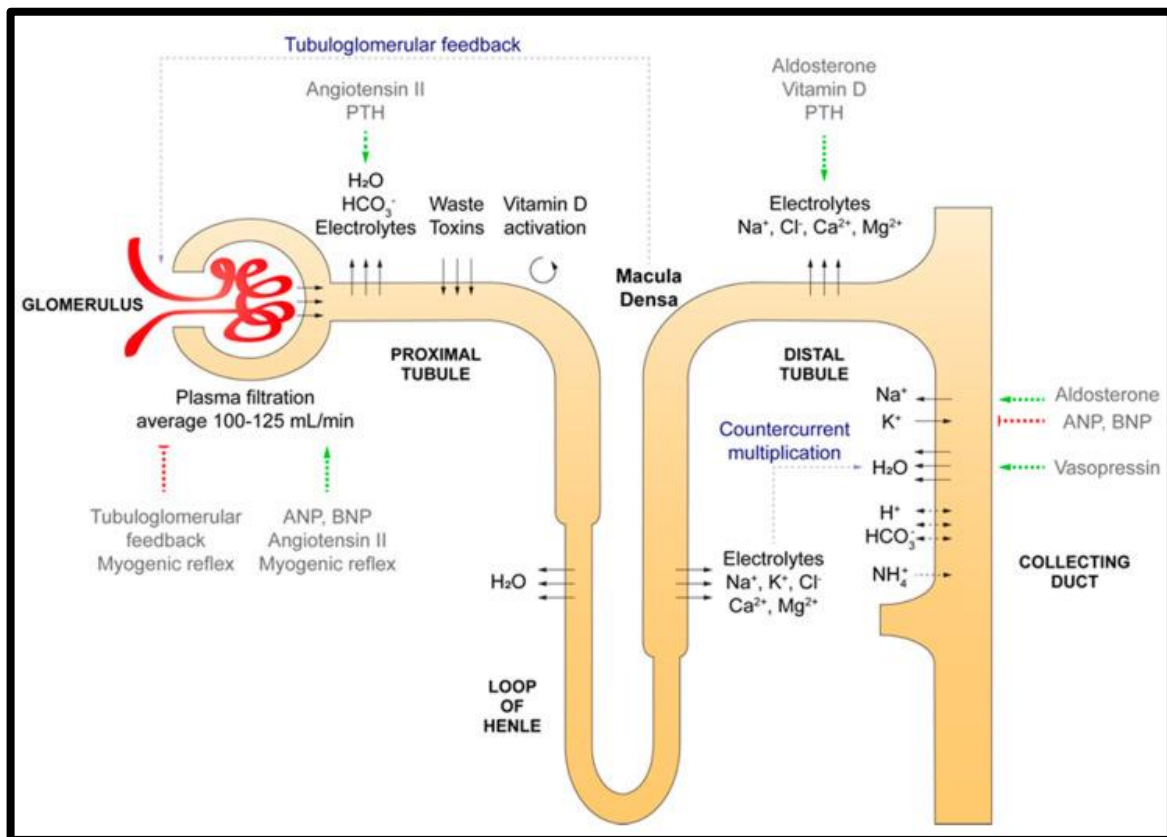


Figure 3: Summary of the main filtration, reabsorption, excretion and endocrine processes across the nephron.

Important hormones and mechanisms that regulate these functions are depicted in grey. ANP = atrial natriuretic peptide. BNP = brain natriuretic peptide. PTH = parathyroid hormone (Yousef Yengej, Jansen et al. 2020).

4.1.3 The cardio-renal interrelationship

Cardiovascular and renal systems are closely related through a synergistic interrelationship in order to keep cardio-renal homeostasis (Ronco, Haapio et al. 2008). The heart is responsible for providing a sufficient amount of blood to all organs through the vasculature, whereas the kidneys maintain acid-base and electrolytes equilibriums (Butler, Forman et al. 2004). This bidirectional interrelationship is fine-tuned by several neurohormonal activities, including the sympathetic nervous system (**SNS**), renin angiotensin aldosterone system (**RAAS**), natriuretic peptides (**NPs**), and

baroreflex systems.

The cardio-renal physiology is of utmost importance for normal body function and hemodynamic stability (Viswanathan and Gilbert 2011). In that regard, while the kidneys are highly affected by cardiac output (**CO**), kidney performance can in return directly affect the heart through fluid and electrolyte homeostasis modifying blood volume, hence cardiac hemodynamics (Viswanathan and Gilbert 2011). This bidirectional interrelationship is controlled by different physiological systems incorporated to regulate the volume status, vascular tone, and CO. It has been documented that the RAAS, the autonomic nervous system (**ANS**), and the natriuretic peptide (**NP**) system are three major conservatives of this cardio-renal crosstalk assisted together in a delicate homeostasis (Boudoulas, Triposkiadis et al. 2017). Stretch receptors in the aortic arch and carotid sinus, otherwise known as the baroreceptors are responsible for the short-term regulation of the blood pressure inducing in seconds to minutes either the sympathetic or the parasympathetic effects (Lanfranchi and Somers 2002). A long-term recovery, importantly involves renal mechanisms such as the SNS and RAAS. Low blood pressure and sympathetic nerve cells acting on the β 1-adrenoreceptors can directly induce renin release from the juxtaglomerular cells in the kidneys; a specialized type of smooth muscle cells that are mainly located in the afferent arterioles and to less extent in the efferent arterioles and plays a major role in synthesis, store, and release of renin, or indirectly by prostaglandins produced from neighboring macula densa cells when sensing low sodium (Sparks, Crowley et al. 2011). With respect to the RAAS, Renin is a protease chopping off the precursor hormone angiotensinogen released by the liver into angiotensin-I. Afterward, angiotensin-I is further metabolized by the angiotensin-converting enzyme (**ACE**) produced by endothelial cells into angiotensin-II (**Ang-II**), the active form, eliciting constriction of blood vessels via AT1 receptors and water and sodium retention by the kidneys (Brewster, Perazella et al. 2003) (Crowley, Gurley et al. 2005). Of note, these strategies for raising blood pressure and maintaining

hemodynamic homeostasis are further sustained by aldosterone and the antidiuretic hormone (**ADH**) secreted through the action of Ang-II on the adrenal and pituitary glands, respectively (Cuzzo and Lappin 2019) (Lumbers 1999). The NP, however, serves as a counter-regulatory mechanism by enhancing vasodilation, inhibiting renin, norepinephrine, and aldosterone release, and exerting natriuretic and diuretic effects on the kidneys (Suzuki, Yamazaki et al. 2001) (**Figure 4**).

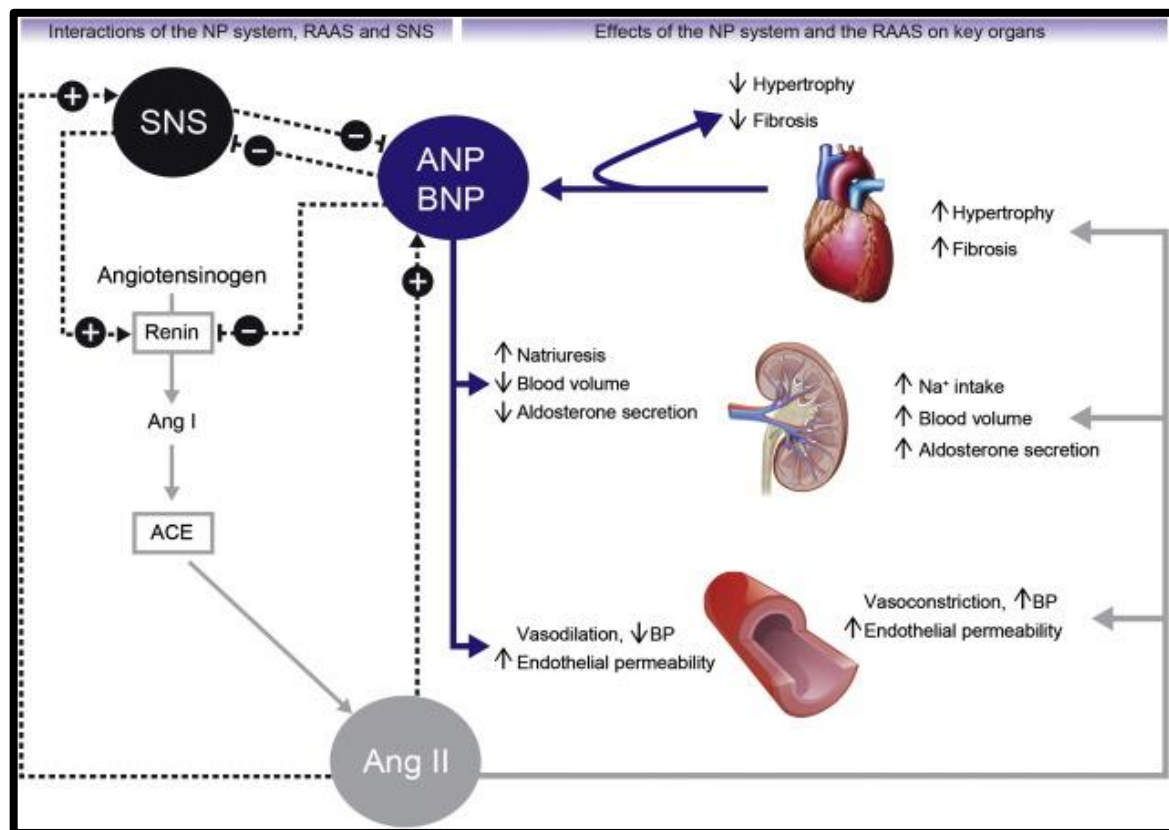


Figure 4: Schematic diagram to show how the NP system, the RAAS and the SNS interact in order to maintain cardio-renal homeostasis, and how the effects of the NP system and the RAAS on key organs are generally counter-regulatory

ACE, angiotensin converting enzyme; Ang, angiotensin; ANP, atrial natriuretic peptide; BNP, B-type natriuretic peptide; BP, blood pressure; NP, natriuretic peptide; RAAS, renin–angiotensin–aldosterone system; SNS, sympathetic nervous system (Volpe 2014).

4.2 THE CARDIO-RENAL SYNDROME

The Acute Decompensated Heart Failure National Registry database indicates that around 30% of patients with cardiac dysfunction develop kidney disease, through the cardiorenal interrelationship, otherwise known as cardio-renal syndrome (**CRS**). Furthermore, in a systemic review of 16 clinical studies, 29% of acute decompensated heart failure (**ADHF**) patients are diagnosed with moderate and severe kidney abnormalities (Smith, Lichtman et al. 2006, Heywood, Fonarow et al. 2007). CRS is a complex pathophysiological condition in which any acute or chronic cardiac and kidney dysfunction overlap (Cowie, Komajda et al. 2006).

4.2.1 CRS classification

Based on whether the initial injury originates in the kidneys or the heart, CRS is classified into five distinct types:

- Type 1 CRS is defined as an acute heart disease such as acute myocardial infarction (MI) causing an acute kidney injury (**AKI**)
- Type 2 CRS is defined as a chronic cardiac disease including congestive heart failure leading to chronic kidney disease (**CKD**)
- Type 3 CRS is defined as an AKI induced acute cardiac dysfunction
- Type 4 CRS is defined as a CKD induced chronic cardiac disease
- Type 5 CRS is defined as a systemic condition (i.e. diabetes mellitus) resulting in a simultaneous injury in both organs the heart and the kidney (McCullough, 2010)

4.2.2 Type I cardio renal syndrome epidemiology

As my thesis research focused on Type I CRS, I will mainly describe this syndrome.

Type I CRS is widely observed in the clinical setting. Although the cardiac etiology was well-clarified, the inconsistency in defining AKI and the heterogeneity of

selected populations resulted in variation in the incidence of type I CRS, the epidemiological studies therefore lacks the precision. In that regard, the incidence of type I CRS in the context of ADHF and acute coronary syndrome ranges from 20-45% and 9-19%, respectively (Goldberg, Hammerman et al. 2005, Cowie, Komajda et al. 2006, Marenzi, Moltrasio et al. 2007, Nohria, Hasselblad et al. 2008). The Acute Dialysis Quality Initiative (**ADQI**) group created therefore a consensus definition for AKI called RIFLE criteria (Bellomo, Ronco et al. 2004), that underwent further modification by the Acute Kidney Injury Network (**AKIN**) (Mehta, Kellum et al. 2007) and Kidney Diseases Improving Global Outcomes (**KDIGO**) group (Khwaja 2012). Based on the initial cause of type I CRS, a systematic review documented a higher incidence of type I CRS following ADHF (47.4%) compared to cardiac surgery (22.1%) and acute coronary syndrome (14.9%) (Khwaja 2012). Of note, although the frequency of type I CRS in the setting of ADHF is higher, type I CRS post-acute coronary syndrome is strongly correlated with higher in-hospital stay and mortality (Eren, Ozveren et al. 2012).

Following MI particularly, kidney injury denotes clinical and prognostic relevance. Around 20% of patients hospitalized with MI develop AKI which significantly increased to more than 50% when complicated with a cardiogenic shock (Marenzi, Assanelli et al. 2010, Eren, Ozveren et al. 2012). AKI has been shown to be an independent predictor of morbidity and mortality in the short and long term (Goldberg, Hammerman et al. 2005, Parikh, Coca et al. 2008, Fox, Muntner et al. 2012) and patients are highly prone to develop adverse cardiovascular outcomes, creating therefore a vicious cycle (Jose, Skali et al. 2006). It has been demonstrated that minor changes in serum creatinine are associated with a substantial increase in long term mortality, longer in-hospital stay, and chronic kidney diseases and end-stage renal disease development and progression (Jose, Skali et al. 2006, Ishani, Xue et al. 2009).

4.2.3 Type I cardio-renal syndrome pathophysiology

The substantial burden of AKI is dramatically increasing in the setting of MI

(Nieminen, Brutsaert et al. 2006, Mendis, Davis et al. 2015). Following MI, a cardiac systolic dysfunction has been reported and shown to be correlated with a substantial decrease in CO and kidney perfusion (Damman, Navis et al. 2007). The compensatory mechanisms mediated by SNS and RAAS are activated post a decrease in the renal afferent arterial flow (Pfeffer and Braunwald 1990, Jernberg, Johanson et al. 2011, Westman, Lipinski et al. 2016). A dilatation in the afferent arteriole mediated by vasodilatory eicosanoids along with a restriction in the efferent arteriole mediated by ANGII have been documented following a reduction in kidney perfusion, as an attempt to keep normal GFR. A decrease in mean arterial pressure to less than 70mmHg (the kidney autoregulatory threshold), however, leads to a decrease in kidney perfusion pressure and a subsequent decline in GFR (Svensen and Rodhe 2019). Additionally, the overactivation of the compensatory mechanisms, overwhelms the endogenous vasodilating effects, and the kidneys become resistant to the NPs, enhancing consequently kidney damage (Brewster, Perazella et al. 2003, Charloux, Piquard et al. 2003). Of note, in the context of MI, left ventricular ejection fraction (**EF**) can be a strong predictor of AKI progression and development (Shacham, Leshem-Rubinow et al. 2015). Additionally, a retrospective study reported that the concomitant decrease in EF along with the elevation of the central venous pressure are high-risk factors that result in a 10-folds increase in the incidence of AKI in the setting of ST segment (the interval between ventricle repolarization and depolarization) -elevated MI (STEMI) patients (Khoury, Steinvil et al. 2018). Recently, investigations have pointed out a more complex multifactorial pathophysiology (Shacham, Leshem-Rubinow et al. 2015, Shacham, Steinvil et al. 2016). Pre-clinical studies have uncovered a tight link between endothelial dysfunction and systemic inflammation and enhanced kidney damage (Fang, Moore et al. 2015). In that regard, Cosentino et al. demonstrated that high C-reactive protein (**CRP**) levels in MI patients at admission is correlated with AKI severity (Cosentino, Genovese et al. 2019). Furthermore, Fujii et al. indicated that elevated CRP levels induce the expression of adhesion molecules, depress vasodilators

such as NO, and impair the antioxidant defense resulting subsequently in exacerbated endothelial dysfunction, a well-documented mechanism in the context of AKI (Fujii, Li et al. 2006). In addition to the crucial role of the inflammatory response, STEMI patients diagnosed with AKI have shown an overactivation of the SNS and an increase in oxidative stress, exerting together a synergistic effect in aggravating AKI pathogenesis (Anzai, Anzai et al. 2010) **(Figure 5)**.

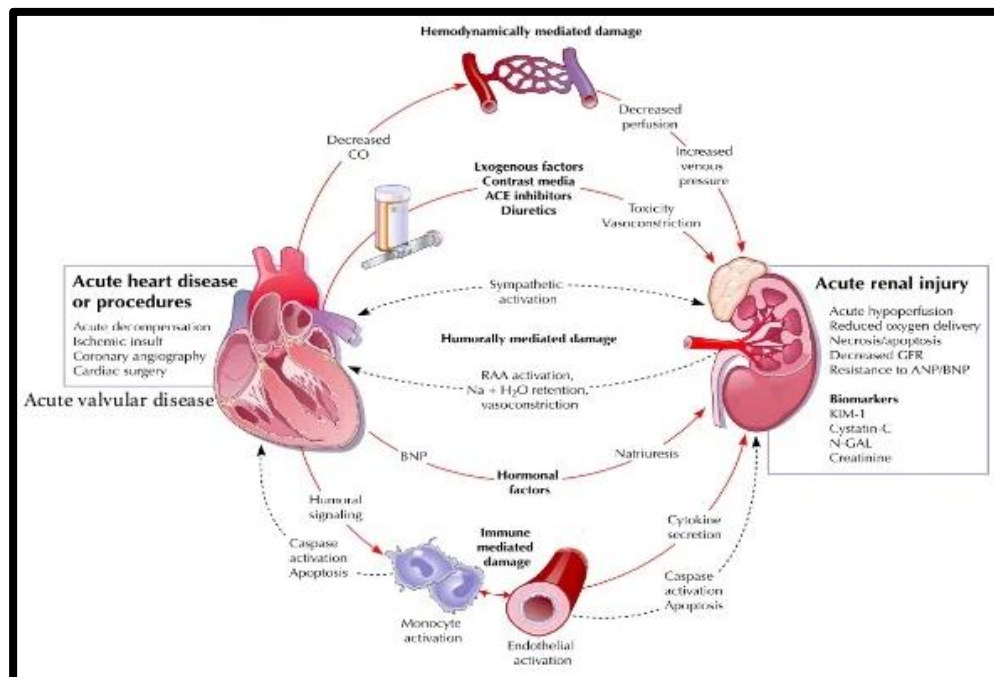


Figure 5: Cardio-renal syndrome (CRS) Pathophysiologic interactions between heart and kidney in type 1 or "acute CRS" (abrupt worsening of cardiac function; eg, acute myocardial infarction, acute cardiogenic shock or acute decompensation of chronic heart failure) leading to kidney injury. Abbreviations: ACE, angiotensin- converting enzyme; ANP, atrial natriuretic peptide; BNP, B-type natriuretic peptide; CO, cardiac output; GFR, glomerular filtration rate; H₂O, water; KIM, kidney injury molecule; Na, sodium; N-GAL, neutrophil gelatinase- associated lipocalin; RAA, renin angiotensin aldosterone. Reproduced from Ronco et al 27 with permission of Elsevier (House, Haapio et al. 2010)

4.2.4 MI prevalence

Myocardial infarction (MI) is a major public health concern and a leading cause of AKI, through the cardiorenal interrelationship, otherwise known as type I CRS. It has been reported that MI is initiated by prolonged ischemia due to a decrease in blood

flow to the heart, leading to the necrosis of the cardiac muscle (Alpert, Antman et al. 2000). The starving muscle consequently dies and a major intervention would be salvaging the infarcted myocardium within a critical time frame (Montalescot, Andersen et al. 2004). According to the World Health Organization (**WHO**). Around 17.5 million deaths annually are attributed to CVDs, with stroke and MI being a major cause of death (<https://www.who.int/health-topics/cardiovascular-diseases/>). Furthermore, the American Heart Association (**AHA**) reported that one American suffers from an MI every 40 seconds, (Benjamin, Muntner et al. 2019).

4.2.5 Myocardial infarction (MI) risk factors:

Multiple factors including atherosclerosis, hypertension, cigarette smoking (**CS**), diabetes mellitus, and obesity are classified as both dependent and independent high risk factors for MI development and progression according to the thrombolysis in myocardial infarction risk factor assessment score (Johnson, Gordon et al. 2000).

4.2.5.1 Atherosclerosis

Atherosclerosis is defined as a chronic inflammatory disease of the arteries, with increased risk of blood clots formation and atherosclerotic plaque instability being leading causes of MI (Libby, Ridker et al. 2002). It has been reported that around 75% of MI cases caused by plaque rupture (Roma Pahwa 2022). Atherosclerotic mouse models exhibited a 14-fold increase in circulating monocytes when compared to the relative control group, which subsequently results in augmenting post-MI inflammatory response (Swirski, Libby et al. 2007). The inflammatory response following MI is characterized by increased serum cytokines, enhanced recruitment of inflammatory cells in the infarcted area, and augmented protease activity in the myocardium (Nahrendorf, Swirski et al. 2007, Panizzi, Swirski et al. 2010). This exacerbated pro-inflammatory response has critical adverse functional consequences. For instance, increased numbers of circulating monocytes post-MI are strongly correlated with a decline in functional recovery and aggravated adverse LV remodeling

and may also impact on peripheral organs such as the kidneys (Maekawa, Anzai et al. 2002, Tsujioka, Imanishi et al. 2009). Furthermore, increased serum cytokines levels are also tightly linked to accelerated progression into heart failure (Andreassen, Nordøy et al. 1998, Aukrust, Ueland et al. 1998). Moreover, increased the pro-inflammatory cytokine (**IL-1 β**) and matrix metalloproteinase-7 following MI may have an impact on the electrophysiological remodeling and vulnerability to develop arrhythmia (De Jesus, Wang et al. 2015)

4.2.5.2 Hypertension

Hypertension is a well-known risk factor for MI development and progression (Yusuf, Hawken et al. 2004). Multiple epidemiological studies and clinical data demonstrated that around 39% of MI cases are attributable to hypertension (Os, Kjeldsen et al. 1993, Jones and Nagpal 2001). Of note, the prevalence of hypertension is higher in males compared to age-matched females. This discrepancy, however, is gradually disappearing post menopause, with a risk of elevated blood pressure increasing to 58% in females aged between 60–70 years old (Hertz, Unger et al. 2005, Maric 2005, McDonald, Hertz et al. 2009, Lacruz, Kluttig et al. 2015). Sustained elevation in BP due to an over activation of RAAS and SNS can stimulate vascular endothelial dysfunction by causing alterations in extracellular matrix turnover, consequently increasing the risk of chronic heart diseases progression. Importantly, serious consequences of persistent increase in BP, such as fibrosis and hypertrophy, may result in enhanced structural and functional alterations in the left ventricle (**LV**) of the heart known as left ventricular hypertrophy (**LVH**) (Tomek and Bub 2017). Eventually, if left untreated, LVH accelerates the risk of ischemic cardiomyopathy and heart failure development (Frohlich 2009).

4.2.5.3 Cigarette smoking (CS)

Mounting body of evidence highlights the role of CS as an independent risk factor in the pathogenesis of IHD (Mendis, Puska et al. 2011). Of note, CS-induced

CVDs is highly correlated with the amount of cigarette smoked and duration of smoking (Burns 2003, Health and Services 2004). Additionally, sex based difference in CVDs following CS exposure has been reported, with a 10 folds increase in the incidence of MI in males versus a 5 folds increase in females, suggesting a cardio protective role of female sex hormone (Tang and Dickinson 1998).

It has been well-documented that CS contains around 5000 potential toxicants such as free radicals, oxidative gases, amid, reactive oxygen species (**ROS**), and carbon monoxide (Huang, Lin et al. 2005). Nicotine, the main alkaloid in CS, can be acquired through both active and passive smoking (Jain and Jaimes 2013). Two pathological mechanisms are implicated in adverse LV remodeling following CS. The first mechanism is mediated by the direct action of CS on the cardiac muscle resulting in enhanced coronary vasoconstriction, fibrosis, and necrosis, leading eventually to metabolic and histological alterations in the heart, known as smoke cardiomyopathy. The second mechanism, however, caused by the indirect actions of CS on the heart via exacerbating the adverse effects of other comorbidities including dyslipidemia and hypertension, that further fuel the high risk impact of CS on cardiac disease development (Hori and Nishida 2009, Altara, Manca et al. 2016).

4.2.5.4 *Obesity*

Obesity is a well-known risk factor for CVDs (Khan, Harvey et al. 2015). A tight link between abdominal obesity and increased risk of the incidence of MI in both men and women have been documented (Schargrodsky, Rozlosnik et al. 1994, Zhu, Su et al. 2014). Obesity is highly correlated with diabetes mellitus (**DM**), atherosclerosis, and hypertension, all of which are major risk factors for IHD progression (Gupta, Rastogi et al. 2007). It is well established that the accumulation of adipose tissues in the vessels exerts a critical role in obesity-induced CVDs (Williams and Tabas 2005). In that regard, it has been shown that in the context of lipid storage diseases, the transendothelial passage of very-low density lipoprotein (**v-LDL**), Intermediate density lipoprotein

(IDL), and low density lipoprotein **(LDL)** from plasma into the vascular intima initiates an robust inflammatory response mediated by macrophages infiltration and monocytes activation (Rajala and Scherer 2003). These inflammatory response, thereafter triggers the release of pro-inflammatory cytokines including interleukin **(IL-1)**, IL-6, endothelin, angiotensinogen, consequently exacerbating CVDs (Williams and Tabas 1998, Wang and Nakayama 2010).

4.2.5.5 *Diabetes Mellitus (DM)*

One of the main complications of DM is coronary heart diseases. Hyperglycemia and insulin resistance fuels the secretion of different pro-inflammatory cytokines including CRP, Tumor necrosis factor-alpha **(TNF- α)**, IL-1, and IL-6, all of which dramatically trigger endothelial dysfunction (Shoelson, Lee et al. 2006, Matheus, Tannus et al. 2013). The pro-inflammatory environment impairs endothelial nitric oxide production, prostacyclin generation, and enhances the uptake of the oxidized LDL in coronary arteries, exacerbating therefore endothelial dysfunction, and increasing subsequently the risk of myocardial ischemia (Chait, Han et al. 2005, Leon and Maddox 2015).

Epidemiological studies reported that the incidence of MI is 2.5 times higher in diabetic males and 3.4 times higher in diabetic females when compared to control subjects (Kannel and McGee 1979). Additionally, clinical data indicated a 4-fold higher risk in diabetic males and 7-fold higher risk in diabetic females of MI-related death when compared to the relative non-diabetic MI subjects (Kannel and McGee 1979, Yusuf, Hawken et al. 2004).

4.2.6 *MI pathophysiology*

Despite the improvement in pharmacological and interventional therapies and the marked increase in survival rates post-MI (60% to ~ 90%), around 25% of MI

patients still undergo adverse left ventricle remodeling (**LVR**) (Velagaleti, Pencina et al. 2008). Of note, cardiac remodeling following MI varies based on multiple factors including the infarct size, the healing process, and the presence or absence of comorbidities (Altara, Manca et al. 2016). A shift in adenosine triphosphate (**ATP**) production to anaerobic glycolysis has been reported following MI, resulting in increased intracellular acidosis and induced cell death by necrosis (Whelan, Kaplinskiy et al. 2010). As a result, dangerous associated molecule patterns (**DAMPs**) including heat shock proteins, RNA, DNA, and ATP are released from dying cardiomyocytes into the surrounding extracellular space, triggering an inflammatory response.

Three interchangeable but overlapping phases are involved in wound LV remodeling post-MI (Rossen, Michael et al. 1988, Frangogiannis, Smith et al. 2002). In animal models of MI, this process has been carefully dissected out. The early inflammatory phase is initiated at the onset of MI and characterized by the recruitment of monocytes and neutrophils to the site of injury resulting in monocytes differentiation into macrophages M1, fibroblast proliferation, secretion of pro-inflammatory cytokines (TNF- α , IL-1, IL-6,,) and extracellular matrix (**ECM**) degradation (Frantz, Bauersachs et al. 2009, Zlatanova et al. 2016)(Epelman, Liu et al. 2015, Altara, Manca et al. 2016), The second phase is the granulation phase that is mediated by the differentiation of macrophages M1 into M2 and the release of anti-inflammatory cytokines such as (IL- 10, TGF-B1) leading to subsidizing the inflammation response, differentiation of fibroblast to myofibroblasts, and ECM deposition (Nian, Lee et al. 2004). Myofibroblast survival along with fibrotic scar formation are the hallmark of the last phase known as the maturation phase (Brown, Ambler et al. 2005) (**Figure 6**). It is well documented that prolonged ischemia and overactivation of the inflammatory response have a crucial impact on cardiomyocytes within both non-infarcted and infarcted areas and is proven to be detrimental in the absence of adequate inflammatory resolution and appropriate transition between remodeling phases. In that regard it has been demonstrated that unresolved inflammation enhances

progressive infarct expansion and border zone extension by eliciting myocyte death and ECM degradation (Whittaker, Boughner et al. 1991, Siwik, Chang et al. 2000). Additionally, following MI, the mechanical workload of the heart has been shown to be shifted to the remote uninfarcted area which responds by increasing the amount of contractile units. Remote hypertrophied myocytes, however, are dysfunctional with reduced systolic sarcoplasmic reticulum calcium (Ca^{+2}) release and desensitization to Ca^{+2} which further aggravates cardiac systolic dysfunction in the setting of MI (Yokoyama, Vaca et al. 1993, Goldhaber 1996) .

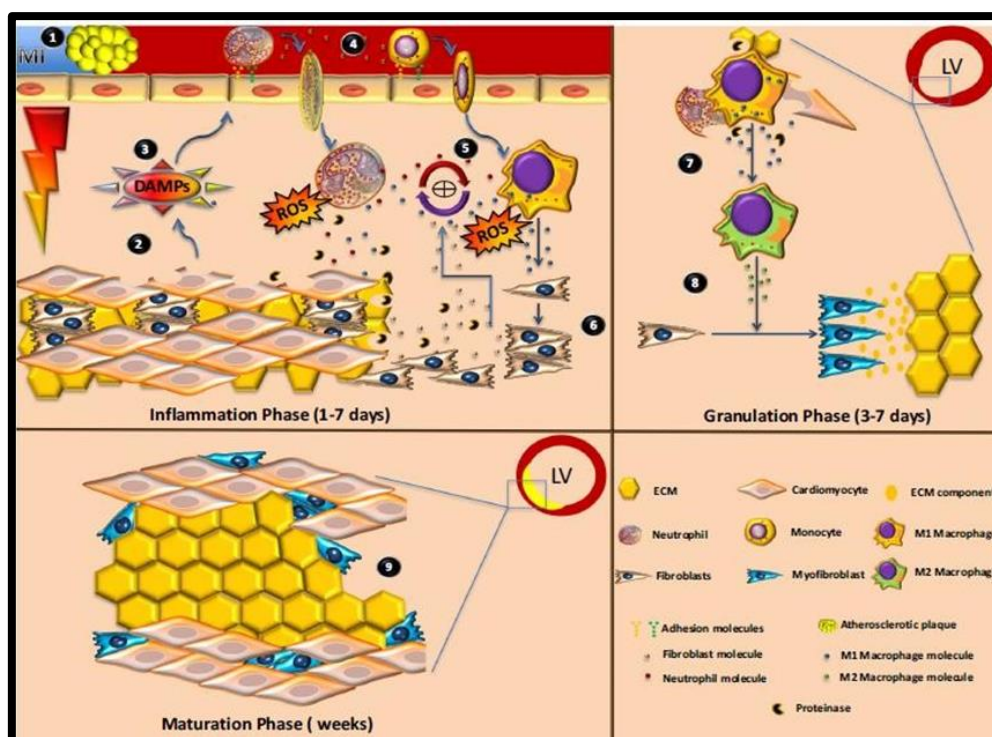


Figure 6: Wound-healing process

Three phases are identifiable in the wound-healing process that follows myocardial infarction. Inflammation phase: (1) Onset of myocardial infarction by obstruction of blood flow in coronary arteries. (2) Hypoxic environment triggers cellular necrosis and the release of DAMPs. (3) Irritated endothelium expresses adhesion molecules and alert circulating innate immune cells. (4) Acute inflammatory response is initiated by neutrophil and monocyte recruitment, activation, and extravasation to the site of injury. (5) Pro-inflammatory environment triggers monocytes to macrophage M1 differentiation and activation. (6) Macrophages stimulate fibroblast proliferation and migration which further boost pro-inflammatory cytokines (i.e. IL-1, IL-6, and $\text{TNF-}\alpha$), chemokines (i.e. MCP-1 and IL-8), release of

proteinases (i.e. MMPs), and ECM degradation. Granulation phase: (7) Macrophage phagocytosis of necrotic debris and apoptotic neutrophils which triggers M2 macrophages activation. (8) M2 molecules (i.e. IL-10 and TGF- β) subside inflammation and stimulate fibroblast to myofibroblast differentiation. Myofibroblast becomes the major producer of ECM components and mediators of ECM deposition. Maturation phase: (9) Prolonged myofibroblast survival along with ECM crosslinking and fibrotic scar maturation and contraction (Altara, Manca et al. 2016).

4.3 AKI MANIFESTATION IN THE CONTEXT OF MI

4.3.1 Functional Alterations

The deterioration in kidney function among hospitalized patients diagnosed with MI could be due to multiple factors such as hemodynamic alterations, drug toxicity such as loop diuretics, and the use of contrast media (Shacham, Steinvil et al. 2016). Of note, serum creatinine and GFR are two main indicators that have been used in the clinical setting for the assessment of kidney function. It has been observed that a mild change in serum creatinine level indicates high prognostic relevance, early detection, however, remains a challenge for timely effective treatment (Newsome, Warnock et al. 2008). Mounting evidence demonstrates that cystatin C and neutrophil gelatinase-associated lipocalin (**NGAL**) are promising markers of kidney injury with prognostic significance in the context of cardiac diseases (Lindberg, Pedersen et al. 2012, Fouad and Boraie 2013). Additionally, microalbuminuria, a measure of kidney damage, has been documented to define high-risk populations in addition to kidney function (Berton, Cordiano et al. 2001, Mok, Ballew et al. 2019). Pre-clinical investigation showed a GFR decrease one week following MI, during which EF markedly decreased (Lekawanvijit, Kompa et al. 2012). Additionally, a clinical study performed on 5395 patients hospitalized with MI showed that 34.5% of MI patients developed a decrease in GFR, increasing mortality rate substantially (de Carvalho, McCullough et al. 2013). Furthermore, a study included 1299 MI patients reported that 16.4% developed AKI at admission, increasing the risk of developing non STEMI and in hospital mortality rate (Wang, Hong et al. 2022).

4.3.2 Structural Alterations

In 1960, Aukland and Krog demonstrated a heterogeneous oxygen tension being lower in the medulla compared to the cortex of healthy dogs (Aukland and Krog 1960). Similarly, a follow up study reported that the tension in the medullary region ranged around 10–20 mm Hg, whereas that in the cortex was 30–60 mm Hg (Brezis, Heyman et al. 1991, Brezis, Agmon et al. 1994, Calzavacca, Evans et al. 2015). Of note, it was later recognized that the variation in oxygen tension was due to general anesthesia, which led to a decrease in renal blood flow (Calzavacca, Evans et al. 2015). This experiment was then performed on non-anesthetized sheep and showed that medullary oxygen tension was around be 30–40 mm Hg, similar in a way to that in the cortex (Calzavacca, Evans et al. 2015). It is worth mentioning that the basal tissue perfusion in the medullary region is substantially less than that in the cortex and thus the decline in oxygenation and perfusion is greater in the medulla following kidney artery occlusion (Calzavacca, Evans et al. 2015). Collectively, these observations indicate that due to its inadequate capability to sustain oxygen homeostasis, the medulla primarily vulnerable to hypoxia under pathophysiological conditions. To further elaborate on this observation, studies using a hypoxic marker, pimonidazole, reported positive staining in both the medulla and corticomedullary regions in kidneys of healthy rats (Manotham, Tanaka et al. 2004, Rosenberger, Rosen et al. 2009). These observations shed the light on the concept that the medullary region is “relatively hypoxic,” when compared to the cortex even if there is no decline in tissue oxygen tension. The kidney's susceptibility to injury is in part correlated with the tubule-blood vessel morphological association. Hypoperfusion-induced vasoconstriction results in a glomerular tuft collapse (Basile, Anderson et al. 2012). To further fortify these observation, Lu Et al. indicated enhanced kidney dysfunction with significant glomerular and tubular swelling and exacerbated fibrosis at day 3 post-permanent ligation of the left coronary artery in a mouse model (Lu, Wang et al. 2012). Additionally, fibrosis represents an independent risk factor that can directly affect the

kidney function regardless of the primary cause (Efstratiadis, Divani et al. 2009). Furthermore, kidney injury molecule-1 (**KIM-1**), a biomarker of tubular injury, has been observed to be upregulated in PT of the MI rats (Lekawanvijit, Kompa et al. 2012).

4.3.3 Molecular Alterations

4.3.3.1 Inflammation

Mounting evidence demonstrates that systemic inflammation serves as a prognostic indicator of mortality in patients diagnosed with AKI (Simmons, Himmelfarb et al. 2004). MI elicits an inflammatory response in the kidneys characterized by enhancing the expression level of multiple inflammatory cytokines (TNF- α , IL-1 β , and IL-6) and VCAM-1 in glomerular endothelial cells, as well as augmenting leukocytes recruitment in the kidneys 24 hours after an MI in a mouse model (Ruparelia, Digby et al. 2013). Increased Vascular cell adhesion protein 1 (**VCAM-1**) level is correlated with endothelial activation that induces endothelial-leukocyte interaction and aggravates local inflammation. Of note, endothelial dysfunction serves as a target of inflammation (Galle, Quaschnig et al. 2003). Therefore, MI induced-acute inflammatory response may aggravate endothelial dysfunction and initiate kidney damage (Akçay, Nguyen et al. 2009, Cosentino, Genovese et al. 2019). Furthermore, Anzai et al. demonstrated a marked increase in white blood cells count and systemic inflammatory markers (CRP, IL-6) in STEMI patients with AKI compared to non-AKI (Anzai, Anzai et al. 2010). Additionally, mounting evidence indicates a link between local and systemic inflammation in MI (such as peripheral monocytes, C-reactive protein, TNF- α , IL-6, and IL-1 β) and adverse cardiac remodeling (Nian, Lee et al. 2004). Collectively, these findings suggest the pivotal role of inflammation in exacerbating kidney injury in the setting of MI.

4.3.3.2 Oxidative stress

While physiological ROS levels are necessary for multiple different biological processes including proliferation, growth, and cell survival, excessive ROS, however,

can initiate or exacerbate kidney injury. In the vasculature, ROS induces endothelial dysfunction and elicits inflammatory cells recruitment (Nian, Lee et al. 2004). By suppressing the endothelial cells' ability to produce NO, which controls vessel relaxation, ROS enhances the vasoreactivity response to the sympathetic nervous system Ang-II, and endothelin-1 (**ET-1**). In parallel, increased ROS levels can enhance kidney damage through reducing tubules Na⁺ reabsorption, increasing glomerular basement membrane permeability, altering gene expression levels, and eliciting cell death (Ratliff, Abdulmahdi et al. 2016). Additionally, strong evidence demonstrates that ROS can contribute to enhanced kidney fibrosis by augmenting the TGF- β /SMAD molecular pathway (Iglesias-De La Cruz, Ruiz-Torres et al. 2001). Furthermore, Anzai et al. reported a higher level of serum Malondialdehyde-Modified Low-Density Lipoprotein (**MDA-LDL**) in patients diagnosed with AKI compared to non-AKI, suggesting a major role of increased ROS levels in exacerbated kidney pathogenesis (Anzai, Anzai et al. 2010). In agreement with this hypothesis, lectin-like oxidized low-density lipoprotein receptor-1 knockout mice subjected to LAD ligation showed an enhancement in cardiac and kidney function along with a suppression in kidney inflammatory response and morphological alteration when compared to the wild type group (Lu, Wang et al. 2012).

4.3.3.3 *Fibrosis*

The pathogenesis of fibrosis involves a complex interplay of exacerbated inflammation, enhanced oxidative stress, and neurohormonal overactivation that together lead to stiffness and loss of function. Of note, interstitial fibrosis represents the hallmark of all progressive kidney diseases. It has been reported that the infiltration of inflammatory cells including monocytes, macrophages, dendritic cells, and lymphocytes, along with enhanced ROS production and profibrotic cytokines secretion, generate a solid platform for the activation and expansion of matrix-producing cells, chiefly fibroblasts (Djudjaj and Boor 2019). In that regard, it has been documented that α -smooth muscle actin expression contributes to the differentiation

of fibroblasts to myofibroblasts, resulting subsequently in increased matrix-components deposition mainly fibronectin and collagen type I and type II. A study performed by Lekawanvijit et al. revealed increased macrophages infiltration and upregulated profibrotic markers including TGF- β and SMAD along with enhanced interstitial fibrosis in kidneys, leading consequently to a deterioration in kidney function one week post-MI (Lekawanvijit, Kompa et al. 2012). Furthermore, in addition to the pro-inflammatory characteristic of Ang-II, it has been shown that it upregulates TGF- β expression level, augments the differentiation of fibroblasts to myofibroblasts, contributing therefore to aggravated kidney interstitial fibrosis (Lekawanvijit, Kompa et al. 2012).

4.3.4 Metabolic impairment

Mounting evidence documents that metabolic disturbances are highly prevalent in the context of MI. Additionally, cellular metabolic impairments in turn were shown to accelerate ROS generation, leading to exacerbated oxidative damage and consequent inflammatory response, creating eventually detrimental vicious cycle (Kaplan, Abidi et al. 2017). Under physiological conditions, low ROS levels control multiple intracellular processes (Mailloux, McBride et al. 2013). Conversely, excessive ROS production alters mitochondria protein synthesis, attenuating therefore mitochondrial redox and ATP synthesis, heightening consequently cellular dysfunction and cell death (Ballinger, Patterson et al. 2000). In addition to its important role in regulating cell signaling and death, mitochondria controls energy production in cardiovascular cells (Knight-Lozano, Young et al. 2002). It has been reported that MI patients with metabolic disturbances (a combination of obesity, hypertension, dyslipidemia) are at 10-fold increased risk to develop AKI (Clavijo, Pinto et al. 2006).

4.4 ACUTE KIDNEY INJURY RISK FACTORS

4.4.1 Aging

Aging is a central contributor to kidney disease development and progression.

Renal aging is characterized by multiple different morphological changes including a decrease in nephron size and number and an increase in interstitial fibrosis, among others (O'Sullivan, Hughes et al. 2017). A proportional relationship between aging and a decline in GFR has also been reported, increasing consequently the incidence of developing AKI and chronic kidney diseases (Chao, Wang et al. 2018).

The prevalence of AKI is dramatically increasing with aging. According to the United States Renal Data System Survey, the incidence of AKI among people aged 66-69 is about 19.1 episodes per 1000, while it is increasing significantly to reach 26.7, 40, 57.8 and 89.7 episodes per 1000 in patients aged 70-74, 75-79, 80-85, and >85, respectively, (USRDS, Annual report, 2017) (Chao, Wang et al. 2018).

4.4.2 Diabetes Mellitus (DM)

A growing body of evidence reports that the incidence of AKI is higher in patients diagnosed with DM. For instance, among 36,136 patients who underwent coronary artery bypass grafting, the adjusted odd ratio (**OR**) for the incidence of developing AKI was 4.89 [95% CI, 3.8-6.2] in type I DM patients, whereas it reached 1.27 [95% CI 1.16–1.40] in type 2 DM patients when compared to the healthy control (Hertzberg, Sartipy et al. 2015). In line with this observation, a meta-analysis study on > 1 million participants documented that decreased eGFR and high urine albumin-creatinine ratio with increased risk of developing AKI was observed in DM patients (James, Grams et al. 2015). Furthermore, it is worth mentioning that AKI-requiring dialysis was 5 times higher in DM patients when compared to patients without DM (Harding, Li et al. 2020).

4.4.3 Myocardial infarction

IHD is a main contributor to mortality worldwide. MI, the most serious type of IHD has been reported to be highly associated with increased risk of AKI development and progression, increasing therefore death rate substantially. Multiple

epidemiological studies revealed that the prevalence of AKI in the setting of MI during hospitalization ranges between 7.1 and 29% (Hwang, Jeong et al. 2011, Shacham, Leshem-Rubinow et al. 2014). Furthermore, the incidence of AKI in the context of MI has been reported to increase mortality rate two to three folds not only during the first month after the acute ischemic event, but also during the first year (Kaltsas, Chalikias et al. 2018). Additionally, the Prevention of Renal and Vascular End Stage Kidney Disease (**PREVEND**) study showed a yearly decrease in kidney function of -2.2 ml/min [95% CI -5.0 to -0.9] per 1.73 m² in MI patients (Eijkelkamp, de Graeff et al. 2007).

4.4.4 Cigarette smoking

CS is a major risk factor for multiple different diseases including CVDs, neurological, and pulmonary diseases (Das 2003). Recent investigations have shed the light on the association between CS and kidney disease progression and development, independent on other well-defined risk factors such as aging, CVDs, and DM. Although CS amount and time are tightly linked to kidney disease pathogenesis, studies showed that heavy smokers remain at high risk for developing kidney diseases even 10 years post-cessation (Fox, Larson et al. 2004, Xia, Wang et al. 2017, Kaplan, Abidi et al. 2020). A study done by our work group in the American University of Bayreuth showed that 6 weeks CS-exposed male mice exhibited increased kidney pro-inflammatory and pro-fibrotic markers release. Additionally, elevated ROS score and kidney fibrosis was observed in this study, indicating the detrimental effects of CS on the kidneys (Kaplan, Abidi et al. 2020). Although the detrimental effects of CS on the kidneys have been well-investigated, the impact of CS exposure in the context of MI-induced AKI is yet to be determined and this would be the first aim of our study.

4.5 DIAGNOSTIC MARKERS

Mounting evidence identifies multiple biomarkers of glomerular and tubular injury such as creatinine, cystatin C, NGAL, and KIM-1 in the diagnosis of type I CRS.

4.5.1 Creatinine

Creatinine is known to be the end product of muscle creatine metabolism and shown to be freely filtered in glomeruli but not reabsorbed in PTs (Shemesh, Golbetz et al. 1985). Of note, a marked amount of creatinine is secreted from PTs into urine, thus this indicator is highly effected by a variation in tubular secretion (Stevens, Coresh et al. 2006). Yet, creatinine clearance has been used for a long time to evaluate kidney function and monitor kidney disease development and progression (normal 110-150 ml/min in men, 100-130 ml/min in women) (Chernecky and Berger 2012). Serum creatinine level in healthy male subjects is around 0.6 to 1.2mg/dL and between 0.5 to 1.1 mg/dL for females. Increased creatinine levels above the normal threshold has been shown to be correlated with decreased GFR, subsequently exacerbated renal dysfunction (Hosten 1990).

Creatinine 1 mg/dL: Normal GFR

Creatinine 2 mg/dL: 50% reduction in GFR

Creatinine 4 mg/dL: 70 to 85% reduction in GFR

Creatine 8 mg/dL: 90 to 95% reduction in GFR

4.5.2 Cystatin C

Cystatin C is generated in all nucleated cells in the body and shown to be freely filtered in glomeruli and then completely reabsorbed from the PT, made it good candidate for estimating renal function. It has been shown that cystatin C is significantly elevated in plasma following kidney disease with reduced GFR. A growing body of evidence has reported that cystatin C is an independent marker of sex, age, and muscle mass and thus it is considered as a more accurate indicator than serum creatinine in the assessment of kidney pathogenesis (Knight, Verhave et al. 2004, Dupont, Shrestha et al. 2013).

4.5.3 Neutrophil gelatinase associated lipocalin (NGAL)

For many years NGAL has been considered as a small protein produced mainly by neutrophils. Recently, it has been shown to be upregulated in many cells including kidney tubular cells in response to insult. NGAL is freely filtered in glomeruli and totally reabsorbed in PCTs. Strong evidence demonstrated that in the context of kidney injury and within 24-48 hours post PT damage, NGAL level is substantially increased in both serum and urine (normal < 20ng/ml), considering it therefore as a promising marker for assessing kidney injury (Mishra, Ma et al. 2003, Mishra, Dent et al. 2005, Soni, Cruz et al. 2010).

4.5.4 Kidney injury molecule 1 (KIM 1)

KIM-1, a type-1 transmembrane protein, is strongly correlated with the severity of kidney injury and shown to pick within 24 hours following PT injury in urine (normal < 200 ng/gCr). Under physiological condition, KIM-1 is minimally expressed in normal PT cells, whereas, it is highly upregulated in the S3 segment of the PT following ischemic kidney diseases, enhancing therefore phagocytosis of apoptotic cells in the kidneys (Han, Wagener et al. 2009, Brisco and Testani 2014).

4.6 SEX-BASED DIFFERENCES IN CARDIAC AND KIDNEY DISEASES

Accumulating evidence has demonstrated that premenopausal women are protected against CVDs and kidney diseases when compared to age-matched men. This protection, however, disappears with age to reach comparable levels post-menopause (Orth 2002). Sexual dimorphisms are potentially attributable to the protective role of female sex hormones, mainly estrogen E2 (17- β Ethinyl Estradiol). Preclinical investigations and clinical studies indicated that E2 effects are mainly mediated by two types of receptors; ER (α and β) and the newly discovered GP receptor 30 (GPR30). In that regard, it has been reported that E2 exerts a cardioprotective role in female rats post-ischemia reperfusion (I/R) injury through decreasing infarct size and enhancing cardiomyocyte contractility when compared to the ovariectomized

female rats (without ovarian E2 production) (Iorga, Cunningham et al. 2017). Furthermore, Zhang et al. revealed that ER α KO female mice exhibited higher incidence of tachycardia, lower coronary blood flow, and increased infarct size when compared to the wild type group (Zhang and Guo 2017). In accordance with the previous investigations, the direct cardio-protective role of ER α was observed in MI female mice by using ER α agonist (PPT), resulting in decreased infarct size and cardiac dysfunction (Mahmoodzadeh, Leber et al. 2014). ER β receptors, on the other hand, exert indirect cardioprotective effects by modulating the autonomic cardiac control and lowering blood pressure (Fagerström 2002). Furthermore, in an animal model of cardiac hypertrophy, GPR30 agonist attenuates cardiac hypertrophy through decreasing cardiac fibrosis and cell death (Wang, Xing et al. 2022). It has been also shown that in the setting of aortic stenosis, males have substantially more adverse cardiac remodeling compared to women, and transcriptome characterization demonstrated that fibrotic and inflammatory genes and pathways are upregulated in males but not in the female counterpart (Kararigas, Dworatzek et al. 2014). Additionally, premenopausal women have been shown to be more protected against I/R injury during open heart surgery compared to the male group (Hayward, Kelly et al. 2000). Importantly, the observed protection against CVDs in females is tightly linked to female sex hormone levels mainly E2 as the incidence and severity of CVDs markedly increased in postmenopausal women (Hayward, Kelly et al. 2000), as well as the prevalence of CVDs has been shown to be greater in premenopausal females who underwent an oophorectomy compared to those with intact ovaries (Parker, Jacoby et al. 2009). In an animal mode of I/R injury, Jeanes et al. has been elucidated that E2 and ER α -specific agonists reduced the infarct size by attenuating myocardial lipid peroxidation (Jeanes, Tabor et al. 2008). In an in vitro model of hypoxia and reoxygenation of rat cardiomyocytes, E2 decreased cardiomyocyte death and ROS overproduction by attenuating MAPK activity (Arias-Loza, Muehlfelder et al. 2013). Estrogen has also been known to decrease the risk of CVDs progression and

development by downregulating inflammatory markers, including cell adhesion molecules and chemokines (Pelekanou, Kampa et al. 2016). E2 has also been documented to exert a protective role on the vasculature (Arnal, Lenfant et al. 2017). In that regard, a study performed on postmenopausal women with hyperlipidemia indicated that estrogen treatment for one year markedly decreased catecholamine levels, blood pressure, while increasing nitrite and nitrate levels (Anagnostis, Bitzer et al. 2020, Xiang, Liu et al. 2021). Taken together, this findings have shed the light on the potential protective role of female sex hormones in the setting of CVDs.

In addition to the well-established cardioprotective effects of estrogen in the context of CVDs, multiple experimental studies have linked E2 to improved kidney function. In that regard, Kummer et al. observed that E2 plays an anti-apoptotic role in podocyte cells through tempering the opening of the mitochondrial permeability transition pore and reducing the release of pro-apoptotic biomarkers into cytosol (Ly, Grubb et al. 2003). Furthermore, Seppi et al. elucidated that PT renewal mechanism is cyclically controlled by female sex hormones, resulting in increased cell turnover, eventually improved PT repair capacity (Seppi, Prajczar et al. 2016). Furthermore, It has been reported that estrogen augments the release of NO resulting in enhanced vasodilation. NO deficiency, however, is known to be associated with exacerbating kidney injury progression and development by reducing vasodilation and enhancing endothelial dysfunction (Cobo, Hecking et al. 2016). A study done by Catanuto, Doublier et al. showed that that administration of estradiol upregulates ER- β expression in the kidneys, leading to prevent glomerulosclerosis and albuminuria in an animal model of type 2 diabetes (Catanuto, Doublier et al. 2009). Additionally, in an animal model of gentamicin-induced AKI, ovariectomized rats exhibited decreased ER- α expression levels in the proximal tubule epithelial cells. Biomarkers of AKI such as cystatin-C and KIM-1 were inversely correlated with ER- α expression levels. The administration of estrogen or estrogen receptor agonist, phytoestrogens, improve kidney function (Abd El-Lateef, El-Sayed et al. 2019). A retrospective clinical study

showed that males (aged between 18-65) are more prone to develop AKI in the intensive care unit when compared to the relative female group. This protection, however, markedly decreased in women above 65, suggesting the potential protective role of E2 (Güzel, Yeşiltaş et al. 2019). Although the protective role of E2 is well-established in the setting of CVDs and kidney diseases, little is known about sexual dimorphism in MI-induced AKI and the impact of E2 deprivation in type I CRS, and thus this topic would be the second aim of our study.

4.7 NAD FUNCTION, SYNTHESIS, AND METABOLISM

Strong evidence has demonstrated that the heart has the highest mitochondrial number and NAD⁺ levels. The kidneys are only second to the heart in term of mitochondrial abundance and among the highest energy demanding organs (Forbes 2016). NAD⁺ is a metabolic and redox substrate that is crucial for all living cells. Investigations have demonstrated that NAD⁺ exerts a central role as a co-substrate for multiple enzymes such as sirtuins, implicated in mitochondrial biogenesis, and poly ADP Ribosyl polymerases (PARP-1), involved in DNA repair, and as a coenzyme in Oxidative phosphorylation reactions (Hershberger, Martin et al. 2017). NAD⁺ depletion is emerging as a fundamental feature of cardiac and renal disease (Mericskay 2016, Tran, Zsengeller et al. 2016). Therapeutic approaches to attenuate cardiac and kidney diseases progression and development either by inhibiting NAD⁺ consuming enzymes and/or boosting NAD⁺ levels via the stimulation of NAD⁺ biosynthesis or supplementation of NAD⁺ precursors is an interesting area for research that can decrease morbidity and mortality rate substantially.

4.7.1 Nicotinamide dinucleotide coenzyme

Nicotinamide dinucleotide (NAD) has been first discovered in 1906 by Arthur Harden and William Young and described as the first cofactor that provokes fermentation in yeast. Hans von Euler-Chelpin purified in 1923 the coenzyme from the yeast extract and identified its chemical characters, for which he earned jointly with

Arthur Harden the Nobel Prize for Chemistry in 1929. The capacity of NAD to transfer hydrogen was discovered by Otto Warburg in 1930, for which he earned the Nobel Prize in Physiology or Medicine 1931 (Warburg, Christian et al. 1935). NAD producing pathways were discovered by Arthur Kornberg, and Preiss and Handler in 1940 and 1950, respectively. The main role of NAD in cell metabolism and its implication in glycolysis, oxidative phosphorylation, and TCA cycle were also identified (Berger and Ramírez-Hernández , Yang and Sauve 2016).

In 1937, the key contributor of NAD metabolism in health and disease was studied by Elvehjem and colleagues in the context of canine black tongue and human pellagra. They found that the heat stable dietary factor was able to cure these diseases which reached epidemic proportions a few centuries ago. The heat stable dietary factor was then identified as niacin, a type of vitamin B3, an NAD precursor, highlighting therefore the critical role of NAD boosting therapies. Of note, Pellagra is a food related disease that was associated in severe cases with NAD depletion and high mortality rate. Decreased NAD levels was then documented in multiple diseases including CVDs, kidney diseases, aging, among others (Xiao, Wang et al. 2018).

NAD homeostasis is a key cofactor in oxidation-reduction reactions of energy metabolism. Furthermore, NAD has been shown to play a major role in sustaining other biological processes through controlling the activity of multiple signaling pathways that consume NAD^+ as a substrate (Houtkooper, Cantó et al. 2010, Katsyuba and Auwerx 2017). It is well-known now that maintaining balanced NAD levels could be achieved either by boosting NAD^+ biosynthetic pathways or inhibiting NAD^+ consuming enzymes. These two strategies could serve as potential promising therapies to target diseases associated with NAD depletion.

NAD is the fusion of two nucleotides, adenine nucleobase and nicotinamide (NAM), joint by a phosphodiester bond (**Figure 7**).

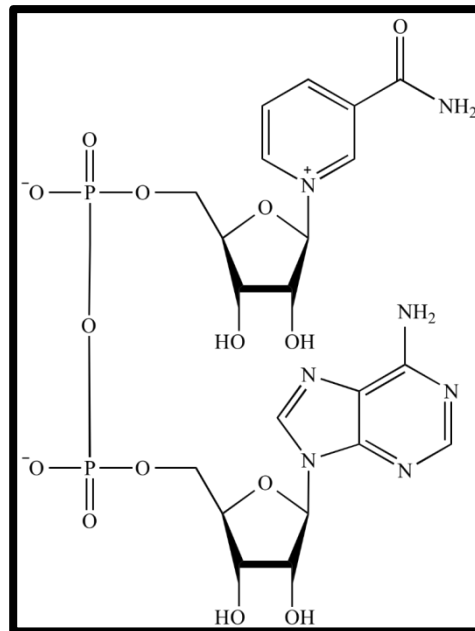


Figure 7: The NAD molecule

Importantly, NAD exists in two forms: the oxidized form NAD^+ and the reduced form NADH. The oxidized form undergoes a reversible reduction of the pyridine cycle of the NAM base by the gain of a hydride ion upon oxidation of substrates by dehydrogenases (Nelson and Cox 2017) (**Figure 8**).

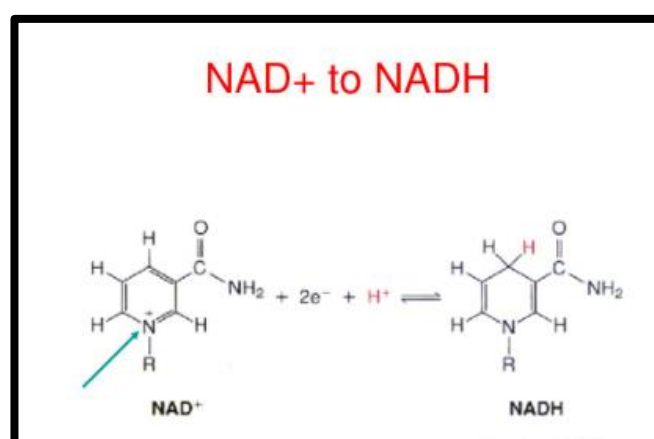


Figure 8: NAD oxidation and reduction

4.7.2 Role of NAD in energy metabolism as a cofactor

The central role of NAD in energy metabolism as a redox cofactor has gained a great attention and extensively studied in health and multiple disease models. Different reducing enzymes such as dehydrogenases and oxidoreductases catalyze the interconversion of NAD^+ to NADH, and reciprocally, depending on the balance of substrates and products. It has been documented that NAD is involved in multiple energy pathways that require electron transfer such as glycolysis, lactate dehydrogenase reaction, Tricarboxylic acid cycle (**TCA**) cycle, pyruvate dehydrogenase, fatty acid β oxidation, and oxidative phosphorylation (Okabe, Yaku et al. 2019). Notably, there is no variation in total NAD backbone quantity, i.e. the sum of NAD^+ and NADH levels, following this type of reaction (Katsyuba and Auwerx 2017).

In the glycolytic pathway that takes place in the cytosol, the glycerol triphosphate (**G3P**) dehydrogenase generates NADH from NAD^+ . To maintain glycolytic potential, NADH is then reoxidized into NAD^+ by the lactate dehydrogenase (**LDH**) reaction. This is especially true in anaerobic condition in most cells, although in the cardiomyocytes a special isoform of LDH-H encoded by the *LDHB* gene has a reaction equilibrium in favor of conversion of lactate to pyruvate, allowing these cells to use lactate as a substrate rather than generating it as a waste product. To maximize energy efficiency, however, the reducing power of NADH should also be transferred from the cytosol to the mitochondrial matrix to feed the electron transport chain (**ETC**). However, mitochondrial inner membrane is not thought to be permeable to NADH and this process is therefore dependent on reducing equivalent shuttles (Stryer L (1995). "Oxidative phosphorylation.". Biochemistry (Fourth ed.). New York: W.H. Freeman and Company. pp. 537–549.). Different reducing equivalent shuttles exist to carry NADH across the mitochondrial inner membrane, the malate-aspartate (**M-A**) shuttle, the malate-citrate shuttle and the α -glycerophosphate shuttle (Lu, Zhou et al. 2008). In cardiac tissues, it has been shown that the major reducing equivalent shuttle is the M-A shuttle, resulting in 10 fold higher NADH flux rate than the α -

glycerophosphate shuttle (Scholz and Koppenhafer 1995). Additionally, the M-A shuttle has been reported to be a central contributor to renal sodium (Na^+) absorption in healthy kidneys (Ross, Silva et al. 1981).

The M-A shuttle contains a transport-transamination-redox cycle in mitochondrial and cytosolic domains (**Figure 9**). In the inner mitochondrial membrane, the glutamate-aspartate transporter, and the malate- α -ketoglutarate transporter are the two antiporter proteins. The glutamate-aspartate transporter is characterized by the outflow of the aspartate from the mitochondria followed by the entry of glutamate and proton into the inner mitochondrial membrane. Furthermore, the malate- α -ketoglutarate transporter is responsible for the interchange between malate and α -ketoglutarate and is highly dependent on the substrate concentration gradient (Lu, Zhou et al. 2008).

In one M-A shuttle cycle, oxaloacetate is converted to malate in the cytosol, under the action of malate dehydrogenase leading to the regeneration of NAD^+ from NADH. The malate moves afterward to the mitochondria and is transformed to oxaloacetate by mitochondrial malate dehydrogenase, which results in the generation of NADH from NAD^+ . The produced oxaloacetate is converted to aspartate under the action of aspartate aminotransferase, which in parallel produces α -ketoglutarate. Notably, both oxaloacetate and α -ketoglutarate flow can flow back to the cytosol or participate to the TCA cycle. Thus, this type of shuttle allows NADH produced by glycolysis in the cytosol to be oxidized back to NAD^+ to be available for another glycolysis round, whereas NAD^+ in mitochondrial matrix is reduced to NADH to be

utilized by the ETC to generate ATP (**Figure 9**).

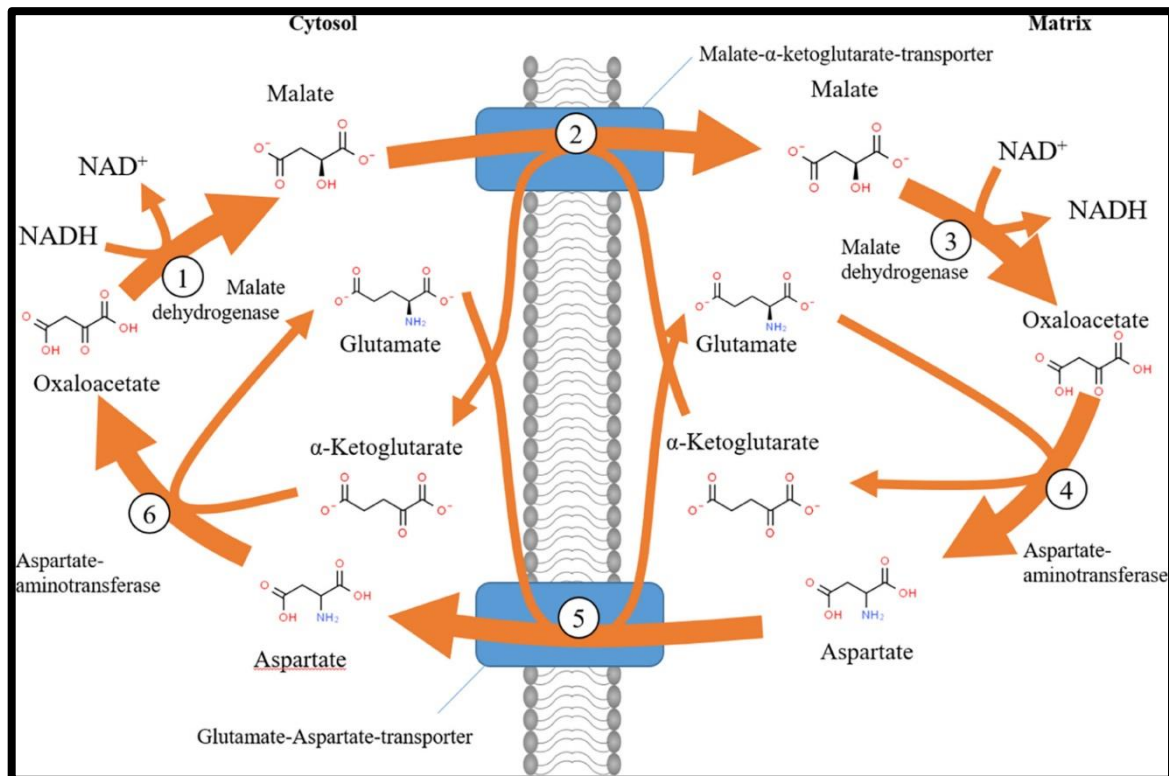


Figure 9: The malate–aspartate shuttle for transporting reducing equivalents from cytosolic NADH into the mitochondrial matrix

Fuel oxidation process in general contribute to NADH generation including the pyruvate dehydrogenase in mitochondria, the FAO pathway, notably the 3-hydroxy acyl CoA dehydrogenase (Barycki, O'Brien et al. 1999), and the β -hydroxybutyrate dehydrogenase involved in the oxidation of ketone bodies (Lopaschuk, Karwi et al. 2020). All these process generate acetyl-coA that feed the TCA cycle, which is ultimately also a major contributor to NADH production leading to the generation of three NADH moieties per cycle through the activity of three enzymes, the α -ketoglutarate dehydrogenase, isocitrate dehydrogenase, and malate dehydrogenase. In addition to NADH production, one molecule of FADH_2 is generated. Therefore NADH is the principal electron donor to the ETC.

The ETC is made up of a chain of protein complexes where electrons coming

from reduced cofactors (NADH and FADH₂) go through to enhance their reduction potential and lead consequently to energy release. Most of the produced energy is dissipated as either heat or utilized to generate a proton gradient by pumping H⁺ from mitochondrial matrix to the intermembrane space. The ETC consists of the following proteins: Complex I (C-I), complex II (C-II), coenzyme Q, complex III (C-III), cytochrome C, and complex IV (C-IV).

C-I consists of NADH dehydrogenase, flavin mononucleotide (FMN), and eight iron sulfate groups. It has been shown that C-I is able to generate and transfer four Hydrogen ions from mitochondrial matrix to the intermembrane space, contributing therefore to the generation of proton gradient (Hirst 2005, Sazanov and Hinchliffe 2006, Hirst 2010, Lencina, Franza et al. 2018). C-II or succinate dehydrogenase, is the second entry point to the ETC and receives electron from succinate, an intermediate of the citric acid cycle. Of note, during this step, there is no movement of hydrogen ions from the mitochondrial matrix to the intermembrane space, therefore less energy is produced compared to C-I (Yankovskaya, Horsefield et al. 2003, Horsefield, Iwata et al. 2004). Other two major contributors to the ETC are G3P and Acyl-CoA. G3P dehydrogenase and acyl CoA dehydrogenase accept electrons from G3P and acyl CoA, respectively, resulting in delivering reducing equivalent to the mitochondrial ETC. (Thorpe and Kim 1995, Geertman, van Maris et al. 2006). Coenzyme Q is defined as an electron carrier, transfer electron to C-III. C-III, otherwise known as a cytochrome C reductase. Cytochrome C then transfers electron to C-IV (Trumpower 1990, Iwata, Lee et al. 1998, Sun, Benlekbir et al. 2018). C-IV also known as cytochrome C oxidase, it oxidizes cytochrome C and transfer electron to O₂, the final electron acceptor, resulting in the reduction of O₂ to form H₂O and the release of four proton ions, enhancing therefore the proton gradient in the mitochondrial intermembrane (Calhoun, Thomas et al. 1994, Schmidt-Rohr 2020). ATP synthase or complex V depends on the proton gradient created in the intermembrane space to generate one

ATP molecule from every four proton ions (Okuno, Iino et al. 2011) (**Figure 10**).

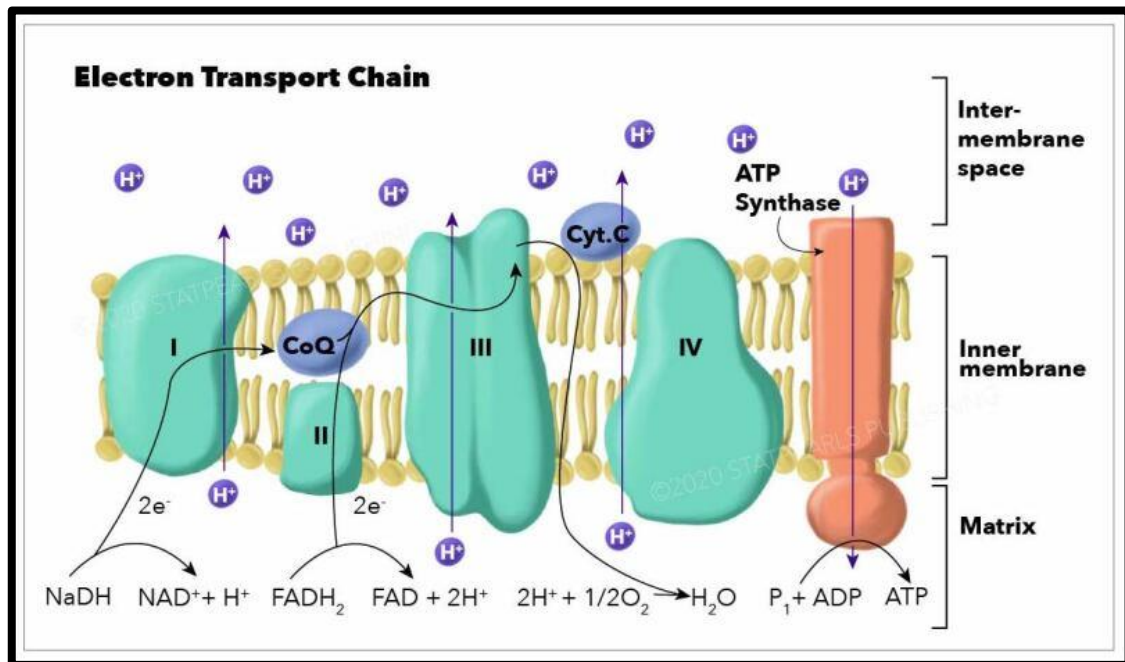


Figure 10 Electron Transport Chain (Ahmad, Wolberg et al. 2018)

4.7.3 Role of the phosphorylated form of NAD

Around 10% of NAD⁺ is phosphorylated by NAD kinases 1 and 2, resulting in the generation of NADP (Xie, Zhang et al. 2020). NADP can in turn be dephosphorylated by NADP phosphatase to form NAD⁺. Mitochondrial NAD(P) transhydrogenase is another way to catalyze the conversion of NADP to NADPH by receiving an electron from NADH allowing the coupling of NADPH production with NADH production by energy metabolism (**Figure 11**).

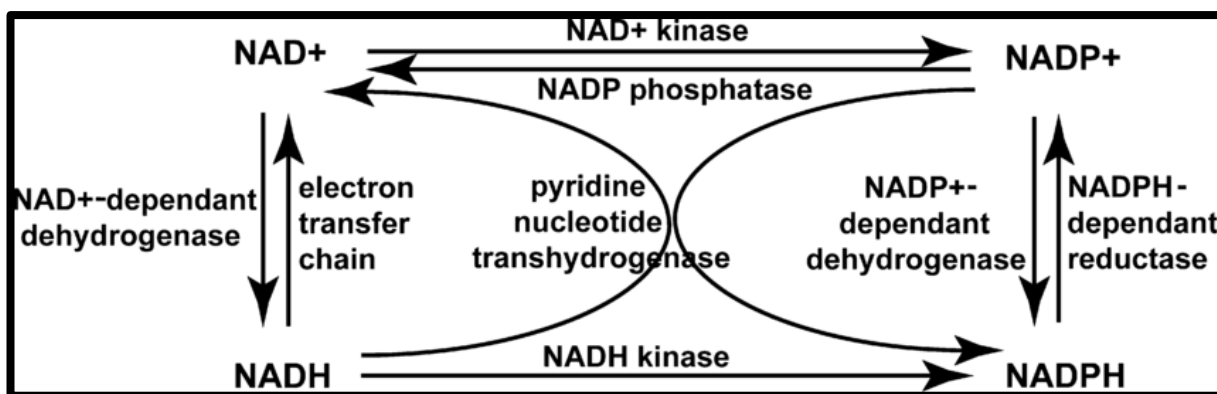


Figure 11: Conversion and regeneration pathway of coenzyme NAD⁺/NADH and NADP⁺/NADPH

Reactions catalyzed by NAD p kinase, NADP phosphatase and NADH kinase are responsible for the conversion between NAD(H) and NADP(H). Reactions catalyzed by NAD p -dependent dehydrogenases and electron transfer chain are responsible for the regeneration of NAD(H), whereas that by NADP p -dependent dehydrogenases and NADPH-dependant reductase are for the regeneration of NADP(H). Reaction mediated by pyridine nucleotide transhydrogenase transfers a hydride from NADH to NADP p with the concurrent production of NADPH and NAD (Shi, Li et al. 2009).

A growing body of evidence reports that NADP is a key coenzyme implicated in cellular electron transfer reactions. It has been observed that cells spend a marked amount of energy to maintain NADP in its reduced form, thus sustaining a pool of electron to reduce oxidized compounds. NADPH is a central contributor to the oxidative defense system and involved in the detoxification and reductive reactions in the cell. In that regard, it has been demonstrated that anabolic reactions in general are reducing process that requires NADPH as a reducing substrate. NADPH has also been shown to be involved in the production of triglyceride, steroids, cholesterol, amino acids, and DNA. Additionally, the generation of anti-oxidant defense systems such as glutathione, thioredoxins catalase superoxide dismutase and glutathione S-transferase requires NADPH as a reducing agent. For instance, NADPH is needed to produce the reduced form of glutathione from the oxidized form, under the action of glutathione reductase, which is crucial for the function of different anti-oxidant systems (Agledal,

Niere et al. 2010) .

Inversely, NADPH can also be used for ROS production through the action of NADPH oxidases. NADPH oxidases known as (NOX) have been reported to transfer electron to oxygen molecule resulting in the production of O_2^- , enhancing ROS generation.

Mitochondrial ETC is a main source of ROS in eukaryotic cells. It has been observed that oxygen can undergo one-electron reduction to produce O_2^- at all enzyme complexes of the respiratory chain (Adam-Vizi and Chinopoulos 2006). Of note, previous publications demonstrated that O_2^- production mainly occurs at complex I and complex III (Murphy 2009). ROS can also be generated by monoamine oxidases (MAOs) and the mitochondrial-localized NADPH oxidase 4 (NOX4) (Bugger and Pfeil 2020). Of note, Mitochondrial NOX4 has been shown to be highly expressed in the kidneys and mainly leads to the production of H_2O_2 rather than O_2^- that is produced by other NOX isoforms. Published investigations reported that NOX4 is mainly expressed in the distal tubule in human, whereas it is highly expressed in the S1 segment of the PT in rodents. Recently, studies have documented a similar expression pattern of NOX4 in both human and rodent model being most abundant in the early part of the PT. Additionally, NOX4 has been indicated to be expressed at a low level in the collecting duct, with almost no expression in glomeruli, under physiological conditions (Geiszt, Kopp et al. 2000, Babelova, Avaniadi et al. 2012, Khodo, Dizin et al. 2012, Rajaram, Dissard et al. 2019).

Under physiological conditions, mitochondrial ROS has been shown to play a critical role as a second messenger to mediate multiple different intracellular pathways. For example, The ROS generated from mitochondrial Complex III are a key contributor for hypoxia inducible factor -1 α stabilization and eventually, for cells proliferation, including endothelial cells and vascular smooth muscle cells (Diebold and Chandel 2016). Furthermore, under hypoxic conditions, ROS mediates the activation of AMPK,

resulting in enhanced cytoprotective autophagy (Emerling, Weinberg et al. 2009). Whether mitochondrial metabolism is a main source of ROS in vivo in normal physiological condition in presence of high levels of NAD^+ remains a debated topic but certainly in the context of disease, notably ischemia-reperfusion injury, increased reverse electron transport together with extra-mitochondrial sources of ROS such as NADPH oxidase contribute to ROS production (Dmitry, Juhaszova et al. 2014). In that regard, after ischemia reperfusion injury, complex I has been shown to be the main source of superoxide generation. This observation has been confirmed by giving a selective pharmacological inhibitor of complex I which leads to protect from reperfusion injury and ROS overproduction (Lesnefsky, Chen et al. 2004). Furthermore, marked amounts of ROS may be produced by complex III. It has also been reported that cardiolipin that is necessary for preserving the activity of ETC complexes, mainly (Complex I/III/IV), is progressively oxidized by ROS production during reperfusion, and this may lead to loss of cardiolipin, resulting in ROS amplification (Paradies, Petrosillo et al. 2004). Disruption of the interaction between cytochrome c with the inner mitochondrial membrane would result in an increase in the oxidized components of the ETC preceding complex IV, enhancing therefore the mitochondrial production of oxygen radicals during perfusion (Bugger and Pfeil 2020).

4.7.4 NAD^+ metabolism

Growing body of evidence has shed the light on multiple different NAD^+ biosynthetic pathways that are implicated in maintaining NAD homeostasis and preventing its depletion. It has been documented that NAD^+ is produced by several extracellular precursors such as: Nicotinamide (**NAM**), nicotinamide riboside (NR), nicotinic acid (**NA**), and nicotinic acid riboside (**NAR**), all of which can be found in the diet and considered as vitamin B3 derivatives (Bogan and Brenner 2008). NR is mainly found in milk, whereas NA presents in regular diet and is produced by algae and plants (Trammell, Schmidt et al. 2016). Furthermore, regular diet provides NAD^+ , NADH, NADP, and NADPH (Bogan and Brenner 2008). Another discovered NAD^+ precursor is

the amino acid tryptophane (**TrP**) that is implicated in NAD⁺ synthesis through the *de novo* pathway.

Pathways involved in NAD⁺ production compensate for the net consumption of NAD⁺ by multiple NAD⁺ dependent enzymes that are involved in post-translational modification including sirtuins (**SIRT**s), poly-ADP ribosyl polymerases (**PAR**P)s, and CD38 (Ziegler and Nikiforov 2020). NAD⁺ cleavage by these enzymes results in the production of NAM, which internally serves as an intracellular source of vitamin B3 (Braidy, Berg et al. 2019) (**Figure 12**).

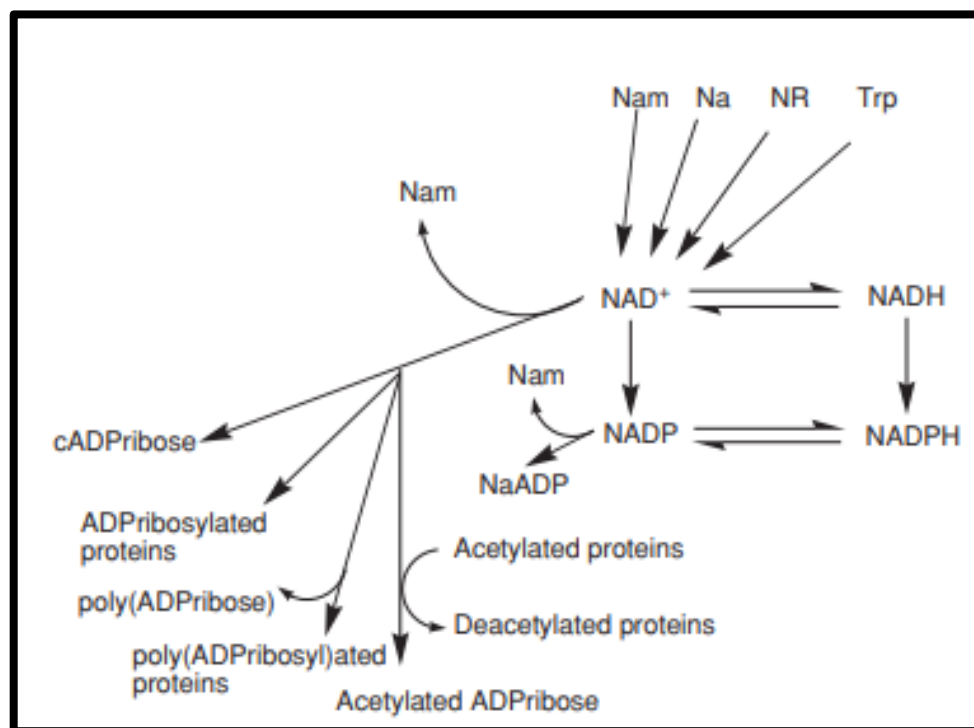


Figure 12: NAD metabolism (Bogan and Brenner 2008)

4.7.5 NAD biosynthetic pathways

A balance between NAD⁺ biosynthesis and consumption is critical to keep intracellular NAD⁺ homeostasis. NAD⁺ biosynthesis pathways exert a major role in maintaining NAD pool. The latter is important not only for boosting redox metabolism but also for fueling NAD⁺ dependent pathways. NAD⁺ dependent signaling pathways

are implicated in the consumption of NAD^+ in order to perform their work, therefor a continuous replenishment of NAD^+ is of utmost importance and can be accomplished by efficient NAD^+ producing enzymes (Nikiforov, Kulikova et al. 2015) . The three well-identified pathways for NAD^+ biosynthesis are: The *de novo* pathway, the Preiss-Handler pathway, and the salvage pathway (Aman, Qiu et al. 2018, Rajman, Chwalek et al. 2018). The generation of NAD and NA adenine dinucleotide (**NAAD**) constitutes a crucial steps in NAD synthesis (Lau, Niere et al. 2009). The enzymes that catalyze this reaction nicotinamide mononucleotide adenyl transferases (**NMNAT**) exists in three isoforms with different localizations. The nuclear isoform NMNAT-1 is highly expressed in the heart, kidneys, and skeletal muscles. The cytosolic isoform NMNAT-2 is mainly expressed in the brain, whereas the mitochondrial isoform NMNAT-3 presents in the spleen and lungs (Raffaelli, Sorci et al. 2002, Berger, Lau et al. 2005, Hikosaka, Ikutani et al. 2014). NMNAT-3 has been reported to be mitochondrial, whereas all other enzymatic activities related to NAD^+ biosynthesis are either nuclear or cytosolic (Nikiforov, Dölle et al. 2011) (**Figure 13**).

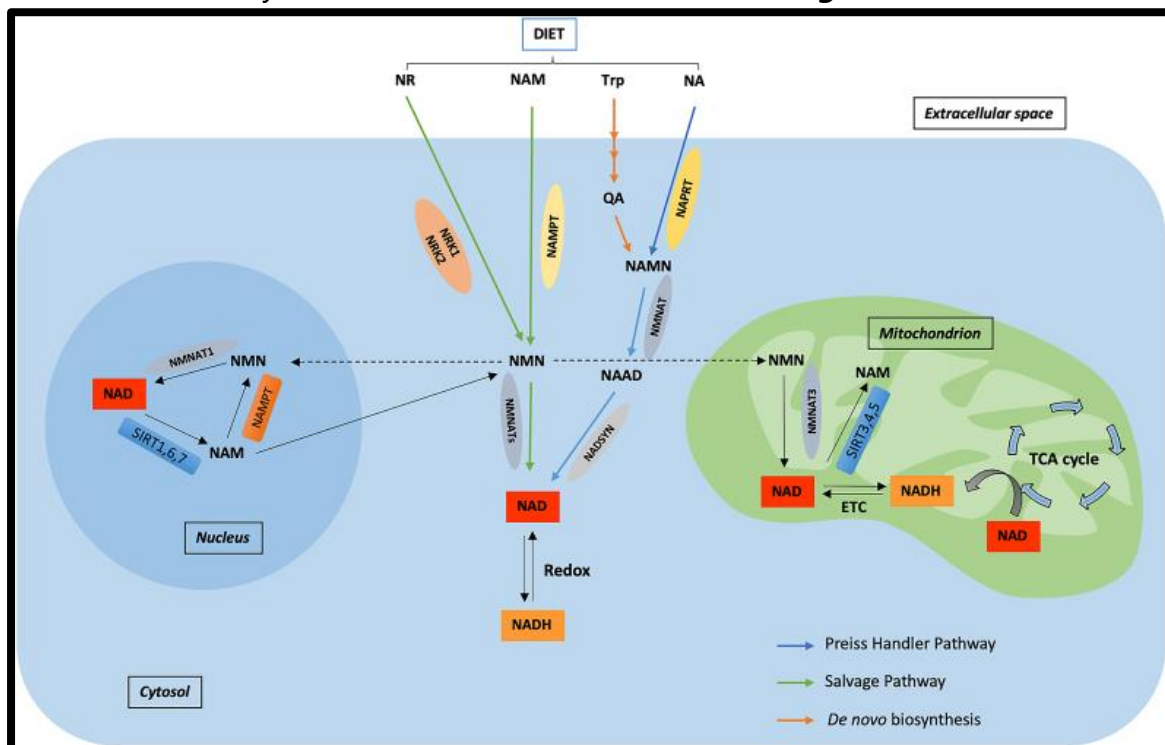


Figure 13: Representation of NAD^+ biosynthetic pathways

Biosynthesis pathways and cellular metabolism of NAD⁺. NAD precursors such as NR, NA, NAM, and Trp provided by diet can be converted into NAD via three pathways. In the Preiss-Handler pathway, NA is converted into NAMN by NAPRT, NAMN is converted into NAAD by NAD by NMNATs, and NAAD is converted into NAD by NADSYN. In the de novo synthesis pathway, Trp is converted into QA in a series of steps, which is then converted into NAD by forming NAMN and NAAD. In contrast, in the salvage pathway, NR and NAM provided by diet are converted into NAD by forming NMN by enzymes NAMPT and NRK [127]. The equilibrium in each subcellular compartment such as the nucleus and mitochondria is determined by NAD/NADH redox ratios. ETC is a significant contributor to the conversion of NADH into NAD. Additionally, NAD-consuming enzymes such as PARPs and sirtuins catalyze NAM production in subcellular compartments, which can be used for NAD synthesis via the salvage pathway [128].

4.7.5.1 *Preiss-handler pathway*

The main substrate of the Preiss-handler pathway is NA. Na can be found in redfish, meats, and nuts. In this pathway, the reaction of NA with phosphoribosyl pyrophosphate leads to the production of NAMN under the action of NA phosphoribosyl transferase (**NAPRT**). NAMN is then converted into NA dinucleotide (**NAAD**). Of note, this reaction is catalyzed by NMNATs. Finally, NAAD is amidated by the NAD synthase to form NAD⁺ (Marletta, Massarotti et al. 2015, Amjad, Nisar et al. 2021).

4.7.5.2 *De novo pathway*

The main de novo precursor of NAD⁺ is Trp through the kynurenine pathway. In human cells, the conversion of Trp to NAD⁺ mainly occurs in the liver and the kidneys and seems to be independent of the activity of the salvage pathway and the presence of NAM. The first step in the do novo pathway is the conversion of Trp to N-formyl kynurenine. The two main enzymes that are responsible for Trp conversion are tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase 1 (IDO-1) (Magni, Amici et al. 2004). TDO has been shown to be widely expressed in the liver and its level is upregulated in the presence of Trp. IDO-1, however, is primarily expressed outside the liver in different tissues including the placenta, small intestine, and the lung. Under physiological condition, IDO-1 has been shown to be expressed at low

levels, however, it is induced in the presence of multiple pro-inflammatory markers. In that regard, it has been documented that following inflammation, IDO is upregulated in dendritic cells and macrophages under the effect of multiple pro-inflammatory cytokines including IL-6, TNF- α and interferon- γ (O'Connor, André et al. 2009). This increase in IDO levels leads to the generation of downstream metabolites that inhibit effector T cells and stimulate the differentiation of regulatory T cells (Mellor and Munn 2004). Furthermore, it has been reported that increased IDO activity enhances renal ischemia reperfusion in a mouse model (Mohib, Wang et al. 2008). Additionally, it has been shown that the IDO metabolite kynurenine is significantly increased in cardiac tissues in MI mice and promotes cardiomyocyte apoptosis through boosting ROS generation (Melhem, Chajadine et al. 2021). Afterward, N-formyl kynurenine is converted to amino-3-carboxymuconate semialdehyde (**ACMS**), which can undergo either a spontaneous cyclization resulting in the generation of quinolinic acid, another NAD⁺ precursor, or can be converted by ACMS decarboxylase to α -amino- β -muconate- ϵ -semi. The latter can in turn undergoes either a complete oxidation to CO₂ and H₂O by the TCA cycle and glutarate pathway or to quinolinic acid (Katsyuba, Mottis et al. 2018). Quinolinic phosphoribosyl transferase catalyzes the conversion of quinolinic acid into NAMN through the Preiss-handler pathway, as previously described in details (Houtkooper, Cantó et al. 2010, Kulkarni and Brookes 2019). This end-product further explain why the de novo pathway mediated by Trp is not the main NAD production pathway, as it will only be involved in NAD production when it can overcome the ACMS decarboxylase activity (**Figure 14**).

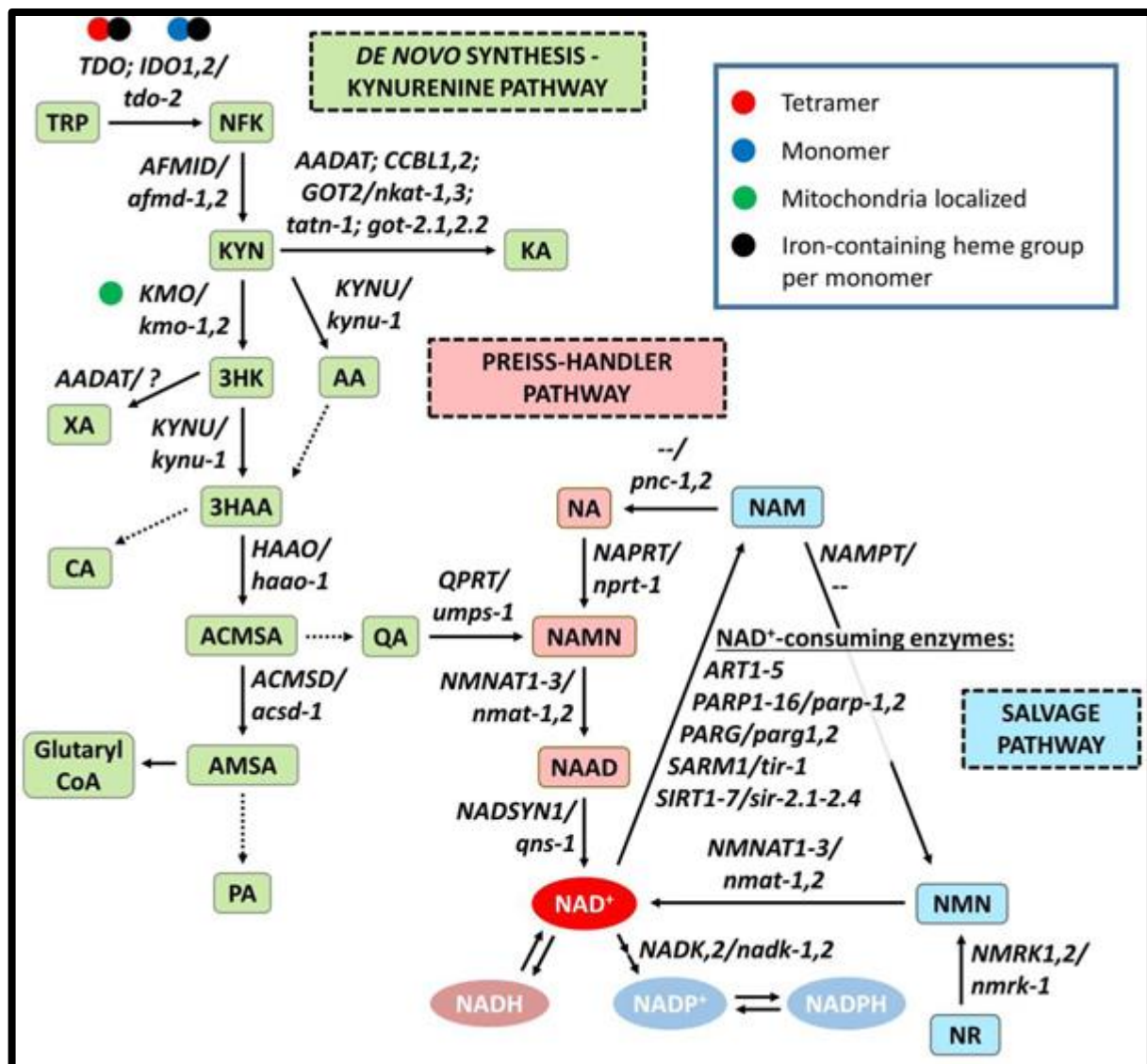


Figure 14: NAD biosynthetic pathways

The kynurenine pathway represents one of three routes for NAD⁺ production or recycling. The enzymatic degradation of the essential amino acid tryptophan (TRP) through the series of reactions catalyzed by rate-limiting enzymes culminates in de novo synthesis of NAD⁺ constitutes one of the two major branches of the kynurenine pathway. The other major branch converts kynurenine (KYN) to the neuroactive metabolite kynurenic acid (KA). Cells can also generate NAD⁺ from nicotinic acid (NA) through the Preiss-Handler pathway, or from nicotinamide riboside (NR) through the salvage pathway. Note that mammalian genomes contain the enzyme nicotinamide phosphoribosyltransferase (NAMPT), which converts nicotinamide (NAM) to nicotinamide mononucleotide (NMN), but not the enzyme nicotinamides (NAMase), which converts NAM to NA, while the invertebrate *C. elegans* and *D.*

melanogaster genomes contain NAMase but not NAMPT. Thus mammals recycle NAM through the salvage pathway, while invertebrates recycle NAM through the Preiss-Handler pathway. Each catalytic step is annotated with the human or *C. elegans* gene encoding the corresponding enzyme. Enzymes (enzyme symbol: human gene/worm gene): indoleamine 2,3-dioxygenase (IDO: IDO1,2/--); tryptophan 2,3-dioxygenase (TDO: TDO2/tdo-2); arylformamidase (AFMID: AFMID/afmd-1,2); kynurenine aminotransferase (KYAT: AADAT, CCBL1,2/nkat-1,3, tatn-1); glutamic-oxaloacetic transaminase (GOT: GOT2/got-2.1,2,2); kynurenine 3-monooxygenase (KMO: KMO/kmo-1,2); kynureninase (KYNU: KYNU/kynu-1); 3-hydroxyanthranilate 3,4-dioxygenase (HAAO: HAAO/haao-1); and aminocarboxymuconate-semialdehyde decarboxylase (ACMSD: ACMSD/acsd-1), quinolinate phosphoribosyl transferase (QPRT: QPRT/umps-1), nicotinate phosphoribosyltransferase (NAPRT: NAPRT/npert-1), nicotinamide nucleotide adenyltransferase (NMNAT: NMNAT1,2,3/nmat-1,2), NAD synthetase (NADSYN: NADSYN1/qns-1), NAD kinase (NADK: NADK,2/nadk-1,2), nicotinamide riboside kinase (NMRK: NMRK1,2/nmrk-1), nicotinamide phosphoribosyltransferase (NAMPT: NAMPT/-), ADP-ribosyltransferase (ART: ART1-5/--), poly(ADP-ribose) polymerase 1-16 (PARP: PARP1-16/parp-1,2), poly(ADP-ribose) glycohydrolase (PARG: PARG/parg-1,2), sterile alpha and TIR motif containing (SARM: SARM1/tir-1), sirtuin NAD-dependent protein deacetylase (SIRT: SIRT1-7/sir-2.1,2.2,2.3,2.4). Metabolites: tryptophan (TRP); Nformylkynurenine (NFK); kynurenine (KYN); kynurenic acid (KA); 3-hydroxykynurenine (3HK); 3-hydroxyanthranilic acid (3HAA); anthranilic acid (AA); xanthurenic acid (XA); 2-amino-3-carboxymuconic semialdehyde (ACMSA); 2-aminomuconic semialdehyde (AMSA); and quinolinic acid (QA); glutaryl-coenzyme A (Glutaryl CoA); picolinic acid (PA); nicotinic acid (NA); nicotinic acid mononucleotide (NAMN); nicotinic acid adenine dinucleotide (NAAD); nicotinamide adenine dinucleotide (NAD⁺/NADH); nicotinamide adenine dinucleotide phosphate (NADP⁺/NADPH); nicotinamide (NAM); nicotinamide mononucleotide (NMN); nicotinamide riboside (NR) (Castro-Portuguez and Sutphin 2020).

4.7.5.3 *The salvage pathway*

NAM and NR are the main two substrates of the salvage pathway that are converted into NMN then NAD⁺ without the need of NADS. This pathway is considered the major NAD⁺ biosynthetic pathway in different tissues including cardiac and kidney tissues. Two main type of enzymes are implicated in this pathway: the nicotinamide phosphoribosyl transferase (**NAMPT**) that uses NAM as a substrate to generate NMN, and the nicotinamide riboside kinase (**NMRK**)-1 and 2 that uses ATP to phosphorylate their substrate NR to generate NMN (Xiao, Wang et al. 2018). Consequently, NMN is

converted into NAD^+ by NMNATs (Amjad, Nisar et al. 2021). In addition, the glycosidic bond of NR can be broken by purine nucleoside phosphorylases (**PNP**), resulting in the formation of NAM that is used then by NAMPT (Amjad, Nisar et al. 2021). The activity of this PNP pathway is still not fully understood in mammalian and requires further investigations. Upon NMN formation either from NAM or NR, the same NMNATs that are used in Preiss-handler and *de novo* pathways convert NMN to NAD^+ .

The salvage pathway mediated by the activity of NAMPT is a key regulator mechanism of NAD^+ , since NAM is a product of different NAD^+ dependent enzymes including SIRT6, CD38, and PARPs. NAMPT is the rate limiting- enzyme in NAD^+ biosynthesis and it is expressed in multiple different tissues, highlighting therefore a critical role in cell metabolism (Revollo, Grimm et al. 2004, Garten, Schuster et al. 2015). Notably, NAMPT presents in two forms; intracellular (nuclear and cytosolic) and extracellular. Intracellular NAMPT is involved in NAD^+ production, whereas the extracellular form excreted (eNAMPT) by different cells such as myocytes and adipocytes is also known as the visfatin cytokine (Revollo, Körner et al. 2007, Pillai, Sundaresan et al. 2013) (**Figure 15**). It remains unclear until these days whether eNAMT(Visfatin) really has a NMN synthesis activity in the extracellular space as it also need ATP for autophosphorylation to perform its catalytic reaction, which could be present only when cell death is occurring in the local environment.

One important difference between the NAMPT and NMRK pathway is that the latter consumes only 2 ATP to generate NAD^+ , whereas the NAMPT signaling pathway requires 4 ATP molecules, suggesting that NMRK is an energy saving pathway for NAD synthesis and in line with this hypothesis, *Nmrk2* gene was shown to be activated by the energy stress sensor AMP-Kinase through recruitment of the PPAR α pathway (Diguët, Trammell et al. 2018). Two NMRK isoforms are identified: NMRK-1 that is highly expressed in different tissues including the kidneys, and NMRK-2 that is mainly

expressed in the heart and skeletal muscles (Bieganowski and Brenner 2004, Diguët, Trammell et al. 2018). It has been reported that NMRK is a rate limiting enzyme in NMRK-mediated NAD^+ production. Additionally, under physiological conditions NMRK-2 is undetectable, whereas following cardiac diseases including MI, a high levels of NMRK-2 (around 75 fold increase) was observed in cardiac tissues (Ahmad, Tomar et al. 2020). Furthermore, it has been demonstrated that in NMRK-2 KO mice following ischemia, a rapid progression into heart failure was observed when compared to the control group and characterized by worsening LV dysfunction, exacerbated LV chamber dilatation, and aggravated fibrotic scar formation. Additionally, the gain of NMRK-2 function was associated with decreased cell death and seems to be mediated by the regulation of $\text{p38}\alpha$ (Ahmad, Tomar et al. 2020). As a result, these findings suggest an important role of NMRK-2 signaling pathway in cardiac diseases progression and development.

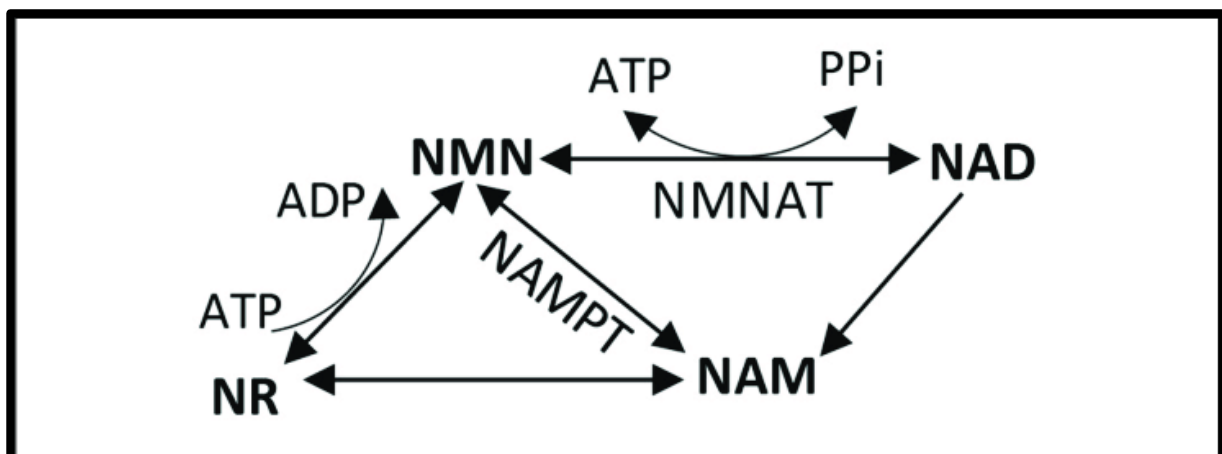


Figure 15: Nicotinamide dinucleotide (NAD⁺) salvage pathway

NAD^+ can be synthesized de novo using tryptophan (not shown), or through a salvage pathway using vitamin precursors niacin (vitamin B3), nicotinamide (NAM), or nicotinamide riboside (NR). In the salvage pathway, NMNAT catalyzes the formation of NAD^+ from NMN and ATP. The reverse reaction converts NAD^+ into NMN, giving off ATP. NMN, nicotinamide mononucleotide; NAD, nicotinamide dinucleotide; NMNAT, nicotinamide mononucleotide adenylyltransferase; NAMPT, nicotinamide phosphoribosyltransferase (Inman and Harun-Or-Rashid 2017)

4.7.6 NAD dependent signaling enzymes

It is well-established that NAD serves as a substrate used by multiple different enzymes, resulting in a net consumption in the cell. SIRT6 (lysine deacetylases), PARPs, and CD38 (Chini 2009, Griffiths, Williams et al. 2020).

4.7.6.1 Sirtuins

Multiple lines of evidence have shed the light on the crucial role of SIRT6 in regulating metabolism and responding to stress in ageing, given that they are implicated in cell longevity and survival, chromatin remodeling, and regulation of different energy metabolism genes (Sato, Stein et al. 2011). SIRT6 are NAD⁺ dependent lysine deacetylases catalyzing the breaking of NAD into NAM and ADP-ribose, enabling them therefore to respond to fluctuations in NAD and NAM levels (Sauve and Youn 2012). Seven families of SIRT6 have been identified in mammalian (sirtuin1-7). Nuclear SIRT-1 has been demonstrated to activate multiple transcription factors such as peroxisome proliferator-activated receptor coactivator 1- α (**Pgc1 α**), involved in energy metabolism, and FoxO-1 that has gained much attention as it is implicated in the induction of autophagy and response to oxidative stress (Wang, Zhao et al. 2015, Jalgaonkar, Parmar et al. 2022). It has been reported that SIRT-1 exerts a cardio protective effect following myocardial ischemia through regulating multiple complex signaling pathways including autophagy, hypoxia inducible factor, NF κ B, and p53 (Yang, Duan et al. 2013). Furthermore, a growing body of evidence has shown that SIRT-1 regulates the progression and development of AKI through exerting anti-inflammatory, anti-apoptotic, and anti-oxidant effects, alleviating therefore AKI pathogenesis (Huang, Jiang et al. 2022). SIRT-2 has also shown to suppress the inflammatory response in rodents through the inhibition of NF κ B signaling pathway. Additionally, it has been indicated that SIRT-2 deletion leads to enhanced hypertrophy by downregulating AMP-activated protein kinase (AMPK) signaling pathway, known to inhibit the protein synthesis in cardiac diseases, resulting subsequently in accelerating

the development to heart failure (Taneja, Ravi et al. 2021). SIRT-3, 4, and 5 are mainly located in mitochondria and implicated in energy homeostasis. In an animal model of isoprenaline-induced cardiac hypertrophy, SIRT-3 has been shown to suppress hypertrophy by inhibiting BNP levels and decrease PARP-1 acetylation (Feng, Wang et al. 2020). In the kidneys of septic mice, SIRT-3 deficiency was associated with exacerbated kidney damage through decreasing the levels of superoxide dismutase and catalase, and enhancing inducible nitric oxide synthesis (Fan, Le et al. 2021).

4.7.6.2 *Poly ADP Ribosyl polymerases (PARPs)*

The second class of NAD⁺-dependent enzyme family is PARPs. PARPs are responsible for catalyzing the formation ADP-ribose polymerase that eventually recruits proteins involved in DNA repair (van Beek, McClay et al. 2021). Overactivation of PARPs mainly PARP-1, however, has been shown to be associated with enhanced inflammation, cell death, and NAD depletion. In that regard, a study performed on PARP-1 (+/+) and PARP-1 (-/-) mice showed that Ang-II induced cardiomyocytes hypertrophy and enhanced interstitial fibrosis in PARP-1(+/+), only. This results were replicated in vitro in cultured cardiomyocytes and findings indicated aggravated hypertrophy, NAD depletion, and cell death in PARP-1 (+/+) cardiomyocytes, highlighting that PARP-1 could be a potential target to be repressed to reduce the deleterious impact of overactivation of angiotensin in cardiac diseases (Pillai, Gupta et al. 2006). At the level of the kidneys, a study performed by Wang et al. demonstrated that in animal model of endotoxic shock, PARP-1 inhibition using 3-AB, decreased kidney damage through suppressing the inflammatory response mediated by the activation of NFκB and intercellular cell adhesion molecule-1 in septic shock mice (Wang, Han et al. 2018).

4.7.6.3 *CD38*

CD38 ectoenzyme is implicated in the cleavage of NAD to produce cyclic ADP-ribose, a secondary messenger involved in calcium signaling. The group of Dr.

Mericskay collaborated to a study showing that CD38 inactivation is beneficial to reduce cardiomyopathy and reduce calcium waves and sparks in cardiomyocytes from *mdx* mice, a model of Duchenne dystrophy and dilated cardiomyopathy (de Zélicourt, Fayssol et al. 2022). In the context of MI, the inhibition of CD38 has been associated with maintaining NADP(H) pool and endothelium-dependent relaxation and preserving nitric oxide generation, leading consequently to enhance contractile function and decreased LV remodeling post-infarction (Reyes, Boslett et al. 2015). Furthermore in a mouse model of LPS-induced AKI, the inhibition of CD38 with quercetine has been shown to decrease kidney morphological alterations and M1 pro-inflammatory macrophages recruitment and NFκB pathway activation (Shu, Feng et al. 2018).

Collectively, the dual role of NAD, both as a co-enzyme involved in energy metabolism and co-substrate consumed by multiple different NAD-dependent enzymes, shed the light on the critical role of NAD in maintaining homeostasis at different levels ranging from energy metabolism to anti-inflammatory pathways and calcium flux. The activation of NAD biosynthetic pathways, therefore is of utmost importance and could attenuate both cardiac and kidney disease development and progression.

4.8 PERTURBATION OF NAD METABOLISM FOLLOWING CARDIAC AND KIDNEY DISEASES

4.8.1 Perturbation of NAD metabolism in the context of CVDs

A drastic decrease in cardiac NAD levels following CVDs has been reported in multiple pre-clinical investigations. It is well documented that energy metabolism disturbances is one of the main key players that accelerates the development and progression of CVDs. In that regard, a study performed in a rat model of cardiac ischemia reperfusion I/R showed that the administration of NAD (10-20 mg/kg , intravenous injection) just before the ischemia leads to a decrease in the infarct size,

downregulation in the apoptotic markers including the active caspase-3 and BAX levels and an upregulation in Bcl2 levels. Furthermore, NAD administration reversed the significant decrease in SOD activity and SOD2 expression levels, providing a cardioprotection in this disease model (Zhang, Wang et al. 2016). Along the same line, it has been reported that following ischemia I/R injury, a substantial decrease in NAD and NAMPT expression levels have been observed. Downregulated NAMPT expression was associated with increased caspase-3 levels and cytochrome c release. Cardiac specific overexpression of NAMPT, however, increased NAD levels, decreased infarct size, and protected against cell death (Hsu, Oka et al. 2009). Furthermore, in non-ischemic dilated cardiomyopathy induced by a cardiac specific serum response factor (**SFR**) HKO mice showed a marked decline in NAD at day 15 along with a significant decrease in NAMPT and a substantial increase in NMRK-1 expression levels. SFR HKO mice were then treated with NR (400 mg/kg/day, dietary supplementation), a precursor of the upregulated NMRK-1 enzyme. NR administration restored NAD pool and decreased adverse LV remodeling.

4.8.2 Perturbation of NAD metabolism in the context of kidney diseases

A drastic decrease in NAD levels following kidney diseases has been well-established. Accumulating experimental studies have demonstrated that boosting NAD levels by regulating NAD biosynthetic enzymes and exogenous administration of NAD precursor could be a potential promising therapeutic strategy for kidney diseases. In this line, a significant decrease in kidney NAD levels along with an upregulation in tubular PARP-1 levels 6 to 12 hours after reperfusion were observed in a rat model of kidney ischemia reperfusion injury (Martin, Lewington et al. 2000). Additionally, a study performed by Vaschetto et al. showed that the inhibition of PARP-1 resulted in less NAD⁺ consumption, preserved eventually renal blood flow in an animal model of ventilator induced lung injury (Vaschetto, Kuiper et al. 2010). A defect in the *de novo* pathway has also been reported following ischemia reperfusion in human. In this

regard, an increase in urinary quinolinic acid, an NAD⁺ precursor, has been reported. The observed increase in urine quinolinic acid suggested the potential downregulation in the enzyme that uses quinolinic acid as a substrate to generate NAD⁺, quinolinate phosphoribosyltransferase (Poyan Mehr, Tran et al. 2018). Furthermore, in an animal model of aged mice with ischemia reperfusion and cisplatin nephrotoxicity, the administration of the NAD⁺ precursor NMN attenuated AKI (Guan, Wang et al. 2017).

4.9 NICOTINAMIDE DINUCLEOTIDE BOOSTING THERAPIES

There are three major approaches that researchers follow to boost NAD levels: 1) The administration of NAD substrate, 2) The activation of NAD biosynthetic enzymes, 3) The inhibition of NAD degrading signaling pathways.

4.9.1 Nicotinamide dinucleotide boosting approaches

A growing body of evidence demonstrates that consecutive boosting of NAD levels is of utmost importance to maintain normal physiological processes. One of the most effective therapeutic approaches to boost NAD levels is the administration of NAD precursor. In that regard, it has been observed that NMN and NR are the molecules of choice in both experimental investigations and clinical studies due to their oral bioavailability (Conze, Crespo-Barreto et al. 2016, Trammell, Schmidt et al. 2016).

NA has been known for long time to reduce cholesterol levels, whereas, its role in boosting NAD levels has been recently come into focus (Altschul, Hoffer et al. 1955, Cantó, Houtkooper et al. 2012). Multiple studies demonstrated that NA treatment enhanced NAD levels, its use, however, was limited due to the development of side effects such as flushing and itching, which could be potentially related to prostaglandin release (Birjmohun, Hutten et al. 2005, Chen and Damian 2014).

An alternative therapeutic approach to enhance NAD levels is to activate NAD

biosynthetic enzymes, especially those which are a rate-limiting step in NAD⁺ production. The NAMPT salvage pathway is the main route implicated in the production of NAD and considered as a rate limiting step, resulting in the conversion of NAM into NAD (Revollo, Grimm et al. 2007). Under physiological conditions, NAMPT is responsible for maintaining NAD levels, whereas following different injuries such as CVDs, kidney diseases, lung injury, NAMPT levels have been shown to decrease. In that regard, preliminary data showed that P7C3 could serve as NAMPT activator, however, further experiments are needed in order to confirm this observation (Wang, Han et al. 2014). Another attractive target is NMNATs because they have dual substrate specificity NMN and NAMN, thus the activation of NMNATs leads to boost both the de novo and salvage pathways (Zhou, Kurnasov et al. 2002). Epigallocatechin gallate, a compound of green tea, has been postulated to activate NMNAT2 by 100%, and NMNAT3 by 42% at a dose of 50 μM (Berger, Lau et al. 2005).

The third approach to increase NAD levels is the inhibition of NAD⁺ consuming enzymes. The main NADase in mammalian is CD38. Recently, several different flavonoids molecules have been shown to inhibit CD38 in vitro and in vivo such as kuromanin, luteolinidin, luteolin, apigenin, and quercetin (Kellenberger, Kuhn et al. 2011, Escande, Nin et al. 2013). For example, it has been indicated that luteolinidin sustain NAD pool and protect myocardial and endothelial function following IHD (Boslett, Hemann et al. 2017). Furthermore, PARP-1 inhibitor could also be a potential therapeutic target leading to increased NAD levels and it has been currently used as an adjuncts for chemotherapy (Brown, Kaye et al. 2016).

4.9.2 NR supplementation

The interest in NAD biology as a moiety implicated in energy metabolism and cellular oxidation-reduction reactions has been gaining momentum. An accumulating evidence indicates declined NAD levels in the setting of CVDs and kidney diseases as previously described in details, suggesting a great necessity to use NAD boosting

therapies. There are multiple different NAD precursors that can be used to increase NAD levels, therefore further investigations are needed to reveal the ideal precursor. NR has been documented as the third discovered NAD precursor that naturally presents in milk. Additionally, NR has been currently described as a favorable NAD precursor since it has not been involved in developing any serious side effects in a randomized, double-blind, placebo-controlled, crossover clinical trial in healthy middle-aged and older adults. (Martens, Denman et al. 2018). Similarly, a clinical study has reported that single oral dose of NR (100, 300, and 1000 mg) can lead to a dose dependent increase in blood NAD⁺ levels without developing side effects (Trammell, Schmidt et al. 2016). On the contrary to niacin (nicotinic acid) for instance, which has been prescribed for its antilipolytic effects is poorly tolerated by patients due to side-effects including flushing, liver damage, glucose intolerance, or gastrointestinal problems (Kamanna, Ganji et al. 2009, Antagonists 2012). Moreover, Oral NR supplementation has been shown to exert protective effects in immortalized human embryonic kidney cells, a murine model of hepatocellular carcinoma, and in immortalized mouse myoblast cell line, mediated by increased NAD levels, decreased mitochondrial dysfunction, and enhanced SIRT activity (Belenky, Bogan et al. 2007, Bolat, Gültepe et al. 2007, Cantó, Houtkooper et al. 2012).

In different model of CVDs including models of cardiomyopathy, the NAMPT enzyme is downregulated (Hsu, Yamamoto et al. 2014), whereas the NMRK-2 enzyme is substantially upregulated (Diguët, Trammell et al. 2018). It was suggested therefore that the activation of NMRK-2 signaling pathway following CVDs could represent a compensatory protective mechanism in response to NAMPT suppression. Of note, although the NMRK-2 pathway has been shown to be activated in the setting of heart failure, the cardiac NAD levels remain depressed, indicating the urgent need of exogenous NR supplementation, the substrate of the upregulated enzyme (Mehmel, Jovanović et al. 2020).

Although altered NAD levels following cardiac and renal diseases and the potential beneficial effect of NR therapies are well documented, the impact of MI on kidney NAD homeostasis is yet to be well fully elucidated. Based on our team investigation (Dr. Tannous et al., stated in the previous section) and based on the strong correlation between the cardiovascular system and the kidneys through the cardio-renal interrelationship, we hypothesis that NR treatment could exert a protective effect on the kidneys, either indirectly through improving cardiac function or directly via its effects on the kidneys and this would be the third aim of our study.

5 OBJECTIVES OF THESIS

IHD is a central contributor to morbidity and mortality in both developed and developing countries. Ischemia and MI have been shown to be the most common and serious types of IHD, with three million people worldwide being subjected to MI each year. AKI is a frequent complication of MI associated with poor prognosis and clinical outcomes, resulting consequently in increased death rate substantially. Multiple different risk factors have been documented to initiate or exacerbate AKI in the context of MI including DM, aging, hypertension, and CS.

Mounting body of evidence demonstrates that CS leads to a 10 folds increase in the incidence of MI in men versus a 5 folds increase in women. Strong published evidence reports that CS resulting in one in every five deaths (~5 million deaths/year) with a 6-15% association with overall healthcare costs. Furthermore, smokers are projected to surpass one billion individuals worldwide (>10 million deaths/year) by 2025 despite the great awareness of CS adverse side effects. Of note, epidemiological studies indicated that the ratio of female-to-male smoking prevalence is progressively and dramatically increasing. Although the adverse impact of CS on CVDs and kidney diseases is well studied in males, the impact of CS on females as well as sex dimorphism in type I cardio-renal syndrome is yet to be fully elucidated.

Multiple recent investigations have shed the light on sexual dimorphism in MI-induced AKI in the presence and absence of risk factors including CS. It has been demonstrated the pre-menopausal females are less prone to develop AKI when compared to age-matched male subjects. Sex-based discrepancies in AKI could be potentially mediated by the cardio and renal protective effects of endogenous estrogen. Endogenous estrogen has been indicated to play a central role in maintaining immune system homeostasis, and reducing arterial pressures, among other mechanisms that are postulated to protect the kidneys.

Although AKI-associated mortality rate declined over time due to the newly developed therapeutic strategies, to date there is no metabolic drug that can treat AKI in the setting of MI. NAD, has emerged as a potential promising target being the main regulator of energy metabolism. Altered NAD biosynthetic enzymes in the setting of MI has been shown to be characterized by an upregulation of NMRK-2 and a downregulation of NAMPT, resulting in a decrease in total NAD pool. NR, an NMRK-1/2 precursor has been recently studied in the context of CVDs and kidney diseases, exerting multiple beneficial effects.

Thus, based on the strong published evidence, we aim at addressing the three following questions:

1. Does pre-menopausal females are more protected against the adverse impact of CS on MI-induced AKI compared to age-matched males?
2. Does endogenous estrogen exert protective effects on MI-induced AKI in pre-menopausal females?
3. Does NR supplementation protects males against AKI at early stage following MI?

6 RESULTS

6.1 SEXUAL DIMORPHISM IN ACUTE MYOCARDIAL INFARCTION-INDUCED ACUTE KIDNEY INJURY AND SUSCEPTIBILITY TO CIGARETTE SMOKING RISK FACTOR

Acute kidney injury (AKI) has been well documented in the setting of acute myocardial infarction (MI), increasing morbidity and mortality rate in both sexes substantially. Passive and active chronic cigarette smoking (CS) remains a national and international epidemic and a major risk factor for cardiovascular disease (CVD) and kidney diseases progression and development. The World Health Organization (WHO) estimates around 5 million deaths annually due to direct CS exposure and more than 600,000 deaths due to second-hand smoke. Current understanding of MI induced-AKI, otherwise known as type I cardiorenal syndrome (CRS), following CS exposure in both males and females is not well elucidated. This is the first study to investigate sexual dimorphism in type I CRS at an early stage post-MI. C57BL6/J male and female mice were exposed to 2 weeks of CS prior to MI induction followed by 1 week of CS exposure in order to assess the impact of CS on kidney damage in the context of MI. Our hemodynamic analysis showed a marked decline in ejection fraction in CS-exposed MI male mice when compared to the relative female group, whereas a comparable decrease in cardiac output in CS-exposed MI mice of both sexes was observed. At the kidney level, morphological alterations were more pronounced in CS-exposed MI male mice when compared with the relative female counterparts. Although reactive oxygen species (ROS) production and glomerular cell death increased to the same extent in CS-exposed MI male and female mice, markers of fibrosis such as alpha-smooth muscle actin (α -SMA) and connective tissue growth factor (CTGF) substantially increased in CS-exposed MI male mice, only. Metabolically, nicotinamide phosphoribosyltransferase (NAMPT) and nicotinamide riboside-1 (NMN-1), NAD⁺ biosynthetic enzymes, significantly upregulated in CS-exposed MI female mice only, whereas sirtuin (SIRT)-1 and SIRT-3, NAD⁺ dependent enzymes, markedly decreased in CS-exposed MI male mice compared with their relative female group. Importantly, kidney NAD levels significantly declined in CS-exposed MI male mice, only. Collectively, our findings reveal that MI female mice exhibited pronounced kidney protection following CS when compared with the relative male group. This work has been performed at Dr. Zouein's lab at the American University of Beirut.

Research Article

Sex-based differences in myocardial infarction-induced kidney damage following cigarette smoking exposure: more renal protection in premenopausal female mice

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The impact of cigarette smoking (CS) on kidney homeostasis in the presence of myocardial infarction (MI) in both males and females remains poorly elucidated. C57BL6/J mice were exposed to 2 weeks of CS prior to MI induction followed by 1 week of CS exposure in order to investigate the impact of CS on kidney damage in the presence of MI. Cardiac hemodynamic analysis revealed a significant decrease in ejection fraction (EF) in CS-exposed MI male mice when compared with the relative female subjects, whereas cardiac output (CO) comparably decreased in CS-exposed MI mice of both sexes. Kidney structural alterations, including glomerular retraction, proximal convoluted tubule (PCT) cross-sectional area, and total renal fibrosis were more pronounced in CS-exposed MI male mice when compared with the relative female group. Although renal reactive oxygen species (ROS) generation and glomerular DNA fragmentation significantly increased to the same extent in CS-exposed MI mice of both sexes, alpha-smooth muscle actin (α -SMA) and connective tissue growth factor (CTGF) significantly increased in CS-exposed MI male mice, only. Metabolically, nicotinamide phosphoribosyltransferase (NAMPT) and nicotinamide riboside-1 (NMRK-1) substantially increased in CS-exposed MI female mice only, whereas sirtuin (SIRT)-1 and SIRT-3 substantially decreased in CS-exposed MI male mice compared with their relative female group. Additionally, renal NAD levels significantly decreased only in CS-exposed MI male mice. In conclusion, MI female mice exhibited pronounced renal protection following CS when compared with the relative male groups.

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Introduction

Cardiovascular diseases (CVDs) remain the leading cause of death in males and females worldwide [1,2]. The World Health Organization (WHO) estimates that more than 17 million CVD deaths annually are due to ischemic heart diseases, involving mostly myocardial infarction (MI) and strokes [3]

(http://www.who.int/cardiovascular_diseases/en/). According to the 2016 heart disease and stroke statistics, one American will suffer an acute MI every 42 s around the clock [4]. The prevalence of MI, however, is lower in females when compared with age-matched males, but picks up with aging to reach comparable levels following menopause [5,6]. For instance, the male to female MI risk ratio under 45 years of age is 10:1, while dropping substantially in those over 75 years of age to 2:1 [7]. Noteworthy, patients diagnosed with MI are not only at high risk of heart failure development, but also of multiple comorbidities including a 29-fold increase in acute kidney injury (AKI) through the cardio-renal interrelationship [8,9]. In fact, MI-induced cardiac systolic dysfunction leads to a decrease in stroke volume and cardiac output (CO) which hypoperfuses the kidney, markedly enhancing the risk of kidney damage [10–12]. Multiple factors including hypertension, diabetes mellitus, and cigarette smoking (CS) are identified as high risk factors for both MI and AKI development [13].

Recent published evidence positively correlates CS to AKI onset and progression via both direct and indirect mechanisms [14–16]. CS contains approximately 5000 potential toxic chemicals including, among others, oxidative gases, free radicals, nicotine, and amide [17]. These chemicals have been shown to enhance reactive oxygen species (ROS) production, mitochondrial dysfunction, fibrosis, and cell death, all of which are hallmark features of AKI development [18–24]. Besides all the undesired chemicals in CS and their harmful effects, nicotine itself is known to induce apoptosis in podocytes and fibrosis in proximal convoluted tubules (PCTs), promoting therefore kidney damage [25,26].

Interestingly, pre-menopausal women are less prone to develop AKI following CS exposure when compared with age matched men [27]. This sexual dimorphism is attributed to the well-known renal protective effects of endogenous estrogen (E2) that is reported in multiple pre-clinical and clinical studies [28,29]. For instance, E2 has been tightly linked to reduced kidney injury following renal ischemia reperfusion through decreasing glomerular mesangial cells damage, albuminuria, and tubulointerstitial fibrosis [30,31]. In contrast, estrogen receptor- α knockout is linked to enhanced podocyte apoptosis in female mice, resulting in extracellular matrix (ECM) accumulation and glomerular hypertrophy, increasing consequently albuminuria and kidney injury [32]. To date, very little is known about the impact of CS on males and female's kidneys in the presence of MI. This is the first study to provide experimental evidence of sex discrepancy between CS-exposed male and female mice with MI, suggesting therefore the potential importance of sex hormones in modulating the pathophysiological responses of this disease model.

Materials and methods

Animal use

All animal experiments in the present study were conducted according to the experimental protocols approved by American University of Beirut Animal Care and Use Committees (IACUC # 18-2-RN560) in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, 8th edition [33]. Age-matched male and female C57BL6/J mice (5 months old) were maintained in the animal care facility at the American University of Beirut Medical Center under optimum conditions with 12 light/12 dark hours cycle with unlimited access to water and standard chow.

Experimental design

Five months old C57BL6/J male and premenopausal female mice were allocated into four groups: control group (3 weeks of air exposure) ($n = 6$), CS exposure (non-MI) group (3 weeks of CS exposure) ($n = 6$), MI group ($n = 6$), and MI+CS group ($n = 10$) (2 weeks of CS exposure followed by MI and another week of CS exposure). Seven days post-MI, MI male and female mice were killed and at the end of week 3, control, CS-exposed (non-MI), and CS-exposed MI mice of both sexes were killed. One hundred microliters heparin (Heparin Sodium 1000 IU/ml) was administrated to mice 30 min prior to sacrifice. Following deep anesthesia with 4% isoflurane, blood was collected by cardiac puncture, centrifuged for 10 min at 2200 rpm and the plasma flash-frozen in liquid nitrogen and stored at -80°C . Mice were then subjected to cervical dislocation. For kidney collection, right kidney was harvested and immediately placed into a cryotube in liquid nitrogen followed by storage at -80°C for molecular work. Left kidney was harvested into 4% zinc formalin tubes for histology. A portion was also used to assess ROS (see below).

CS exposure protocol

Conscious C57BL6/J aged matched male and female mice were retained and exposed to mainstream tobacco cigarette smoke using nose only exposure apparatus (ONARES, CH Technologies, U.S.A.). 3R4F scientific cigarettes (University of Kentucky, Lexington, KY, U.S.A.), scientifically prepared cigarettes concentrated with toxins, were used and placed into the cigarette puffer to generate puffs at a constant frequency of one puff/50 s, a volume of 2 ml/puff, and a duration of 2.5 s/puff allowing a total particle matter (TPM) concentration of approximately 100

mg/cm³/mouse/session, 9.4 mg tar, and 0.726 mg nicotine per cigarette. Mice were exposed to two 90-min sessions daily (10 cigarettes/session), 7 days/week either for 3 weeks or for 2 weeks before inducing MI, then one additional week thereafter.

Myocardial infarction

MI was induced by left anterior descending (LAD) coronary artery ligation. Heart rate, body temperature, and respiratory rate were assessed in order to reduce any surgical complications. The mouse was placed on a heating pad to prevent anesthesia-induced hypothermia. Fifteen min prior to the surgery, tramadol (0.05–0.1 mg/kg i.p.) and isoflurane (2–3% in oxygen) inhalation were given to induce analgesia and general anesthesia. Orotracheal intubation was performed to maintain normal respiratory rate by placing a needle into the trachea and connecting it to a mini automated ventilator (Harvard Apparatus). LAD coronary artery, left ventricle, and left atrium were exposed by excising between the ribs of the left thorax. MI was induced by LAD ligation with 7-0 polypropylene suture at 1–3 mm underneath the left atrium appendage. Successful MI induction was confirmed by the blanching of the tissue downstream of the ligation site, by ECG, and by echocardiography 24 h after surgery. Immediately following successful LAD ligation, the chest was closed and mice were placed on a warm pad for recovery. Once they groomed freely, MI mice were caged back individually and monitored on daily basis for full recovery.

Echocardiography

Vevo 2100™ High-Resolution Imaging System (Visual Sonics, Toronto, Canada) was used to perform echocardiography according to the American Society of Echocardiography guidelines. In order to induce general anesthesia, mice were exposed to isoflurane (1.5% in oxygen) inhalation. Body temperature was maintained at 37°C using rectal thermostat and warming plate. The transducer was then placed on the left thorax and ultrasound beam was 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. Ejection fraction (EF) and CO were measured at baseline, at the end of week 2 (before the induction of MI), and at the end of week 3 (before killed).

Hematoxylin and eosin staining

Hematoxylin and eosin (H&E) staining was used to highlight glomerular retraction for all experimental groups. Four micrometers thick renal sections from each mouse were stained with H&E according to standard laboratory protocol. Briefly, kidney tissues were first fixed in 4% formalin, then dehydrated and embedded in paraffin. Dewaxing and hydration steps of paraffin-embedded kidneys were performed, and then the tissue was stained with H&E and examined under light microscope with 40× magnification. Glomeruli that were sectioned at midpoint with commonly visible afferent and efferent arterioles were included in the analysis. The ones that had deformation in shape and borders and were sectioned close to the pole of the glomerular sphere were excluded.

Periodic acid Schiff staining

Four µm thick kidney sections from each mouse were stained with periodic acid Schiff (PAS) according to standard laboratory protocol to assess PCT dilatation. Briefly, paraffin embedded kidneys underwent dewaxing and hydration steps. Dehydration with increasing percentages of ethanol (75, 95% and 100%) was performed, kidney tissues were then stained with 0.5% PAS for 10 min, followed by Schiff reagent for 10–20 min, and washed for 5 min. Finally, slides were mounted using mounting medium and observed under light microscope at 40×. PCT cross-sectional area was measured using Image J software (<https://imagej.nih.gov/ij/>)

Masson's trichrome staining

Masson's trichrome (MTC) staining was used to assess total renal fibrosis. Paraffin embedded kidneys underwent dewaxing and hydration steps, soaked in Bouin solution for one h at 56°C, then washed and rinsed in distilled water. Kidney tissues were incubated for 10 min with hematoxylin, then washed and stained in biebrich scarlet acid fuchsin. After washing, kidney sections were differentiated in phosphomolybdic-phosphotungstic acid solution for 10 min, transferred to aniline blue solution, stained for 5 min and observed under light microscopy at 10× magnification. Total renal fibrosis was measured using Image J software (<https://imagej.nih.gov/ij/>). Images were converted to gray scale followed by threshold modification in order to measure red-stained fibrosis.

Table 1 List of primers used in the present study

Primer	Forward	Reverse
GAPDH	TGTGTCCGTGGATCTGA	TTGCTGTTGAAGTCGCAGGAG
NAMPT	ACCAGCGGGGAACCTTGTTA	ACATAACAACCCGGCCACAT
NMRK-1	CTTGAAGCTTGCTCTGCGAC	GTGTCGTCTTCCCTCCGTTT
SIRT-1	CGGCTACCGAGGTCCATATAC	ACAATCTGCCACAGCGTCAT
SIRT-3	GATTCGGATGGCGCTTGAC	TCTCCACCTFTAACTCTCCC
PARP-1	ACACCACAAAACCTCAGCCA	ACAAACCACAAACAACCGGC
α -SMA	CAGCGGGCATCCACGAAA	GGCCCAGCTTCGTCGTATT
CTGF	ACCAACTATGATGCGAGCC	GGTAACTCGGTGGAGATGC

Dihydroethidium staining

Total ROS score was assessed using dihydroethidium (DHE) staining (Calbiochem, Darmstadt, Germany) and performed on whole kidney tissue sections. Ten μ M DHE was used to incubate unstained sections under dark condition in a humidified chamber at room temperature for 45 min. Images of kidney tissues were acquired using Laser Scanning Fluorescent Microscope (Zeiss Axio) under 20 $\times$ magnification.

Terminal deoxynucleotidyl transferase dUTP nick end labeling

Glomerular DNA fragmentation was evaluated using Click-It Plus TUNEL Assay (Invitrogen). Briefly, kidney tissues underwent dewaxing and rehydration steps, then DNA fragmentation detection kit was used to perform terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining as described by manufacturer's instructions. Laser Scanning Fluorescent Microscope (Zeiss Axio) was used to assess glomerular DNA fragmentation under 40 $\times$ magnifications. Glomeruli with green nuclear labeling were considered as TUNEL positive.

Immunofluorescence

The protein expression level of alpha-smooth muscle actin (α -SMA) in kidney tissues was evaluated using immunofluorescence (IF). Kidney slides were placed in an antigen retrieval buffer at 95°C for 15 min, followed by two washes with tris-buffered saline (TBS)-Triton 0.025%, 5 min each. To block non-specific binding of α -SMA antibody, slides were incubated with 10% normal goat serum (NGS) and with 1% bovine serum albumin (BSA) in TBS for 2 h at room temperature. Kidney slides were then incubated with a dilution of α -SMA antibody (Abcam, ab5694), in TBS (1:200) at 4°C overnight, and washed twice with TBS-T 0.025%, 5 min each. Incubation with a dilution of secondary antibody (FITC) (Abcam, ab97050) in TBS (1:100) for 1 h was performed, followed by two washes with TBS, 5 min each. Dapi was then added, and slides were washed twice with phosphate-buffered saline (PBS), 5 min each. Laser Scanning Fluorescent Microscope (Zeiss Axio) was used to detect renal α -SMA protein expression under 20 $\times$ magnification.

RNA extraction and RT-qPCR

Snap frozen kidney tissues were crushed under liquid nitrogen, then total RNA was extracted using Trizol according to manufacturer's instructions (Thermo Fisher Scientific, Grand Island, NY, U.S.A.). RNA was quantified using NanoDrop ND-1000 UV-Vis Spectrophotometer. Differences in mRNA expression levels were quantified using real time q-PCR. During reverse transcriptase (RT) steps, a c-DNA synthesis kit (QuantiTect Reverse Transcription Kit, Qiagen) was used to synthesize c-DNA from 1 μ g RNA, followed by real time PCR analysis in a CFX96 real-time PCR system (Bio-Rad, Germany). Afterward, the synthesized c-DNA was loaded in duplicate with 0.05 μ l of the forward and reverse primers of the following genes: nicotinamide phosphoribosyltransferase (NAMPT), nicotinamide riboside kinase-1 (NMRK1), sirtuin-1 (SIRT-1), sirtuin-3 (SIRT-3), poly [ADP-ribose] polymerase (PARP-1), connective tissue growth factor (CTGF), and α -SMA and mixed with SYBR[®] Green for qPCR steps. To normalize gene expression between different samples, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used. No-template (water without DNA) was used to check for non-specific amplification. Normalized fold expression relative to the control was calculated and plotted by Biorad CFX-manager to compare differential gene expression. Primer sequences were used as listed in Table 1

Table 2 Baseline plasma estrogen levels comparison between female mice groups in the presence of CS and MI

Female mice group	Mean $\pm$ SEM (pg/ml)	Mice/group	P value
Ctrl	31.678 $\pm$ 6.051	3	0.5051
CS	38.211 $\pm$ 2.646	5	
MI	34.106 $\pm$ 2.045	5	
MICS	35.412 $\pm$ 1.907	5	

Data were analyzed for significance using one-way ANOVA. All bars represent mean $\pm$ SEM ($n = 3-5$); CS: cigarette smoking; Ctrl: Control; MI: myocardial infarction.

Enzyme-linked immunosorbent assay

Assessment of plasma TNF- α levels

Enzyme-linked immunosorbent assay (ELISA) Stemcell Technologies kit (catalog #02030) was used for the detection and measurement of mouse TNF- α in plasma. Plasma samples were diluted 1:1 in ELISA diluent for a total minimum volume of 250 μ l per dilution. One hundred microliters of diluted samples were then loaded in duplicate in ELISA strip plates pre-coated with the cytokine of interest and incubated at room temperature for 2 h. Afterward, each well was washed five times with 300 μ l washing buffer. One hundred microliters/well of diluent detection antibody was added and the plate was incubated at room temperature for 1 h. Each well was then washed five times with 300 μ l washing buffer and 100 μ l/well of diluted streptavidin horseradish peroxidase (SA-HRP) was added and the plate was incubated at room temperature for 1 h. After washing steps, 100 μ l/well of 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added to all wells and the plate was incubated for 15 min at room temperature. One hundred microliters/well of stopping solution was then added to each well and the absorbance was measured at 450 nm in a microplate reader.

Assessment of plasma 17- β estradiol levels

ELISA Abcam kit (catalog#108667) was used for the detection and measurement of baseline 17- β estradiol in plasma, to verify that all female mice are in the premenopausal phase [34] (Table 2). Twenty-five microliters of samples were loaded in duplicate, 17- β Estradiol-HRP conjugated was then added, and the plate was incubated at room temperature for 2 h. Afterward, each well was washed three times with 300 μ l washing buffer. After washing steps, 100 μ l/well of TMB substrate was added to all wells and the plate was incubated for 30 min at room temperature. One hundred microliters/well of stopping solution was then added to each well and the absorbance was measured at 450 nm in a microplate reader.

Total NAD extraction and quantification

Twenty milligrams of kidney tissues were extracted using 75% ethanol and 25% HEPES (10 mM pH 7.1) and diluted 1:20 in distilled water for a total minimum volume of 50 μ l per dilution to reach a concentration within the standard curve. Twenty five microliters of NAD samples were then loaded in duplicate in 96-well microtiter-plate. Afterward, 100 μ l/well of reaction buffer [600 mM ethanol, 0.5 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2 mM phenazine ethosulfate (PES), 120 mM Bicine (pH7.8), yeast alcohol dehydrogenase (SIGMA A3263 > 300 μ g/mg)] was added to the extract. The kinetic of the reaction (OD at 550 nm, every 30 s for 20 min) was followed up on LB 942 multimode reader and NAD was then quantified using linear regression curve equation method between NAD standard concentration and the slope of the regression line.

Statistical analysis

To perform statistical analysis, Graphpad Prism7 software was used. Statistical comparisons were performed using one-way ANOVA or two-way ANOVA and Sidak's multiple comparisons test. Results were expressed as mean $\pm$ standard error of the mean (SEM). A P-value of $P < 0.05$ (*) was considered significant.

Results

CS exposure decreased EF but not CO in MI male mice when compared with the relative female subjects

CS-exposed MI mice of both sexes experienced a significant decrease in EF when compared with non-exposed and CS-exposed (non-MI) mice. CS-exposed MI male mice exhibited a marked decrease in EF when compared with the

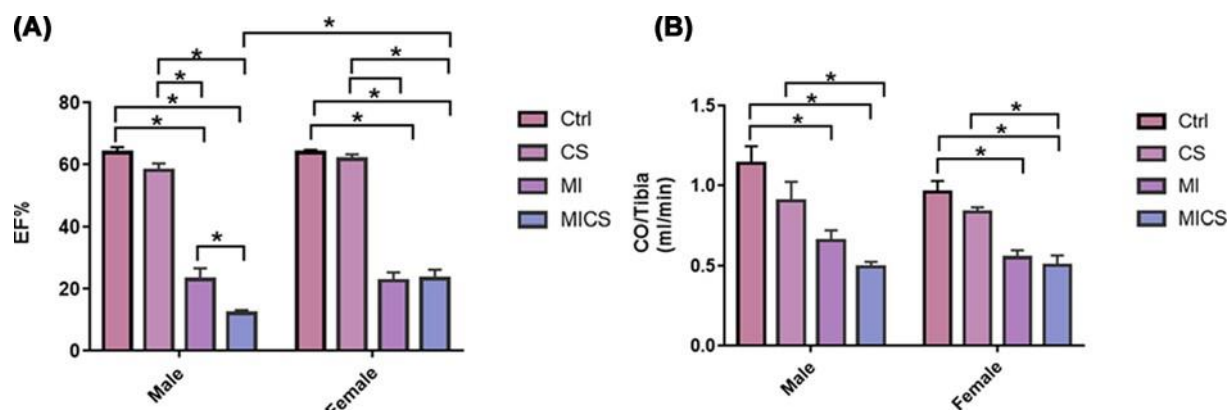


Figure 1. EF and CO comparison between male and female mice following CS exposure in the presence of MI
Cardiac functional assessment. EF markedly decreased in CS-exposed MI males when compared with their relative female subject (A). (B) Co significantly decreased in CS-exposed MI mice of both sexes. Data were analyzed for significance using two-way ANOVA. All bars represent mean $\pm$ SEM (* P value < 0.05; n = 5–9); CS: cigarette smoking; Ctrl: Control; MI: myocardial infarction.

MI group. No significant change in CS-exposed MI female mice in EF when compared with MI female mice, however, was observed. CS-exposed (non-MI) mice of both sexes showed no significant change in EF when compared with their relative non-exposed groups, whereas MI mice of both sexes experienced a substantial decrease in EF when compared with non-exposed and CS-exposed (non-MI) groups. Cross-sex analysis revealed a marked decrease in EF in CS-exposed MI males when compared with the relative female group. No significant difference between control male and female mice was observed (Figure 1A). As for the impact of CS and MI on CO, CS-exposed MI mice of both sexes experienced a comparable decrease in CO when compared with non-exposed and CS-exposed (non-MI) groups. CS-exposed (non-MI) male and female groups, on the other hand, showed no significant change in CO when compared with their relative non-exposed groups. MI mice of both sexes, however, exhibited a substantial comparable decrease in CO when compared with non-exposed groups. No significant difference between control male and female mice was observed (Figure 1B).

Plasma TNF- α was undetectable in all mice groups of both sexes

The effect of CS and MI on systemic inflammation was assessed in terms of plasma TNF- α levels. Plasma TNF- α was measured using Stem Cells Technologies TNF- α ELISA kit. Plasma TNF- α protein levels were undetectable in all groups of both sexes in the presence and absence of CS and MI.

CS exposure increased glomerular retraction in MI male mice compared with the relative female subjects

Glomerular retraction was assessed by H&E staining. Figure 2 shows a significant increase in glomerular retraction in CS-exposed MI mice of both sexes compared with the relative non-exposed, CS-exposed (non-MI), and MI groups. CS-exposed (non-MI) and MI male mice experienced a marked increase in glomerular retraction when compared with non-exposed groups. However, no significant change in glomerular retraction in CS-exposed (non-MI) and MI female mice when compared with the non-exposed group was observed. Cross-sex analysis revealed a substantial increase in glomerular retraction in CS-exposed (non-MI), MI, and CS-exposed MI male mice when compared with the relative female groups. No significant difference between control male and control female mice was observed.

CS exposure increased PCT cross-sectional area in MI male mice compared with the relative female subjects

PCT cross-sectional area was assessed by PAS staining. Figure 3 reveals an enhanced PCT cross-sectional area in CS-exposed (non-MI), MI, and CS-exposed MI male mice when compared with the non-exposed group. All female mouse groups, however, exhibited no significant change in PCT dilatation. Cross-sex analysis revealed a substantial increase in PCT cross-sectional area in CS-exposed (non-MI), and CS-exposed MI male mice when compared with the relative female groups. No significant difference between control male and female mice was observed.

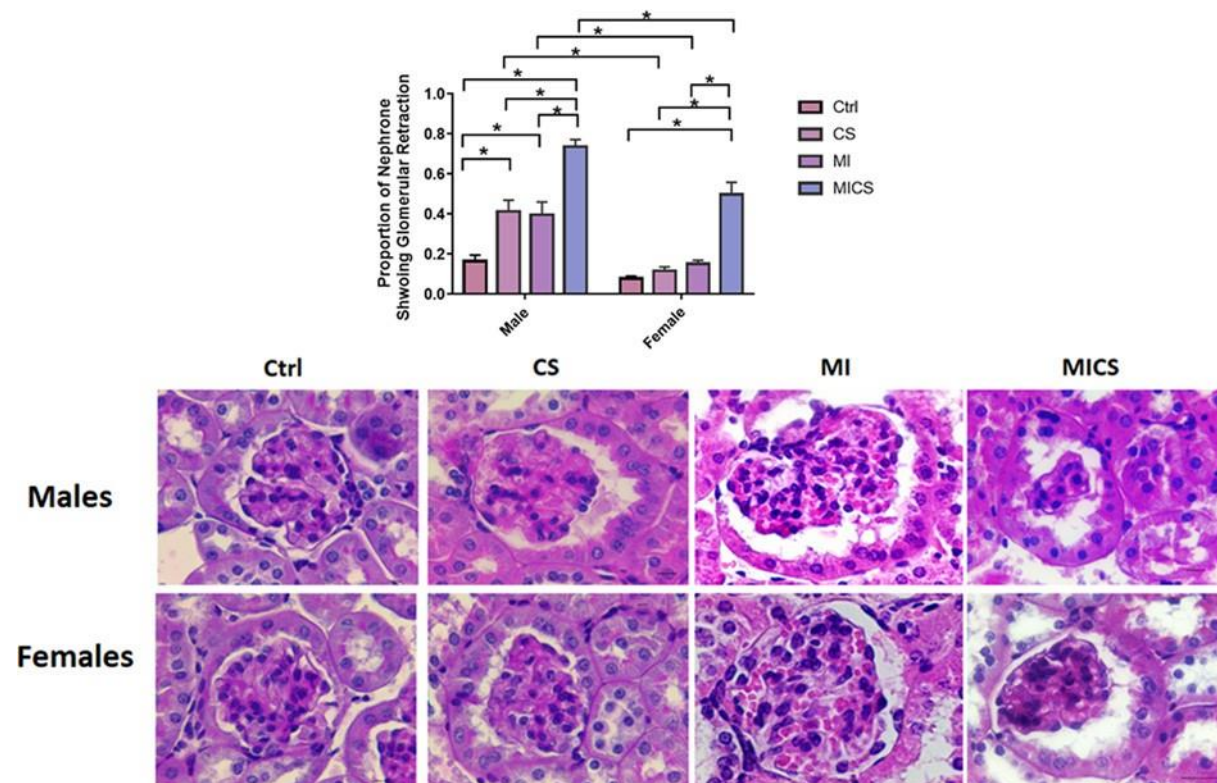


Figure 2. **Glomerular retraction comparison between male and female mice following CS exposure in the presence of MI** Enhanced glomerular retraction was observed in CS-exposed MI males when compared with the relative female subject. Representative image of each H&E stained group is shown. Data were analyzed for significance using two-way ANOVA. All bars represent mean $\pm$ SEM (**P* value < 0.05; *n* = 3–9); CS: cigarette smoking; Ctrl: Control; MI: myocardial infarction.

CS exposure increased total renal fibrosis but not α -SMA and CTGF in MI male mice compared with the relative female subjects

Total renal fibrosis was assessed by MTC staining. Figure 4A reveals a significant increase in total renal fibrosis in CS-exposed MI mice of both sexes when compared with the relative non-exposed, CS-exposed (non-MI), and MI groups. A marked increase in total renal fibrosis in CS-exposed (non-MI) mice when compared with the non-CS exposed group was only seen in male mice. Additionally, MI male mice experienced a significant increase in total renal fibrosis when compared with non-exposed and CS-exposed groups. Cross-sex analysis revealed a significant increase in total renal fibrosis in CS-exposed (non-MI), MI, and CS-exposed MI male mice when compared with the relative female mice. No significant difference between control male and female mice was observed. Figure 4B shows a marked increase in renal CTGF mRNA expression levels in MI and CS-exposed MI male mice when compared with non-exposed and CS-exposed mice. No significant change in renal CTGF mRNA expression levels in all female mice, however, was observed. Cross-sex analysis revealed a substantial increase in renal CTGF mRNA expression levels in MI male mice when compared with the relative male group. Figure 4C shows a significant increase in renal α -SMA mRNA expression levels in CS-exposed MI and MI male mice when compared with non-exposed groups. MI male mice experienced a significant increase in renal α -SMA mRNA expression levels when compared with CS-exposed mice. No significant change in all female mice, however, was observed. Cross-sex analysis revealed a substantial increase in renal α -SMA mRNA expression levels in MI male mice when compared with the relative female group. Our finding is further fortified with IF data showing an accumulation of α -SMA in the peritubular space in MI and CS-exposed MI male mice, only (Figure 4D).

CS exposure increased total ROS score and glomerular DNA fragmentation in MI mice of both sexes

Figure 5A shows a significant and comparable increase in total ROS in CS-exposed MI mice of both sexes when com-

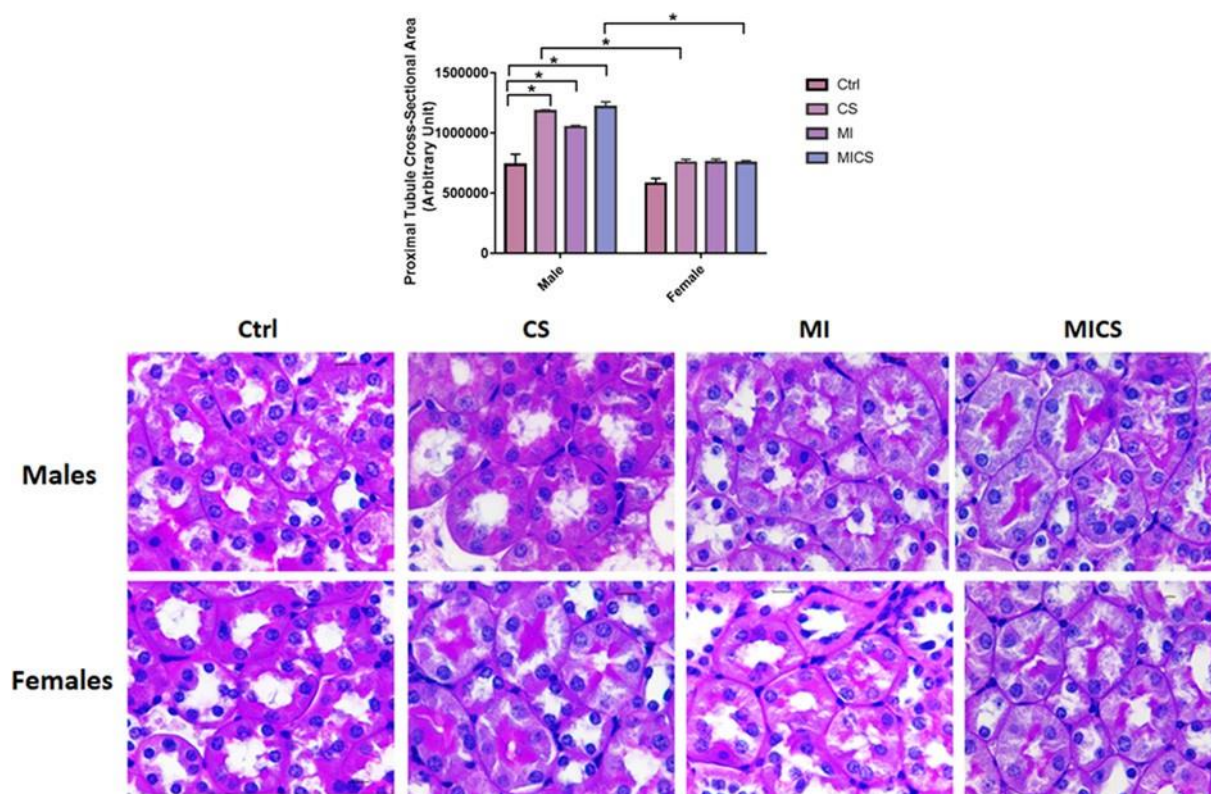


Figure 3. CS exposure increased PCT cross-sectional area in MI male mice compared to the relative female subjects
PCT dilatation was substantially increased in CS-exposed MI males when compared with the relative female subjects. Representative image of each PAS stained group is shown. Data were analyzed for significance using two-way ANOVA. All bars represent mean $\pm$ SEM (* P value < 0.05 ; $n = 3-8$); CS: cigarette smoking; Ctrl: Control; MI: myocardial infarction.

pared with non-exposed and CS-exposed (non-MI) groups. A significant increase in total ROS score in CS-exposed MI mice when compared with the MI group was observed in female mice, only. CS-exposed (non-MI) male and female mice, however, showed no significant change in total ROS score when compared with their relative non-exposed groups. A marked increase in total ROS score in MI mice when compared with the non-exposed group was observed in male mice, only. No significant difference between control male and female mice was observed. Figure 5B shows a comparable significant increase in glomerular DNA fragmentation in CS-exposed MI mice of both sexes when compared with non-exposed and CS-exposed (non-MI) mice. A significant increase in glomerular DNA fragmentation in CS-exposed MI mice when compared with the MI group was observed in female mice, only. CS-exposed (non-MI) male and female mice, however, showed no significant change in glomerular DNA fragmentation when compared with their relative non-exposed groups. A marked increase in glomerular DNA fragmentation in MI mice when compared with the non-exposed group and CS-exposed groups was observed in mice of both sexes. No significant difference between control male and female mice was observed.

CS exposure altered NAD pathway enzymes in MI mice of both sexes

Figure 6A shows a significant increase in renal NAMPT mRNA expression levels in CS-exposed MI female mice when compared with non-exposed and CS-exposed (non-MI) mice. CS-exposed (non-MI) female mice experienced a marked increase in NAMPT mRNA expression levels when compared with non-exposed mice. Coherently, MI female mice showed a substantial increase in NAMPT mRNA expression levels when compared with non-exposed and CS-exposed (non-MI) groups. No significant change in NAMPT mRNA expression levels, however, in all male mice was observed. Cross-sex analysis revealed a significant increase in NAMPT mRNA expression levels in MI and CS-exposed MI female groups when compared with the relative male mice. Figure 6B reveals a marked increase in renal NMRK-1 mRNA expression levels in CS-exposed MI female mice when compared with non-exposed and CS-exposed (non-MI) female groups. No significant change in renal NMRK-1 mRNA expression levels in CS-exposed

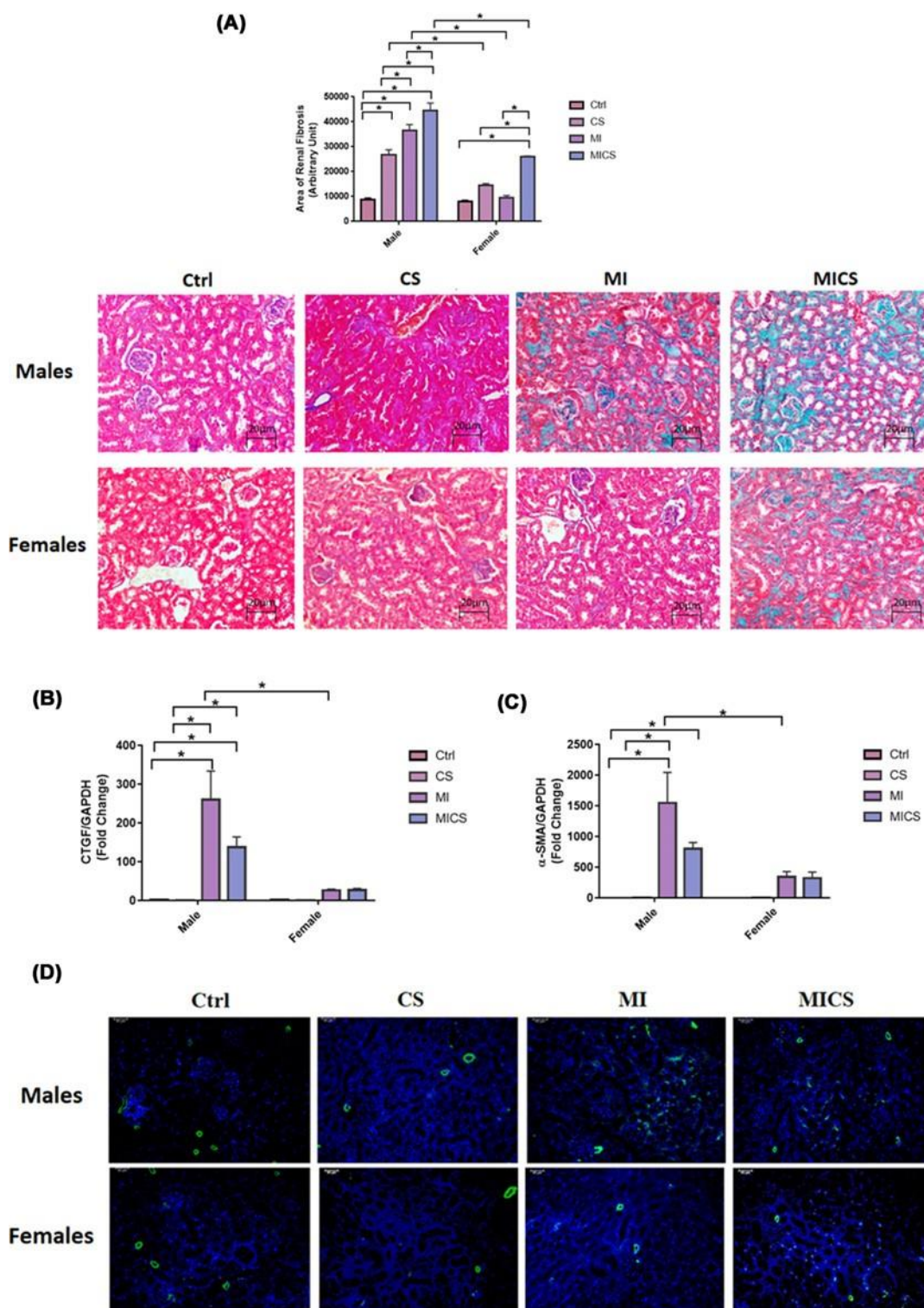


Figure 4. **CS exposure increased total renal fibrosis but not α -SMA and CTGF in MI male mice compared to the relative female subjects**

Total renal fibrosis significantly increased in CS-exposed MI males when compared with the relative female subjects (A). Representative image of each MTC stained group is shown. α -SMA and CTGF significantly increased in CS-exposed MI male mice, only (B,C). (D) α -SMA accumulation in the peritubular space of CS-exposed MI male mice, only. Representative image of each IF stained group is shown. Data were analyzed for significance using two-way ANOVA. All bars represent mean $\pm$ SEM (* P value < 0.05; n = 3–8); CS: cigarette smoking; Ctrl: Control; MI: myocardial infarction.

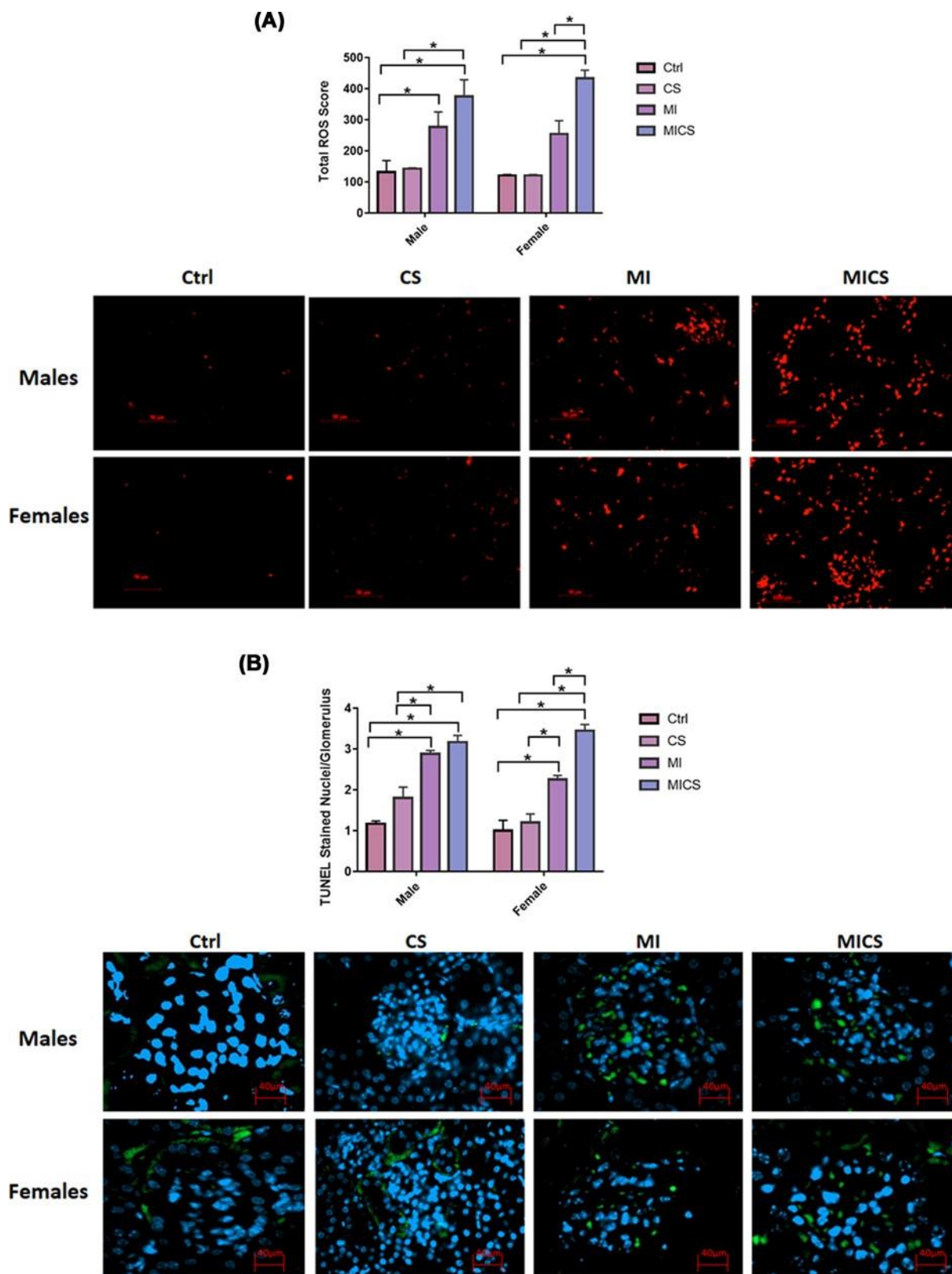


Figure 5. Total ROS score and glomerular DNA fragmentation comparison between male and female mice following CS exposure in the presence of MI

Total ROS score (A) and glomerular DNA fragmentation (B) significantly increased in CS-exposed MI mice of both sexes. Representative image of each DHE and TUNEL stained group is shown. Data were analyzed for significance using two-way ANOVA. All bars represent mean $\pm$ SEM (**P* value < 0.05; *n* = 3–5); CS: cigarette smoking; Ctrl: Control MI: myocardial infarction.

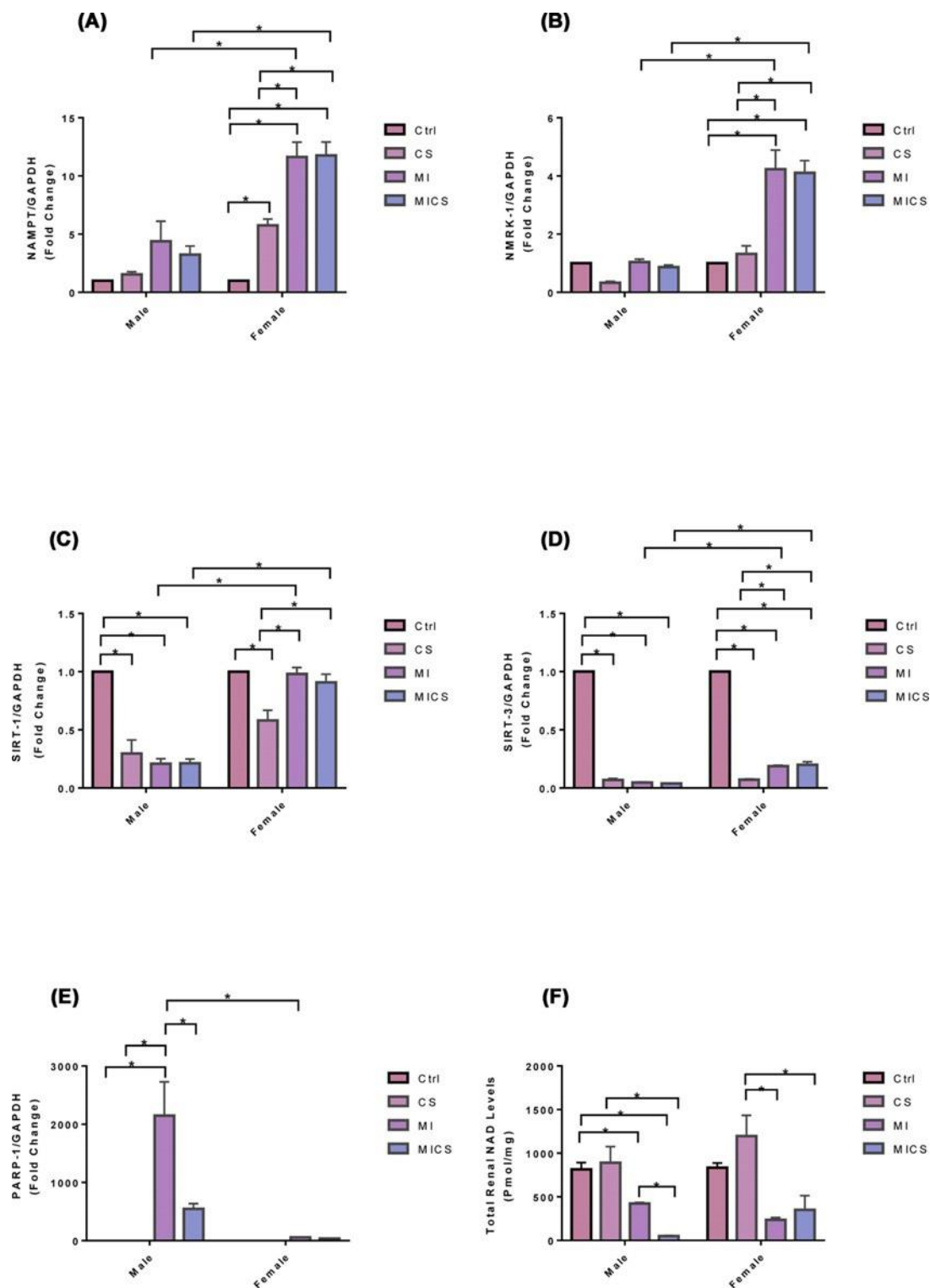


Figure 6. NAD biosynthetic, NAD dependent, total renal NAD levels comparison between male and female mice following CS exposure in the presence of MI

NAMPT and NMRK-1 mRNA expression levels significantly increased in CS-exposed MI female mice when compared with the relative male subjects (A,B). SIRT-1 and SIRT-3 mRNA expression levels markedly decreased in CS-exposed MI male mice when compared with the relative female subjects (C,D). PARP-1 mRNA expression levels and total renal NAD levels substantially decreased in CS-exposed MI male mice when compared with the MI group (E,F). Data were analyzed for significance using two-way ANOVA. All bars represent mean $\pm$ SEM (**P* value < 0.05; *n* = 3–10).

(non-MI) mice when compared with non-exposed mice of both sexes, however, was observed. A marked increase in renal NMRK-1 mRNA expression levels in MI female mice when compared with non-exposed and CS-exposed (non-MI) female groups was also observed. No significant change in NMRK-1 mRNA expression levels in all male mice groups, however, was seen. Cross-sex analysis revealed a substantial increase in renal NMRK-1 mRNA expression levels of MI and CS-exposed MI female mice when compared with the relative male groups. Figure 6C shows a significant decrease in renal SIRT-1 mRNA expression levels in CS-exposed, MI, and CS-exposed MI male mice when compared with the non-exposed group. CS-exposed female mice exhibited a marked decrease in renal SIRT-1 mRNA expression levels when compared with non-exposed mice. Conversely, MI and CS-exposed MI female mice showed a significant increase in renal SIRT-1 mRNA expression levels when compared with the CS-exposed group. Cross-sex analysis revealed a significant increase in renal SIRT-1 mRNA expression levels in MI and CS-exposed MI female mice when compared with the relative male mice. Figure 6D shows a significant decrease in renal SIRT-3 mRNA expression levels in CS-exposed (non-MI), MI, and CS-exposed MI mice of both sexes when compared with the non-exposed groups. Both MI and CS-exposed MI female mice exhibited a marked increase in renal SIRT-3 mRNA expression levels when compared with CS-exposed (non-MI) female mice. Cross-sex analysis revealed a substantial increase in renal SIRT-3 mRNA expression levels in MI and CS-exposed MI female mice when compared with the relative male groups. Figure 6E shows a significant increase in renal PARP-1 mRNA expression levels in MI male mice when compared with non-exposed and CS-exposed (non-MI) groups. CS-exposed MI-male mice experienced a marked decrease in renal PARP-1 mRNA expression levels when compared with MI mice. No significant change in all female mice, however, was observed. Cross-sex analysis revealed a substantial increase in renal PARP-1 mRNA expression levels in MI male mice when compared with the relative female group. As for total renal NAD levels, Figure 6F reveals a significant decrease in total renal NAD levels in CS-exposed MI males compared with non-exposed, CS-exposed (non-MI), and MI mice. A marked decrease in total renal NAD levels in CS-exposed MI female mice when compared with the CS-exposed (non-MI) group was also observed. No significant change in total renal NAD levels in CS-exposed (non-MI) groups when compared with non-exposed mice in both sexes was observed. MI male mice experienced a substantial decrease in total renal NAD levels when compared with the non-exposed group, whereas MI female mice showed a marked decrease in total renal NAD levels when compared with the CS-exposed (non-MI) group. No significant difference between control male and female mice was observed.

Discussion

Findings of the present study are novel and of utmost importance. Numerous studies reported a strong association between CS, CVDs, and adverse kidney events [3,21,35,36]. In the present study, mice were primarily exposed to CS for 2 weeks, followed by the induction of MI by ligating the LAD artery, then exposed to CS for 1 week after in order to investigate the effect of CS on kidneys damage post-MI. Hemodynamic analysis revealed a significant decrease in EF in CS-exposed MI mice of both sexes, being more pronounced in males. CO, however, compensated for EF differences between sexes and comparably decreased in CS-exposed MI male and female mice. This finding indicates that the observed sexual dimorphism in kidney damage is not due to differences in kidney perfusion between both sexes, at least at the systemic level. Similarly, inflammation was at its lowest levels in CS-exposed groups 7 days post-MI, with plasma TNF- α being below the detectable limit (<5pg/ml) in both sexes, ruling out therefore any potential positive correlation between systemic inflammation and kidney damage. These findings are in accordance with previous investigations highlighting that pro-inflammatory cytokines such as TNF- α are released 4–6 weeks following CS exposure [3,37]. Additionally, TNF- α secretion has been shown to be markedly enhanced in the early inflammatory phase post-MI and down-regulated 24–72 h thereafter [38,39].

With the absence of pronounced differences between CS-exposed MI mice of both sexes in cardiac hemodynamic parameters and systemic inflammation, sex-based kidney damage discrepancy was tracked down to the kidney molecular and structural levels. Histologically, our findings revealed increased renal morphological alterations in CS-exposed MI mice of both sexes but to a greater extent in male mice. For instance, glomerular retraction, renal fibrosis, and PCT-cross-sectional area markedly increased in CS-exposed male mice post-MI when compared with their relative female group. These findings correlate with a study done by Kobeissy et al. demonstrating that PCT swelling and glomerular retraction, markedly increased in CS-exposed male mice 7 post-MI [40]. Additionally, it has been reported that renal interstitial fibrosis significantly increased following both CS exposure and MI [41,42]. Our findings were further fortified with the pro-fibrotic markers CTGF and α -SMA. Both renal CTGF and α -SMA mRNA expression levels substantially increased in CS-exposed MI male mice, only. Multiple studies demonstrated a pivotal role of CTGF in enhanced kidney fibrosis. For instance, Riser et al. reported increased fibrosis in mesangial cells exposed to recombinant CTGF [43]. Additionally, a clinical study has reported a strong correlation between increased

plasma CTGF levels and decreased glomerular filtration rate, subsequently enhanced kidney disease progression and development [44]. Similarly, α -SMA, the hallmark of fibroblast differentiation into myofibroblast, has been reported to increase in the interstitium and glomeruli, resulting therefore in enhanced fibrogenesis in the kidneys [45,46]. Coherently, IF data showed accumulation of α -SMA in the peritubular space in the kidneys of MI and CS-exposed MI male mice, only. MI/CS-induced kidney damage is majorly attributed to substantial ROS production [37,47,48]. Our study revealed a comparable increase in renal ROS generation in CS-exposed MI mice of both sexes. This outcome indicates that the protection observed in CS-exposed female mice post-MI is not due to the attenuation of ROS production, but rather due to a mechanism downstream of ROS-induced kidney damage [49,50]. Prolonged ROS production due to exogenous or endogenous stimuli is tightly linked to mitochondrial dysfunction, energy imbalance, and cell death [47,51,52]. Our TUNEL assay showed a comparable increase in glomerular DNA fragmentation in CS-exposed MI mice of both sexes, a finding consistent with previous studies supporting the glomerular cell death stimulating effects of CS and MI [53,54]. DNA fragmentation, however, is a common feature of both apoptosis and necrosis. To further characterize the type of cell death involved, kidney NAD depletion was assessed as a marker of cell death by necrosis and energy failure [55]. NAD levels significantly decreased in CS-exposed MI male mice only when compared with the MI group, supporting a potential necrotic type of cell death linked to mitochondrial dysfunction. [56,57]. Multiple studies report a strong correlation between decreased NAD levels and enhanced kidney injury [56,57]. NAD in the kidney is mainly produced via the amidated pathway through two enzymes NAMPT and NMRK-1. NAMPT converts nicotinamide (NAM) into nicotinamide mononucleotide (NMN), whereas NMRK-1 uses nicotinamide riboside (NR) as a precursor to generate NMN. NMN is then converted through nicotinamide mononucleotide adenylyltransferase (NMNAT) into NAD [57]. NAD has recently emerged as an important moiety used by different enzymes involved in the regulation of energy metabolism and mitochondria biogenesis including SIRT-1 and SIRT-3 as well as enzymes implicated in DNA repair such as PARP-1 [58]. Our findings revealed that unlike CS-exposed MI male mice, CS-exposed MI female mice showed a significant increase in NAD⁺ biosynthetic salvage enzymes expression, including NMRK-1 and NAMPT. Veer et al. and Piali et al. demonstrated that enhanced NAMPT expression level extends cellular life span through inducing SIRT-1 mediated mitochondrial protein deacetylation [59,60]. Enhanced NAMPT levels subsequently induce SIRT1 expression and activity through replenishing NAD levels, and improving consequently mitochondrial bioenergetic function and cellular integrity [61–64]. In the present study, both NAMPT and SIRT1 expression levels significantly increased in CS-exposed female mice post-MI when compared with their relative male group and are in line with previous findings [63–65]. Of note, SIRT-1 has been shown to protect against ROS-induced injury through the activation of class O of forkhead box (FOXO) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α), mitochondrial transcription factors, and subsequently enhancing the expression of mitochondrial targeted antioxidant enzymes [66]. However, NAD and SIRT1 levels were markedly decreased in the CS-exposed MI male group when compared with their relative female group. SIRT-3 substantially decreased in CS-exposed MI mice of both sexes, being more pronounced in male mice. Our data are consistent with multiple studies highlighting the link between kidney injury and decreased SIRT-3 mRNA expression levels. SIRT-3 activity significantly decreased in an animal model of diabetic nephropathy resulting in enhanced mesangial cells hypertrophy [67]. Similarly decreased SIRT-3 mRNA levels and increased mitochondrial protein hyperacetylation are reported in an animal model of cystatin-induced AKI [68]. PARP-1, on the other hand, substantially decreased in CS-exposed MI male mice when compared with the MI group, while remained unchanged in all female mice groups. It has been demonstrated that PARP-1 is activated in response to ROS overproduction, decreasing therefore DNA damage, in line with our outcomes indicating enhanced kidney damage in CS-exposed MI male mice [58]. The differential expression in NAMPT, NMRK-1, SIRT-1, and SIRT-3 levels along with the comparable increase in ROS levels in the kidneys of CS-exposed MI mice of both sexes, could explain the sex-biased difference in kidney protection downstream of ROS injury. [69].

Conclusion and future directions

The importance of the findings revealed in the present study is twofold: (1) CS exacerbated cardiac systolic dysfunction following MI in both sexes; (2) kidney damage was more pronounced in CS-exposed male mice post-MI when compared with the relative female group; and both sexes were characterized by renal morphological alterations, being more pronounced in CS-exposed MI male mice. The observed sex-based kidney damage in the present study seems to be independent of cardiac dysfunction and systemic inflammation, but triggered downstream ROS generation given that both CS-exposed MI male and female mice exhibited similar levels of renal ROS production. For instance, a marked decrease in renal NAD levels and mitochondrial bioenergetic enzymes along with a significant increase in

pro-fibrotic markers were observed in CS-exposed MI males, suggesting, potentially enhanced mitochondrial dysfunction, necrotic cell death, and fibrosis when compared with the relative female group. The protection observed in females is potentially linked to the well-known protective impact of estrogen on the cardiovascular system in general and the kidneys in particular. For instance, estrogen receptor- α is expressed in podocytes and exerts renoprotective effects against experimentally induced cell death [70]. Additionally, both tubular injury and macrophage infiltration increased substantially in female mice following estrogen depletion [71], whereas the administration of E2 to aging Dahl salt sensitive rat attenuates tubulointerstitial fibrosis and glomerulosclerosis [72,73]. Mechanistically, E2 seems to enhance expression of both SIRT-1 and NAMPT in response to prolonged ROS production, resulting subsequently in decreased cell death through the inhibition of the pro-apoptotic biomarker caspase 3 [74,75]. Future experiments tailored to detect the protein expression levels of NAD biosynthetic and dependent enzymes and the downstream affected pathways are warranted. Determining the relationship between NAD depletion and the associated apoptosis/necrosis and inflammation may also be needed. Additionally, using SIRT1 and SIRT-3 knock out male mice may be needed to detect whether the observed exacerbated kidney damage in CS-exposed MI male mice is due to decreased SIRT-1 and SIRT-3 levels, consequently potential enhanced mitochondria dysfunction. Last but not least, conducting the same experiments on ovariectomized CS-exposed female mice post-MI is necessary to reveal whether estrogen is primarily or partially involved in the observed protective effects.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contribution

F.A.Z., N.J.H., and A.Y. contributed to the study design and conception. N.J.H., A.M., A.K., R.G., H.A., C.T., A.H., A.J., R.A., G.W.B., A.Y., and F.A.Z. participated in the acquisition, analysis, or interpretation of the data. N.J.H. performed and supervised all the experiments. N.J.H. and F.A.Z. drafted the manuscript. All authors judiciously revised the manuscript and gave their final approval for all the data presented, ensuring reliability and integrity.

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Abbreviations

AKI, acute kidney injury; CO, cardiac output; CS, cigarette smoking; CTGF, connective tissue growth factor; CVD, cardiovascular disease; DHE, dihydroethidium; EF, ejection fraction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IF, immunofluorescence; LAD, left anterior descending; MI, myocardial infarction; MTC, Masson's trichrome; NAMPT, nicotinamide phosphoribosyltransferase; NMRK-1, nicotinamide riboside-1; PAS, periodic acid Schiff; PCT, proximal convoluted tubule; ROS, reactive oxygen species; TBS, tris-buffered saline; α -SMA, alpha-smooth muscle actin.

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6.2 EFFECTS OF OVARECTOMY ON ACUTE MYOCARDIAL INFARCTION-INDUCED ACUTE KIDNEY INJURY

Cardiovascular diseases (CVDs) remain the leading cause of death in both sexes worldwide. According to the World-Health-Organization, around 31% of CVDs related death is due to ischemic heart diseases, most notably myocardial infarction (MI). Acute kidney injury (AKI) is one of the most common complications in the setting of MI through the cardiorenal interrelationship, otherwise known as type I CRS, increasing therefore mortality rate substantially. Accumulating studies have shown that premenopausal females are less susceptible to adverse left ventricular (LV) remodeling following MI and thus type I CRS progression and development when compared to age matched-males with undefined mechanisms. MI prevalence, however, substantially increased with age to reach comparable level post-menopause. For example, the male to female risk ratio under the age of 45 is 10:1, while increasing significantly above the age of 75 to 1:2. Sex dimorphism in MI-induced AKI remodeling is potentially attributed to the protective effects of the endogenous estrogen (17- β Estradiol). In this study we aimed at investigating the mechanisms along with the potential involvement of estrogen in the observed sex-based differences between premenopausal female and age-matched male mice in AKI post-MI. Five months old males and females C57BL6/J mice were employed in this study and divided into 5 groups: Male control, female control, MI-male, MI-female, and MI ovariectomized females. MI was induced by ligating the left anterior descending artery, whereas, estrogen depletion was induced by bilateral ovariectomy. Mice were sacrificed 7days post-MI. Our hemodynamic analysis revealed exacerbated adverse LV remodeling in MI mice of both sexes, with female mice being more protected. Enhanced cardiac dysfunction post-menopause, however, was observed and seen to be mediated by marked decrease in ejection fraction and cardiac output. At the kidney level, both sexes exhibited kidney dysfunction and a marked decrease in total kidney NAD levels. Unlike MI female mice, MI males showed aggravated histological alterations with increased proinflammatory, profibrotic, and proapoptotic, biomarkers. The expression of NAD⁺ biosynthetic enzymes NAMPT and NMRK-1 was increased in MI females only, while males showed a substantial increase in NAD⁺ consuming enzyme PARP-1. With OVX, MI females developed a swelling of proximal convoluted tubules, enhanced proinflammatory cytokine expression levels, and a further deterioration in kidney function. Our findings demonstrate that MI-induced AKI in both sexes with pre-menopausal female mice being more protected. Ovariectomy exacerbates aspects of AKI in females after MI. In conclusion, our findings revealed enhanced AKI in male and female mice post- MI, with female mice being more protected. This work had been performed at Dr. Zouein 's lab at the American University of Beirut in collaboration with Dr. Merisckay at Paris Saclay University.

Sexual dimorphism in acute myocardial infarction-induced acute kidney injury: cardiorenal deteriorating effects of ovariectomy in premenopausal female mice

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Acute kidney injury (AKI) is a common complication of cardiovascular diseases (CVDs) in both males and females, increasing mortality rate substantially. Premenopausal females appear to be more protected, suggesting a potential protective role of female sex hormones. Here, we tested the hypothesis that ovariectomy (OVX) eliminates the beneficial effect of female sex on renal protection following acute myocardial infarction (MI). Seven days post-MI, both sexes exhibited worsened kidney function and a substantial decrease in total kidney NAD levels. Unlike MI female mice, MI males showed exacerbated morphological alterations with increased proinflammatory, proapoptotic, and profibrotic biomarkers. The expression of NAD⁺ biosynthetic enzymes NAMPT and NMRK-1 was increased in MI females only, while males showed a substantial increase in NAD⁺ consuming enzyme PARP-1. OVX did not eliminate the female-sex protection of glomerular morphology but was associated with swelling of proximal convoluted tubules with MI as in males. With OVX, MI females had enhanced proinflammatory cytokine release, and a further decrease in creatinine clearance and urine output was observed. Our findings suggest that MI induced AKI in both sexes with pre-menopausal female mice being more protected. Ovariectomy worsens aspects of AKI in females after MI, which may portend increased risk for development of chronic kidney disease.

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Introduction

Cardiovascular diseases (CVDs) remain the leading cause of death in both sexes with tremendous health and economic burdens worldwide [1–3]. According to the world health organization (WHO), 4 out of 5 CVD deaths are attributed to myocardial infarction (MI) and stroke (<http://www.who.int/cardiovascular-diseases/en/>) [4]. Epidemiological studies revealed that one American is subject to an MI every 60 s [5]. Sex discrepancies in CVDs have been well established, with premenopausal women being less prone to develop MI compared with age-matched men [2,6]. MI prevalence, however, increases with age to reach comparable levels after menopause [7,8]. For instance, the female-to-male MI risk ratio is 1:10 under the age of 45, while increasing dramatically to 1:2 over the age of 75 [9].

Of note, patients diagnosed with MI are not only at a 30-fold increase in risk of heart failure development but also at a 25-fold increase in risk of cardiorenal syndrome (CRS) through the cardiorenal inter-relationship, increasing therefore the mortality rate substantially [10,11]. In fact, MI-induced left ventricle (LV) systolic dysfunction leads to decreased stroke volume (SV) and cardiac output (CO), and a subsequent decline in renal perfusion [12–14]. In addition to altered cardiac hemodynamics, systemic inflammation post-MI can aggravate kidney damage progression and development independently of compromised cardiac systolic function [15]. Evidence indicates that acute kidney injury (AKI) is a common occurrence with ST-segment elevation MI (STEMI) and is correlated with poor prognosis [16].

Accumulating preclinical and clinical studies report sexual dimorphism in kidney damage pathogenesis, being less frequent in premenopausal females [17,18]. A recent study reported that AKI occurs more often in women, presumably postmenopausal, than in men with MI undergoing percutaneous coronary intervention (PCI), although the basis for this was not defined [19].

Sex differences are potentially attributable to the nephroprotective effects of endogenous estrogen, 17β Estradiol (E2) [20–22]. To date, our understanding of the impact of cardiac dysfunction as early as 7 days post-MI on male and female kidney damage is very limited. In the present study, we asked the question of whether the sexual dimorphism in MI-induced AKI between male and age-matched premenopausal female mice is reversed by ovariectomy.

Materials and methods

Additional methods may be found in the Supplementary Material.

Animal use

C57BL6/J mice were employed in the present study according to the experimental protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the American University of Beirut (AUB) and in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, 8th edition [23]. The approved IACUC protocol number was #18-2-RN560. Five months old age-matched male and female mice (equivalent to a 30-year-old human) were maintained in the animal care facility of the AUB Medical Center under optimum conditions with 12 light/12 dark hours cycle and access to unlimited water and standard chow. Surgical procedures and experiments with animals were performed in the animal care facility of the American University of Beirut Medical Center (AUBMC).

Experimental design

Five months old C57BL6/J mice were allocated into five groups: male control (MC), female control (FC), MI-male (MMI), MI-female (FMI), and ovariectomized MI-female (FMIOVX). For MI groups, MI was induced by ligation of the left anterior descending (LAD) coronary artery. Mice of both sexes were sacrificed 7 days post-MI. Bilaterally ovariectomized female mice (FOVX) were caged individually for 3 weeks post-operation and monitored on a daily basis until full recovery. At the end of week 3, FOVX mice underwent MI (FMIOVX) and were killed 7 days later. Thirty minutes prior to sacrifice, 100 μ l heparin (heparin sodium 1000 IU/ml) was administered to mice and deep anesthesia induced with 4% isoflurane. Cardiac puncture was used to collect blood followed by centrifugation at 2200 rpm for 10 min; plasma was collected, mixed with protease inhibitor cocktail, and flash frozen in liquid nitrogen and stored at -80°C . Mice were then subjected to cervical dislocation. Left kidney was harvested and immediately placed into a cryotube in liquid nitrogen followed by storage at -80°C for molecular work. Right kidney was harvested into 4% zinc formalin tubes for histology.

Surgical procedures

MI was induced by permanent ligation of the LAD coronary artery. In order to reduce any surgical complication, HR, respiratory rate, and body temperature were monitored throughout the procedure. Analgesia and general anesthesia were induced by tramadol (0.05–0.1 mg/kg i.p.) and isoflurane (2–3% in oxygen) inhalation, respectively, and prior to surgery. Anesthesia-induced hypothermia was controlled by a heating pad. Orotracheal intubation was induced by placing a Y-tube into the trachea and connecting it to a mini-automated ventilator (Harvard Apparatus) in order to maintain normal respiratory rate. LV, left atria, and LAD coronary artery were exposed by excising between the ribs of the left thorax. MI was induced by LAD ligation with 7-0 polypropylene suture at 1–3 mm underneath the left atrium appendage, then the chest was sutured and the mouse placed on a warm pad for recovery. Successful MI was established by blanching of tissue downstream of the ligation, by ECG, and by echocardiography 24 h post-surgery. After grooming freely, MI mice were caged back and monitored daily for full recovery. Control mice underwent sham

Table 1 List of antibodies for WB analysis

Antibodies	Catalog number	Dilution
IL-1 β	Abcam; cat# ab9722	1/500
Caspase-3	Abcam; cat# ab4051	1/500
IL-4	Abcam; cat# ab9728	1/1000
α -SMA	Abcam; cat# ab5694	1/200

surgery. For ovariectomy, mice were anesthetized with 2% isoflurane, placed in a supine position, and maintained on a heating pad to avoid heat loss. The ventral area was shaved, cleaned with 70% ethanol, and the peritoneal cavity accessed with a 1 cm midline incision performed below the bottom of the ribcage. Adipose tissue located under the right kidney was pulled away to locate and identify the uterine horn and the right ovary. Using blunt forceps, the ovarian fat surrounding the ovaries was retracted and the oviduct exposed and isolated with a 6.0 prolene suture to prevent bleeding. Using small sterile scissors, the oviduct was gently severed and the uterine horn returned back to the abdominal cavity. The same procedure was repeated to remove the left ovary. The peritoneum and skin were closed in layers using sterile 6.0 prolene sutures. Mice were finally housed on cleaned and dried bedding and allowed to recover for a period of 3 weeks before LAD ligation. Estrogen levels were measured 4 weeks following ovariectomy (Supplementary Table S1).

Histology

Glomerular retraction was assessed using hematoxylin and eosin (H&E) staining. Briefly, kidney tissues were fixed with 4% formalin, dehydrated, and embedded in paraffin. Paraffin-embedded kidney tissues were sectioned into 4 μ m thickness followed sequentially by dewaxing, hydration, and H&E staining. Glomerular retraction was observed using a light microscope under 10 $\times$ magnification and quantified by counting the number of retracted glomeruli over the number of total glomeruli. Representative figures were obtained at 40 $\times$. Glomeruli with visible vascular pole and afferent and efferent arterioles were included in the analysis, while glomeruli with deformations in shape were excluded.

Proximal convoluted tubule (PCT) cross-sectional area (CSA), glomerular capillary area, and Bowman's capsule area were assessed using periodic acid–Schiff (PAS) staining. Briefly, kidney tissues were fixed with 4% formalin, dehydrated, and embedded in paraffin. Paraffin-embedded kidney tissues were sectioned into 4 μ m thickness followed by dewaxing and hydration. The slides were stained with 0.5% PAS for 10 min, incubated with Schiff reagent for 20 min, and then washed for 5 min according to manufacturer's protocol (Periodic Acid Schiff Stain Kit, Mucin Stain, Abcam, ab150680). Slides were mounted and observed under light microscope at 40 $\times$. PCT cross sectional area, glomerular capillary area, and Bowman's capsule area were measured using ImageJ software (<https://imagej.nih.gov/ij/>). For glomerular capillary area and Bowman's capsule area analysis, glomeruli with visible vascular pole and afferent and efferent arterioles were included, whereas glomeruli with deformation in shape were excluded.

Total renal fibrosis was assessed using Masson's trichrome (MTC) staining. Briefly, kidney tissues were fixed with 4% formalin, dehydrated, and embedded in paraffin. Paraffin-embedded kidney tissues were sectioned into 4 μ m thickness followed by dewaxing and hydration. Kidney tissues were then soaked in Bouin solution at 56°C for 1 h, washed, and rinsed in distilled water. Kidney sections were stained in hematoxylin for 10 min, washed, and incubated with Biebrich scarlet acid fuchsin. Finally, kidney tissues were washed again, differentiated in phosphomolybdic-phosphotungstic acid solution for 10 min, and stained with aniline blue solution for 5 min. Total renal fibrosis was observed under light microscopy at 10 $\times$ magnification and measured using ImageJ software.

Protein extraction and Western blots

Snap-frozen kidneys were crushed under liquid nitrogen and homogenized using extraction buffer composed of 100 mM dithiothreitol, 1% sodium dodecyl sulfate (SDS), 0.9% NaCl, and 80 mM Tris hydrochloride (pH 6.8). Kidney tissues were then heated at 95°C for 10 min and left on a rocking shaker overnight at 4°C. Protein samples (150 μ g) were loaded on to 15% gels for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were separated and transferred to nitrocellulose membranes. Membranes were blocked in 5% fat-free milk dissolved in TBS with 0.1% Tween 20 (TBST 0.1%) for 1 h at room temperature. Membranes were immunoblotted overnight at 4°C with antibodies against interleukin (IL)-1, IL-4, uncleaved caspase-3, and α -SMA (Table 1). TBS with 0.02% Tween 20 (TBST 0.02%) was used to wash the membranes (4 $\times$ 5 min). The membranes were incubated with 1:40,000 biotin-conjugated secondary antibody at room temperature for 1 h followed by four washes with TBST 0.02% for 5

Table 2 List of primers used for RT-qPCR analysis

Primer	Forward	Reverse
GAPDH	TGTGTCCGTGGATCTGA	TTGCTGTTGAAGTCGCAGGAG
NAMPT	ACCAGCGGGGAACCTTGTGA	ACATAACAACCCGGCCACAT
NMRK-1	CTTGAAGCTTGCTCTGCGAC	GTGTCGTCTTCCCTCCGTTT
PARP-1	ACACCACAAAACCTCAGCCA	ACAAACCACAAACACCGGC

min each, then incubated with 1/200,000 HRP-conjugated streptavidin for 30 min. After washing with TBST 0.02% and finally TBS (2×5 min), bands were visualized using a Chemidoc MP imaging system (Bio-Rad) and ECL Chemiluminescence kit (Bio-Rad). To mitigate loading differences, protein expression levels were normalized to total protein per lane using Coomassie blue staining [24,25]. Total protein bands were obtained using the Chemidoc MP and analyzed via ImageJ software. A freshly prepared mild stripping buffer (200 mM glycine, 0.1% SDS, 1% v/v Tween 20, pH 2.2) was used for membrane stripping. Membranes were incubated 2 × 10 min with the buffer at room temperature, followed by 2 × 5 min washes with 0.02% TBST and then 2 × 5 min with TBS.

RNA extraction and real time-quantitative polymerase chain reaction

Snap-frozen kidney tissues were crushed under liquid nitrogen followed by total RNA extraction using TRIzol according to the manufacturer's instructions (Thermo Fisher Scientific, Grand Island, NY, U.S.A.). A NanoDrop ND-1000 was used to quantify RNA. Differences in mRNA expression levels were quantified using real-time q-PCR. Briefly, cDNA was synthesized from 1 µg RNA using the QuantiTect Reverse Transcription Kit (Qiagen), followed by real-time PCR in a CFX96 instrument (Bio-Rad, Germany). Samples were loaded in duplicate with 0.05 µl of the forward and reverse primers (Table 2) of the genes for the following: nicotinamide phosphoribosyltransferase (NAMPT), nicotinamide riboside kinase-1 (NMRK1), and poly (ADP-ribose) polymerase 1 (PARP-1) and mixed with SYBR® Green. Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). To check for non-specific amplification, no-template (water without DNA) was used. Normalized fold-expression relative to the control was calculated and plotted by Biorad CFX-manager to compare differential gene expression.

Total NAD extraction and quantification

Ten volumes (µl/mg) of buffered 75% ethanol and 25% HEPES (10 mM, pH 7.1) were used to extract 20 mg of kidney tissues. Extracts were diluted 1:20 in distilled water for a total minimum volume of 50 µl per dilution to reach a concentration within the standard curve. Afterward, 25 µl of NAD samples were loaded in a 96-well microtiter-plate in duplicate. Ten µl/well of reaction buffer (600 mM ethanol, 0.5 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT]), 2 mM phenazine ethosulfate (PES), 120 mM bicine (pH 7.8) was added. Yeast alcohol dehydrogenase (Sigma-Aldrich A3263 > 300 units/mg) was then added to the NAD samples. The kinetics of the reaction (OD at 550 nm, every 30 s for 20 min) was examined on an LB 942 multimode reader and NAD was quantified using a linear regression curve equation method between NAD standard concentration and the slope of the regression line.

Statistical analysis

GraphPad Prism 9 software was used for statistical analysis. Results are expressed as fold change or mean[±] standard error of the mean (SEM). Statistical comparisons were performed using two-way ANOVA and a Tukey's or Sidak's multiple comparisons test, or by unpaired *t*-test or Mann–Whitney test. For the two-way ANOVA, capillary area, glomerular retraction, and PCT-CSA were significant for interaction (MI sex) and sex; ejection fraction (EF), IL-1β, IL-4, NAD, creatinine clearance, glomerular ROS, and urine output were significant for MI only; all other parameters were significant for interaction, MI, and sex. A *P*-value of <0.05 was considered significant.

Results

OVX enhanced the decrease with MI in ejection fraction

Figure 1A shows a comparable decrease in EF in MI mice of both sexes compared with their respective controls (*P*<0.05). As for the impact of OVX on FMI mice, Figure 1B shows an exacerbated decrease in EF in FMIOVX mice compared with the FMI group (*P*<0.05).

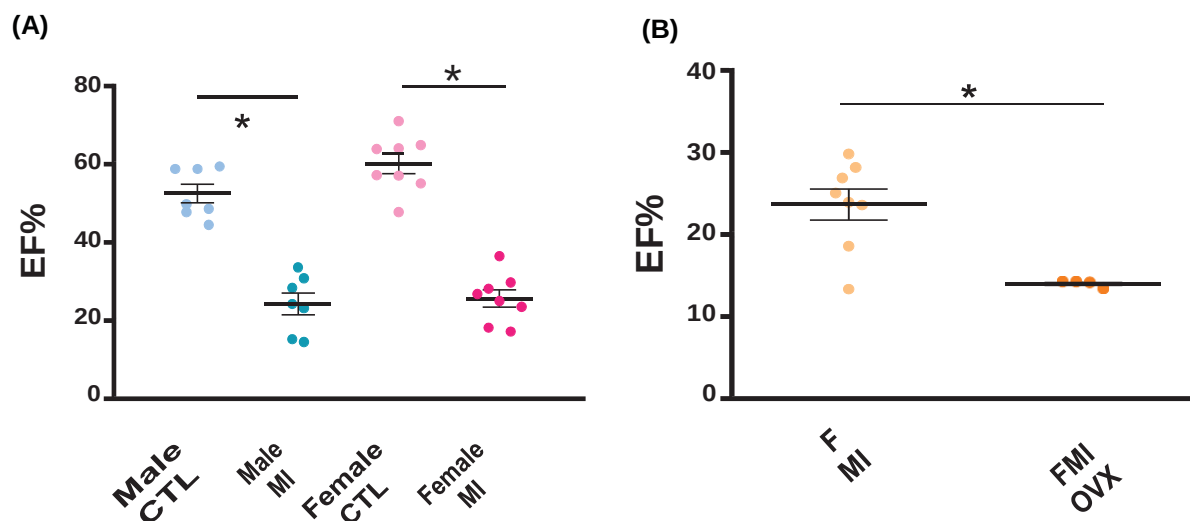


Figure 1. **Comparison of EF between MMI and FMI mice, and between FMI and FMIOVX**

EF markedly decreased in MI mice of both sexes compared with their relative control groups (A). EF significantly decreased in FMIOVX mice compared with the FMI group (B). Data were analyzed for MMI and FMI using two-way ANOVA, and for FMI and FMIOVX using unpaired *t*-test. All bars represent mean $\pm$ SEM (* P <0.05; n =4–13); MI, myocardial infarction; OVX, ovariectomy.

OVX did not eliminate the female-sex protection of glomerular morphology but did cause swelling of proximal convoluted tubules with MI

Figure 2A–C shows an increase in glomerular retraction and in the Bowman's capsule area, and a decrease in glomerular capillary area, in MI male mice compared with their control group and female mice (P <0.05). OVX did not have any impact on kidney morphology (glomerular retraction, Bowman's capsule area, and glomerular capillary area) in FMI mice (Figure 2D–F).

Figure 3A shows enhanced proximal convoluted tubule cross-sectional area (PCT-CSA) in MI male mice compared with their controls and female mice (P <0.05). Females did not show a significant increase with MI; however, with OVX there was a significant increase in PCT-CSA in FMIOVX mice compared with the FMI group (P <0.05) (Figure 3B).

OVX eliminated the attenuated increase in inflammatory IL-1 β with MI but did not alter caspase-3 or anti-inflammatory IL-4 levels

We assessed the effect post-MI of OVX on differences between males and females in pro- and anti-inflammatory cytokines and caspase-3, which has a role in apoptosis by both the extrinsic and intrinsic pathways. Figure 4A–C reveals increased IL-1 β , uncleaved caspase-3, and IL-4 protein levels in kidneys of male mice with MI compared with controls (P <0.05). Female mice, however, show no changes in uncleaved caspase-3 protein levels in the kidneys post-MI. Female mice showed an increase with MI in IL-1 β and IL-4 protein levels, but this did not reach significance. As for the impact of OVX, Figure 4D shows a significant increase in IL-1 β protein levels in the kidneys of FMIOVX mice compared with the FMI group (P <0.05), whereas no significant change in caspase-3 nor IL-4 protein levels in the kidneys of FMIOVX mice compared with the FMI group was observed (Figure 4E,F). The effect of MI and OVX on systemic inflammation was assessed in terms of plasma TNF- α levels, which were under the detection limit (<5 pg/ml) in all groups.

OVX had mixed effects on female-related increases in NAD⁺ generating enzymes with MI but did not protect NAD levels

We next assessed the effect of OVX on sex-based differences in NAD⁺ biosynthetic enzymes of the kidney after MI. NAD⁺ is a major coenzyme for glycolysis and mitochondrial oxidative phosphorylation [26]. Furthermore, NAD⁺ is an essential substrate of crucial enzymes involved in regulating metabolism, cellular repair, and maintaining cellular

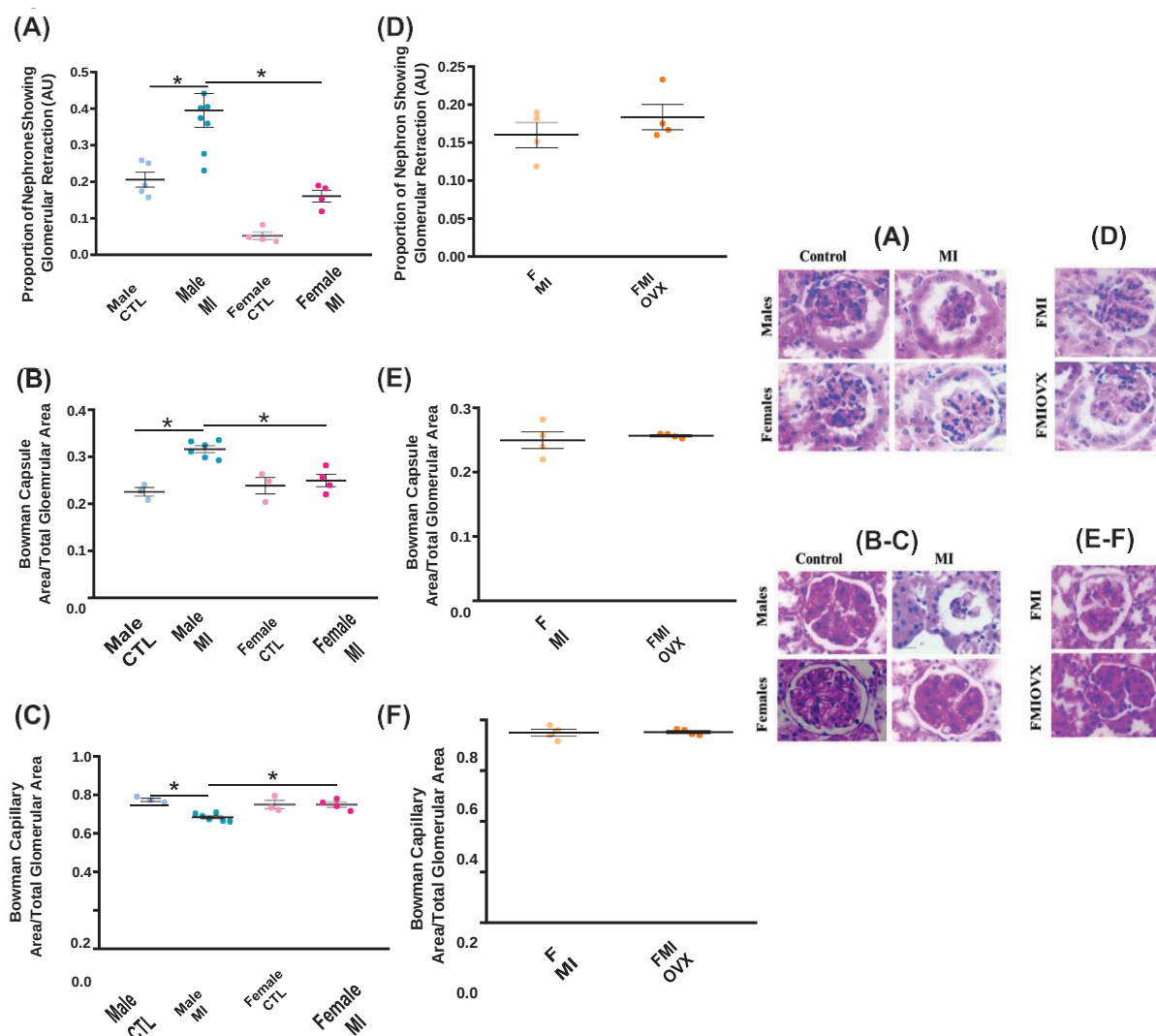


Figure 2. Glomerular alterations in MMI, FMI, and FMIOVX mice

Glomerular retraction and the Bowman's capsule area markedly increased in MI males, but not in MI females (A,B). Glomerular capillary area decreased in MI male mice but not MI females (C). There was no change in glomerular retraction, Bowman's capsule area, and glomerular capillary in FMIOVX mice compared with FMI mice (D–F). Representative image of each stained group is shown. Data were analyzed for MMI and FMI using two-way ANOVA, and for FMI and FMIOVX by unpaired *t*-test. All bars represent mean $\pm$ SEM (* $P < 0.05$, $n = 3–7$).

homeostasis [27]. Figure 5A,B show a significant increase in NAMPT and NMRK-1 mRNA expression levels in the kidneys of MI female mice compared with their controls ($P < 0.05$). Male mice, however, showed no change in renal NAMPT and NMRK-1 mRNA expression levels post-MI ($P < 0.05$). A significant difference between male and female MI mice was observed. Figure 5C shows a comparable decrease in total NAD levels in the kidneys of mice of both sexes after MI ($P < 0.05$). As for the impact of OVX, Figure 5D shows a significant decrease in NAMPT mRNA expression levels in the kidneys of FMIOVX mice compared with the FMI group ($P < 0.05$). In contrast, NMRK-1 mRNA expression levels markedly increased in the kidneys of FMIOVX mice compared with the FMI group ($P < 0.05$) (Figure 5E). However, Figure 5F shows no significant change in total NAD levels in the kidneys of FMIOVX mice compared with the FMI group.

PARP-1 can lead to NAD depletion. Figure 6A shows a marked increase in PARP-1 mRNA expression levels in kidneys of males with MI ($P < 0.05$). Female mice, however, show no change in PARP-1 mRNA expression levels in the kidneys post-MI. There was no significant change in PARP-1 mRNA expression levels in the kidneys of FMIOVX mice (Figure 6B).

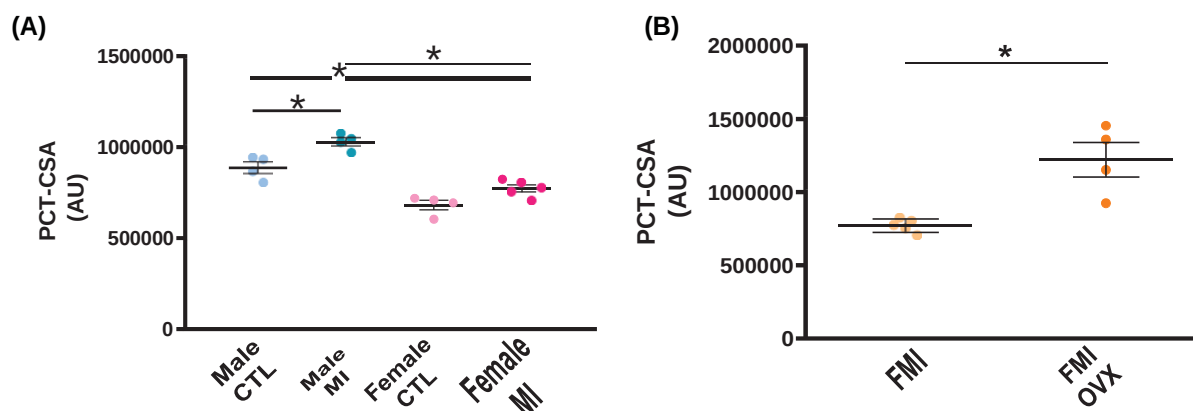


Figure 3. PCT-CSA comparison among MMI, FMI, and FMIOVX mice

PCT-CSA significantly increased with MI in male mice compared with females (A). PCT-CSA substantially increased in FMIOVX mice compared with the FMI group (B). Data were analyzed for MMI and FMI using two-way ANOVA, and for FMI and FMIOVX by unpaired *t*-test. All bars represent mean ± SEM (**P* < 0.05, *n* = 3–7).

Females have lower kidney fibrosis after MI regardless of OVX

The α -SMA protein is a marker of myofibroblasts and fibrosis. Figure 7A shows a significant increase in α -SMA protein levels in the kidneys of male mice following MI (*P* < 0.05). Female mice, however, had no significant change in α -SMA protein levels in the kidneys post-MI. A significant difference between MI males and MI females was observed. As for the impact of OVX, Figure 7B shows no significant change in α -SMA protein levels in the kidneys of FMIOVX mice compared with the FMI group. Our data were further supported by IF showing an accumulation of α -SMA in the peritubular space of the kidneys of MI male mice only (Supplementary Figure S2).

Figure 8A shows a significant increase in total renal fibrosis in male mice with MI compared with their control group and female MI mice (*P* < 0.05). No significant difference between control male and female mice was observed. As for the impact of OVX, Figure 8B shows a modest increase in total renal fibrosis in FMIOVX mice compared with the FMI group (*P* < 0.05).

Ovariectomy exacerbates kidney damage following MI

Figure 9A shows a comparable decrease in creatinine clearance (CrCl) in both sexes post-MI (*P* < 0.05). Whereas as shown in Figure 9B, there was a marked decrease in urine output in male mice post-MI (*P* < 0.05), there was no significant change in urine output in MI female mice compared with their controls. No significant difference in CrCl and urine output between control male and female mice was observed. Figure 9C reveals no significant change in urine protein concentration in either sex post-MI, although a significant difference in urine protein concentration was observed between male and female mice regardless of MI (*P* < 0.05). As for the impact of OVX, Figure 9D,E shows a marked decrease in CrCl and urine output in FMIOVX mice compared with the FMI group (*P* < 0.05). No significant change in protein concentration of urine was observed in FMIOVX mice compared with the FMI group (Figure 9F).

Discussion

Accumulating experimental and clinical studies report a positive correlation between acute cardiac injury and kidney disease development and progression [28–30]. In the present study, we affirmed that male mice are in general more susceptible to alterations in renal function and structure following MI than age-matched premenopausal female mice (Table 3). Comparable sex differences in some of these parameters were reported previously in a study examining the consequences of tobacco smoking [31]. Here, we extend those findings and address the potential contribution of estrogen to this protection by performing ovariectomy (OVX). Notably, a number of parameters related to kidney structure and function were not affected by ovariectomy. These include changes strongly observed in males with MI, such as increased glomerular retraction and Bowman's capsule area, decreased glomerular capillary area, and increased renal IL-4, PARP-1, and α -SMA expression. Total NAD was depressed by MI in both sexes and was not further

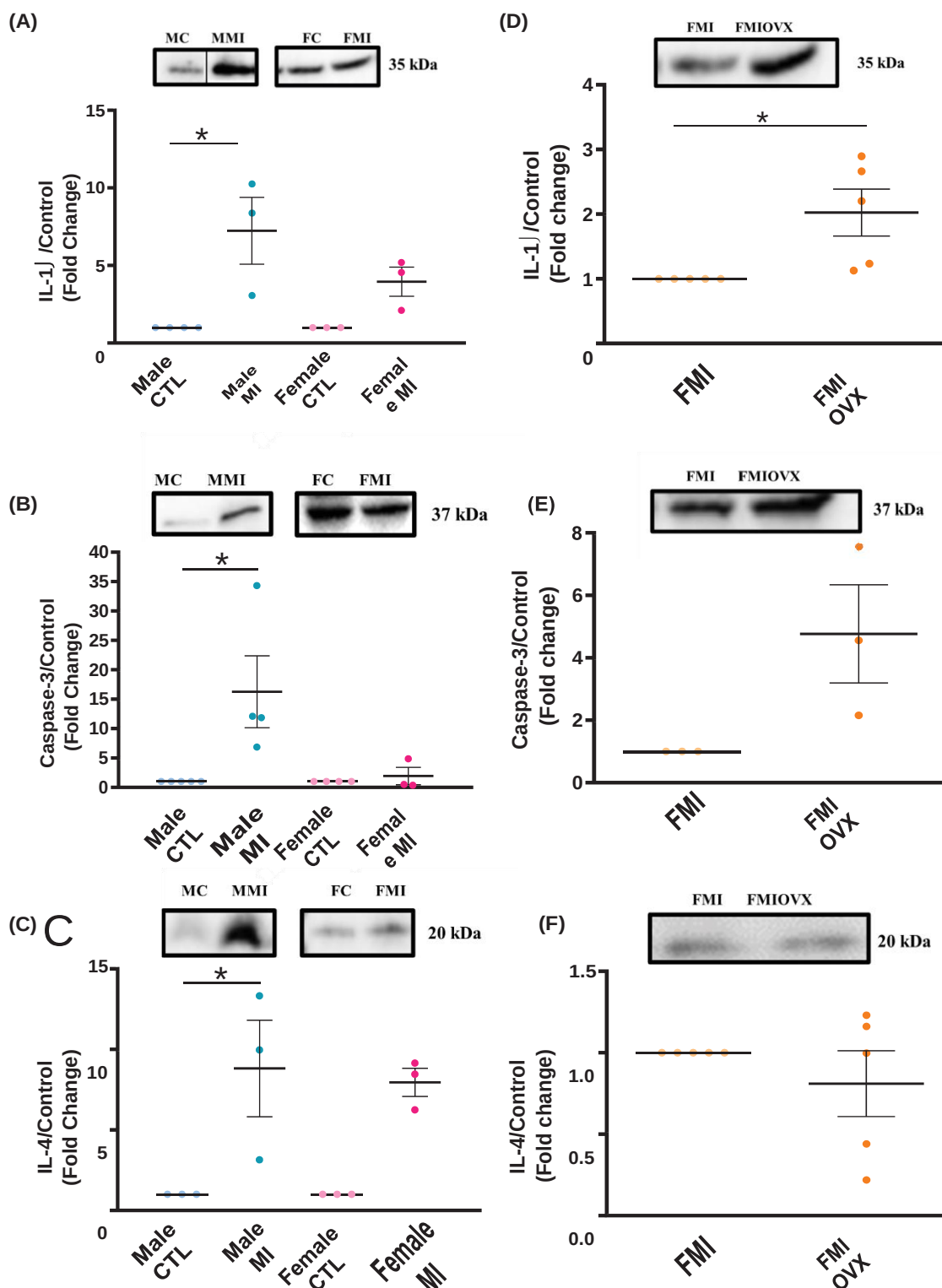


Figure 4. Renal cytokine and caspase-3 comparisons in MMI, FMI, and FMIOVX mice

IL-1 β protein levels significantly increased in the kidneys of MI male mice compared with their controls (A). Uncleaved caspase-3 markedly increased in the kidneys of MI male mice but not in females (B). IL-4 protein levels substantially increased in the kidneys of MI male mice compared with their controls groups (C). IL-1 β but not caspase-3 and IL-4 substantially increased in FMIOVX mice compared with the FMI group (D–F). Representative image of each blot is shown. IL-1 β and caspase-3 protein levels were normalized to total protein (Supplementary Figure S1). Data were analyzed for MMI and FMI using two-way ANOVA, and for FMI and FMIOVX using unpaired *t*-test. All bars represent mean $\pm$ SEM (**P* < 0.05, *n* = 3–5).

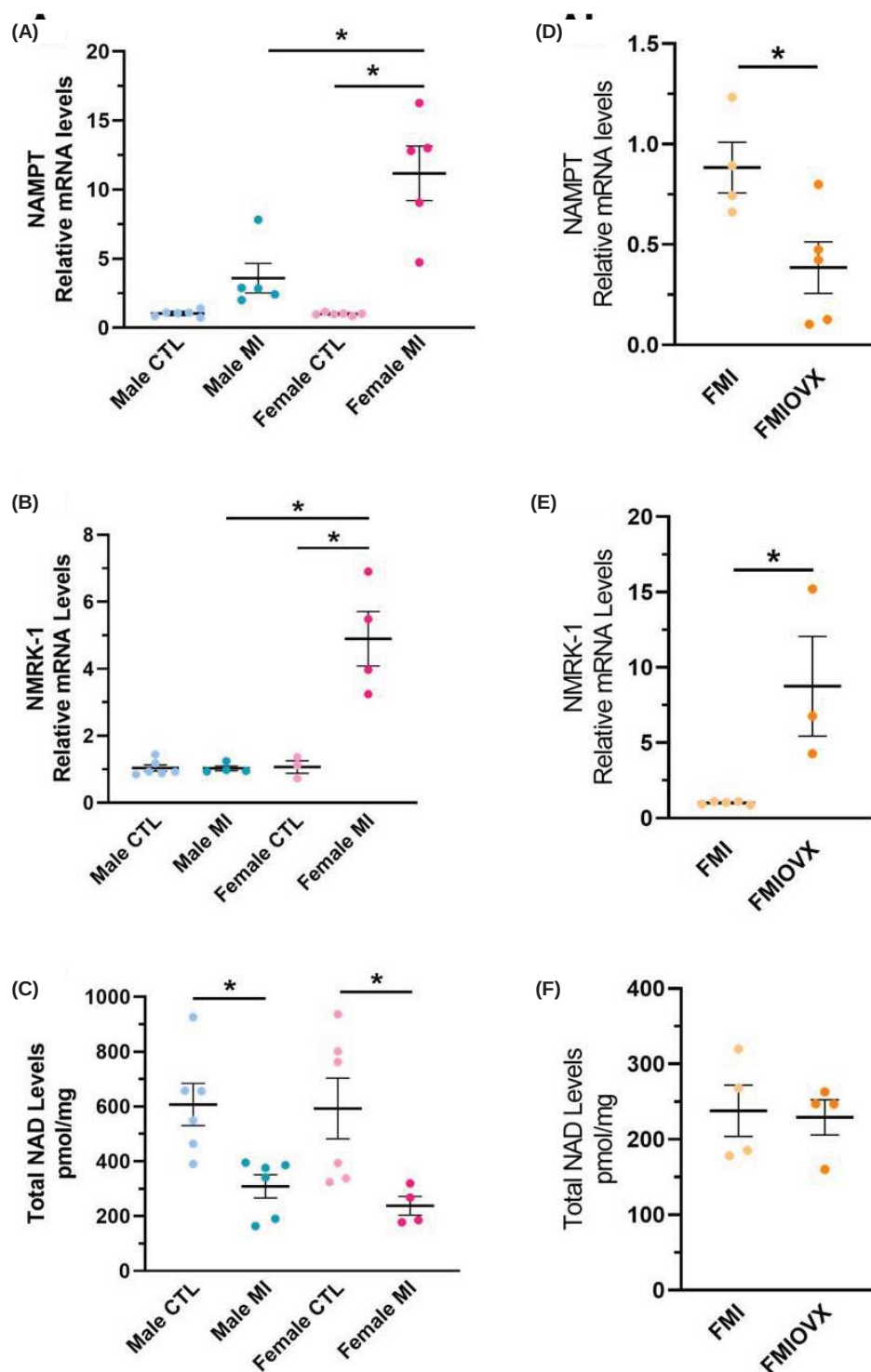


Figure 5. Comparison of NAD⁺ biosynthetic enzymes and total renal NAD levels between MMI and FMI mice, and between FMI and FMIOVX

NAMPT and NMRK-1 mRNA expression levels significantly increased in the kidneys of MI female mice compared with the relative male group (A,B). Total renal NAD levels decreased in both sexes with MI (C). NAMPT mRNA expression levels decreased in the kidneys of FMIOVX mice compared with the FMI group (D), whereas NMRK-1 mRNA expression levels significantly increased in the kidneys of FMIOVX mice compared with the FMI group (E). No significant change in total NAD levels in the kidneys of FMIOVX mice compared with the FMI group was observed (F). Data were analyzed for MMI and FMI using two-way ANOVA, and for FMI and FMIOVX using unpaired *t*-test. All bars represent mean $\pm$ SEM (**P*<0.05).

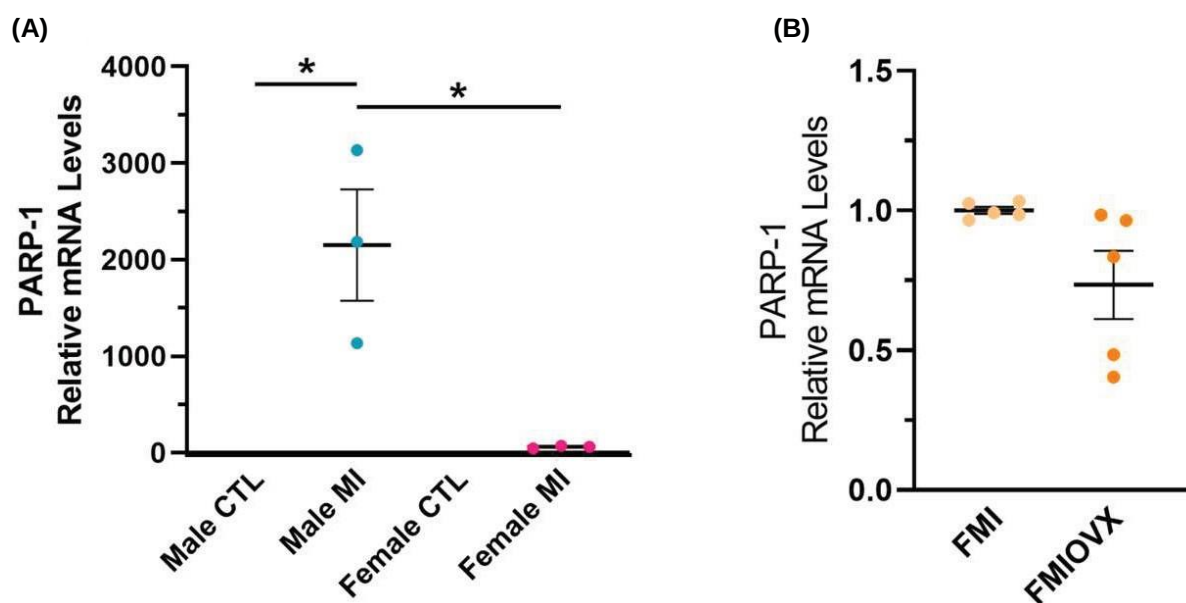


Figure 6. The effects of MI and OVX on PARP-1 in the kidney

PARP-1 mRNA expression levels significantly increased in the kidneys of male mice with MI compared with females (A). No significant increase in PARP-1 mRNA expression levels was observed in the kidneys of FMIOVX mice compared with the FMI group (B). Data were analyzed for MMI and FMI using two-way ANOVA, and for FMI and FMIOVX using unpaired *t*-test. All bars represent mean $\pm$ SEM (**P* < 0.05).

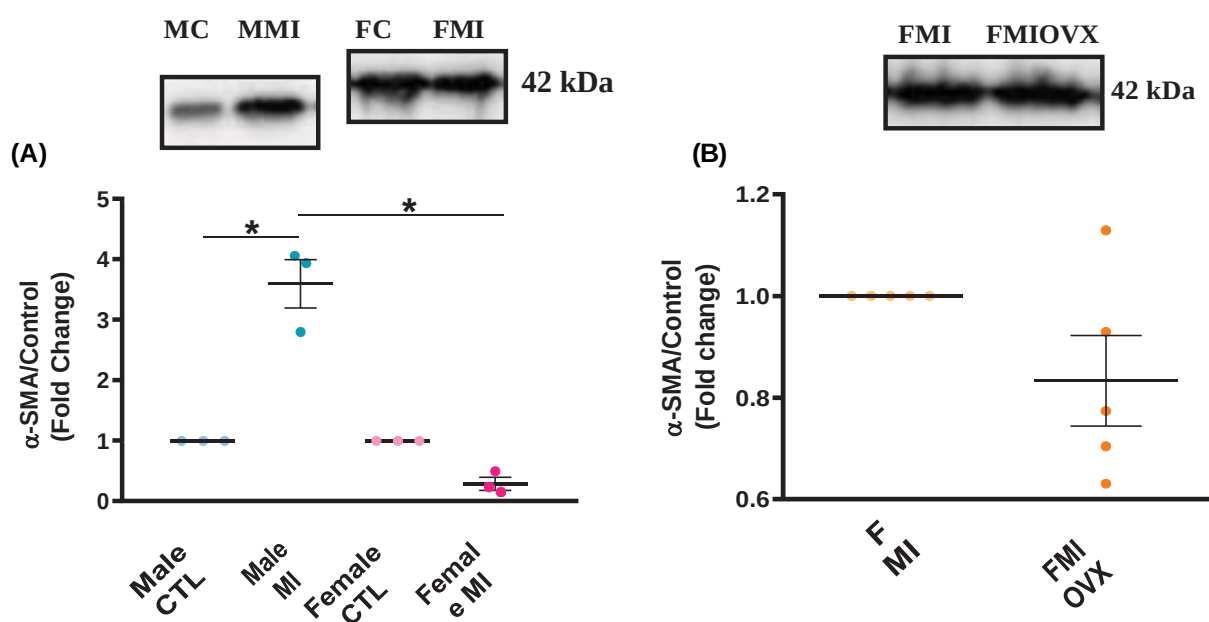


Figure 7. The effects of MI and OVX on α -SMA protein levels

α -SMA protein significantly increased in the kidneys of MI male unlike females (A). No significant change in α -SMA protein levels in the kidneys of FMIOVX mice compared with the FMI group was observed (B). α -SMA protein levels were normalized to total protein (Supplementary Figure S3). Data were analyzed for MMI and FMI using two-way ANOVA, and for FMI and FMIOVX using unpaired *t*-test. All bars represent mean $\pm$ SEM (**P* < 0.05, *n* = 3–4).

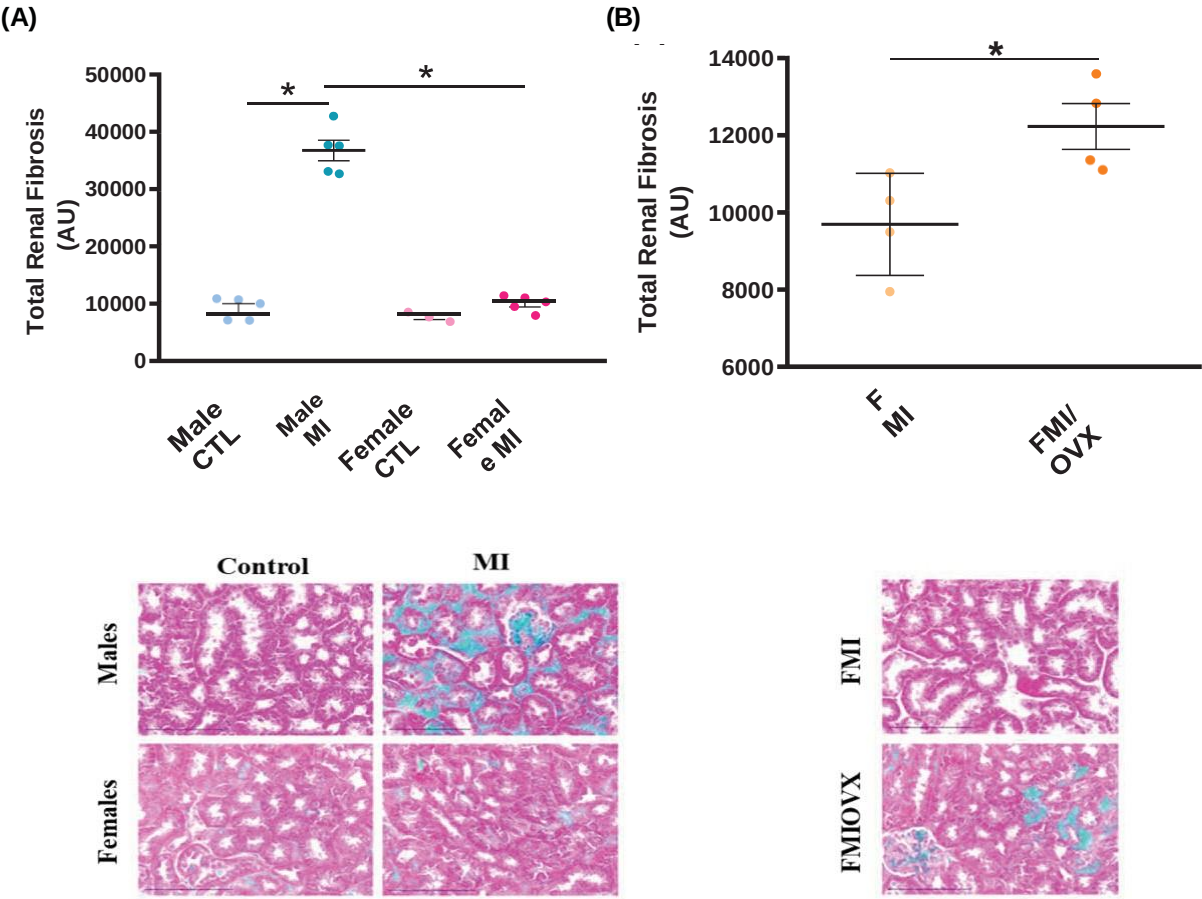


Figure 8. **The effects of MI and OVX on total renal fibrosis**

Total renal fibrosis significantly increased in male mice with MI compared with females (A). Total renal fibrosis increased in FMIOVX mice compared with the FMI group (B). Representative image of each MTC stained group is shown. Data were analyzed for MMI and FMI using two-way ANOVA, and for FMI and FMIOVX using unpaired *t*-test. All bars represent mean $\pm$ SEM ($*P < 0.05$, $n = 3-7$).

Table 3 **Key findings**

Parameter	MMI	FMI	FMIOVX vs. FMI
Glomerular retraction	↑↑	~ ↑	nc
Bowman's capsule area	↑	nc	nc
Glomerular capillary area	↓	nc	nc
PCT-CSA	↑	nc	↑
IL-1β	↑↑	~ ↑	↑
Caspase-3	↑↑	nc	~ ↑
IL-4	↑↑	~ ↑	nc
NAMPT	~ ↑	↑↑	↓
NMRK-1	nc	↑↑	↑↑
Total NAD	↓↓	↓↓	nc
PARP-1	↑↑	nc	~ ↓
α-SMA	↑↑	nc	nc
Renal fibrosis	↑↑	nc	↑
Creatinine clearance	↓↓	↓↓	↓↓
Urine output	↓	nc	↓
Urine protein	nc	nc	~ ↓

Abbreviations: ~, trend; nc, no change; PCT-CSA, proximal convoluted tubule cross-sectional area.

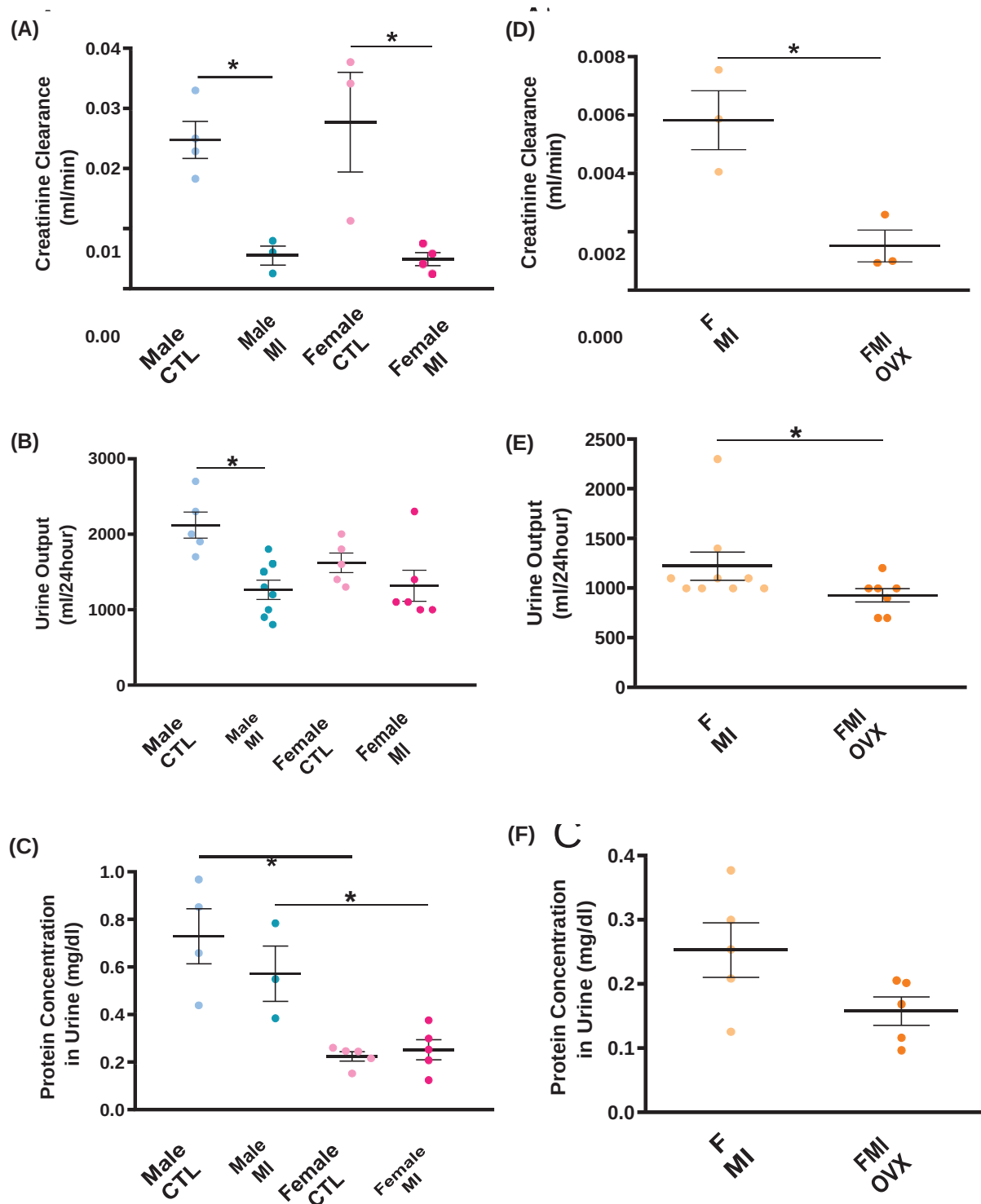


Figure 9. Effects of MI and OVX on CrCl, urine output, and urine protein concentration

CrCl was decreased with MI mice in both sexes (A). Urine output was markedly decreased in MI male mice compared with controls (B). No change in urine protein concentration with MI mice of both sexes was observed (C). CrCl and urine output decreased in FMIOVX mice compared with the FMI group (D,E), whereas no change was observed in urine protein concentration in FMIVX mice compared with the FMI group (F). Data were analyzed for MMI and FMI using two-way ANOVA, and for FMI and FMIOVX using unpaired *t*-test. All bars represent mean $\pm$ SEM (* $P < 0.05$, $n = 3-9$).

affected by OVX. However, levels of NAD generating enzymes NAMPT and NMRK-1 were increased in females with MI (but not males), and OVX attenuated and enhanced these changes, respectively. A good number of parameters were indeed changed in a more male-like direction by OVX, including increased PCT-CSA, increased renal IL-1 β , caspase-3, and fibrosis, and decreased creatinine clearance, and urine output. OVX also tended to decrease urine protein, which otherwise was not seen in females or in males. These findings suggest that while estrogen provides some protection to the kidney after MI, other factors come into play as well, such as pre-existing structural differences. In addition, a further decrease in EF after MI with OVX may negatively impact on renal function and structure. In this regard, Cavašin et al. also reported that OVX decreased EF in mice post-MI, leading to worsen cardiac function and aggravated LV adverse remodeling [32]. The further decrease in EF seen with OVX might be attributed to the harmful effects of increased sympathetic drive on the injured heart in female mice [33–35]. Moreover, increased sympathetic drive may have had direct harmful effects on the kidney, so that any harmful effects of OVX may reflect an indirect effect of estrogen loss.

Hemodynamic analysis showed a marked decrease in EF, indicating cardiac dysfunction and enhanced adverse LV remodeling, 7 days post-MI occurring equally in both sexes. The absence of systemic inflammation as observed with undetectable plasma TNF- α levels in mice of both genders, not only correlates with previous studies [36,37] but also rules out any potential correlation between kidney damage and persistent systemic inflammation at 7 days post-MI. With the absence of any pronounced or evident differences between male and premenopausal female mice in systemic hemodynamics and inflammation at 7 days post-MI, the observed sexual dimorphism in MI-induced kidney damage was tracked down to kidney molecular, metabolic, structural, and functional levels.

Our data indicated pronounced renal structural alterations in MI mice of both sexes but to a greater extent in males. For instance, glomerular retraction, glomerular capillary area, Bowman's capsule area, PCT-CSA, and renal fibrosis significantly increased in MI male mice compared with their female counterparts. These outcomes are in accordance with non-sex-based studies. For instance, it has been reported that PCT-CSA was markedly enhanced in male mice 7 days following MI [38]. Additionally, Sasak et al. showed that changed glomerular capillary area to the Bowman's capsule area is tightly linked to the initial phase of glomerular injury [39]. In agreement, Munshi et al. indicated that glomerular retraction is the histological hallmark of ischemic glomerular damage [40]. Similarly, Lekawanvijit et al. demonstrated that male Sprague–Dawley rats developed renal interstitial fibrosis post-MI, resulting therefore in enhanced kidney dysfunction [41].

The proinflammatory cytokine IL-1 β and proapoptotic marker caspase-3 significantly increased in MI male mice, while remaining unchanged in female mice post-MI. Of note, we did not see an increase in cleaved caspase-3 in MI male mice probably because of timing (data not shown); we postulate that the increase in uncleaved caspase 3 represents a compensatory response. Moreover, increased caspase 3 and IL-1 β may reflect aspects of pyroptosis-related inflammasome activity in male MI mice [42].

IL-1 β has been implicated in exacerbated kidney pathology [43,44]. Additionally, it has been shown that caspase-3, an executioner caspase implicated in DNA fragmentation, induces kidney apoptosis and fibrosis [45]. A marked increase in IL-4 was observed in MI male mice, only. The role of IL-4 in the setting of kidney diseases including AKI is still controversial. For instance, it has been demonstrated that IL-4 stimulates the polarization of the proinflammatory M1 phenotype into the anti-inflammatory M2 phenotype, thereby decreasing tubular injury [46]. In contrast, a study performed by Kim et al. indicated that overexpression of IL-4 enhanced glomerular injury and kidney damage [47].

Inflammation is known to play a central role in the development of fibrosis and cell death, key elements contributing to kidney disease development [48,49]. Unlike MI females, α -SMA significantly increased in MI male mice. Multiple studies have reported that α -SMA is the hallmark of extracellular matrix deposition, resulting in enhanced fibrosis [50,51].

NAD⁺ salvage biosynthetic, dependent, consuming enzymes, and total NAD levels were assessed as markers of potential mitochondrial dysfunction and necrotic cell death. Both NAMPT and NMRK-1 levels, NAD⁺ biosynthetic salvage enzymes, substantially increased in MI female mice compared with the male counterparts. NAMPT and NMRK-1 are involved in converting nicotinamide (NAM) and nicotinamide riboside (NR) respectively, to nicotinamide mononucleotide (NMN) and subsequently NAD⁺ [52,53]. Unlike in females, PARP-1, an NAD⁺ consuming enzyme, markedly increased in male mice with MI. It has been reported that overactivation of PARP-1 leads to NAD⁺ depletion, subsequently enhanced energy loss and cell death [54]. Total renal NAD levels, however, decreased in MI mice of both sexes to the same extent. Previous evidence reported a positive correlation between altered NAD⁺ biosynthetic and dependent enzymes and exacerbated NAD⁺ depletion [55–57]. For instance, it has been demonstrated that enhanced NAMPT levels, the main NAD⁺ biosynthetic enzyme, results in increased NAD⁺ levels and enhanced cellular function [56,58]. Thus, a more extensive time course is needed to establish whether NAD levels recovered (more so) in female versus male mice after MI.

Our findings indicate enhanced renal structural alterations including increased PCT-CSA and interstitial fibrosis with MI following OVX. Notably, there were no changes with OVX in the lack of an effect in females with MI on glomerular retraction, glomerular capillary area, and Bowman's capsule area. Multiple studies are in accordance with our outcomes [22,59,60]. NAMPT markedly decreased in MI female mice following OVX, whereas NMRK-1 substantially increased, although total NAD levels were still depressed. Annie et al. reported that administration of estrogen to ovariectomized mice led to a boost in NAMPT level in mouse uterus [61].

The observed MI-induced structural and molecular alterations in the kidneys may contribute to deterioration in kidney function. Both sexes experienced a comparable decrease in CrCl 7 days post-MI, while urine output markedly decreased in MI male mice only. A pronounced decline in CrCl and urine output was also seen in MI female mice following OVX. CrCl reflects glomerular filtration rate (GFR) [62,63], indicating a potential decrease in GFR. Decreased urine output is a key determinant of the severity of AKI [64]. Proteinuria was also measured, showing no significant change in both sexes post-MI, a finding consistent with a previous study reporting that proteinuria is a pathogenic mechanism of chronic kidney disease and end-stage renal failure [65]. Our findings would indicate an early stage of decreased GFR without a loss of glomerular barrier function. Of note, a number of studies have demonstrated that CKD may develop in patients without proteinuria [66]. A limitation of our study is that we only used functional and damage biomarkers to assess AKI. Future studies will be done to measure novel serum and urinary proteins that have been identified as biomarkers of AKI.

Conclusion and future direction

The present study shows both sexes are prone to develop kidney damage post-MI, with premenopausal female mice being more protected against certain aspects of MI-induced kidney injury at least during the first 7 days post-MI. OVX worsens kidney dysfunction in female mice following MI, indicating a renal protective effect of ovarian estrogen. Exploration of the factors that might contribute to AKI with MI need to be better defined, such as those released from the injured heart (e.g., exosomes), activated immune cells, and stimulation of the sympathetic and renin–angiotensin–aldosterone systems. Hypoperfusion with oxygen deprivation and build-up of metabolic waste may play a role as well. Future experiments aimed at highlighting the effect of an aromatase inhibitor on MI-induced kidney damage in the FMIOVX group to decrease extra-gonadal estrogen synthesis is warranted. Additionally, characterizing the role of testosterone in the observed kidney injury in male mice post-MI by castration is needed.

Clinical perspectives

- MI is associated with elevated risk of AKI, through the cardiorenal interrelationship. Preclinical investigations and clinical studies showed that females are less prone to develop AKI following CVDs.
- Using a mouse model of MI-induced AKI, our data show that both male and premenopausal female mice experienced kidney deterioration at molecular, structural, and functional levels, with males being more affected. Ovariectomy negatively altered the observed kidney protection in females post-MI.
- The identification of sex-dependent molecular pathways involved in MI-mediated AKI renders promising targets to attenuate kidney injury post-MI.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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CRedit Author Contribution

Nada J. Habeichi: Conceptualization, Resources, Data curation, Software, Formal analysis, Supervision, Validation, Investigation, Visualization, Methodology, Writing—original draft, Project administration, Writing—review & editing. **Rana Ghali:** Conceptualization, Data curation, Software, Formal analysis, Supervision, Validation, Methodology, Project administration. **Ali Mroueh:** Data curation, Software, Supervision, Validation, Investigation, Methodology. **Abdullah Kaplan:** Conceptualization, Formal analysis, Validation, Visualization, Methodology. **Cynthia Tannous:** Data curation, Formal analysis, Validation. **Abdo Jurjus:** Formal analysis, Methodology. **Ghadir Amin:** Data curation, Supervision, Writing—review & editing. **Mathias Mericskay:** Conceptualization, Software, Formal analysis, Supervision, Validation, Visualization, Project administration, Writing—review & editing. **George W. Booz:** Conceptualization, Formal analysis, Validation, Visualization, Writing—original draft, Project administration, Writing—review & editing. **Ahmed El-Yazbi:** Conceptualization, Resources, Data curation, Software, Formal analysis, Supervision, Validation, Methodology, Writing—original draft, Project administration. **Fouad A. Zouein:** Conceptualization, Supervision, Funding acquisition, Methodology, Project administration, Writing—review & editing.

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Abbreviations

CrCl, creatinine clearance; CRS, cardiorenal syndrome; CVD, cardiovascular disease; EF, ejection fraction; GFR, glomerular filtration rate; MI, myocardial infarction; NAMPT, nicotinamide phosphoribosyltransferase; NMRK1, nicotinamide riboside kinase-1; OVX, ovariectomy; PARP-1, poly (ADP-ribose) polymerase 1; PCT-CSA, proximal convoluted tubule cross-sectional area.

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6.3 NICOTINAMIDE RIBOSIDE: A PROMISING THERAPY FOR MI-INDUCED ACUTE KIDNEY INJURY

Nicotinamide adenine dinucleotide (NAD) is the central coenzyme in boosting oxidation and oxidative phosphorylation. The cellular levels of NAD are a crucial regulator of metabolism and bioenergetics that link cellular energy status to a wide variety of processes which regulate cell survival. In the setting of myocardial infarction (MI), NAD level is decreased and cellular hypertrophy occurs in response to increased burden of stress and needs. Nicotinamide-Riboside-Kinase2 (NMRK-2) is an enzyme highly expressed in cardiac tissues and involved in NAD salvage pathway. Based on preliminary evidence NMRK-2 which requires an exogenous B3 vitamin (nicotinamide riboside (NR)) supplementation to synthesize NAD, is highly upregulated following MI. Acute kidney injury (AKI) is a main complication of MI, increasing morbidity and mortality rate substantially, through the cardiorenal syndrome, otherwise known as type I cardiorenal syndrome (CRS). We aim in this study at assessing the impact of NR supplementation, a substrate for NMRK-2 in the heart and NMRK-1 in the kidneys, on MI- induced AKI in order to reveal if NR treatment can protect against type I CRS either indirectly through enhancing cardiac function and/or directly through its impact on the kidneys. MI was induced by the left anterior descending coronary artery ligation in 2 months old male mice and NR (400mg/kg/day) was administrated to mice either for 4 or 7 days. Our findings reveal no significant impact of NR on cardiac function at day 4 and 7 post-MI, whereas NR enhances kidney function, decreases kidney metrological alteration and cell death. The protective effect of NR on MI-induced AKI seems to be mediated by increased kidney NAD levels through upregulating nicotinamide phosphoribosyltransferase mRNA expression levels. This work has been initiated at Dr. Zouein's lab at the American University of Beirut and continued at Dr. Meriskay' Lab at Paris Saclay University.

Nicotinamide riboside: A promising therapy for MI-induced acute kidney injury by upregulating nicotinamide phosphoribosyltransferase-mediated increased NAD levels

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Abstract

Acute kidney injury (AKI) is one of the most common complications of myocardial infarction (MI), and is considered an independent risk factor increasing substantially short- and long-term mortality. Nicotinamide dinucleotide (NAD^+) is an important coenzyme in oxidative phosphorylation and a substrate for multiple NAD^+ -dependent enzymes such as Sirtuins and poly-ADP ribose polymerases, which are involved in mitochondrial biogenesis and DNA repair following cardiac and kidney injury. Decreased cardiac NAD levels along with an upregulation in nicotinamide riboside kinase 2, an NAD^+ biosynthetic enzyme that uses nicotinamide ribose (NR) to generate NAD^+ , has been reported following MI. Therefore, we hypothesized that NR treatment could be a potential promising therapy for MI-induced AKI. In this study, MI was induced by ligating the left anterior descending coronary artery in 2 months old C57BL6 mice, following by the administration of NR (IP injection, 400mg/kg/day) for four and seven days. Our findings show no significant improvement in cardiac ejection fraction following NR treatment at day 4 and 7 post-MI, whereas the administration of NR significantly enhanced kidney function and decreased kidney morphological alterations and cell death. The observed renal protection seems to be mediated by an up-regulation of nicotinamide phosphoribosyltransferase-mediated increase in renal NAD levels, notably in distal tubules and a global reduction in cell death. In conclusion, our findings indicate that NR could be a potential promising therapy for AKI following an early stage of MI.

Introduction

Overwhelming epidemiological evidence links cardiovascular diseases (CVDs) to acute kidney injury (AKI) (Palazzuoli and Ruocco 2018). According to the World Health Organization (WHO), ischemic heart diseases, including myocardial infarction (MI) and stroke, are responsible for 31% of CVD deaths annually (http://www.who.int/cardiovascular_diseases/en/). MI is a major public health concern and a leading cause of CVD-induced AKI through the cardiorenal interrelationship, otherwise known as type I cardiorenal syndrome (CRS) (Cai, Xiao et al. 2022). Clinical studies demonstrated that between 7.1-29.3% of hospitalized MI patients developed AKI (Hwang, Jeong et al. 2011, Shacham, Leshem-Rubinow et al. 2014). Furthermore, It has been reported that MI patients are not only at a 30-fold increased risk of heart failure progression and development, but also up to 29-fold increased risk of CRS, increasing significantly short- and long-term mortality rate (Smith, Lichtman et al. 2006, Heywood, Fonarow et al. 2007). In this regard, it has been documented that 2.31% of patients diagnosed with MI undergo hemodialysis during hospitalization (Wang, Hong et al. 2022).

A growing body of evidence demonstrates that CVDs, mainly ischemic heart diseases are associated with a manipulation in cardiac energy metabolism characterized by a shift in energy production from oxidative phosphorylation (OXPHOS) of fatty acid, carbohydrates, amino acids and ketone bodies due to oxygen shortage to anaerobic glycolysis to spare the limited oxygen (Lee, Horowitz et al. 2004, Zuurbier, Bertrand et al. 2020). Nicotinamide adenine dinucleotide (NAD) is emerging as a metabolic target in different CVDs as a major coenzyme in fuel oxidation and OXPHOS, and as a signaling molecule in stress responses through its consumption as a substrate by Sirtuin deacetylases (SIRT-1 to 7) and poly(ADPribose) polymerases, notably PARP-1 (Mericskay 2016, Tannous, Booz et al. 2021). Altered NAD levels have been reported in several models of heart diseases including ischemia reperfusion. For instance, a study performed by Di Lisa et al. showed that 30 min of cardiac ischemia resulted in a marked decrease in mitochondrial and tissue NAD⁺ levels in male wistar rats (Di Lisa, Menabo et al. 2001). However, little is known about NAD homeostasis in the context of type I CRS post-MI. In that regard, our published work showed a significant decrease in kidney NAD levels in C57BL6/J male and female mice at day 7post-MI. Of note, decreased NAD levels was exacerbated in the presence of risk factors such as cigarette smoking (Habeichi, Mroueh et al. 2020). Additionally, a marked downregulation in kidney SIRT-1 and SIRT-3 mRNA expression

levels along with a substantial upregulation in PARP-1 mRNA expression levels were observed at day 7 post-MI, suggesting the urgent need of metabolic drugs that can boost NAD levels and correct the observed alteration in NAD signaling pathways (Habeichi, Ghali et al. 2023) .

To date, there are no metabolic drugs that can target type I CRS or limit its development or progression. Nicotinamide riboside (NR), the nucleoside form of the vitamin B3, has recently come into focus as an effective NAD precursor with superior bioavailability (Trammell, Schmidt et al. 2016). A study performed by Diguët et al. reported a significant decrease in cardiac NAD⁺ levels in a mouse models of dilated cardiomyopathy and transverse aorta constriction (Diguët, Trammell et al. 2018). NR supplementation in food (450 mg/kg/day) attenuated the development and progression of heart failure by rescuing myocardial NAD⁺ levels (Diguët, Trammell et al. 2018). To date, the potential impact of NR on AKI in the setting of MI is not known. Here we provide for the first time experimental evidence on the protective impact of NR on AKI at early stage post-MI.

Materials and Methods

Experimental design. Two months old C57BL6/J male mice were used in this study according to the experimental protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the American University of Beirut (AUB) and in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, 8th edition (Council 2010). The approved IACUC protocol number was #18-2-RN560. Mice were randomly divided into 2 groups: SHAM and MI. MI was induced morning by ligation of the left anterior descending coronary artery and the mice sacrificed morning 4 and 7 days thereafter. Heparin 100 µl (heparin sodium 1,000 I.U/ml) was administrated 30 minutes prior to sacrifice and 4% isoflurane was used to induce anesthesia. Blood was collected using cardiac puncture, and plasma separated by centrifugation at 2200 rpm for 10 minutes, mixed with protease inhibitor cocktail, and flash-frozen in liquid nitrogen and stored at -80°C. Afterward, mice were subjected to cervical dislocation. Upon sacrifice, kidneys were collected. The left kidney was immediately placed into a cryotube in liquid nitrogen followed by storage at -80°C for molecular analysis. The right kidney was placed in 4% zinc formalin tubes for histological analysis.

Myocardial infarction (MI). The MI model was established by permanent ligation of the left anterior descending coronary artery. Briefly, an intraperitoneal injection of Tramadol

(50 mg/kg) was given to mice for analgesia 15 minutes prior to surgery. Mice were anesthetized with 2%-3% isoflurane inhalation. After securing the mice on a heating pad, anesthesia was maintained by mask inhalation of isoflurane 2%-3%, and the temperature was monitored with a rectal thermostat. Mice were intubated orotracheally and ventilated with a MiniVent ventilator (HSE Harvard, Germany). An incision was made between the left third and fourth intercostal space. The pericardium was cut open, the LV exposed, and the LAD artery ligated with a 7-0 prolene suture (Ethicon, Norderstedt, Germany) 2 to 3 mm below left atria. Instant blanching of the LV area below the ligation site and the ST-segment elevation on the echocardiogram indicated successful ligation. The same procedure was performed for sham-operated mice, but without LAD ligation. Ribs, muscles, and skin were closed in layers using 6-0 prolene sutures (Ethicon, Norderstedt, Germany). The mouse was then weaned from isoflurane anesthesia, flushed with oxygen, extubated, and allowed to warm under an infrared lamp until full consciousness.

NR administration. Phosphate buffered solution (PBS) vehicle or NR diluted in PBS solution was administrated at a dose of 400 mg/kg/day in a single intraperitoneal (IP) injection. IP injection was administrated to mice 30 min after the surgery as an immediate intervention and repeated every 24 hours until the day of sacrifice.

Echocardiography. Transthoracic echocardiography was performed using the Visual Sonics Echo system (Vevo 2100, VisualSonics, Inc., Toronto, Canada) equipped with a 22–55 MHz (MS550D) linear transducer according to the American Society of Echocardiography guidelines to assess cardiac function. Mice were anesthetized using 2% isoflurane and placed on a temperature-controlled platform with body temperature controlled at 37°C using rectal probe, in a supine position. After shaving the chest, the ultrasonic gel was applied above the thoracic area. Parasternal 2-dimensional M-mode and B-mode echocardiograms were obtained by directing the probe along the long axis of the LV. Measurements were performed at baseline, day 1 post-LAD ligation, and on the day of euthanasia (4 or 7 days). Heart rate was monitored and maintained constant (450–550 beats/min). At least three consecutive cardiac cycles were measured and averaged.

Periodic acid Schiff (PAS) staining. The PAS stain was applied on 4µm thick paraffin-embedded kidney sections to assess the changes in the area of the glomerular capillary,

Bowman's capsule, and proximal convoluted tubule. The microscopic slides were dewaxed and hydrated using xylene followed by alcohol of decreasing concentration (100, 95, and 70 %). Slides were incubated in 0.5% periodic acid solution for 5 minutes. After rinsing well in distilled water, kidney tissues were covered with Schiff's reagent for 15 minutes, and then washed for 5 minutes. Slides were mounted and observed under the light microscope at 40× magnification. Measurements were performed using the ImageJ software (<https://imagej.nih.gov/ij/>).

Massons' Trichrome (MTC) Staining. Masson's trichrome (MTC) staining was used to determine renal interstitial fibrosis and tubular casts. Dewaxing and rehydration steps were completed, and kidney tissues were treated with Bouin's fixative for 1 hour at 56 °C, washed and rinsed in distilled water. Tissues were stained with Weigert's Hematoxylin for 10 minutes. After washing, tissues were incubated with Biebrich scarlet-acid fuchsin for 10 minutes followed by application of phosphomolybdic-phosphotungstic acid solution. Afterward, sections were directly transferred to aniline blue solution and washed with 1% acetic acid. Tissues were observed under the light microscope at 10× magnification and interstitial fibrosis and tubular casts were measured using ImageJ software.

Immunohistochemistry (IHC) staining. IHC was performed to detect NAMPT protein expression levels in kidney tissues using EnVision FLEX Mini Kit High pH : K8023 (Dako). Slides were incubated with xylene for 10 min, then rinsed with alcohol of decreasing concentration (100% and 70%) and double distilled water. Afterward, slides were incubated with 1X retrieval buffer and washed with 1X PBS for 5 min. To decrease background, FLEX peroxidase blocking reagent (provided by the kit) was applied for 10 min at RT. Slides were then blocked with EnVision FLEX blocking reagent (provided by the kit) + triton-X100 (0.2%). NAMPT (ab2449;1:100) antibody was then diluted in FLEX blocking reagent (provided by the kit) +triton-X100 0.02% and applied on the slides overnight. Three times washing steps with 1X PBS were performed and the secondary antibody (EnVision FLEX HRP, anti-rabbit IgG,) was added to the slides and incubated at RT for 20 min. Washing steps with 1X PBS were again performed and one drop of DAB was added to 1ml EnVision FLEX substrate buffer. Slides were counterstained with hematoxylin for 1 min 30 sec and then rinsed with mineral water, alcohol of increasing concentration (70% and 100%), xylene. Mounting medium was added and slides were observed under the light microscope. Slides were scanned on the digital scanner NanoZoomer 2.0-RS (Hamamatsu))

RNA Extraction and RT-qPCR. Total RNA was isolated from kidney tissues using TriReagent (Thermo Fisher Scientific, Grand Island, NY, USA). Purity (A260/A280 ratio ≥ 1.8) and concentration were measured using the NanoDrop® ND-1000 UV-Vis Spectrophotometer. RNA was reverse transcribed into cDNA, according to the manufacturer's instructions (QuantiTect Reverse Transcription Kit, QIAGEN). mRNA levels of the target genes, nicotinamide phosphoribosyltransferase (NAMPT), nicotinamide riboside kinase-1 (NMRK1), and sirtuin (SIRT)-1 were quantified using SYBR Green (QuantiFast SYBR Green PCR Kit, QIAGEN, Germany) and analyzed in a CFX96 real-time PCR instrument (Bio-Rad, Germany). For each 10 μ l PCR mixture, 4 μ l cDNA was used with 0.9 μ l DNase free water, 5 μ l SYBR green, and 0.05 μ l of forward and reverse primers. Each reaction was performed in duplicate and the mRNA expression level was normalized against the level of YWHAZ in the same sample. The $\Delta\Delta$ CT method was used to analyze data. Results are expressed as fold changes relative to the mean of individual values obtained in the control Sham + vehicle group which is set to 1. Primer sequences used are listed in table 1.

List of primers used for RT-qPCR analysis

Primer	Forward	Reverse
YWHAZ	AGA CGG AAG GTG CTG AGA AA	GAA GCA TTG GGG ATC AAG AA
NAMPT	ACCAGCGGGGAACCTTTGTTA	ACATAACAACCCGGCCACAT
NMRK-1	CTTGAAGCTTGCTCTGCGAC	GTGTCGTCTCCCTCCGTTT
SIRT-1	CGGCTACCGAGGTCCATATAC	ACAATCTGCCACAGCGTCAT

TUNEL assay. Apoptosis levels were determined in situ with the use of Click-iT Plus TUNEL Assay (Invitrogen C10617). Tissue sections were deparaffinized and rehydrated, then permeabilized using proteinase K. TdT reaction was performed to incorporate dUTP modified with an alkyne group to the DNA fragments. Detection was achieved by a click reaction between the alkyne group and Alexa Fluor picolyl azide fluorescent dye. DAPI (4',6-diamidino-

2-phenylindole) was used to identify nuclei. Apoptosis was detected by co-localization of DAPI and TUNEL-red staining [after image](#) overlay using ZEISS Axio observer and was analyzed using ImageJ software.

Enzyme-linked immunosorbent assay (ELISA). Urinary cystatin-C and kidney injury molecule-1 (KIM-1) were assessed using ELISA kits ab#201280 and ab#213477, respectively. Urine samples were diluted 1:500 for cystatin-C and 1:16 for KIM-1 in the NS diluent provided. 50ul of all standard curve points or urine samples were added to 50 µl of antibody cocktail in the 96 wells plate to reach final volume of 100 µl. Plate was incubated on a plate shaker for 1 hour at RT. Three washing steps with 350 µl washing buffer 1X were performed, followed by the addition of 100 µl TMB and incubation on a plate shaker for 10 min at RT in the dark. 100 µl of stop solution was finally added and the plate was read at 450 nm. All samples were analyzed in duplicate.

NAD extraction and Quantification. Buffer composed of 75% ethanol and 25% HEPES 10 mM pH7.1 was used to extract kidney tissues (20 µl/mg tissue). Extracts were diluted (1:20) in water. A volume of 25 µl per well was used (duplicate). A volume of 100 µL of reaction buffer (600 mM ethanol, 0.5 mM 3-(4,5dimethylthiazol-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) 0.05mg/ml was added to the extracts. Kinetics of the reaction was followed on LB 942 Multimode Reader (OD at 550nm, every 30 seconds for 20 minutes). All NAD samples were assayed in duplicate and compared to a range of standard NAD concentrations using linear regression curve equation method between NAD standard concentrations and the slope of the reaction. The NAD cycling assay detects both NAD⁺ and NADH, measuring the global pool of cellular NAD.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 7 software. Results are expressed as fold change or mean ± standard error of the mean (SEM). Statistical comparisons were performed using two-way ANOVA and Tukey multiple comparisons test. A P-value of <0.05 was considered significant.

Results

NR does not preserve cardiac function

Adverse left ventricular remodeling is a consequence of MI (Pfeffer 1994). To validate our model of MI and assess the impact of NR on cardiac dysfunction, echocardiography was performed in MI mice at day 4 and 7. Our data show a comparable increase in left ventricular end systolic diameter (LVESD) and left ventricular end diastolic diameter (LVEDD) at day 4 and 7 post-MI in the presence and absence of NR treatment compared to the SHAM+ vehicle (V) group (Figs. 1A and A' and Figs. 1B and 1B'). A decrease in ejection fraction (EF) and cardiac output (CO) at day 4 and 7 post-MI in the presence and absence of NR treatment compared to the SHAM+V groups was observed (Figs. 1C and 1C' and Figs. 1D and 1D'), showing no protective effect of NR treatment on cardiac function at this early stage post-MI.

NR preserves kidney function

To assess the impact of NR on kidney function, we measured urine output as an indicator of exacerbated kidney injury and a predictor of in-hospital mortality (Md Ralib, Pickering et al. 2013). To further confirm our finding, urinary cystatin-C and kidney injury molecule-1 (KIM-1) were quantified. Cystatin-C and KIM-1 are well known diagnostic indicators of enhanced kidney injury and are considered early makers used in assessing AKI (Bagshaw and Bellomo 2010, Jin, Shao et al. 2017). MI significantly decreased urine output at day 4 and 7, while it remained unchanged in the MI + NR group (Figs. 2A and 2A'). No significant alteration in protein concentration of urine at day 4 and 7 in the presence and absence of NR treatment was observed after MI as expected as proteinuria is more often associated with chronic kidney diseases than acute dysfunction (Figure 2B and 2B'). Urinary cystatin-C markedly increased at day 4 and 7 post-MI, while it remained unchanged following NR treatment (Figure 2C and 2C'). Urinary KIM-1 increased at day 4 post-MI, whereas it remained unchanged following NR treatment. No significant change in urinary KIM-1 level was observed at day 7 among all mice groups (Fig. 2D and 2D'). Collectively, our data indicate decreased injury level and improved kidney function at day 4 and 7 post-MI following NR administration compared to MI + V group.

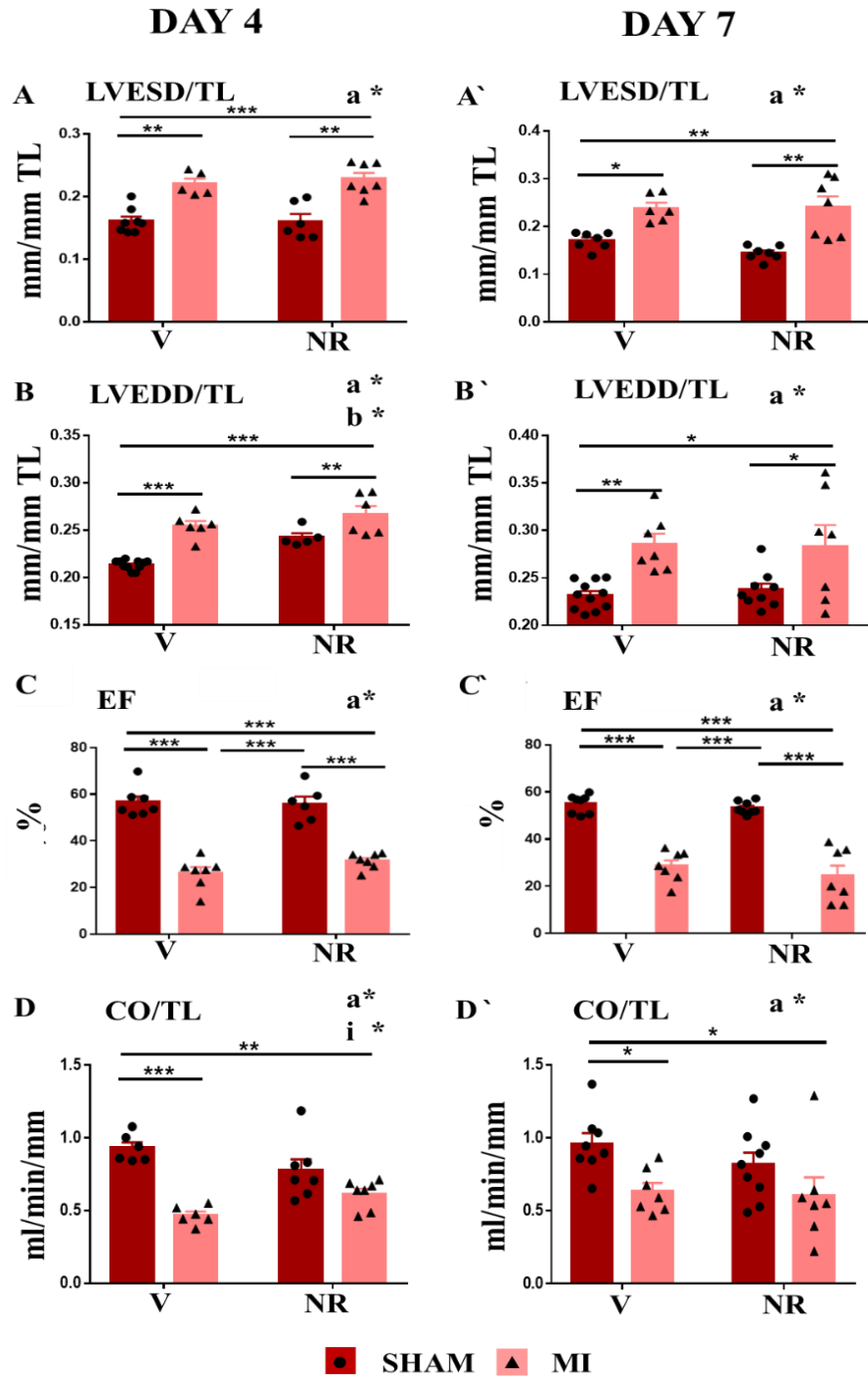


Figure 1: Cardiac function at 4 and 7 days post myocardial infarction with and without NR treatment. A-D: day 4 post-MI, A'-D': day 7 post-MI. Left-ventricular end-systolic diameter (LVESD), Left-ventricular end-diastolic diameter (LVEDD), ejection fraction (EF), and cardiac output (CO) were normalized to tibia length. MI: myocardial infarction, V: Vehicle; NR: Nicotinamide riboside. Values are represented as mean $\pm$ SEM. Two-Way-ANOVA statistical analysis; a^* : MI factor effect <0.05 , b^* : NR factor effect <0.05 , i^* : Interaction effect <0.05 , $n=5-13$ /group; Tukey post-hoc test: $*P$ value <0.05 , $***P$ <0.001 , $****P$ <0.001 ;

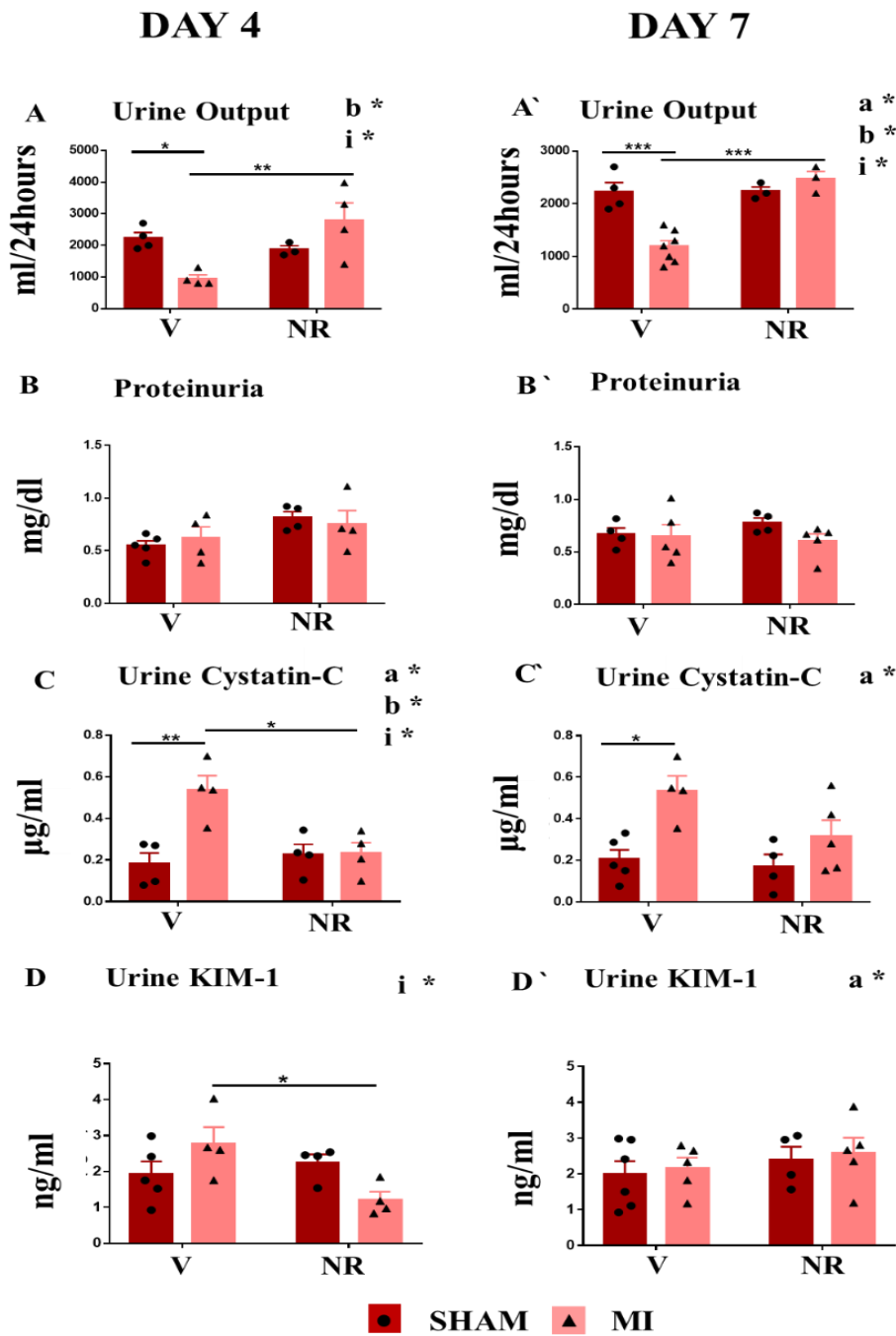


Figure 2: Kidney function at 4 and 7 days post myocardial infarction with and without NR treatment. A-D: day 4 post-MI, A'-D': day 7 post-MI. KIM-1: Kidney injury molecule-1; MI: myocardial infarction, V: Vehicle; NR: Nicotinamide riboside. Values are represented as mean $\pm$ SEM. Two-Way-ANOVA statistical analysis; a*: MI factor effect <0.05 , b*: NR factor effect <0.05 , i*: Interaction effect <0.05 , $n=3-5/\text{group}$; Tukey post-hoc test: *P value <0.05 , ***P <0.001 , ****P <0.001 .

NR reduces kidney morphological alterations

Strong evidence has demonstrated a tight link between ischemic heart diseases including MI and enhanced kidney structural alterations that result in exacerbated kidney dysfunction (Niizuma, Nakamura et al. 2011). To evaluate the impact of NR on kidney damage, periodic acidic Schiff staining was performed to assess glomerular and proximal tubules damage (Figure 3A-A' and E-E'). Figures 3B, 3C and 3D show an increase in glomerular corpuscle area, the tuft area, and Bowman space at day 4 post-MI, whereas they remained unchanged following NR treatment. Bowman space was still increased at day 7 post-MI, while it was preserved with NR treatment (Figure 3D'). Proximal convoluted tubule cross-sectional area (PCT-CSA) was substantially enlarged at day 4 and 7 post-MI, but dilation did not take place following NR treatment (Figs. 3F and 3F'). Our findings reveal decreased post-MI signs of kidney damage following NR treatment.

NR attenuates kidney fibrosis and cell death

AKI is associated with renal fibrosis pathogenesis (Yang 2019). Additionally, apoptosis in the context of AKI has been shown to contribute to parenchymal cell loss, enhancing thereby kidney damage (Priante, Ganesello et al. 2019). In order to assess the impact of NR on kidney fibrosis and cell death, MTC and TUNEL staining were performed (Figure A and A' and C and C'). Our data show a significant increase in interstitial fibrosis at day 4 and 7 post-MI, whereas it remained unchanged following NR treatment (Fig. 4B and 4B'). Similarly, cell death identified by fluorescent TUNEL labeling markedly increased at day 4 and 7 post-MI, while it decreased following NR treatment (Figure 4D and 4D'). Visualization of TUNEL labeling by immunohistochemistry showed that cell death particularly important in the medullary region (Figure 4E). Collectively, our findings reveal a protective role of NR on the kidneys mediated by decreased kidney fibrosis and cell death at early stage post-MI.

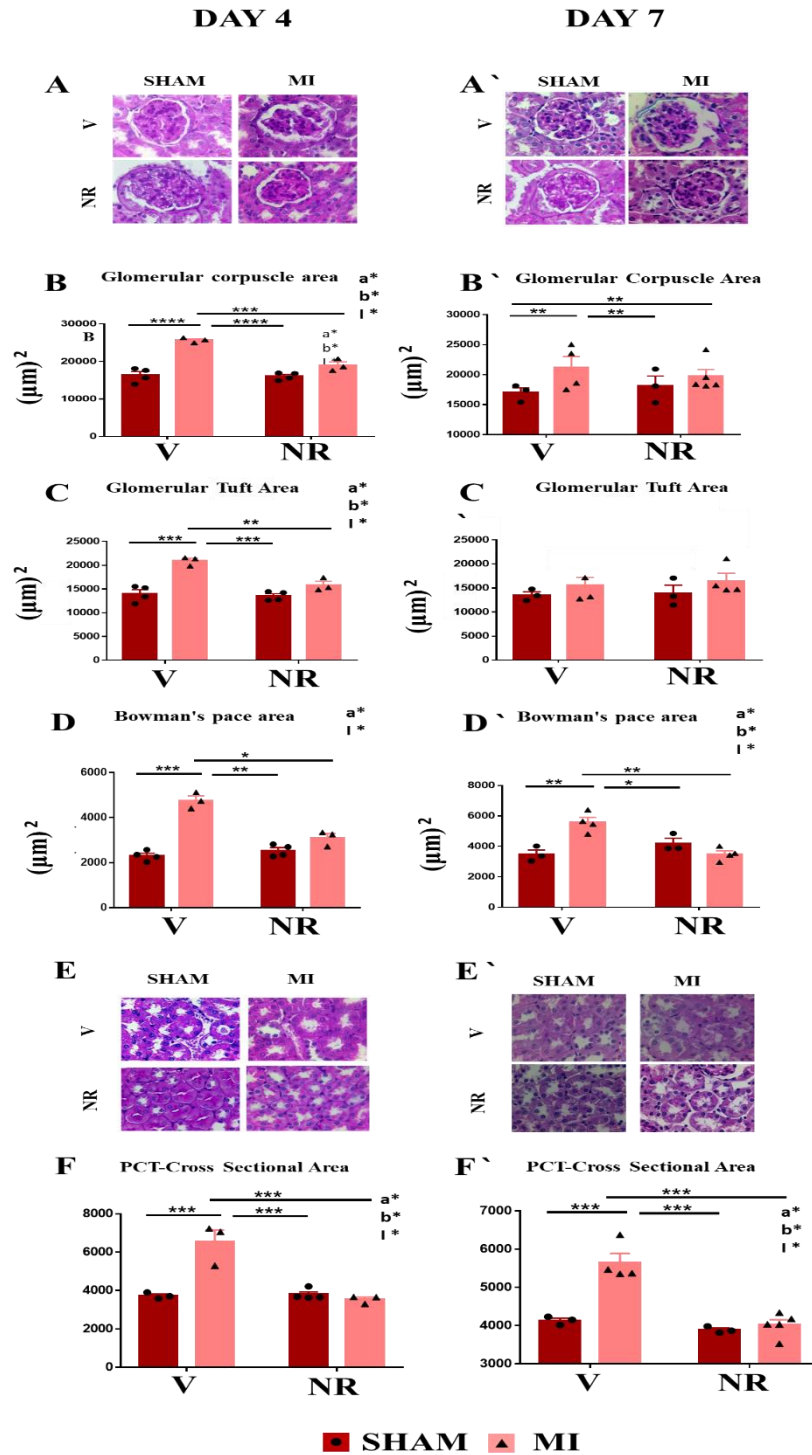


Figure 3: Kidney histology at 4 and 7 days post myocardial infarction with and without NR treatment. A-F: day 4 post-MI, A'-F': day 7 post-MI. PCT: proximal convoluted tubules; MI: myocardial infarction, V: Vehicle; NR: Nicotinamide riboside. Values are represented as mean $\pm$ SEM. Two-Way-ANOVA statistical analysis; a*: MI factor effect <0.05 , b*: NR factor effect <0.05 , i*: Interaction effect <0.05 , $n=3-5/\text{group}$; Tukey post-hoc test: *P value <0.05 , ***P <0.001 , ****P <0.001 ; Scale bar in (A and A') and (E and E') is 20 μm .

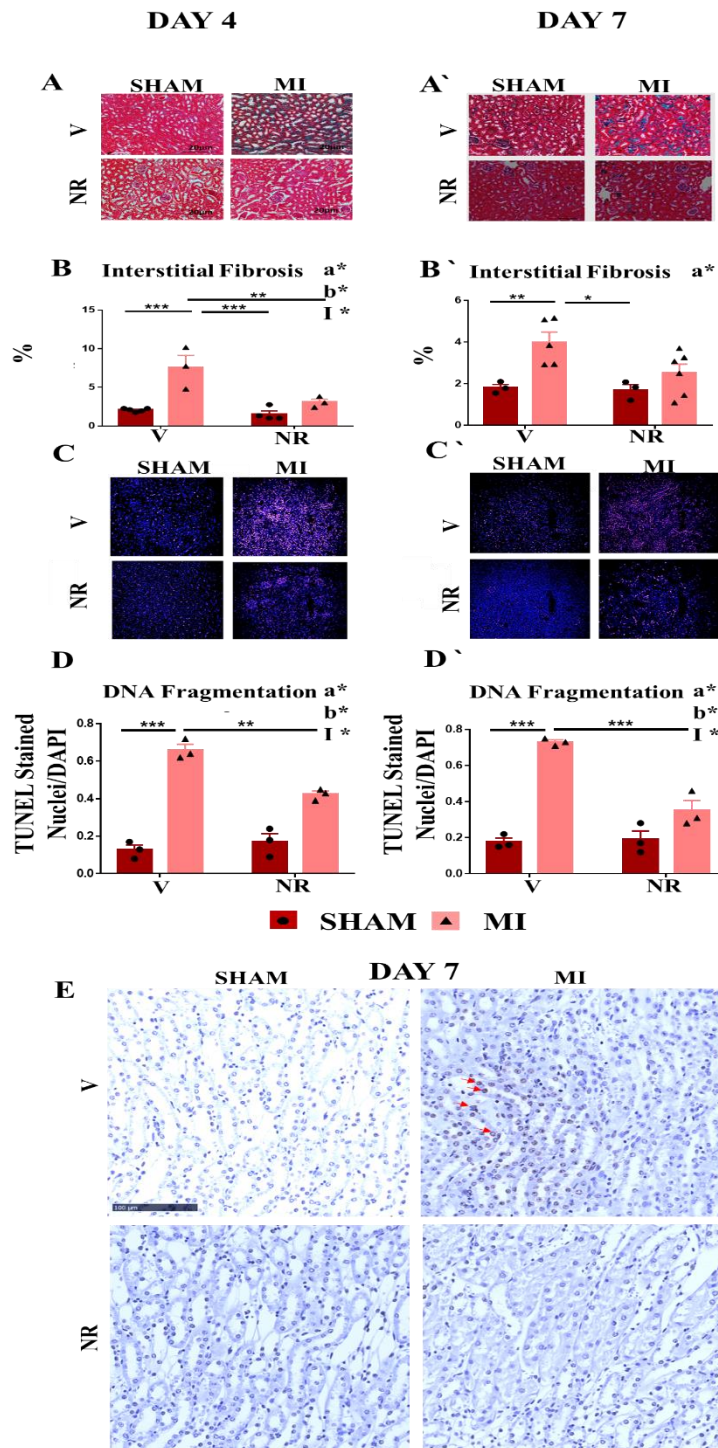


Figure 4: Interstitial fibrosis and cell death at 4 and 7 days post myocardial infarction with and without NR treatment. A-D: day 4 post-MI, A-D': day 7 post-MI. A-A' Masson trichrome staining. B-B': Quantification of fibrosis. C-C': Fluorescent TUNAL labeling. C-C': Quantification of TUNAL labeling. E. TUNEL labeling revealed by immunohistochemistry. MI: myocardial infarction, V: Vehicle; NR: Nicotinamide riboside. Values are represented as mean $\pm$ SEM. Two-Way-ANOVA statistical analysis; a*: MI factor effect <0.05 , b*: NR factor effect <0.05 , i*: Interaction effect <0.05 , $n=3-5/\text{group}$; Tukey post-hoc test: * P value <0.05 , *** P <0.001 , **** P <0.001 ; Scale bar in (A and A') and (C and C') is 20 μm . Scale bar in E is 100 μm .

NR preserves total NAD levels and enhances NAD⁺ signaling pathway

The role of NAD in regulating metabolism and modulating cellular homeostasis is well established (Xie, Zhang et al. 2020). NAMPT and NMRK-1, NAD⁺ biosynthetic enzymes, play a major role in NAD production in the kidneys (Hershberger, Martin et al. 2017). Total NAD levels (sum of NADH and NAD⁺) in the kidneys shows no significant change at 4 and 7 days post-MI, whereas administration of NR increased its levels in the MI group but not the Sham group (Figs. 5A and 5A'). No alteration in *Nampt* mRNA expression levels was observed at day 4 and 7 post-MI, whereas the administration of NR transiently increased this level at day 4 in the MI group only (Fig. 5B and 5B'). *Nmrk1* mRNA expression increased at day 7 following MI (Figs. 5C and 5C'). *Sirt1* mRNA expression levels, an NAD⁺ dependent enzyme, showed no significant change at day 4 and 7 post-MI among all mice groups (Figs. 5D and 5D'). Our findings indicate that NR administration increased kidney NAD levels as early as 4 and up to 7 days post-MI, and this elevation is mediated by upregulating *Nampt* expression levels as soon as day 4 as well as *Nmrk1* at day 7.

NR increases NAMPT protein levels in a cell type specific manner

Immunohistochemistry was performed to confirm the observed increase in NAMPT expression at day 4 post-MI following NR treatment and assess in which cell type elevation occurred. In the cortex, NAMPT is present in the nucleus of a few cells located in the glomeruli (Figs. 6A, B, C and D) and the vast majority of proximal tubule epithelial cells. In distal tubules, NAMPT is present both in the nucleus and cytosol. Interestingly, in the medullary area which is enriched in distal tubules, NR treatment induced the most striking increase in NAMPT signal (Fig. 6D').

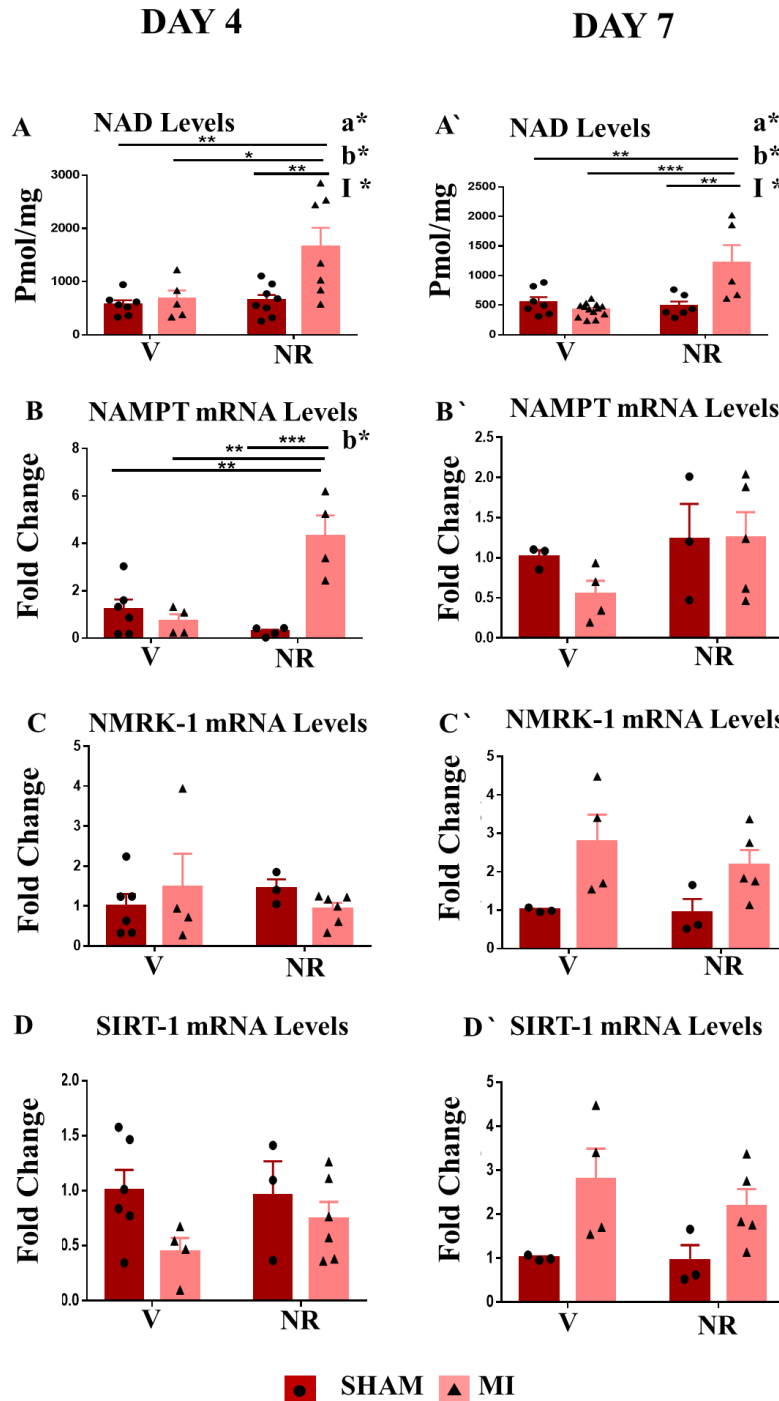


Figure 5: NAD levels and NAD⁺ signaling pathway at 4 and 7 days post myocardial infarction with and without NR treatment. A-D: day 4 post-MI, A'-D': day 7 post-MI. *Nampt*: nicotinamide phosphoribosyltransferase, *Nmrk1*, nicotinamide riboside kinase 1, *Sirt1*: Sirtuin 1; MI: myocardial infarction, V: Vehicle; NR: Nicotinamide riboside. Values are represented as mean $\pm$ SEM. Two-Way-ANOVA statistical analysis; a*: MI factor effect <0.05, b*: NR factor effect <0.05, i*: Interaction effect <0.05, n=3-6/group; Tukey post-hoc test: *P value<0.05, ***P<0.001, ****P<0.001;

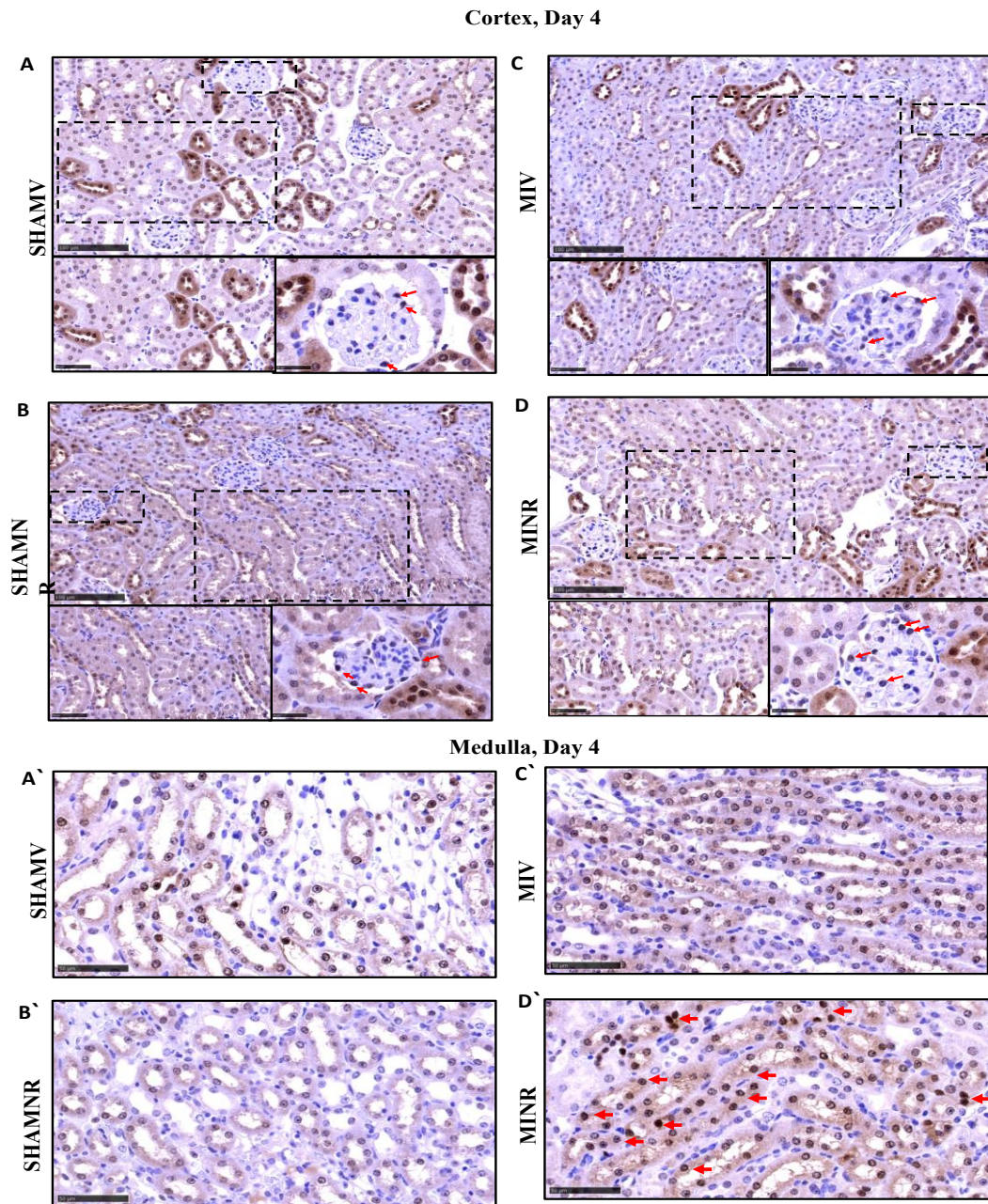


Figure 6: IHC analysis of NAMPT changes in the kidney with NR. Representative images of NAMPT showing an increase in NAMPT protein levels at day 4 following NR treatment at the level of distal tubules. A-D: Cortex region. A'-C': Medulla region. n=3/group. Scale bar = 100 μ m.

Discussion

Mounting evidence reports a close link between acute cardiac diseases and AKI development and progression (Windt, Eijkelkamp et al. 2006, Fan, Li et al. 2018, Wang, Pei et al. 2019). The main objective of this study was to investigate the impact of NR treatment on MI-induced AKI at early stage post-MI. MI was induced in male mice by ligating the LAD coronary artery followed by sacrifice at either 4 or 7 days post-MI. NR, an NAD precursor, was delivered by intraperitoneal injection (400mg/kg/day), during a therapeutic window starting 30 minutes after the surgery up to day of sacrifice (4 and 7 days post-MI).

Strong evidence demonstrates a crucial role of NAD⁺ and NAD⁺ biosynthetic and dependent enzymes in protecting against aggravated oxidative stress and cell death, mitochondrial dysfunction, and enhanced inflammatory response (Cantó, Menzies et al. 2015, Cameron, Castoldi et al. 2019, Klimova, Fearnow et al. 2020, Covarrubias, Perrone et al. 2021, Tannous, Booz et al. 2021). In this study, we report enhanced kidney dysfunction and morphological alterations and increased cell death and fibrosis, at day 4 and 7 post-MI. Post-MI administration of NR, the substrate of NMRK2 in cardiac tissues and NMRK-1 in the kidneys, nearly doubled kidney NAD levels, improved kidney function, decreased kidney morphological alterations, and protected against cell death and fibrosis. As EF and CO were not improved in this MI model following NR treatment for 4 and 7 days, we therefore conclude that the decrease in kidney injury is rather due to a direct effect of NR on the kidneys.

The alteration of NAD homeostasis has been reported in several experimental models of AKI including ischemia reperfusion injury (Morevati, Egstrand et al. 2021). In this study we observed no change in total NAD pool in the kidneys as early as 4- and 7-days post-MI. This observation could be due to the age of the mice which is 2 months old. Our previous work -, which had showed a significant decrease in NAD levels in the kidneys at day 7 post-MI was done in 5 month-old mice. Multiple studies indicated that NAD⁺ metabolism is drastically and progressively impaired by aging (Verdin 2015, Peluso, Damgaard et al. 2021). However, NR administration post-MI still markedly increased NAD levels in the kidneys. Of note, NMRK-1, an NAD⁺ biosynthetic enzyme that uses NR as a substrate to generate NAD⁺ increased at day 7 post-MI. On the other hand, NAMPT, an NAD⁺ biosynthetic enzyme that relies on

nicotinamide (NAM) to generate NAD^+ , increased earlier at day 4 post-MI but only following NR treatment. NAMPT protein mainly increased at the level of the medulla. In light with this observation, a study performed by Kropotov et al. indicated that NR after entering the cells, is in part metabolized to NAM under the action of purine nucleoside phosphorylase (PNP), leading to the intracellular accumulation of NAM in the tissues, including kidneys (Kropotov, Kulikova et al. 2022). In this study, inhibition of PNP using Immucillin H resulted in increased NAD synthesis from NR and high level of NR in the kidneys and blood two hours after injection (Kropotov, Kulikova et al. 2022).

In order to reveal if the observed increase in NAD levels following NR administration exerts a protective effect against MI-induced kidney injury, kidney functional, morphological, and molecular parameters were assessed. Our data revealed a decrease in urine output reflecting deterioration in kidney function at day 4 and 7 post-MI. The administration of NR reverse this dysfunction and decrease levels of markers of kidney injury, including KIM-1 and cystatin-C. In that regard, KIM-1 has gained interest as a prognostic maker for renal tubule damage and decline in renal function (Waanders, van Timmeren et al. 2010). Moreover, urinary cystatin-C has been identified as a better and earlier predictive marker of AKI than creatinine, not being affected by sex, age, or muscle mass (Soto, Coelho et al. 2010, Bongiovanni, Magrini et al. 2015).

Morphologically, our findings demonstrated aggravated kidney structural alterations at day 4 and 7 post-MI, whereas the administration of NR reversed this damage. In a different model of kidney injury, administration of NR, 10 days prior to induction of AKI and until the day of sacrifice (400 mg/kg/day diet), was previously documented to lessen kidney dysfunction and decrease tubular injury analyzed at 48 hours post-renal IRI-induced AKI and 72 hours following cisplatin-induced AKI (Faivre, Katsyuba et al. 2021). This administration was given preventively whereas our study demonstrates that NR can be administrated as an immediate therapeutic intervention post-MI, establishing a strong potential for translation into clinics in type I CRS. We also found that NR treatment decreased kidney interstitial fibrosis and cell death. In line with this observation, it has been shown that NR administration also attenuates hepatic fibrosis by enhancing the activity of SIRT-1 and supressing expression of P300 (Jiang, Zhou et al. 2019). Furthermore, in a study performed by Pham et al. the administration of NR (400 mg/kg/day) for 20 weeks decreased liver fibrosis through inhibiting collagen deposition

in a mouse model of high fat diet-induced hepatic fibrosis (Pham, Bae et al. 2019).

Conclusion and Future direction

This is the first study performed in the context of type I CRS following MI to assess whether boosting NAD levels with NR treatment exerts protective effects on the kidneys. The effects of NR administration in maintaining kidney NAD levels, preserving mitochondrial function, attenuating exacerbated inflammatory response, and most importantly improving overall survival, establish a solid rationale for NR as a promising therapy for lessening AKI pathogenesis in the setting of large MI. As NR has been shown to be well tolerated in humans with no adverse effects reported on kidney or cardiac function including in patients with heart failure (Trammell, Schmidt et al. 2016, Conze, Brenner et al. 2019, Wang, Airhart et al. 2022), we believe that this therapeutic approach should now be tested in a controlled clinical trial for type I CRS.

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6.5 Nicotinamide riboside modulates the inflammatory response in myocardial infarction-INDUCED TYPE I CARDIO-RENAL SYNDROME

6.5.3 Introduction

Ischemic heart disease is a critical contributor to mortality worldwide (Vos, Lim et al. 2020). Myocardial infarction (MI) is the most serious manifestation of cardiovascular diseases and a main cause of acute kidney injury (AKI) (Hwang, Jeong et al. 2011, Shacham, Leshem-Rubinow et al. 2014). The occurrence of AKI in the setting of MI is an independent risk factor of increased in-hospital and long term mortality following MI (Cai, Xiao et al. 2022).

Multiple signaling pathways have been implicated in the pathogenesis of AKI. Inflammation has been shown to be a common player in multiple different AKI models. It is well-established that ischemia among other models results in structural and functional alterations in vascular endothelium and tubular epithelium cells. These changes lead to immune cells infiltration including leukocytes, macrophages, and lymphocytes into the injured kidneys. Consequently, increased secretion of inflammatory markers such as cytokines and chemokines by the endothelial and tubular cells eventually exacerbate kidney pathogenesis (Akca, Nguyen et al. 2009).

The NAD⁺ precursor, nicotinamide riboside (NR), a type of vitamin B3, has been shown to exert anti-inflammatory effects. In that regard, an in vitro study performed on alcohol induced inflammation in bone-marrow derived macrophages showed that administration of NR (1 mM for 72 hours) substantially reversed the observed significant increase in tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β , and IL-6 following alcohol administration (Kang, Park et al. 2021). The anti-inflammatory role of NR in MI-induced AKI is yet to be elucidated. In this chapter we assessed if NR exerts protective effects in the kidneys through attenuating systemic inflammation as early as 4 and 7 days post-MI.

6.5.3 Materials and Methods

Enzyme-linked immunosorbent assays (ELISAs)

Assessment of plasma tumor necrosis factor-alpha (TNF- α) and interleukin (IL)-4 levels.

ELISA STEMCELL Technologies kits (#02030 and #02038A) were used to detect Plasma TNF- α and plasma IL-4, respectively according to the manufacturer's instructions. Plasma samples were added to ELISA diluent 1:1 to reach a total minimum volume of 250 μ L. One hundred μ L

of diluted sample was loaded in duplicate in the ELISA strip plate pre-coated with capture antibodies specific for the cytokine TNF- α and IL-4 incubated 2h at room temperature. Five rinses with 300 μ L washing buffer were performed and biotinylated detection antibody was added, followed by incubation for 1 h. After 5 washes (300 μ L each), diluted streptavidin horseradish peroxidase (SA-HRP) was added (100 μ L/well), and the plate incubated for 1 h at room temperature. Wells were washed, and 100 μ L of 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added to each well and the plate incubated at room temperature for 15 min. Finally, stopping solution finally added (100 μ L/well) and the absorbance measured at 450 nm in a microplate reader.

6.5.3 Results

The impact of NR on the pro-inflammatory cytokine TNF- α and the anti-inflammatory cytokine IL-4

Systemic inflammatory response has been well documented in patients with acute myocardial infarction. Deregulated immune response in the setting of MI is characterized by the secretion of pro-inflammatory cytokines, chemokines, and cell adhesion molecules. To assess if MI is associated with altered systemic inflammatory response at day 4 and 7 we measured plasma TNF- α and IL-4 as markers of pro and anti-inflammatory cytokines, respectively. Our findings indicate that plasma TNF- α levels are under the detection limit at day 4 and 7 post-MI in the presence and absence of NR treatment. The anti-inflammatory cytokine IL-4 shows no significant change at day 4 and 7 post-MI, whereas the administration of NR significantly increased its levels at both time points, suggesting enhanced anti-inflammatory response following NR treatment in this disease model.

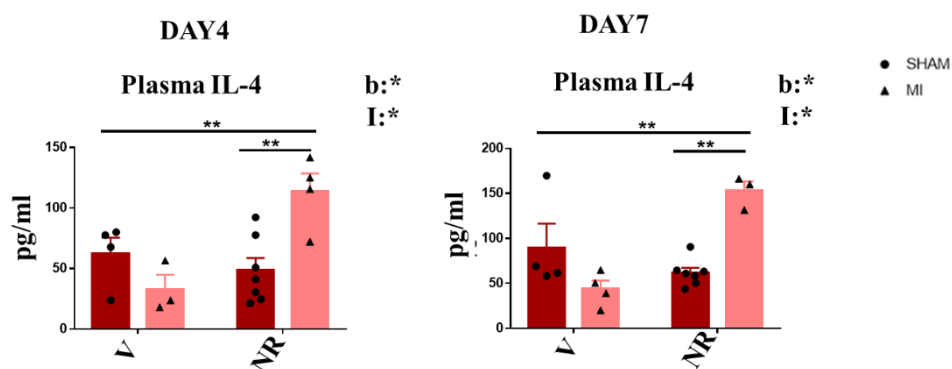


Figure 1: Impact of NR on plasma IL-4: No significant change in plasma IL-4 levels in MI mice at day 4 and 7 was observed, whereas, the administration of NR significantly increased its levels. V: Vehicle; NR: Nicotinamide riboside; b*: NR effect <0.05, n=3-7/group; I*: Interaction effect <0.05; Two-Way-Anova statistical analysis; Tukey post-hoc test; Values are represented as mean $\pm$ s.e.m.; *P value<0.05, **P<0.01, ***P<0.001, ****P<0.0001, n=3-6/group.

6.5.3 Discussion

Basic science and clinical research reported a strong correlation between MI and inflammation. AKI is very common in the setting of ischemic cardiac diseases. The pathophysiology of MI-induced AKI is very complex and multifactorial (Makris and Spanou 2016). One of major causes of MI-induced AKI is imbalanced inflammatory response (Bonventre 2007). Here we assessed the impact of MI-induced AKI on systemic inflammation. Systemic inflammation was assessed in term of TNF- α and IL-4 plasma levels. Our findings revealed that plasma TNF- α was under the detection limit in all mice groups at day 4 and 7 post-MI, which is in correlation with previous investigations indicating that plasma TNF- α picks at day 3 post-MI during the inflammatory phase (Halawa, Salomon et al. 1999). IL-4 plasma levels, however, was within the detection limit and showed no change between SHAM and MI group at day 4 and 7. Importantly, NR treatment almost doubled plasma IL-4 levels at both time points post-MI.

Multiple investigations have reported that NR exerts an anti-inflammatory role. In that regard, it has been shown that NR administration (stomach gavage for six weeks) to diabetic mice reduced the inflammatory response in the brain through attenuating TNF- α , IL-1 β , and IL-6 levels (Lee and Yang 2019). Another study performed by Lee et al. demonstrated that the administration of NR (10 μ M and 10 mM) for 24 hours in palmitic acid-treated hepatocyte to induce hepatocyte steatosis was associated with decreased TNF- α , and IL-6, pro-inflammatory cytokines (Lee and Yang 2019). Furthermore, It has been documented that oral administration of NR (1g/day for 21 days) to aged men in a placebo-controlled, randomized, double-blind, crossover trial, resulted in increased muscle NAD⁺ metabolome such as NAD and NAM and decreased plasma proinflammatory cytokines including IL-6, IL-5 and TNF- α , suggesting a protective role of NR treatment in aged humans (Elhassan, Kluckova et al. 2019). Moreover, Zhou et al. demonstrated that peripheral blood mononuclear cells (PBMCs) isolated from stage D heart failure patients exhibited reduced respiratory capacity and enhanced pro-inflammatory cytokines release such as TNF- α , IL-1 β , and IL-18 mRNA. PBMCs isolated from stage D heart failure patients treated with NR for 5-9 days (250 mg twice a day for day 1, 500 mg twice a day for day 2, and 1000 mg twice a day or day 3 on), however, showed an improvement in respiratory capacity and up to 30 fold decrease in TNF- α , IL-1 β , and IL-18 mRNA expression levels (Zhou, Wang et al. 2020). To the best of our knowledge, our study is

the fto evaluate the correlation between NR and the anti-inflammatory cytokine IL-4, suggesting that NR does not only dampen the pro-inflammatory response but also boost the anti-inflammatory cytokine secretion.

Conclusion and future directions

NR treatment promotes the anti-inflammatory response as early as 4 and 7 days post-MI through enhancing the systemic anti-inflammatory cytokine IL-4. Further experiments aiming at assessing the impact of NR on systemic and local pro-inflammatory cytokine levels such as IL-1 β and IL-6 are needed

6.6 MITOCHONDRIAL FUNCTION IN THE SETTING OF AKI AT EARLY STAGE POST-MI

6.6.4 Introduction

Acute kidney injury (AKI) is a main complication of ischemic heart diseases, increasing mortality rate substantially (Akca, Nguyen et al. 2009). Multiple complex mechanisms are involved in AKI pathogenesis including mitochondrial dysfunction (Bonventre and Zuk 2004). Strong published evidence demonstrated that the kidneys are one of the most energy-demanding organs and reported that both the kidneys and the heart have the highest resting metabolic rates (Wang, Ying et al. 2010). Furthermore, it has been indicated that the kidneys are only second to the heart in term of mitochondrial abundance (O'Connor 2006, Pagliarini, Calvo et al. 2008). The high resting metabolic rate in the kidneys has been attributed to the fact that the kidneys rely on mitochondria to provide sufficient energy to be able to perform multiple complex tasks such as removing waste from the blood, reabsorbing nutrients and glucose, and maintaining acid-base balance, among others (Bhargava and Schnellmann 2017).

One major role of mitochondria in the kidneys is to provide energy to the Na⁺-K⁺-ATPase, located in proximal tubules (PT), distal tubules, loop of Henle, and collecting ducts, which all of these parts of the kidneys need active transport to reabsorb ions (Soltoff 1986).

A great body of evidence has shed the light on the critical role of mitochondrial dysfunction in initiating and exacerbating AKI (Ishimoto and Inagi 2016). In that regard, a decline in mitochondrial content of kidney PT following ischemia reperfusion has been well documented, suggesting enhanced mitochondrial damage (Lan, Geng et al. 2016). Additionally, decreased ATP levels and enhanced mitochondrial dysfunction have been attributed to the loss of mitochondrial protein in PT (Funk and Schnellmann

2012, Parikh 2013). Moreover, a disruption of the oxidation and transportation of fatty acid has been observed in the setting of AKI and led to the accumulation of fatty acids in the cytoplasm, consequently decreased ATP levels in multiple animal model of AKI including sepsis and ischemic diseases (Ruidera, Irazu et al. 1988, Simon and Hertig 2015). In that regard, it has been reported that damaged electron transport chain is not capable to oxidize NADH to regenerate NAD^+ , a main cofactor implicated in fatty acid metabolism following AKI (Portilla 1999). Importantly, under physiological conditions, PT relies mainly on oxidative phosphorylation to generate ATP, following AKI, however, increased lactate and pyruvate levels and glycolytic enzymes have been observed, suggesting a shift in the kidneys metabolic substrate in attempt to maintain normal kidney function (Weinberg, Venkatachalam et al. 2000, Smith, Stallons et al. 2014, Zager, Johnson et al. 2014, Venkatachalam, Weinberg et al. 2015, Lan, Geng et al. 2016). In this chapter, we aimed at evaluating the impact of MI on kidney mitochondrial function as early as 4 and 7 days via assessing the activity of complex I, II, III, IV activity.

6.6.4 Materials and Methods

Oxygen Consumption Rate Assay in Permeabilized Kidney Cortex Tissues

Kidney cortex and medulla tissues were isolated and permeabilized using saponin. Mitochondrial respiration was assessed using O2K respirometer (Oroboros instruments, Innsbruck, Austria). Respiration solution was made from CaK_2EGTA (100mM) diluted to 2.77 mM, K_2EGTA (100mM) diluted to 7.23 mM, and 6.56 mM MgCl_2 to obtain 100 nM free Ca^{2+} and 1 mM free Mg^{2+} , 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 kidney was harvested then the cortex and medullary regions (2-4mg) were isolated manually and washed with PBS 1X. The protocol started with adding a thin slice of cortex/medulla tissues (2-4mg) to a 2 mL chamber containing the respiration solution supplemented with pyruvate/malate (1 mM and 4 mM,

respectively), followed by sequential addition of ADP (100uM and 2 mM), succinate (15 Mm, to activate complex II), amytal (2 mM, to inhibit complex I), and finally tetramethyl-p-phenylenediamine (TMPD, 0.5 mM, to activate complex IV) with ascorbate (2 mM) to avoid auto-oxidation of TMPD.

Enzymatic Activities

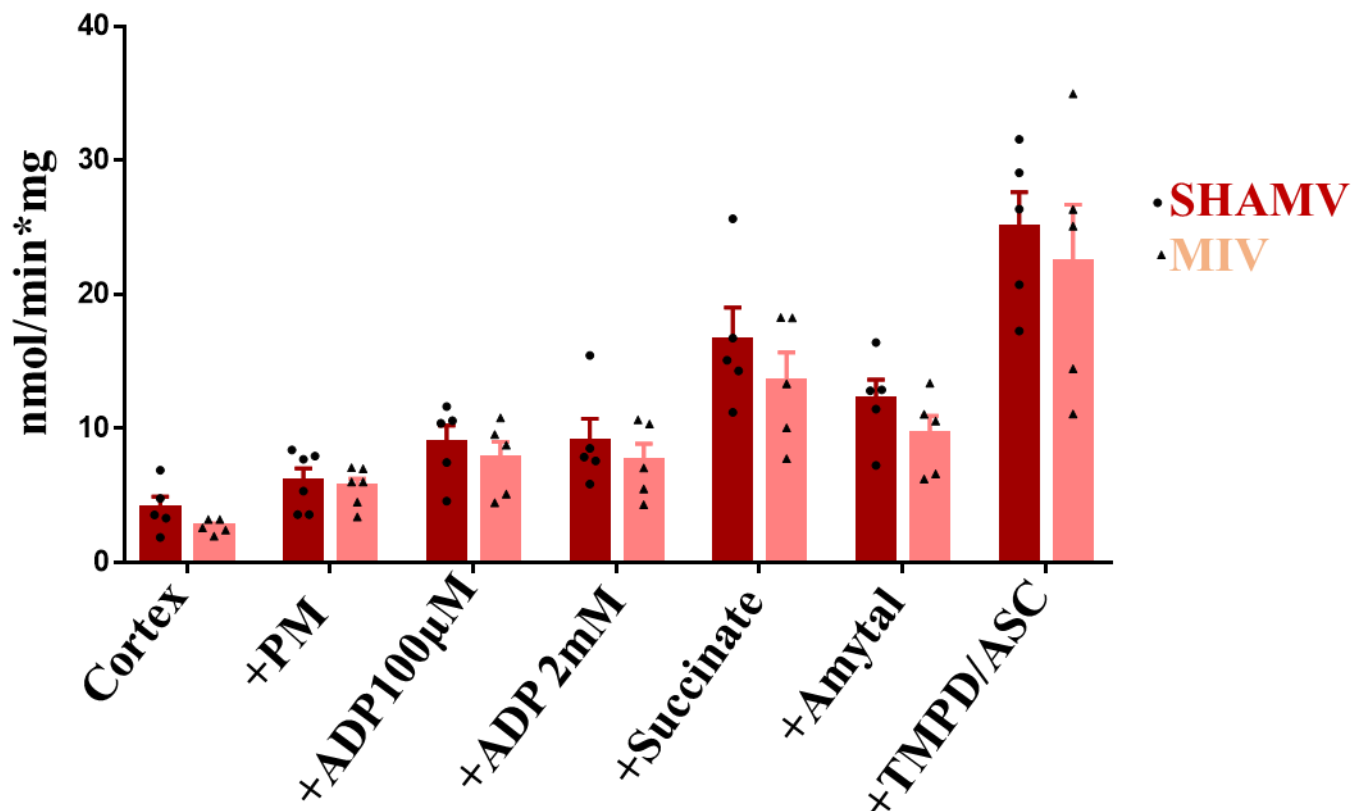
Frozen tissue samples were weighed, homogenized with a Precellys 24 (Bertin Instruments) in ice-cold buffer (50 mg/ml) containing 5 mM HEPES (pH 8.7), 1 mM EGTA, 1 mM DTT and 0.1% Triton X-100. Citrate synthase (CS) activity was determined in homogenized kidney cortex. The activity of CS was assessed using standard spectrophotometric assays. Around 0.5 µg of protein was added to 1 mL Buffer M (100 mM Trizma (pH 8), 0.1 mM 5,5'-dithiobis-(2-nitrobenzenoic acid), 0.3 mM acetyl-CoA, 0.5mM oxaloacetic acid) and the absorbance was measured at 412 nm for 3 min. CS activity was calculated using an extinction coefficient of $13600\text{M}^{-1}\cdot\text{cm}^{-1}$ and rates were given in international unit/g protein.

6.6.4 Results

1. The impact of MI on kidney mitochondrial function at day 4 and 7

In order to know if MI is associated with altered mitochondrial function in the kidneys as early as 4 and 7 days, mitochondrial oxidative capacities were measured in permeabilized kidney cortex and medulla tissues. We first harvested kidney cortex tissues to analyze mitochondrial function. At day 7, in the presence of PM activator of complex I, the respiration rate measured in the presence of increasing ADP concentration (100uM, and 2mM) remained below 10 nmol/min*mg and showed no significant change in MI mice when compared to the SHAMV group. Succinate (activator of complex II) increased respiration rate but did not show a change between MI mice and the SHAM group. Since we had observed low complex I dependent respiration in the cortex we decided to focus on the measurement of complex II-

dependent respiration which still requires the addition of amytal to offset any



remaining complex I activity. Amytal barely decreased respiration rate when compared to the succinate alone with no difference between MI mice and the SHAM group. TMPD was then added to measure maximal complex IV-dependent respiration and showed an enhancement of the respiration rate but did not show change between groups (**Figure 1**). Collectively, our data does not suggest major mitochondrial dysfunction in the cortex at 7 days post-MI.

Figure 1: Effect of MI on the mitochondrial oxygen consumption rate (VO₂) in kidney cortex of SHAM and MI mice. No significant alteration in the mitochondrial oxygen consumption rate (VO₂) in cortex tissues was observed at day 7 post-MI when compared to the SHAM group. (Oxygen consumption rate is normalized to the cortex dry weight. Each value represents the mean $\pm$ SEM. n=3-6/group; Data were analyzed using Two way ANOVA. All bars represent mean $\pm$ SEM (P <0.05); V: Vehicle; MI: myocardial infarction; NR: Nicotinamide riboside.

Since our findings revealed no change in oxygen consumption rate in cortex tissues 7 days post-MI. Therefore, we then aimed at assessing if there was mitochondrial dysfunction at earlier stage (4 days post-MI) in both cortex and medullary regions. In cortex tissues and at day 4, in the presence of PM activator of complex I, the respiration rate measured in the presence of increasing ADP concentration (100uM, and 2mM) remained below 10 nmol/min*mg and showed no significant change in MI mice in the presence and absence of NR treatment when compared to the SHAMV group. Succinate (activator of complex II) increased respiration rate but did not show a change between MI mice in the presence and absence of NR and the SHAM group. Since we had observed low complex I dependent respiration in the cortex we decided to focus on the measurement of complex II-dependent respiration which still requires the addition of amytal to offset any remaining complex I activity. Amytal barely decreased respiration rate when compared to the succinate alone with no difference between MI mice in the presence and absence of NR treatment and the SHAM group. TMPD was then added to measure maximal complex IV-dependent respiration and showed an enhancement of the respiration rate but did not show change between groups (**Figure 2**). Collectively, our data does not suggest major mitochondrial dysfunction in the cortex at 4 and 7 days post-MI.

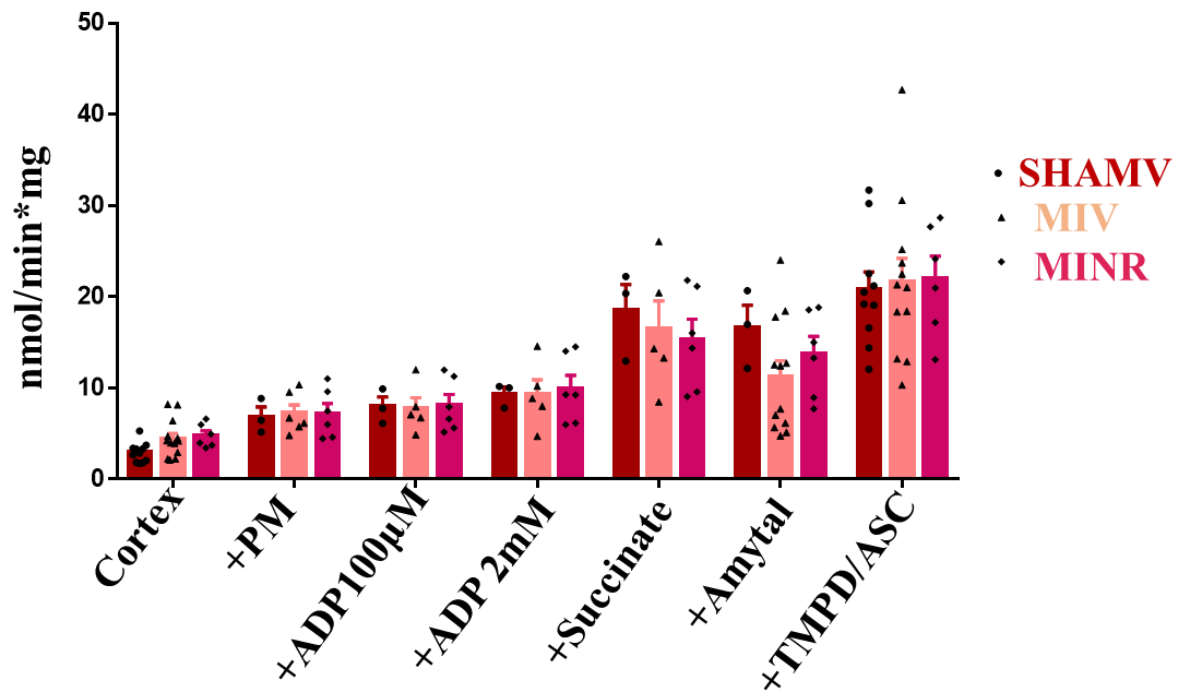


Figure 2: Effect of MI on the mitochondrial oxygen consumption rate (VO₂) in kidney cortex of SHAM and MI mice in the presence and absence of NR treatment. No significant alteration in the mitochondrial oxygen consumption rate (VO₂) in cortex tissues was observed at day 4 post-MI in the presence and absence of NR treatment, when compared to the SHAM group. (Oxygen consumption rate is normalized to the cortex dry weight. Each value represents the mean $\pm$ SEM. n=3-13/group; Data were analyzed using Two way ANOVA. All bars represent mean $\pm$ SEM (P <0.05); V: Vehicle; MI: myocardial infarction; NR: Nicotinamide riboside. All bars represent mean $\pm$ SEM (P <0.05); n=3-13/group; Data were analyzed using Two Way ANOVA; MI: myocardial infarction, NR: nicotinamide riboside.

Recent preclinical investigations indicated that the basal tissue perfusion in the medullary region is markedly less than that in the cortex and thus any minor decrease in oxygenation and perfusion following kidney artery occlusion could affect more severely the medullary region rather than the cortex which may lead to exacerbated

mitochondrial dysfunction (Calzavacca, Evans et al. 2015). Furthermore, it has been documented that a hypoxic marker, pimonidazole, presents in both the medullary and corticomedullary regions in kidneys of normal healthy rats (Rosenberger, Rosen et al. 2009). Collectively, the medulla is “relatively hypoxic,” when compared to the cortex even under physiological condition, and thus it is more susceptible to injury following hypoxia (Sugahara, Tanaka et al. 2020). Therefore, we harvested medulla tissues at day 4 post-MI in the presence and absence of NR treatment and compared the mitochondrial oxygen consumption rate (VO₂) to the SHAMV group. In medullary tissues and at day 4, in the presence of PM activator of complex I, the respiration rate measured in the presence of increasing ADP concentration (100uM, and 2mM) remained below 10 nmol/min*mg and showed no significant change in MI mice in the presence and absence of NR treatment when compared to the SHAMV group. Succinate (activator of complex II) increased respiration rate but did not show a change between MI mice in the presence and absence of NR and the SHAM group. Since we had observed low complex I dependent respiration in the medulla, we decided to focus on the measurement of complex II-dependent respiration which still requires the addition of amyntal to offset any remaining complex I activity. Amyntal barely decreased respiration rate when compared to the succinate alone with no difference between MI mice in the presence and absence of NR treatment and the SHAM group. TMPD was then added to measure maximal complex IV-dependent respiration and showed an enhancement of the respiration rate but did not show change between groups (**Figure 3**). Collectively, our data does not suggest major mitochondrial dysfunction at day 4 post-MI in the medullary region.

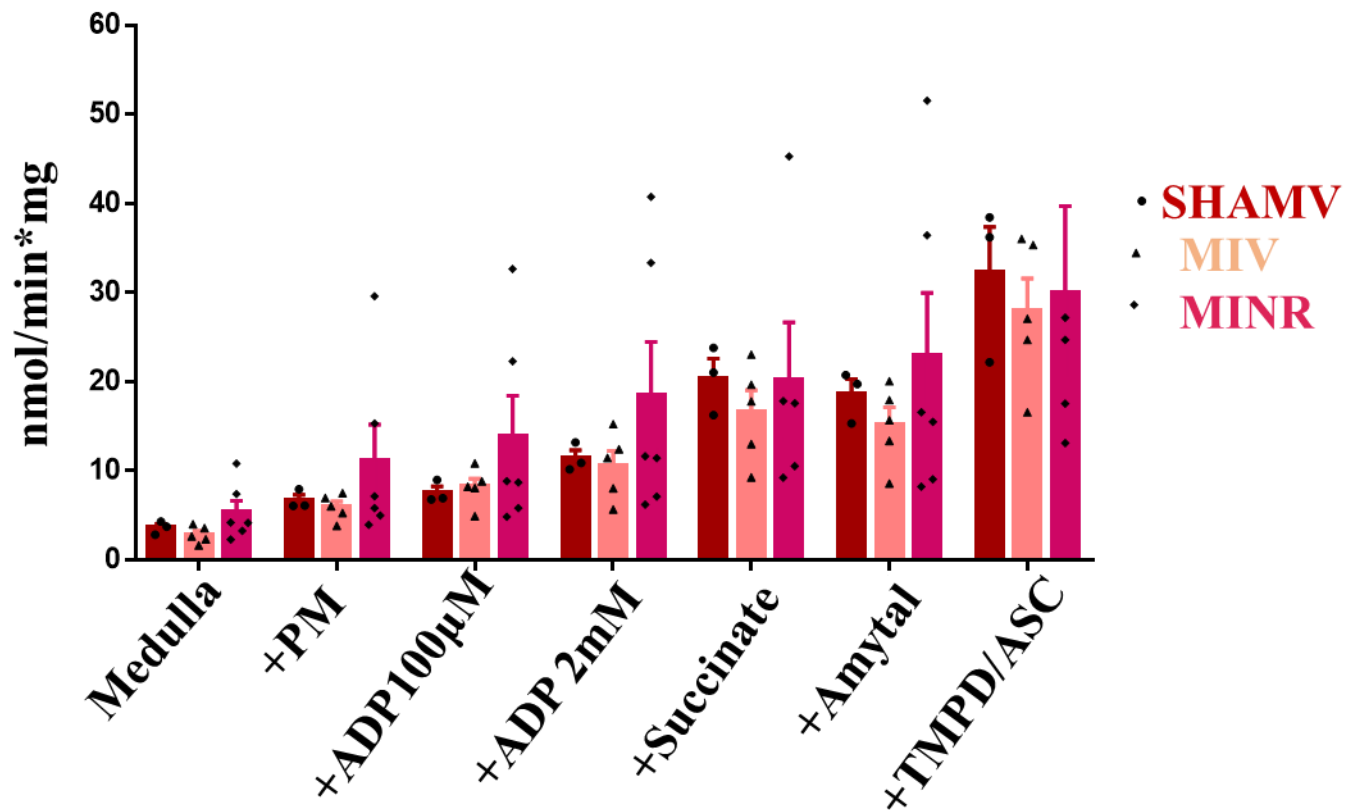


Figure 3: Effect of MI on the mitochondrial oxygen consumption rate (VO₂) in kidney medulla of SHAM and MI mice in the presence and absence of NR treatment. No significant alteration in the mitochondrial oxygen consumption rate (VO₂) in medullary tissues was observed at day 4 post-MI in the presence and absence of NR treatment when compared to the SHAM group. (Oxygen consumption rate is normalized to the cortex dry weight. Each value represents the mean $\pm$ SEM. n=3-13/group; Data were analyzed using Two way ANOVA. All bars represent mean $\pm$ SEM (P <0.05); V: Vehicle; MI: myocardial infarction; NR: Nicotinamide riboside. All bars represent mean $\pm$ SEM (P <0.05); n=3-6/group; Data were analyzed using Two Way ANOVA; MI: myocardial infarction, NR: nicotinamide riboside.

The impact of MI on kidneys citrate metabolism at day 4 and 7

Citrate activity is mainly used to assess mitochondria mass and integrity (Diguët, Trammell et al. 2018). We observed no significant alteration in citrate synthase activity

in the kidneys at day 4 and 7 post-MI when compared to their relative control groups, suggesting there was not a significant loss of mitochondria in the cortex of the kidneys at an early stage following MI.

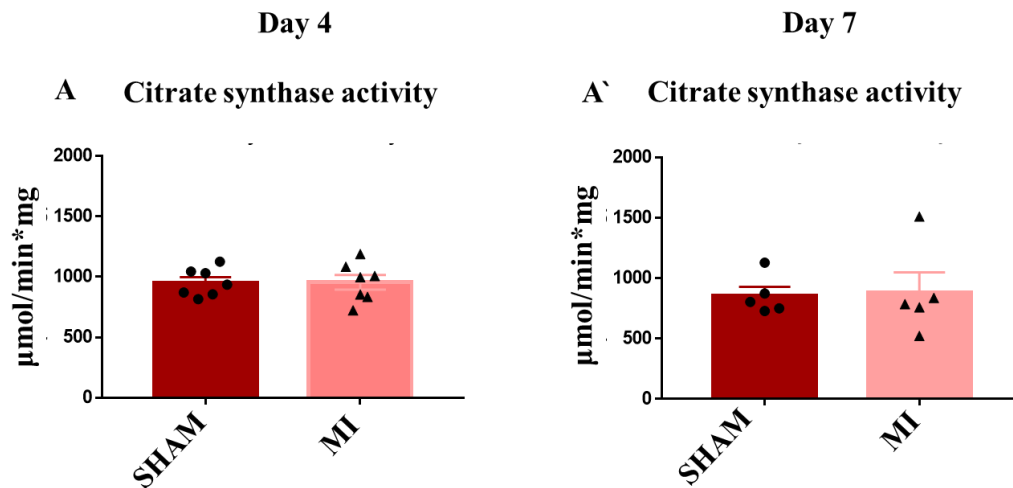


Figure 6: The impact of MI on citrate synthase activity: No significant alteration in citrate synthase activity in the kidneys was observed at day 4 (A) and 7 (A') post-MI. n=5-7/group; Data were analyzed using unpaired t-test. All bars represent mean $\pm$ SEM ($P < 0.05$); MI: myocardial infarction

6.6.4 Discussion

Strong published evidence demonstrated that the occurrence of AKI is frequently common in patients hospitalized with MI and identified as an strong risk factor linked to a substantial increase in-hospital and long term mortality rate (Kosaki, Wakabayashi et al. 2021). AKI pathogenesis is multifactorial and complex. Mitochondrial dysfunction has been shown to be a major player implicated in worsening kidney diseases development and progression (Bhatia, Capili et al. 2020). In this chapter, we aimed at assessing the impact of MI on kidney mitochondrial function as early as 4 and 7 days.

Our findings reveled no significant alteration in mitochondria respiration rate in

term of complex I, II, III, and IV-dependent respiration in both cortex and medulla tissues at early stage post-MI in the presence and absence of NR treatment. Additionally, citrate synthase activity was used as a marker of mitochondrial mass and showed no significant change in the kidney cortex at day 4 and 7 post-MI.

Mitochondrial dysfunction has been well-documented in the multiple different animal model of AKI. In that regard, following kidney ischemia reperfusion injury, damaged mitochondria and enhanced mitochondrial permeability-associated necrosis have been reported and shown to be proportionally correlated with worsening AKI pathogenesis (Duann, Lianos et al. 2016). Furthermore, a decrease in mitochondrial mass, mtDNA depletion, and decline in cytochrome C activity have been observed following the exposure to nephrotoxins such as cisplatin (Mukhopadhyay, Horváth et al. 2012). In our study we assessed mitochondrial function in the kidneys at early stage post-MI. Four and seven days following MI could be a short period to observe mitochondrial dysfunction, but we chose this time period because we focus on type I CRS where **acute** MI develops AKI. Furthermore, our disease model is characterized by indirect injury to the kidneys mediated by MI, which could be not strong enough to initiate mitochondrial dysfunction and observe a protective effects of NR treatment at this early stage.

Conclusion and Future Direction

Our findings indicate no impact of NR treatment on mitochondrial respiration rate in both cortex and medulla tissues of mice at early stage post-MI. Further experiments aim at assessing the chronic impact of MI and NR on kidney mitochondrial function are warranted.

7 GENERAL DISCUSSION OF THESIS RESULTS

CVDs, mainly acute MI is one of the leading causes of death in both developing and developed countries. Epidemiological studies revealed that around three million deaths annually are attributed to MI (Alaour, Liew et al. 2018, Barberi and van den Hondel 2018, Nascimento, Brant et al. 2019). Although MI outcomes have substantially improved due to the widely used percutaneous coronary interventions, MI-induced AKI is still in the rise and negatively affects short and long terms survival following MI (Wang, Hong et al. 2022). The bidirectional interrelationship between the heart and the kidneys are well-documented and studies showed that a dysfunction in one organ may lead to an injury in the second organ and this is called CRS (Rangaswami, Bhalla et al. 2019)

Multiple risk factors including CS and gender have been shown to accelerate the development and progression of AKI. Metabolic impairment following AKI has also been reported and notably characterized by a decrease in total NAD levels. (Hershberger, Martin et al. 2017, Mehr and Parikh 2017, Morevati, Fang et al. 2022). The renoprotective role of NAD has been indicated to be mediated by the regulation of NAD⁺-dependent enzymes activity such as sirtuins (SIRTs) and PARPs. For instance, in the setting of non-MI induced AKI, SIRT-3 mRNA levels markedly decreased, leading to increased ROS score and enhanced tubule-interstitial inflammation (Hui, Zuo et al. 2015). Additionally, in the setting of renal ischemia, PARP-1 overactivation depletes NAD stores and ATP levels, fueling therefore inflammation, cell death and subsequently kidney damage (Devalaraja-Narashimha, Singaravelu, & Padanilam, 2005, (Wang, Han et al. 2018). Until now, the precise impact of sex hormones on type I CRS in the setting of MI and in the presence and absence of CS remained to be determined in experimentally controlled condition as in preclinical models. Additionally, the potential promising effect of NAD boosting therapies in this disease

model was yet to be fully understood. Therefore, we aimed at assessing the impact of CS on type I CRS in the setting of MI in male and female C57BL6/J mice and assessing the role of female sex hormones in this syndrome. Furthermore, we aimed at evaluating the potential impact of NR, a substrate for NMRK1-2, an NAD⁺ biosynthetic enzyme, on MI-induced AKI.

First, we assessed the impact of CS on MI-induced AKI in 5 months old C57BL6/J male and female mice. In this study mice of both sexes were exposed to two 90-min sessions daily (10 cigarettes/session), 7 days/week either for 3 weeks or for 2 weeks before inducing MI, then one additional week. Our findings showed that both male and female mice were prone to develop AKI following MI with female mice being more protected. CS exacerbated MI-induced AKI in both genders through enhancing cell death and ROS score, decreasing NAD levels and altering NAD⁺ biosynthetic and dependent enzymes. In that regard, it has been reported that depending on the intensity and duration of CS, the beneficial effect of endogenous estrogen could be partially or completely lost. CS has been shown to cancel the beneficial impact of estrogen on lipid metabolism and the cardiovascular system through decreasing the level of high-density lipoprotein-cholesterol (Michnovicz, Hershcopf et al. 1989, Ruan and Mueck 2015). Furthermore, CS enhances ROS production, induces fibrosis and inflammation all these proatherogenic effects double the 10-year risk of fatal events in smokers when compared to non-smokers (Gallucci, Tartarone et al. 2020).

In order to reveal if the observed protection in female mice is mediated, at least partially, by female sex hormones, bilateral ovariectomy was performed in C57BL6/J female mice. Our findings revealed exacerbated MI-induced AKI following OVX. This deterioration has been characterized by aggravated kidney dysfunction, enhanced kidney morphological alteration, heightened pro-inflammatory cytokine release, and altered NAD biosynthetic enzymes. Our findings are in accordance with the well-known protective effect of endogenous estrogen on the kidneys. A study performed by

Kummer et al. demonstrated that estrogen receptor- α , expressed in podocytes, exerts protective impact on experimentally induced cell death (Kummer, Jeruschke et al. 2011). Furthermore, Kan et al. reported that tubular injury and immune cells infiltration mainly macrophages markedly increased in female mice post estrogen deprivation (Kang, Lee et al. 2014). Additionally, it has been indicated that treating salt sensitive aging dahl rats with 17β ethinyl estradiol decreased kidney fibrosis and glomerulosclerosis (Maric, Sandberg et al. 2004, Salminen, Kaarniranta et al. 2013). Furthermore, in a rat model of gentamycin induced-AKI, ovariectomy led to a decrease in kidney ER α levels, mainly in PT, resulting consequently in increased serum kidney injury markers including KIM-1 and cystatin C (Abd El-Lateef, El-Sayed et al. 2019). Moreover, the administration of estrogen suppressed endothelin-1 deteriorating effects on the kidney. Endothelin-1 is widely known to exacerbate AKI and the transition to CKD (Gohar, Daugherty et al. 2020). In the uninephrectomized rat model of IR/I, estrogen has been observed to exert a renoprotective effects mediated by the activation of PI3K/Akt pathway, resulting subsequently in increased endothelial nitric oxide synthase in the affected kidneys (Satake, Takaoka et al. 2008). Another proposed protective mechanism of estrogen is that the activation of GPR30 protected the kidney from IRI through regulating NO production in renal interlobular artery smooth muscle and endothelial cells and enhancing glomerular endothelial barrier function (Chang, Han et al. 2019). Interestingly, stimulation of NAD metabolism could also be involved in the protective effect of E2. Indeed, E2 has been shown to modulate the expression of NAD⁺ dependent and biosynthetic enzymes such as SIRT-1 and NAMPT. In that regard, Shen et al. reported that E2 increased SIRT-1 expression levels in response to prolonged ROS overactivation, leading to decreased cell death through the inhibition of caspase 3 (Shen, Ding et al. 2014).

In order to correct the metabolic impairment characterized by decreased NAD levels and altered NAD⁺ dependent enzymes, 3 months old C57BL6/J male mice were treated with NR, an NAD precursor for either 4 or 7 days post-MI, (400 mg/kg/body

weight, IP injection, 30 min after the induction of MI). Interestingly, our data revealed no significant change in NAD levels in the kidneys of 2 months old C57BL/J males at day 4 and 7 post-MI on the contrary to what we had observed previously (Habeichi, Mroueh et al. 2020, Habeichi, Ghali et al. 2023). We hypothesize that the observed difference in NAD levels following MI might be due to the age factor. In the first two studies we used 5 months old C57BL6/J mice (middle aged mice) whereas in this study we used 3 months old (young aged mice) C57BL6/J mice. In that regard, a study performed to assess NAD⁺ levels in hippocampus showed a gradual decrease in NAD⁺ levels between 3 months and 6 months old mice. Whereas no further reduction in NAD⁺ levels was observed between 6 months (middle aged mice) and 12 months (old aged mice) (Stein and Imai 2014). Additionally, a clinical study done on males and females showed a 60% reduction in skin NAD⁺ levels in middle aged participants when compared to young subjects (Massudi, Grant et al. 2012). Further experiment might be needed to quantify NAD levels in the kidneys of aged mice compared to young mice to know the exact impact of age on the kidneys in this disease model.

Of note, in our three studies we worked on C57BL6/J mice, a strain that has a naturally occurring deletion in the gene encoding nicotinamide nucleotide transhydrogenase (**Nnt**) gene, resulting eventually in a complete loss in the Nnt protein. It has been shown that NNT protein is a redox driven proton pump that is implicated in the reduction of NADP⁺ via the action of nicotinamide dinucleotide (**NADH**) and the conversion of NADH to NAD⁺. Along this line, it has been documented that NNT is a strong generator of NADPH that is needed by glutathione reductase to reduce glutathione in order to maintain physiological ROS score and prevent ROS burst (Hoek and Rydström 1988, Newgard and McGarry 1995). The C57BL6/J strain, therefore, exhibited impaired ATP production and increased ROS levels (Ripoll, Meadows et al. 2012). This could be one of the limitation of our studies that affects NAD levels in the kidneys following MI. Further experiments aim at assessing the impact of MI on AKI in C57BL6/N compared to C57BL6/J mice might be

needed in order to better understand the impact of the *Nnt* mutation on this disease model.

Despite the apparent lack of renal NAD deficiency after MI in young mice, the administration of NR still proved to be beneficial and actually significantly increased NAD levels at both time points after MI. The observed increase in NAD levels in MI mice following NR treatment seems to be mediated by an upregulation in NAMPT mRNA expression levels, an NAD⁺ biosynthetic enzyme. NR mediated increased NAD levels resulted in a decrease in kidney morphological alteration and cell death and improved kidney function. It has been reported that NAD is a major source of ATP and is a substrate for multiple enzymes such as PARP-1, an NAD⁺ dependent enzyme implicated in DNA repair. A study performed on a mouse model of ischemic brain injury showed that NAMPT overexpression had a neuroprotective effect through decreasing cell death, which is in line with our study, although the studied tissue was different. Furthermore, a study performed on two months old male Wistar rats of renal I/RI induced AKI, showed a significant decreased in NAD⁺ levels (Morevati, Egstrand et al. 2021). In this study, prophylactic administration of NR (500mg/kg/day, oral gavage, 2 weeks before the surgery until day one post-surgery) resulted in restoring renal NAD levels. On contrary, our findings revealed no significant change in NAD levels in the kidneys of 2 month old C57BL6/J male mice. The discrepancies could be due to the different rodent types, different disease model, and different mode of NR administration. In our study we characterized AKI in the setting of MI which is indirect injury to the kidneys, whereas in Morevati, Egstrand et al. study, authors used an animal model of renal I/RI which is a direct injury that can lead to more severe energy loss in the kidneys. Furthermore, in a 2 months old C57BL male mouse model of LPS-induced AKI, a significant decrease in NAD levels was observed. Prophylactic treatment with NMN (500mg/kg) for seven days before the administration of LPS protected NAD levels, decrease apoptosis, oxidative stress, and kidney damage (He, Gao et al. 2022). One limitation of this study is that authors did not specify the genetic background

which could affect the observed findings. By comparison to these prophylactic approaches, our approach based on administration of NR after injury brings new promising perspectives on the translational potential of this molecule into clinical application. Additionally, the mode of administration selected was expected to bypass high degradation rate of NR in the intestinal tract, that accelerates pharmacokinetics of NR delivery to the tissues, which could be a key factor for treating acute model such as the MI model [20].

Importantly, we would have expected to observe an upregulation in NMRK-1 levels, an NAD⁺ biosynthetic enzyme that uses NR as a substrate to generate NAD, following NR treatment. Our findings, however, revealed an upregulation in NAMPT mRNA levels, an NAD⁺ biosynthetic enzyme that uses NAM as a substrate to generate NAD. The potential hypothesis behind this interesting finding is that we may have an upregulation in purine nucleoside phosphorylase (PNP) enzyme that is responsible for the conversion of NR to NAM. Further experiments therefore, aim at assessing the expression level of PNP and NAM levels are warranted.

Conclusion

Our study showed that both sexes are prone to develop kidney damage at early stage post-MI in the presence and absence of CS, with premenopausal female mice being more protected against certain aspects at least during an early stage post-MI. OVX aggravated kidney dysfunction in female mice post-MI through increasing the pro-inflammatory response, exacerbating kidney morphological alterations and hence global injury level, suggesting a protective effect of ovaries-derived estrogen on the kidneys. Although the protective role of estrogen in CVDs and kidney diseases have been extensively- studied, progesterone can also be a player in the observed protection in MI female mice and this require further investigations. NR treatment improves kidney function and attenuates kidney morphological alterations and cell death in MI male mice through increasing NAD levels and upregulating NAMPT mRNA

expression levels in the kidneys

Future Directions

Regarding the first study, we aim at highlighting the impact of ovariectomy on MI female mice following CS exposure in order to confirm the potential implication of endogenous estrogen in the observed protection in MI female mice following CS exposure. The anti-estrogenic effect of CS exposure has been well documented. In that regard, it has been shown that chronic CS exposure affects either estrogen production or estrogen metabolism (Gu, Caporaso et al. 2013). It has been reported that CS exposure inhibits aromatase activity, the enzyme responsible for the conversion of androgen to estrogen, resulting therefore in a decrease in circulating estrogen level (Baron, Nichols et al. 2021). Additionally, it has been indicated that CS exposure induces CYP450 (CYP1A2 and CYP3A4) that are known to induce estrogen metabolism, resulting in increasing 2 hydroxyestrogen, an estrogen metabolite (Michnovicz, Hershcopf et al. 1989). Therefore, based on the strong published evidence, further investigations aim at assessing the expression levels of aromatase and CYP1A2 and CYP3A4 as well as measuring plasma estrogen level and urinary 2 hydroxyestrogen levels are warranted.

With respect to the second study, we aim at treating MI female mice in the presence and absence of OVX with NR, since MI female mice exhibited a significant upregulation in NMRK-1 mRNA levels, an NAD⁺ biosynthetic enzyme that has the ability to use NR as a substrate to generate NAD⁺, in the kidneys at day 7 post-MI. In addition, estrogen receptors ER α and ER β as well as the newly discovered GPR30 have been shown to be expressed in the kidneys and play protective role following kidney diseases. In that regard, ER α has been shown to be expressed in glomeruli, mesangial cells, podocyte and renal tubule and to exert a protective role following kidney diseases through decreasing inflammation, ROS production, and augmenting endothelial NO production (Satake, Takaoka et al. 2008, Wu, Chang et al. 2016, Yu,

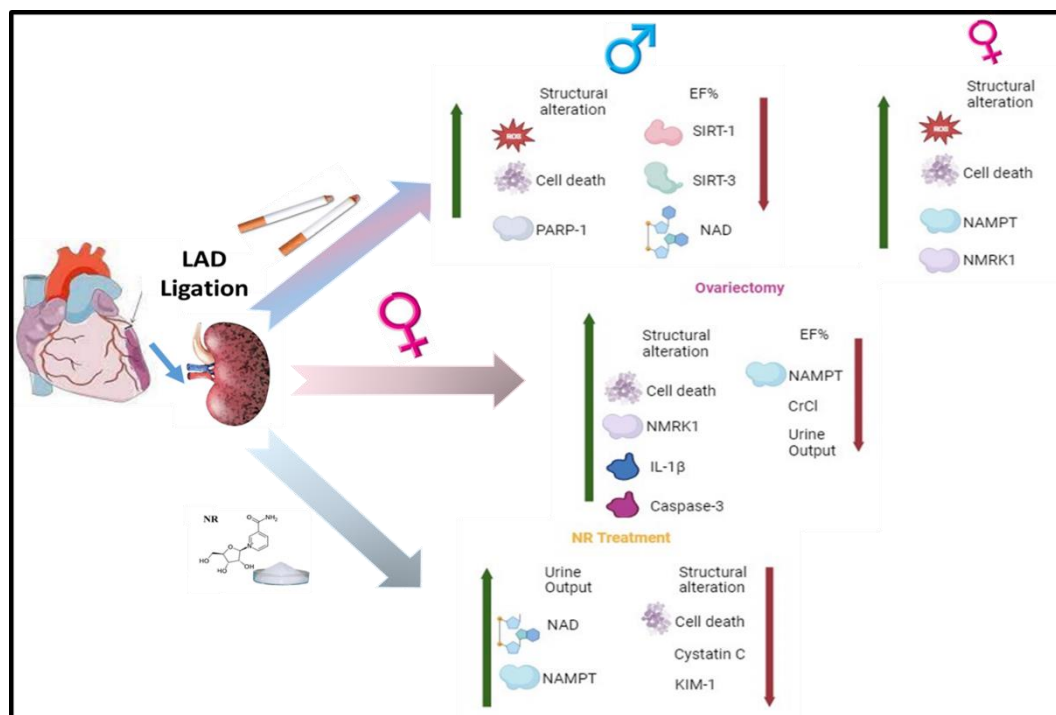
Zhao et al. 2020). ER β has been detected in proximal tubules and shown to exert protective effect following kidney diseases by inhibiting TGF- β -SMAD3 mediated kidney fibrosis (Cao, Su et al. 2023). As for GPR30, has been shown to be expressed in tubular cells mainly distal tubules and to a lower extent in proximal tubules (Rajkumar and Pluznick 2017). The protective role of GPR30 following kidney diseases has been documented and show to be mediated by the interaction with aldosterone and stimulation of the release of nitric oxide, and reduction in inflammation and oxidative stress (Singh, Nasci et al. 2022). Therefore, further experiments aim at assessing which ER is involved in the observed protection in female mice by using a knock out mouse model of ER α , ER β , and GPR30 are warranted.

Finally, for the third study, it has been demonstrated that NR is rapidly degraded in plasma 10% after 10 min and 60% after 1 hour through purine nucleoside phosphorylase and converted into NAM (Ratajczak, Joffraud et al. 2016, Kropotov, Kulikova et al. 2022). We aim therefore at evaluating the expression level of purine nucleoside phosphorylase to further confirm whether the protective effect of NR is mediated by the conversion of NR to NAM, subsequently increased NAD and upregulated NAMPT levels. In addition, we aim at using a kidney NAMPT knock out mouse model to know if the observed increase in NAD level at day 4 and 7 post-MI is mediated through this pathway. NR is also known to have an anti-inflammatory effects. In this regard a study done by Zhou et al. indicated that an accelerating dose of NR (500 to 2000 mg daily) for 5-9 days decreased IL1 β and IL-18, pro-inflammatory cytokines, in PBMCs (Zhou, Wang et al. 2020). Our findings are in consistent with previous publications demonstrating the significant increase in plasma IL-4, an anti-inflammatory cytokine, 4 and 7 days post-MI. IL-4 is known to enhances the differentiation of the pro-inflammatory macrophages M1 to the anti-inflammatory macrophages M2 (Smith, Tse et al. 2016, Yao, Xu et al. 2019). Further experiments evaluating the impact of NR administration on the systemic and local inflammatory response are needed. Recent publications shed the light on the role of that the

reduced form of NR (NRH) in boosting NAD levels. It has been demonstrated that NRH is more potent and faster NAD⁺ precursor than NR. Importantly, NRH uses different steps and enzymes to produce NAD⁺, thus highlighting a new NRK1-independent pathway for NAD⁺ synthesis. Furthermore, NRH is orally bioavailable and not degraded in plasma in mice and prevents cisplatin-induced acute kidney injury (Giroud-Gerbetant, Joffraud et al. 2019). Therefore, assessing the impact (NRH) on MI-induced AKI and the molecular pathway that are involved are of utmost importance.

Clinical Perspective

The identification of sex-dependent molecular pathways involved in MI-mediated AKI renders promising targets to mitigate kidney damage post-MI. Multiple clinical studies have been conducting to assess the role of NR in multiple diseases including kidney diseases after being clinically tested and showed no serious side effects. Our preclinical study has shed the light on the protective effects of NR in type I CS in the setting of MI to move from bench to bedside, given that NR was administrated following the LAD ligation, mimicking therefore the clinical setting.



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

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Abstract

Aims. Myocardial infarction (MI) is a major cause of death. Nicotinamide adenine dinucleotide (NAD⁺) is a coenzyme in oxidative phosphorylation and substrate of sirtuins and poly-ADP ribose polymerases, enzymes critical for cardiac remodeling post-MI. Decreased NAD⁺ 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⁺ 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 months old male mice. NR was administered daily (1 μ mole/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⁺ and overall survival of 61%. NR restored NAD⁺ 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 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.

1. INTRODUCTION

Despite significant advancement 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^+) equilibrium in the failing heart. NAD^+ 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^+ is an essential substrate of crucial enzymes like sirtuin (SIRT) deacetylases and poly-ADP ribose polymerases (PARPs). SIRT6s 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 SIRT6s 1 and 3 [7, 6].

In the normal physiological state, the consumption of NAD^+ by signaling pathways is counterbalanced by NAD^+ biosynthetic pathways relying on NAD^+ precursors (vitamins B3) and dietary tryptophan. However, in pathological conditions, insufficient NAD^+ precursors are not able to sustain cardiac NAD^+ synthesis, hence the necessity of NAD^+ precursor supplementation to correct this defect. Altered NAD homeostasis has been reported in several models of heart disease including ischemia reperfusion [8]. However, little is known about NAD homeostasis in the context of MI. Here, we show that NAD^+ 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^+ synthesis. NMRK2 kinase is involved in the NAD^+ 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.

2. MATERIALS AND METHODS

2.1 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 μ mole/g of body weight) was administered intraperitoneally (i.p.) right after sham operation or left anterior descending (LAD) coronary artery ligation (starting day 0) and continued for 4 or 7 days thereafter. In the sham and MI groups treated with vehicle, a single daily injection of PBS (200 μ l/day) was administered i.p. right after sham operation or LAD coronary artery ligation (starting day 0) and continued for 4 or 7 days thereafter. For experimental procedures, mice are

anesthetized with 2-4% 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.

2.2 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 min prior to surgery, tramadol (0.05-0.1 mg/kg i.p.) and isoflurane (2-3% in oxygen) inhalation were given to induce analgesia and general anesthesia. Orotracheal intubation was performed to maintain normal respiratory rate by placing a needle into the trachea and connecting 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 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 hours after surgery. Immediately following successful LAD coronary artery ligation, the chest was closed, and the mice placed on a warm pad for recovery. The same procedure was applied to the SHAM group but without LAD coronary artery ligation. Once they groomed freely, MI mice were caged back individually and monitored on a daily basis for full recovery.

2.3 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 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. Mean calculation of cardiac parameters was measured at baseline, at day 1 following the MI induction surgery, and before sacrifice.

2.4 Myocardial Strain Measurements

Using speckle-tracking based strain analysis of 2D gray scale echocardiographic images acquired from the parasternal long-axis view, strain and strain rate were quantified in the longitudinal, radial, and circumferential axes; in accordance with myocardial fiber orientation at varying levels of the LV wall, longitudinal strain is most representative of myocardial shortening at the level of the endocardium, whereas radial and circumferential strains are more reflective of shortening at the level of the myocardium.

2.5 NAD Extraction and Quantification

We used the NAD cycling colorimetric assay which detects both NAD⁺ 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 10mM pH 7.1) as previously described [12]. Extracts were cooled on ice for 20 min and centrifuged 10 min at 13000 g. Extracts were diluted 1:20. For each sample, 25 µL of NADt extract was quantified in duplicate by addition of 100µL of reaction buffer (600 mM ethanol, 0.5 mM 3-(4,5-dimethylthiazol-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 seconds for 40 minutes) 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.

2.6 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 $R=2\Delta Ct$ (Ref Cp-target Cp), with HPRT used as a reference. Primers were designed using the NCBI Primer Blast Software.

2.7 Western Blot Analysis

Proteins were homogenized in a lysis buffer (RIPA buffer) containing protease inhibitors. 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 (1:10000) 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 1. Band densities of the target proteins were measured using ImageJ and normalized to the density of the total protein bands.

2.8 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 [13]. Respiration solution was made from CaK₂EGTA (100mM) diluted to 2.77 mM, K₂EGTA (100mM) diluted to 7.23 mM, and 6.56 mM MgCl₂ to obtain 100 nM free Ca²⁺ and 1

mM free Mg^{2+} 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. Rates of respiration are given as nmoles O_2 /min/mg of cardiac tissue dry weight.

2.9 Enzymatic Activities

Frozen tissue samples were weighed and homogenized with a 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 [14]. Activities of enzymes were determined by spectrophotometric assays. For citrate synthase (CS), approximately 0.5 μ g 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, 0.5mM 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 μ g of protein in 1 mL of phosphate buffer (50 mM K_2HPO_4 (pH 7.4)) containing 50 μ M 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.

2.10 Enzyme-Linked Immunosorbent Assay (ELISA)

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

2.11 Sirius 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 Sirius Red to visualize fibrosis. Images were acquired on an Olympus CX41 microscope (total scan of 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 fibrotic area to the total area of myocardium.

2.12 Statistics

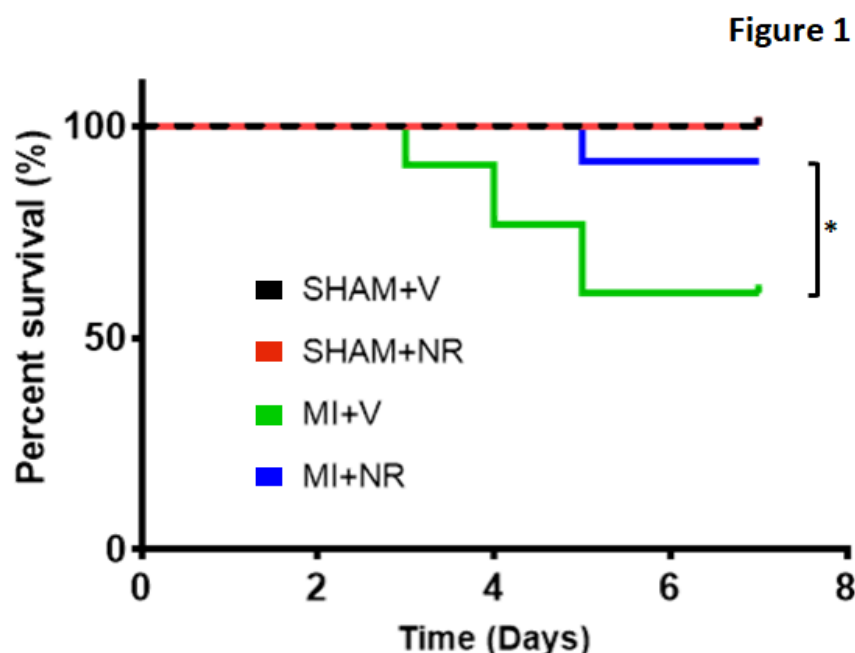
We performed statistical analyses using Prism 8 software (<https://www.graphpad.com>). For survival curve analysis, we used the Gehan-Breslow-Wilcoxon test that is adapted to short term survival studies with early mortality. We used unpaired two-tailed Student's t-test for comparison of two groups or one-way analysis of variance (ANOVA) for 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 $P < 0.05$.

3. RESULTS

3.1 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 started dropping as soon as D3 post-MI to reach a survival percentage of 61% at 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 (Figure 1).

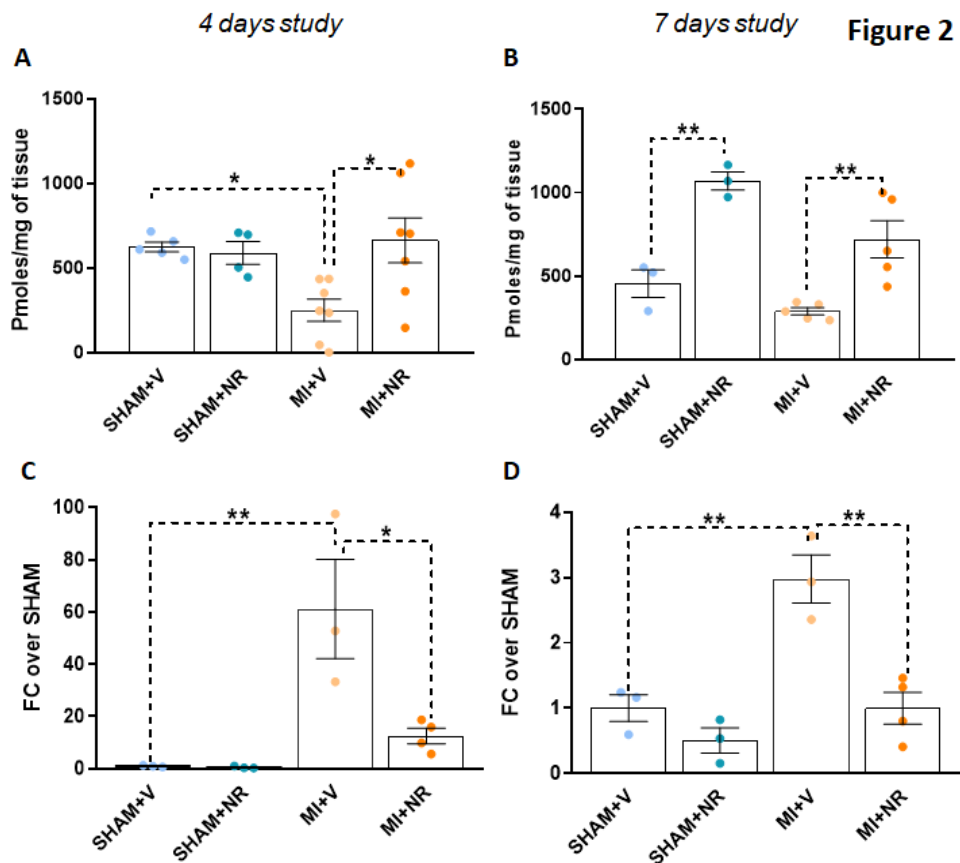


3.2 NAD levels and NMRK2 pathway are affected in the failing heart of MI mice

We assessed the consequence of MI on 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 (Figure 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 (Figure 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 (starting Day 0) and for 4 days thereafter. Consistent with our hypothesis,

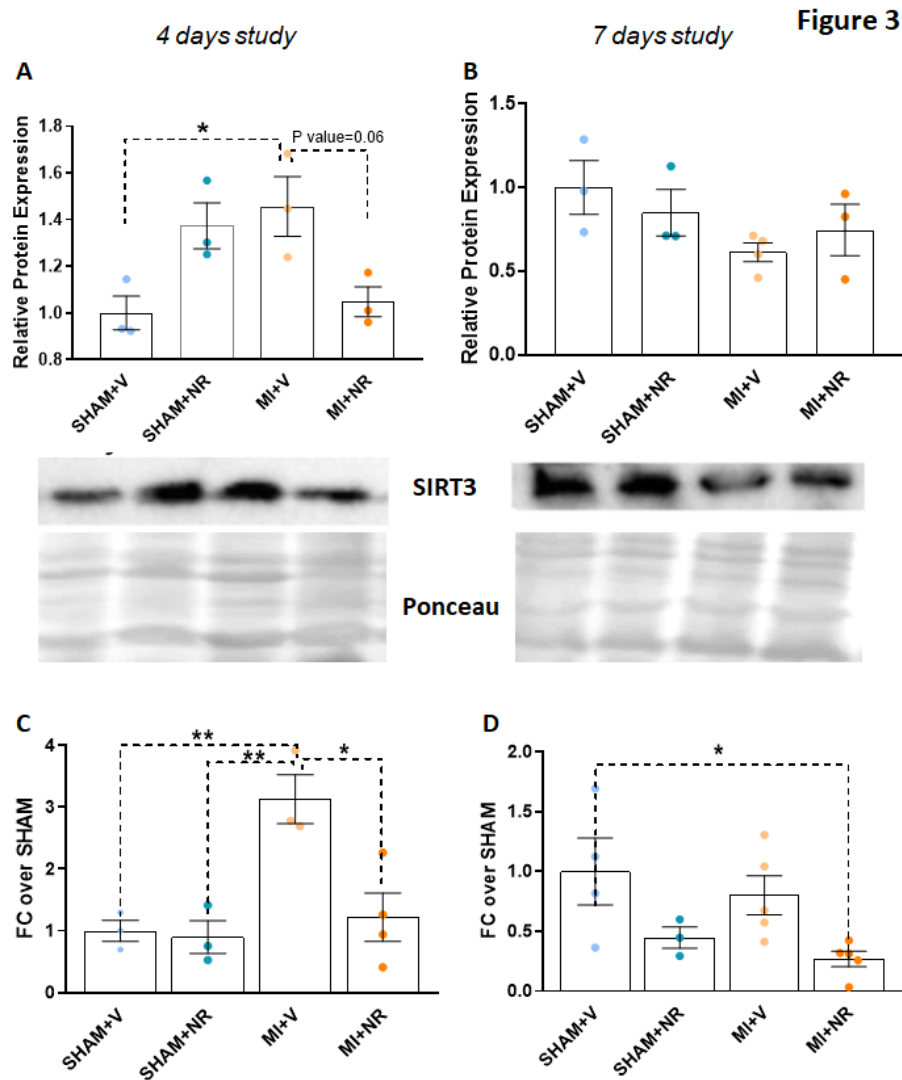
we found that myocardial NAD levels were preserved by NR injection in the MI group, with NAD levels comparable to those in the SHAM group (Figure 2A).

At day 7, myocardial NAD pools dropped by 53% in the vehicle treated-MI group compared to the vehicle treated-SHAM mice (Figure 2B). The mRNA expression levels of *Nmrk2* were significantly increased by 3-fold in the MI group treated with vehicle compared to the SHAM group (Figure 2D), although this was significantly less than at day 4. Intraperitoneal injection of NR right after LAD coronary artery ligation (starting Day 0) and for 7 days thereafter, resulted in significantly increasing myocardial NAD levels compared to the vehicle-treated MI group (Figure 2B). However, the expression level of NAMPT, the enzyme converting nicotinamide (NAM) to nicotinamide mononucleotide (NMN), a direct precursor of NAD, was reduced after MI as reported, although this did not reach statistical significance (data not shown).



3.3 NAD consuming enzymes are affected post-MI

The role of NAD is established as a main substrate of sirtuins (SIRTs) and poly-ADP ribose polymerases (PARPs), enzymes involved in regulating metabolism and maintaining cellular homeostasis [15]. SIRT3 protein levels tended to increase at 4 days post-MI (Figure 3A). Interestingly, while NR increased SIRT3 levels in the SHAM animals, it prevented the increase in MI group. However, at day 7, SIRT3 protein levels did not show significant changes among groups (Figure 3B). Our data demonstrate a substantial increase (3-fold) in the myocardial mRNA expression level of *Parp1* in the vehicle treated MI group at 4 days post-surgery (Figure 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 one in the SHAM groups (Figure 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 (Figure 3D).



3.4 NR improves mitochondrial respiration rate in isolated cardiac fibers when given for 4 days post-MI

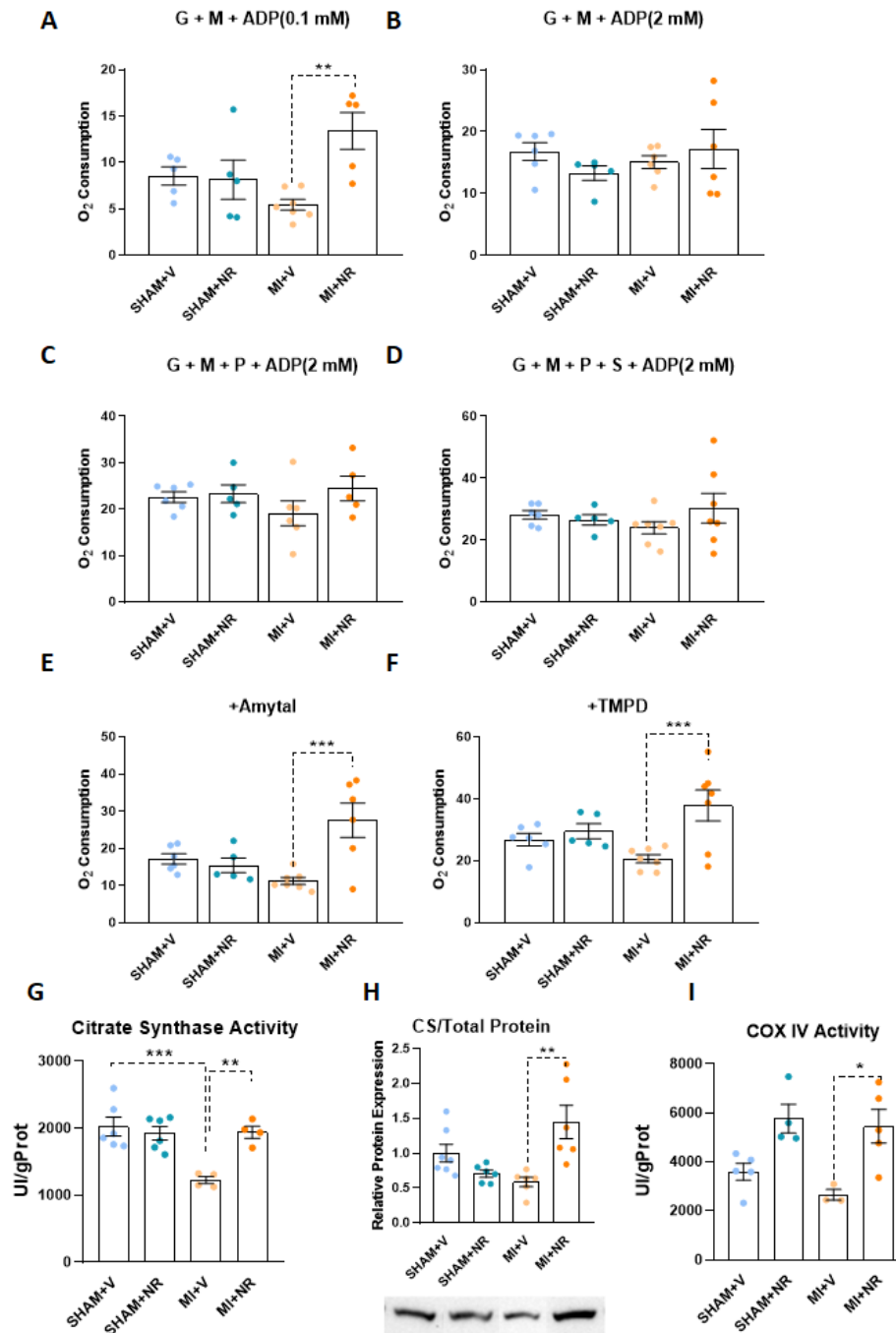
In order to know if the deregulation in NAD homeostasis observed in the MI group is associated with alterations of mitochondrial function, mitochondrial oxidative capacities were measured in permeabilized cardiac fibers from the left ventricle (Figure 4). We harvested tissue from the remote LV posterior 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 (Figure 4A). However, when increasing ADP to saturating levels (2 mM), respiration rates were not different between groups (Figure 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 (Figure 4B-D). However, the subsequent addition of amytal to inhibit complex I revealed a decrease of complex II dependent-respiration rate in the MI vehicle group and NR administration significantly restored this rate (Figure 4E). TMPD was added to measure maximal complex IV-dependent respiration, which was reduced in the vehicle-treated MI group compared to sham group and was increased in NR-treated MI group (Figure 4F).

3.5 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 [16]. CS enzymatic activity was reduced by 39.35% in vehicle-treated MI group. NR administration protected against this decline of CS activity in the MI group (Figure 4G). In addition, CS protein level showed a similar pattern as activity (Figure 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 (Figure 4I).

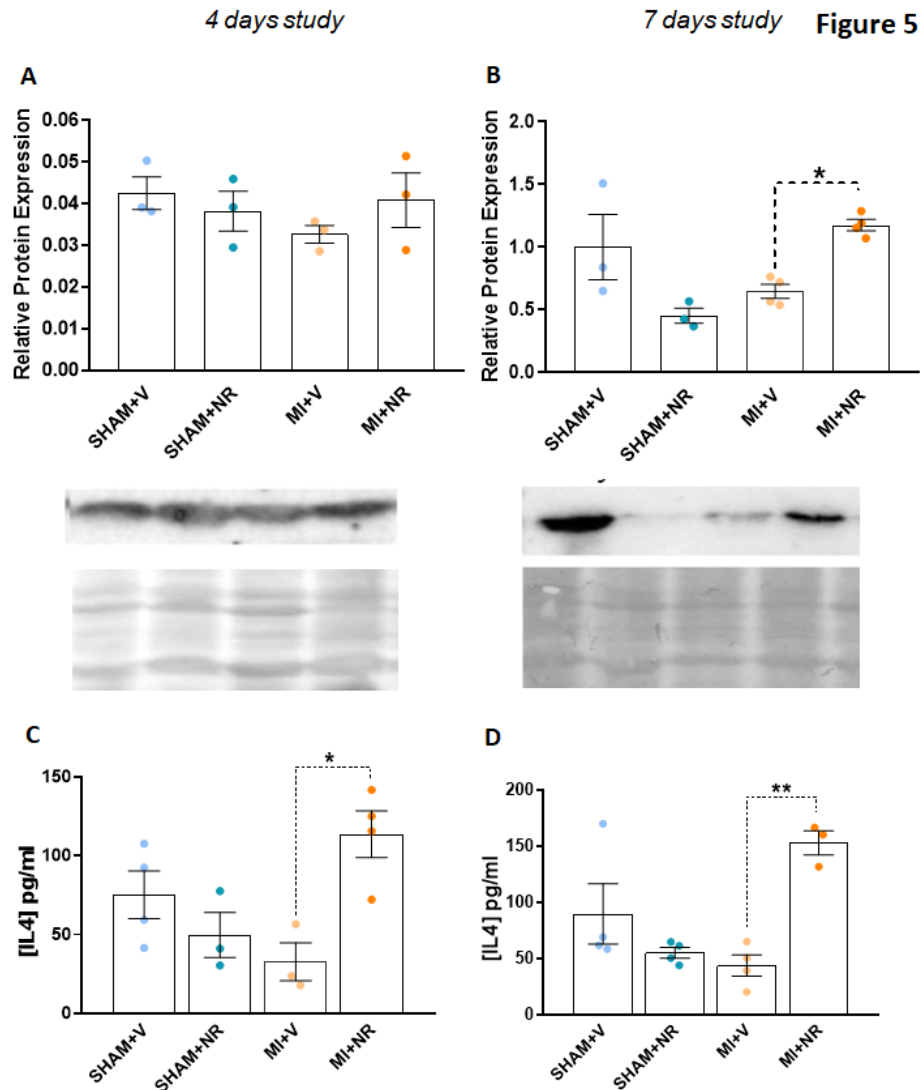
Figure 4



3.6 NR treatment affects plasma and myocardial level of IL-4 cytokine post-MI

Western blot analysis of myocardial levels of IL-4 showed a trend towards an increase at day 4 of NR treated MI group that was significantly amplified at day 7 post-MI (Figure 5A-B). In

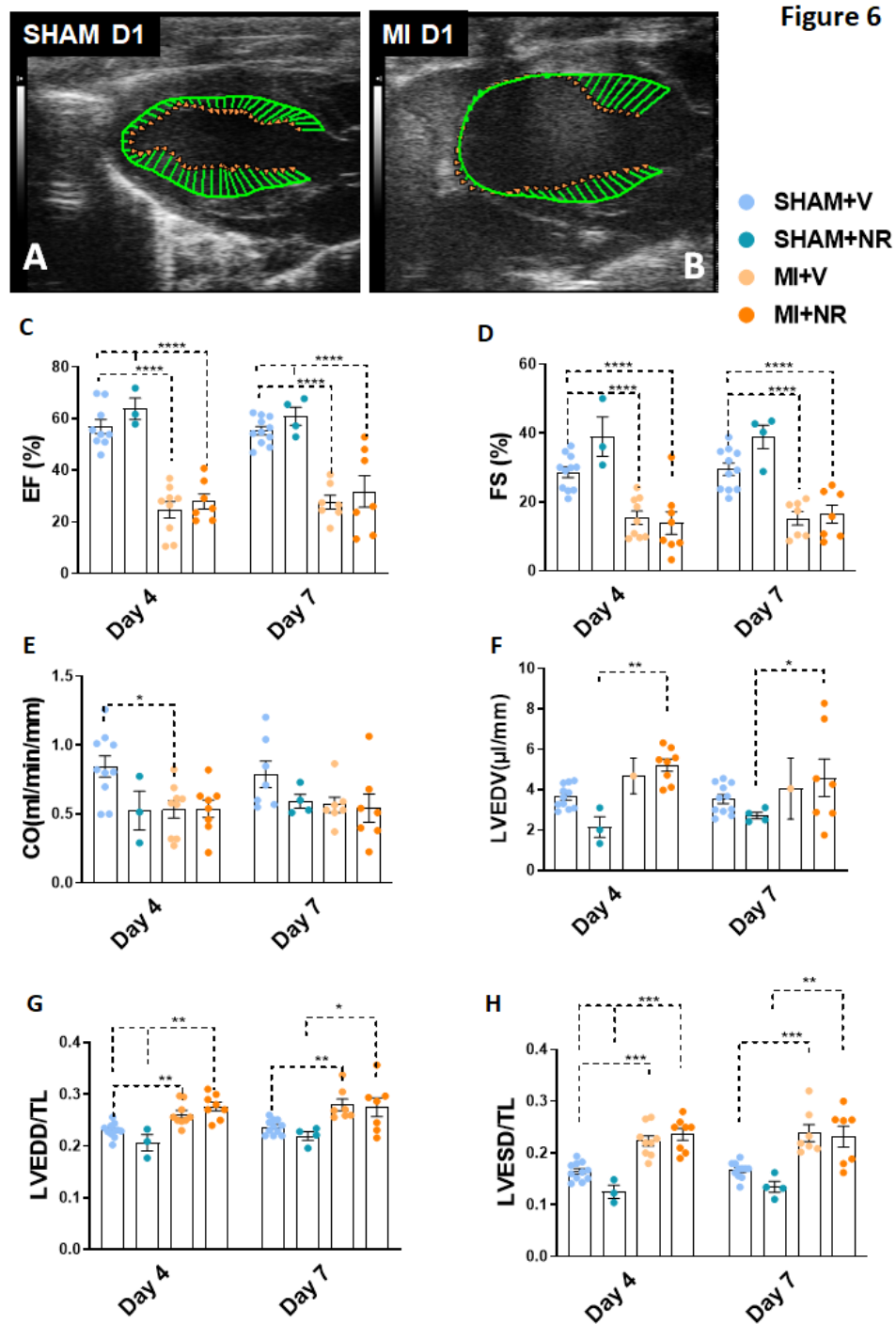
the plasma, a similar pattern was observed with NR administration robustly increasing IL-4 levels in the MI group at days 4 and 7 post-MI (Figure 5C-D).



3.7 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 (Figure 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 (Figure 6C-E). A similar decrease in all hemodynamic parameters (EF, FS and CO) was observed in both 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 (Figure 6F-H). NR treatment had no effect on cardiac fibrosis post-MI (Suppl. Figure I).

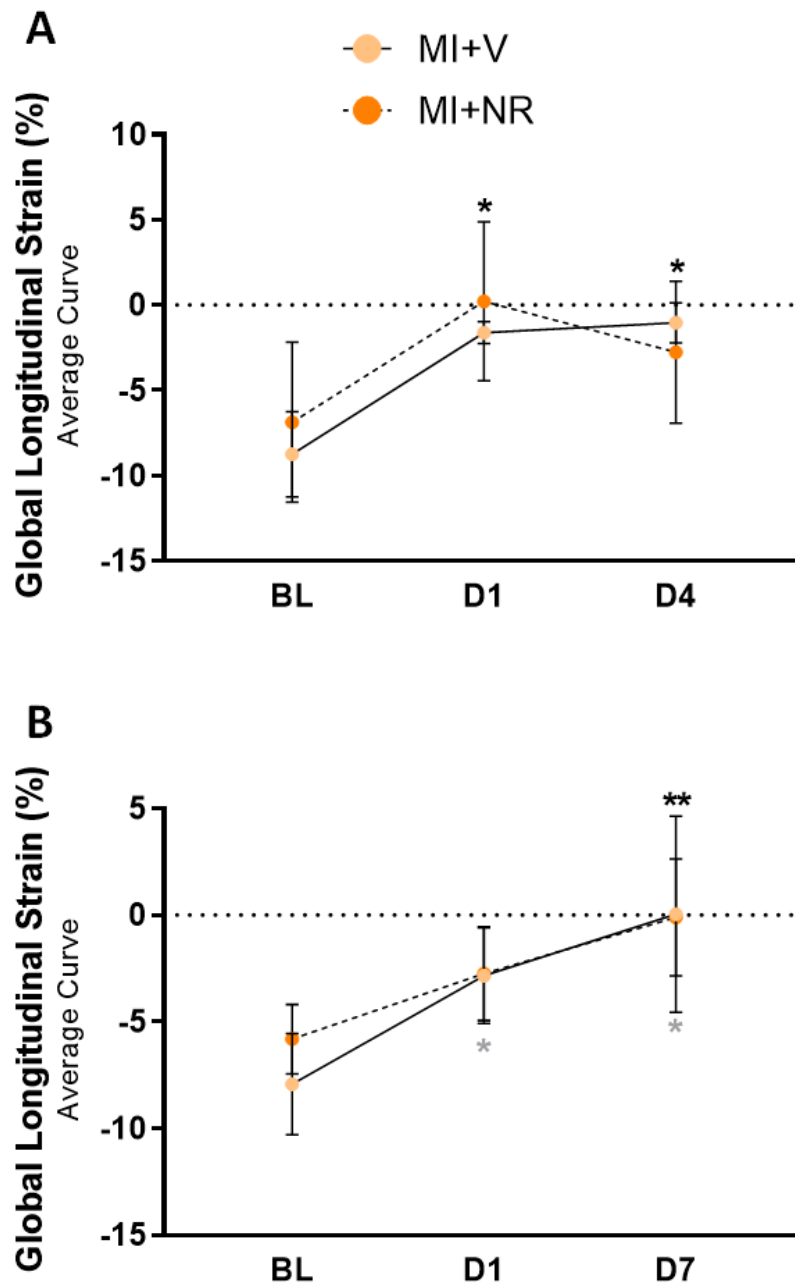


3.8 Global longitudinal strain is moderately normalized with NR treatment following LAD ligation

We assumed that myocardial strain would indicate cardiac dysfunction earlier and more consistently compared to standard M-mode measurements of EF and FS. We measured the

longitudinal myocardial strain at 3 time points (BL, D1 and D4/D7). At Day 1 following LAD ligation, a significant increase in global longitudinal strain was observed in the vehicle-treated-MI group and NR treated-MI group, in both studies (4 and 7 days following LAD coronary artery ligation) (Suppl. Figure II). As MI affects specific regions of the LV, longitudinal strain was analyzed in 6 sub-regions of the LV and expressed as a difference from respective baseline in the same region. Longitudinal strain increased in all regions of the heart in vehicle-treated MI group at days 4 and 7 (Figure 7 and Suppl. Figure III). There was a trend for NR to reduce and stabilize the strain between days 1 and 4 in several regions of the heart, notably in the anterior wall from the base to middle region and in the posterior wall from the middle to the apex level (Figure 7). A similar trend was observed at day 7 (Suppl. Figure III).

Figure 7



3.9 Supplementary Material

The three supplementary figures cited in the results section of this manuscript are provided in a separate supplementary file as following: 1) sinus red staining findings for fibrosis, 4 and 7 days post-MI (Supplementary Figure I); 2) Speckle-tracking derived longitudinal measurements of systolic function for myocardial deformation analysis, 4 and 7 days post-MI

(Supplementary Figure II); and 3) Speckle-tracking derived measurements of systolic function in posterior base, posterior mid and posterior apex regions, 7 days post-MI (Supplementary Figure III).

4. DISCUSSION

Several studies have shown 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 [17-21]. 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⁺ precursor and substrate of NMRK2, preserves myocardial NAD levels and mitochondrial respiration, and significantly increases survival after a large MI. Although ejection fraction and fibrosis deposition are not improved in this MI model, NR tended to reduce the increase in myocardial strain, which could contribute to subclinical improvement of cardiac function and reduction of wall rupture. The link between the increase of myocardial strain following MI and cardiac rupture has been reported in several studies: In patients who died from acute MI, wall rupture has been associated with infarct thinning and triggered by a physical strain [22]. Furthermore, due to sustained arterial hypertension, the strain in the infarcted zone may favor cardiac wall rupture [23-25].

The alteration of NAD homeostasis has been reported in several models of cardiac diseases including DCM [12, 26], pressure overload [27], and hypertension [28]. 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 formation of phosphoribosyl pyrophosphate. Thus, the shift from the NAMPT to the NMRK2 routes for NAD⁺ 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 [29], 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.

Mitochondrial dysfunction is a key feature following cardiac ischemic lesions [30]. 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⁺ extend beyond that of a coenzyme. NAD⁺ links cellular metabolism status to inflammation and immune response [20]. 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 [31]. The utility of our approach is supported by a recent study, which showed that NR supplementation in heart failure patients boasted NAD levels of peripheral blood mononuclear cells (PBMCs), thereby suppressing their inflammatory activation [32]. 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 [33].

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 large number of vehicle-treated MI animals died before day 7, which reduced statistical power in terms of functional analysis. A serial echocardiography between day 1 and day 7 could have given important insights into the evolution of cardiac strain developments with NR administration. Also, a higher number of mice is needed to discern difference at the level of myocardial strain. 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. However, a tendency towards a reduction in myocardial strain is visible with NR treatment at both day 4 and day 7 post MI. 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 preserve cellular homeostasis by correcting NAD levels following MI, restoring mitochondrial function, and improving overall survival. Future studies are warranted in order to discern the underlying mechanisms behind these interesting findings.

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AUTHOR DECLARATIONS SECTION

o Funding (information that explains whether and by whom the research was supported)

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o Conflicts of interest/Competing interests (include appropriate disclosures)

The authors have no conflict of interest or competing interest to disclose.

o Availability of data and material (data transparency)

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

o Code availability (software application or custom code)

Not applicable

o Authors' contributions

Cynthia Tannous: Conceptualization, Methodology, Investigation, Software, Writing- Reviewing and Editing, Original draft preparation. **Rana Ghali, Ahmed Karoui:** Methodology, Software, Data curation, Investigation, Resources. **Nada J. Habeichi, Ghadir Amin:** Methodology, Software, Investigation, Data Curation. **George W. Booz, Mathias Mericskay, Marwan Refaat:** Investigation, Validation, Methodology, Original draft preparation, Formal Data Analysis, Writing- Reviewing and Editing. **Fouad A. Zouein:** Conceptualization, Methodology, Writing- Reviewing and Editing, Funding acquisition, Project administration, Supervision.

o Ethics approval (include appropriate approvals or waivers)

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)

o Consent to participate (include appropriate statements)

Not applicable.

o Consent for publication (include appropriate statements)

All authors on this submission made substantial contributions to the conception and development of the manuscript and have approved the final submission.

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Table 1: Antibodies used for Western Blot analysis

Target Protein	Antibody Supplier, Catalog #	Dilution
Sirtuin 3 (SIRT3)	Abcam, ab86671	1:1000
Nicotinamide phosphoribosyltransferase (NAMPT)	Abcam, ab24149	1:500
Poly-ADP-Ribose-Polymerase-1 (PARP-1)	Abcam, ab32138	1:1000
Interleukin-4	Abcam, ab9728,	1:1000
Citrate Synthase	Abcam, ab96600	1:1000
OxPhos Complex IV subunit (COX IV)	Mitosciences, MS604	1:1000

Figure Legends

Figure 1. Effect of NR administration on survival post-MI. Kaplan Meir 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 and n=12 in the NR treated LAD-ligated group).

Figure 2. Quantification of NAD content in the heart of SHAM and MI mice and expression level of genes involved in NAD salvage and biosynthesis 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. (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 on *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: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Figure 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 vehicle treated SHAM group.

Figure 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. (A) glutamate and malate, (B) ADP, (C) pyruvate, (D) succinate, (E) amytal inhibitor of complex I and (F) TMPD and ascorbate were successively added to permeabilized myocardial fibers from the left ventricle (n=7 per group). 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_2 , oxygen consumption. (G) Citrate synthase activity, (H) Citrate synthase protein expression level and (I) COX activity in the left ventricle. Data are presented as means $\pm$ SEM. Statistical analysis: One Way ANOVA for independent samples; Tukey's multiple comparisons test: * $p\leq 0.05$, ** $p\leq 0.01$, *** $p\leq 0.001$.

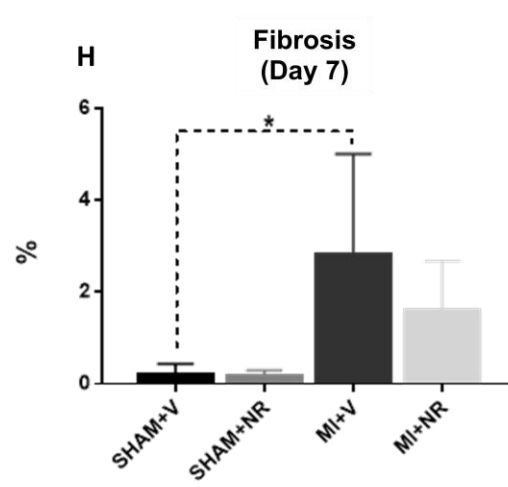
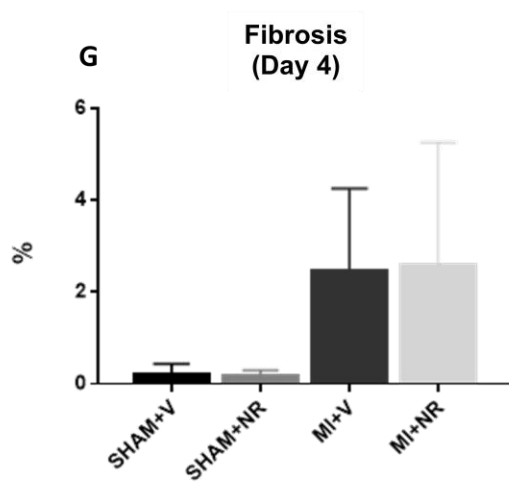
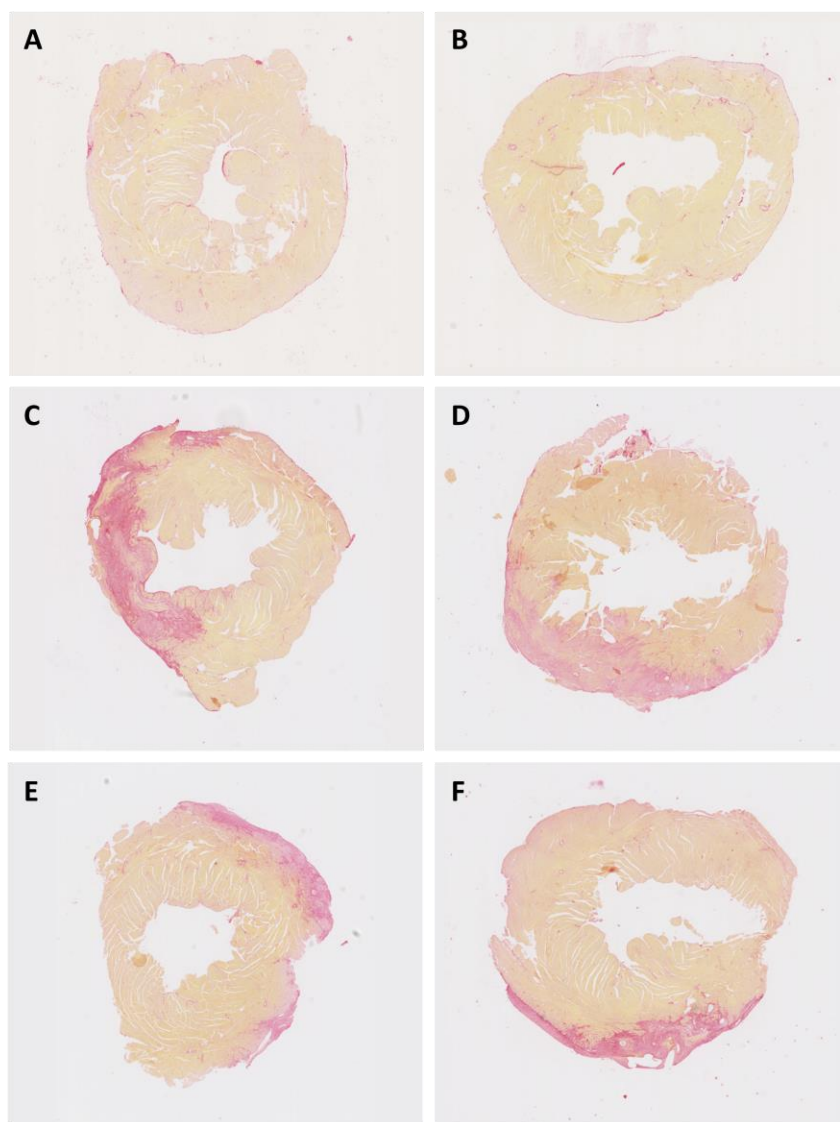
Figure 5. Plasma and myocardial levels of IL-4 post-MI. (A-B) Western Blot analysis of IL-4 in cardiac tissue (n=4 per group). **Quantitative ELISA:** Plasma protein concentrations of (C-D) IL-4 of SHAM and MI groups at 4 and 7 days post following NR treatment (n=4-5 per group). Data are presented as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

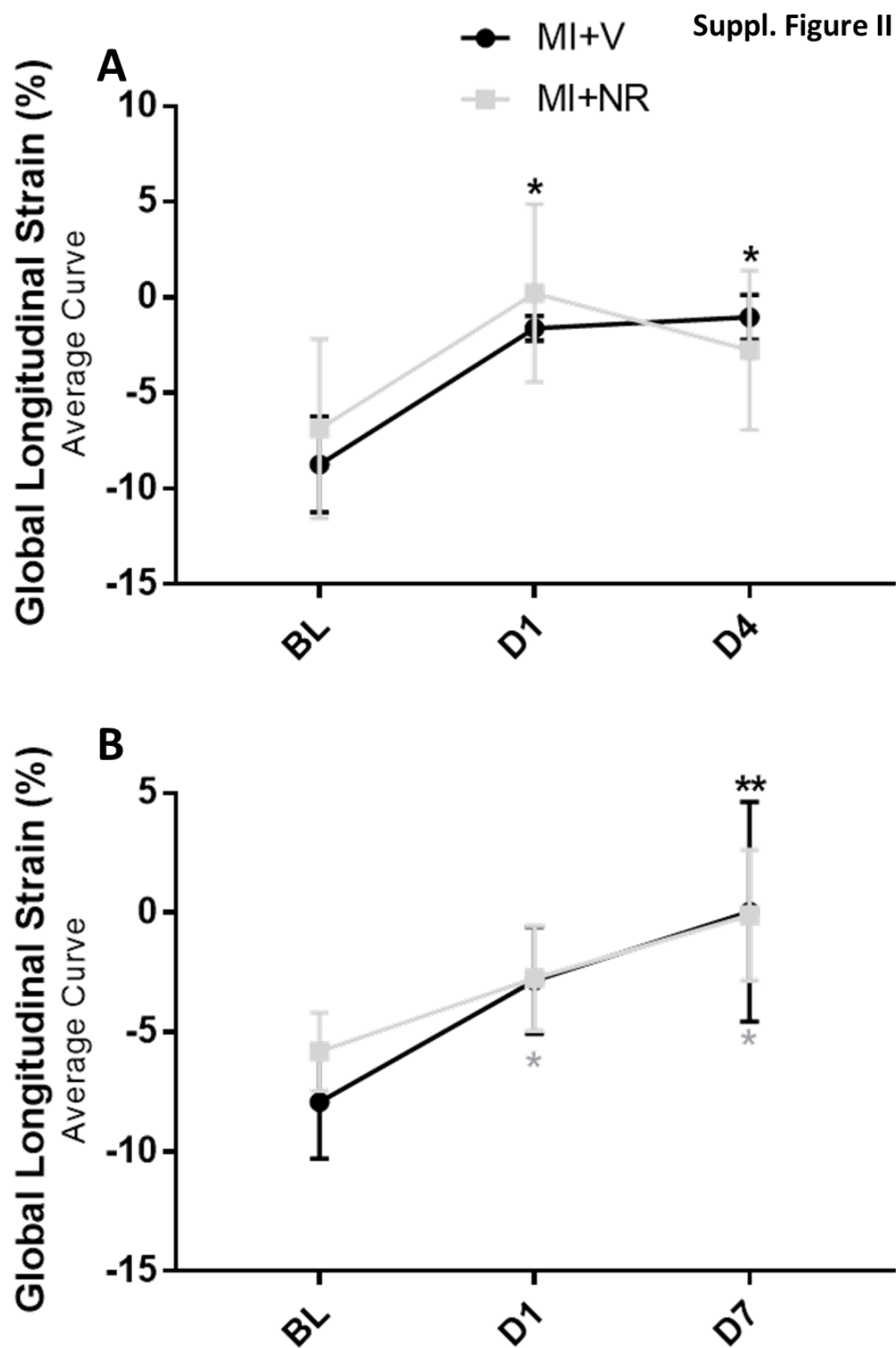
Figure 6. Quantification of cardiac function using B-mode and M-mode echocardiography of SHAM and MI mice, 4 and 7 days after surgery. (A-B) Representative echocardiogram images (B-Mode, parasternal Long Axis), 1 day after surgery (SHAM and MI). Cardiac dysfunction and left ventricle dilation takes place following LAD ligation. (C) Ejection fraction (EF), (D) Fractional Shortening (FS), (E) Cardiac Output (CO), (F) LV End-Diastolic Volume (LVEDV), (G) Left Ventricular End-Diastolic Diameter (LVEDD), (H) Left Ventricular End-Systolic Diameter (LVESD). 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: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Figure 7. Regional speckle-tracking–derived measurements of myocardial deformation 4 days post-MI. Speckle-tracking derived measurements of systolic function in posterior base, posterior mid and posterior apex regions (**A, C and E**) and anterior base, anterior mid and anterior apex (**B, D and F**) Data are expressed as a difference from respective baseline in the same region (n=5 per group). Data are presented as means $\pm$ SEM. Statistical analysis: Two Way ANOVA for independent samples; Tukey's multiple comparisons test: * $p \leq 0.05$, ** $p \leq 0.01$.

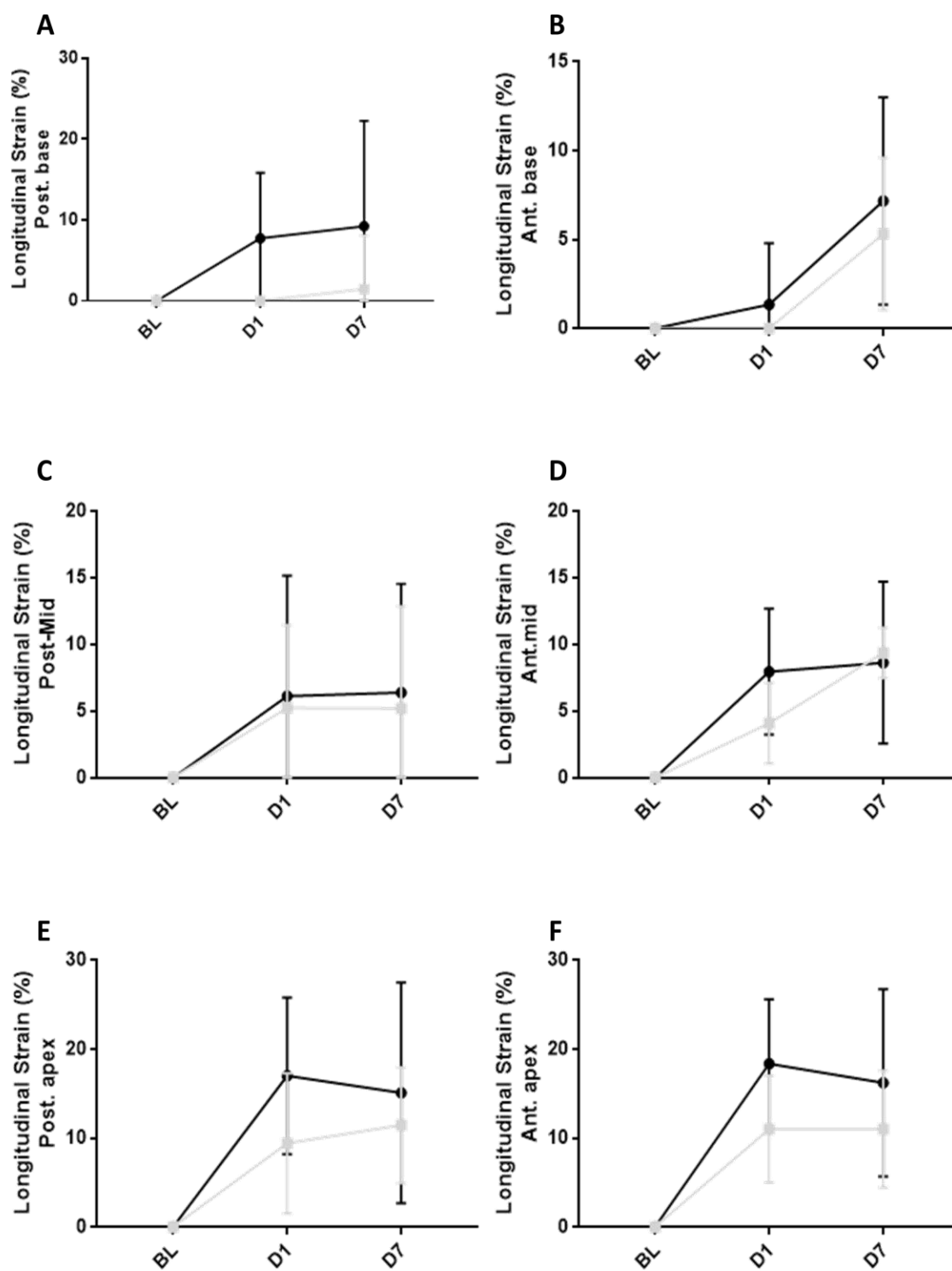
Suppl. Figure I





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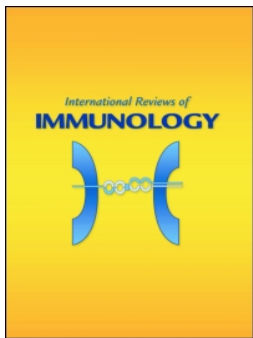
Suppl. Figure III



Suppl. Figure I. Sirius Red staining. Representative cross-section of Sirius Red stained hearts. Left panels: SHAM mice with vehicle, MI mice with vehicle during 4 days and MI mice with vehicle during 7 days (A, C, E). Right panels: SHAM mice with NR treatment, MI mice with NR treatment during 4 days and MI mice with NR treatment during 7 days (B, D, F) (n=3 per group). Statistical analysis: One Way ANOVA for independent samples. Tukey's multiple comparisons test: *p≤0.05.

Suppl. Figure II. Speckle-tracking–derived measurements of myocardial deformation. Speckle-tracking derived measurements of systolic function. **(A-B)** Myocardial strain in the longitudinal planes 4 and 7 days following surgery (n=5 per group). Data are presented as means ± SEM. Statistical analysis: Two Way ANOVA for independent samples. Tukey's multiple comparisons test: *p≤0.05, **p≤0.01.

Suppl. Figure III. Regional speckle-tracking–derived measurements of myocardial deformation 7 days post-MI. Speckle-tracking derived measurements of systolic function in posterior base, posterior mid and posterior apex regions **(A, C and E)** and anterior base, anterior mid and anterior apex **(B, D and F)** Data are expressed as a difference from respective baseline in the same region (n=5 per group). Data are presented as means ± SEM. Statistical analysis: Two Way ANOVA for independent samples. Tukey's multiple comparisons test.



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Insights into the modulation of the interferon response and NAD⁺ in the context of COVID-19

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ABSTRACT

The COVID-19 pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has resulted in dramatic worldwide mortality. Along with developing vaccines, the medical profession is exploring new strategies to curb this pandemic. A better understanding of the molecular consequences of SARS-CoV-2 cellular infection could lead to more effective and safer treatments. This review discusses the potential underlying impact of SARS-CoV-2 in modulating interferon (IFN) secretion and in causing mitochondrial NAD⁺ depletion that could be directly linked to COVID-19's deadly manifestations. What is known or surmised about an imbalanced innate immune response and mitochondrial dysfunction post-SARS-CoV-2 infection, and the potential benefits of well-timed IFN treatments and NAD⁺ boosting therapies in the context of the COVID-19 pandemic are discussed.

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Introduction

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), first emerged in Wuhan, China in December 2019 and rapidly spread as a pandemic, leading to increased global morbidity and high mortality [1, 2]. The clinical manifestations of COVID-19 range from an asymptomatic course to severe lethal pneumonia or acute respiratory distress syndrome (ARDS) [3]. Although the name suggests that the first and main target of SARS-CoV-2 is the lung, basic investigations and clinical data document multiple organ damage that includes the heart, blood vessels, and brain [2–4]. Clinical reports demonstrate that all age groups are susceptible to infection by SARS-CoV-2, but exhibit different degrees of symptoms, with the elderly or those having comorbidities such as hypertension, cardiovascular diseases, diabetes, and lung diseases being more vulnerable to developing severe symptoms [5, 6].

Although multiple proteins can serve as a gateway to SARS-CoV-2, evidence indicates that angiotensin-

converting enzyme 2 (ACE2) is the main attachment protein for entry into human cells [7, 8]. Besides inducing cell death, SARS-CoV-2 can trigger an imbalanced innate inflammatory response characterized by stimulation of pro-inflammatory cytokine release and inhibition of interferon (IFN)-I and IFN-III production, exacerbating thusly disease severity [9]. In addition to suppression of the IFN response, novel “attack” mechanisms that are mediated by enhanced mitochondrial dysfunction and nicotinamide adenine dinucleotide (NAD⁺) depletion are being suggested in the context of SARS-CoV-2 infection [10, 11].

Response of the innate immune system to SARS-CoV-2

Accumulating preclinical and clinical investigations highlight the pivotal role of the innate immune system, which serves as the first line of defense against pathogens, in SARS-CoV-2 progression and development. The innate immune response senses viral infection by recognizing their pathogen-associated

molecular patterns (PAMPs) with pattern recognition receptors (PRRs). These include the retinoic acid-inducible gene I-like receptors (RLRs), Toll-like receptors (TLRs), the nucleotide oligomerization domain-like receptors (NLRs), and cytosolic DNA sensors [12, 13]. The contribution of PRRs in the context of SARS-CoV has been investigated in a genetic study indicating increased susceptibility to viral infection in the absence of RIG-I and melanoma differentiation-associated protein 5 (MDA-5), cytosolic RNA sensors, and their specific signaling pathways [14]. Although SARS-CoV-2 replicates in the cytosol, strong evidence suggests that the replicated viral genome can be recognized by cytosolic RNA sensors, including melanoma differentiation-associated protein 5 (MDA-5) and RIG-I, and endosomal sensors such as TLR3 and TLR7 [14, 15]. A study by Li et al. indicated that both MDA-5 and RIG-I are involved in sensing coronavirus mouse hepatitis virus (MHV) infection, consequently inducing production of IFNs so as to inhibit viral replication [14]. Furthermore, Cervantes-Barragan et al. and Scheuplein et al. reported that TLR7 plays a central role in the recognition of coronaviruses such as murine hepatitis virus and subsequent induction of IFN- α [15, 16]. Similarly, TLR4 deficient mice were shown to be more susceptible to SARS-CoV infection [17, 18]. Recent published evidence in the context of COVID-19 demonstrated that the binding of the SARS-CoV-2 spike protein to TLR4 could be involved in the initiation of the cytokine storm, resulting in the development and progression of COVID-19 [19]. It has been reported in an *in silico* study performed by Choudhury and Mukherjee that the spike protein has the strongest interaction with TLR4 compared to other TLRs [20]. Additionally, the interaction of SARS-CoV-2 with TLR4 has been observed to cause dysregulation in the TLR4 signaling pathway, leading to the activation of the pro-inflammatory MyD88-dependent pathway rather than the TIR domain-containing adaptor-inducing interferon- β /TRIF-related adaptor molecule (TRIF/TRAM)-dependent anti-inflammatory and interferon pathways [19].

Viral recognition by the innate immune system stimulates activation of NF- κ B-mediated secretion of pro-inflammatory cytokines and IFN regulatory factor (IRF), such as IRF-3 and IRF-7, mediated production of type I IFNs (IFN- α and IFN- β) and type III IFNs (IFN- λ 1, IFN- λ 2, IFN- λ 3, and IFN- λ 4) [13, 21]. With respect to the pro-inflammatory response, it has been observed that following viral infection PRRs, including TLRs and RIG-Like receptors, recognize PAMPs. Upon binding to PAMPs, TLRs recruit myeloid

differentiation primary response gene 88 (MyD88) and Toll/IL-1 receptor (TIR), whereas RIG-like receptors exert an effect via the MAVS pathway [22–24]. Consequently, these molecules recruit TNF-receptor associated factor (TRAF), catalyzing the synthesis of K63-linked polyubiquitin chains, subsequently recruiting and activating Ikappa B kinase complex (IKK), phosphorylating and degrading thereafter the inhibitory molecule I κ B [25, 26]. The destruction of I κ B activates the NF- κ B dimer, leading to translocation to the nucleus and release of pro-inflammatory cytokines that constitute an anti-viral innate immune response [27]. The secreted IFNs, on the other hand, activate cell membrane receptors that couple to JAK/STAT signaling and nuclear translocation of IRFs, e.g., IRF-9. The latter activate genes containing interferon-stimulated response elements (ISREs) in their promoters, which causes expression of a set of IFN-stimulated genes (ISGs) that establish an antiviral state [21, 28, 29]. This innate immune response mediated by the release of IFNs in the context of SARS-CoV-2, however, seems to be deficient leading to development of deadly manifestations, essentially fatal respiratory failure. Accumulating evidence demonstrates that SARS-CoV-2 leads to dysregulated immune response and delayed IFN production, contributing therefore to the development and progression of COVID-19 [30, 31]. The underlying pathological mechanism, however, is yet to be fully elucidated. It has been reported that SARS-CoV-2 RNA stimulates RIG-I-MAVS-dependent IFN signaling pathway [32]. The accessory protein ORF9b has been shown to be upregulated within 24 hours following COVID-19 infection, during which the delayed IFN- β secretion was seen, suggesting a major role of ORF9b in the response to SARS-CoV-2 [30, 31]. A study performed by Jing et al., indicated that ORF9b accumulates in primary human pulmonary alveolar epithelial cells and antagonizes the antiviral effect of the type I IFN response following SARS-CoV-2 infection [32].

Production and potential modulation of IFN response by SARS-CoV-2

Substantial evidence documents that interferons exert a central importance in the innate immune response to viral infection [33]. It has been reported that IFN-I and IFN-III are induced following viral recognition by the innate immune system [13, 34]. IFN-I has been shown to be widely expressed and to induce a potent inflammatory antiviral effect, leading to the release of pro-inflammatory cytokines such as tumor necrosis

factor- α (TNF- α), interleukin (IL)-6, and IL-17 [35]. IFN-III, however, is mainly secreted by epithelial cells; it exerts a local antiviral effect, thus eliciting less inflammatory damage [36, 37]. Despite the powerful anti-viral response in the setting of SARS-CoV-2, mainly mediated by activation of the innate immune system, SARS-CoV-2 remains highly pathogenic. In light of this observation, recent evidence demonstrates that SARS-CoV-2 can interfere with the following processes of the innate response: (1) innate sensing, (2) ISG function, and (3) IFN production and signaling pathways, increasing thereby the severity of the disease [13] (Figure 1).

To begin with, coronaviruses such as SARS-CoV have been shown to be replicated in interior double membrane vesicles increasing therefore the virulence of the virus [38]. Chean et al. demonstrated that SARS-CoV nonstructural protein (Nsp) 14 has

guanine-N7-methyltransferase activity that can mimic this cap on viral RNA [39]. Additionally, Nsp16 has been observed to further modify the viral cap with its 2'-O-methyl-transferase activity, preventing eventually viral recognition by MDA5 [40]. This observation was further substantiated in a study performed by Menachery et al. showing that in a mouse model of Middle East respiratory syndrome coronavirus (MERS-CoV) infection, mutating Nsp16 attenuated viral virulence and disease severity [41]. Although this evasion strategy is likely used by SARS-CoV-2, since Nsp16 of SARS-CoV and SARS-CoV-2 share 92% amino acid sequence homology [42], recent published investigations indicated that SARS-CoV-2 stimulates the RIG-I-MAVS-dependent IFN signaling pathway, but leads to the accumulation of ORF9b, an accessory protein, consequently antagonizing the host-virus response mediated by IFN release [32].

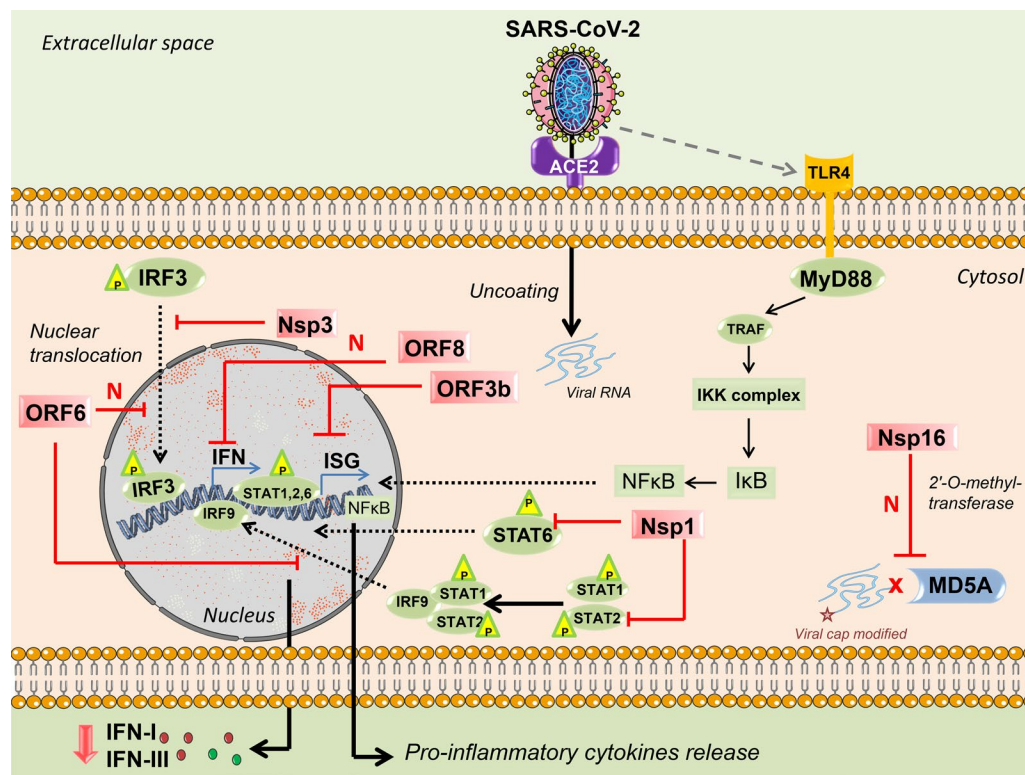


Figure 1. Diagram depicting the impact of SARS-CoV-2 on the innate immune system mediated by IFN secretion. Various ways that SARS-CoV-2 evades IFN signaling are schematically represented, including: SARS-CoV-2 RNA recognition by the cytoplasmic RNA sensor MDA5 which leads to IFN induction and subsequent IFN signaling in surrounding cells, resulting in the expression of ISGs. ORFs in association with nucleocapsid proteins of SARS-CoV-2 inhibit the expression of IFN and the activation of ISGs. SARS-CoV-2 proteins that have been reported to interfere with these pathways are indicated in red rectangles. Furthermore, SARS-CoV-2 was reported to induce the TLR4 signaling pathway leading to the activation of the pro-inflammatory MyD88-dependent pathway and NF- κ B activation triggering the gene expression of pro-inflammatory cytokines. ACE2, Angiotensin Converting Enzyme2; IFN, interferon; IRF, IFN regulatory factor; ISG, IFN-stimulated gene; MDA5, melanoma differentiation-associated gene 5; N, nucleocapsid protein; Nsp, nonstructural protein; ORF, open reading frame; P, phosphate; RIG-I, retinoic acid-inducible gene 1; SARS-CoV, severe acute respiratory syndrome coronavirus; STAT, signal transducer and activator of transcription; TLR4, toll like receptor 4; MyD88, myeloid differentiation primary response gene 88; TRAF, TNF-receptor associated factor; IKK, I kappa B kinase complex; NF- κ B, Nuclear Factor kappa.

With respect to the inhibition of IFN-I and IFN-III production, Siu et al. reported that the membrane protein M of SARS-CoV has the ability to sequester innate sensors in membrane-associated cytoplasmic compartments, evading therefore the innate response [43]. Multiple studies report that MERS M protein and SARS-CoV nucleocapsid (N) protein, Nsp3, and papain-like protease domain can inhibit the phosphorylation and nuclear translocation of IRF-3, thus disrupting IFN-I anti-viral response [44–46]. These findings were further strengthened by a study performed by Blanco-Melo et al. highlighting that low levels of IFN-I and IFN-III are the hallmark of SARS-CoV-2 [47].

Lastly, SARS-CoV Nsp1 has been shown to block phosphorylation of the transcription factor of ISGs, STAT6 [48]. Additionally, SARS-CoV accessory protein ORF3b inhibits transcription of ISGs, resulting in decreased IFN-I and IFN-III levels [45]. A cell culture experiment showed that the open reading frame 6 (ORF6), ORF8, and nucleocapsid (N) proteins of SARS-CoV-2 are able to inhibit expression of IFN- β and activation of ISGs [21]. These proteins exhibited strong inhibition of the type I IFN (IFN- β) promoter and NF- κ B element. All three were able to inhibit the ISRE after infection of cells with Sendai virus, while ORF6 and ORF8 could inhibit the ISRE after treatment with IFN- β . SARS-CoV-2 ORF6 was found to block STAT1 and STAT2 nuclear translocation so as to impair transcriptional induction of ISGs [49]. Of note, however, SARS-CoV-2 ORF6 was reported to interfere less efficiently than SARS-CoV ORF6 with human IFN induction and IFN signaling [50]. Xia et al. identified multiple SARS-CoV-2 proteins that antagonize the IFN-I response [51]. Three proteins antagonize IFN-I production: ORF6 binds importin Karyopherin α 2 (KPNA2) to inhibit IRF3 nuclear translocation; Nsp6 suppresses IRF3 phosphorylation by binding TANK binding kinase 1 (TBK1); and Nsp13 binds and blocks TBK1 phosphorylation. Two groups of proteins were found to antagonize IFN-I signaling by blocking STAT1/STAT2 phosphorylation or nuclear translocation. In this case, Nsp1 and Nsp6 of SARS-CoV-2 more efficiently suppressed IFN-I signaling than their counterparts from SARS-CoV or MERS-CoV [51]. Collectively, SARS-CoV-2 proteins interfere with the innate immune system leading to delayed anti-viral response mediated by IFNs secretion and thus facilitate virus replication [2]. These findings would be helpful to unveil the molecular pathways that may be targeted in the setting of SARS-CoV-2.

Consistent with this observation, the serum of SARS-CoV-2 patients showed a similar trend with marked increase in pro-inflammatory cytokine release and evidence for inhibition of IFN-I and IFN-III production [52]. Other investigations suggested a delayed release of IFNs rather than complete absence. For example, in an animal model of SARS-CoV, IFN-I was undetectable in the lung until several hours after the viral load peak [53].

Despite the well-established anti-viral role of IFNs, contradicting findings are reported in the settings of coronavirus infections. Cameron et al. reported a positive correlation between increased plasma IFN- α levels and disease severity [54]. Similarly, in a clinical study of patients diagnosed with SARS-CoV-2, elevated IFN- α levels were accompanied by increased viral load, and thus enhanced disease progression and development [55].

Fine tuning mitochondrial innate immune response in the setting of viral infection

Besides the well-established role of mitochondria in regulating multiple metabolic processes, including redox reactions and regulation of NAD⁺ and cellular metabolism [56], it is becoming increasingly recognized that mitochondria are fundamentally implicated in the innate immune response following viral infection including by HIV, hepatitis B, and herpes simplex [57–60] (Figure 2). These viruses cause a robust inflammatory response mediated by the release of mtDNA, resulting in enhanced tissue damage [61–63]. Mitochondrial DNA triggers an innate immune response by inducing cyclic GMP-AMP formation that activates in turn the endoplasmic reticulum (ER)-resident protein, stimulator of interferon genes (STING). STING subsequently induces TANK-binding kinase-1 phosphorylation of IRF-3 and STAT6, which dimerize and induce the transcription of hundreds of ISGs, all of which exert a pivotal role in the inhibition of viral replication [64–67]. In addition to the induction of ISGs, circulating mtDNA has been observed to play a critical role in the stimulation of pro-inflammatory response in COVID-19 patients via its ability to activate cGAS and TLR-9 [57]. The release of mtDNA from the cell has been shown to occur during formation of the neutrophil extracellular trap, a pathogenic step implicated in lung tissue injury and blood clotting formation in COVID-19 patients

Additionally, it has been shown that mice deficient in mitochondrial antiviral signaling protein (MAVS)

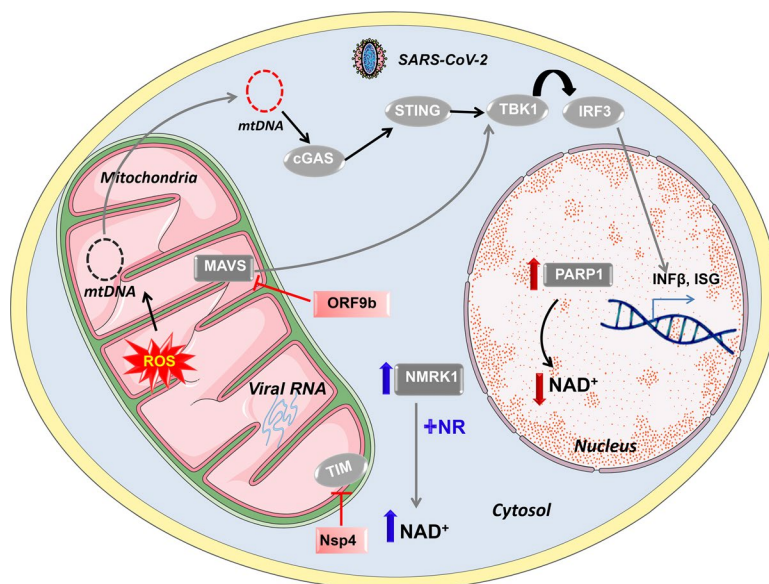


Figure 2: Putative mechanisms of mitochondria-related immune functions controlled by SARS-CoV-2 and their effect on NAD⁺ levels. SARS-CoV-2 encodes a virulence factor known as ORF-9b, which localizes to mitochondria and triggers MAVS degradation. SARS-CoV-2 localizes to mitochondria and increases ROS production. As a result, after SARS-CoV-2 infection, PARP1 is activated to repair DNA fragmentation. PARP-1 activation leads to decreased NAD⁺ levels. On the other hand, the expression of NMRK-1, an NAD biosynthetic enzyme, is upregulated. Red rectangles represent viral proteins. Please see the text for additional details. cGAS, cyclic GMP-AMP Synthase; IFN, interferon; ISG, IFN-stimulated gene; MAVS, mitochondrial antiviral signaling protein; Nsp, nonstructural protein; ORF, open reading frame; P, phosphate; STING, stimulator of interferon genes; SARS-CoV, severe acute respiratory syndrome coronavirus; TANK, TRAF family member associated NF- κ B activator; TBK1, TANK-binding kinase 1; ROS, reactive Oxygen Species; NMRK1, nicotinamide riboside kinase 1; NAD⁺, nicotinamide adenine dinucleotide; NR, nicotinamide riboside; mt DNA, mitochondrial DNA.

after being infected by vesicular stomatitis virus (VSV), a negative-strand RNA virus, exhibit a diminished ability to produce IFN-I, resulting in higher susceptibility to viral infection [68]. A study done by Cao et al. highlighted that methylcrotonyl-CoA carboxylase, an enzyme located in mitochondria, interacts with MAVS, increasing IFN-I levels and consequently enhances the anti-viral response [69]. It has also been documented that following the recognition of viral RNA by RIG-1/MDA5, a signaling complex is triggered on the outer membrane of the mitochondria, defined as MAVS/TRAF3/TRAF6/TOM70, resulting in the induction of a potent anti-viral response [70, 71]. Furthermore, activation of PRR in the setting of viral infection has been shown to switch cell metabolism from oxidative phosphorylation to glycolysis, leading thusly to the stimulation of IFN- α secretion and enhancing eventually an anti-viral defense mechanism [72, 73]. Notably, NAD⁺, a central metabolic coenzyme that exerts a key role in mitochondrial function and longevity, has been reported to regulate innate immune cell function, including that of macrophages [59]. Additionally, NAD⁺ is involved in ATP generation that has been shown to be implicated in the regulation of the immune system [74].

Modulation of mitochondria by SARS-CoV-2

SARS-CoV-altered mitochondria-associated mechanisms implicated in the innate immune response have been proposed in multiple investigations, yet the exact mechanism is still poorly understood. For instance, it has been reported that SARS-CoV encodes a virulence factor known as open reading frame (ORF)-9b, which localizes to mitochondria, induces mitochondria elongation, and triggers MAVS degradation (Figure 2). This severely suppresses the innate immune response by inhibiting production of IFNs [75]. Although the number of basic investigations reporting SARS-CoV-2-induced mitochondrial manipulation are relatively scant, Bojkova et al. demonstrated that SARS-CoV-2 downregulates MAVS in human colon epithelial carcinoma cells CaCo-2 [30]. Additionally, recent computational work by Wu et al. indicated that SARS-CoV-2 localizes to mitochondria, resulting potentially in increasing reactive oxygen species (ROS) production and thereby suppressing mitochondrial function [76]. These findings were further supported by Gordon et al. who showed that SARS-CoV-2 interacts with the TIM complex, a component of the mitochondria import machinery,

and Nsp8 interferes with mitochondria ribosome [77]. It has also been shown that SARS-CoV-2 upregulates genes implicated in the process of NF- κ B regulation and ROS production and downregulates genes involved in mitochondria organization and respiration [78].

Interest in NAD⁺ in the setting of SARS-CoV-2 infection is expanding. SARS-CoV-2 triggers a maladaptive immune response and aggravates DNA damage. As a result, the innate immune response is stimulated to fight the infection and poly (ADP-ribose) polymerase 1 (PARP-1), an NAD⁺ consuming enzyme, is activated to repair DNA fragmentation and inhibit viral replication [79, 80]. PARP's anti-viral effect, however, is opposed by the ADP-ribosylhydrolase macrodomain of viral Nsp3, whose activity is necessary for virulence [81].

Recent evidence demonstrated disturbed NAD⁺ metabolism and NAD⁺ biosynthetic and consuming enzymes post-SARS-CoV-2 infection [11]. For instance, expression of nicotinamide riboside-1 (NMRK-1), an enzyme involved in NAD⁺ biosynthesis was upregulated, whereas the expression of nicotinamide N-methyltransferase (NNMT), an enzyme eliminating nicotinamide from cells, was downregulated, suggesting a compensatory mechanism in infected cells to maintain NAD⁺ levels [11, 80]. Additionally, it has been demonstrated that SARS-CoV-2 increases expression of several PARPs, leading to NAD⁺ depletion and dysregulation of the immune response in the lungs of patients diagnosed with SARS-CoV-2 [11]. Taken together, replenishing NAD⁺ levels through NMRK-1 may restore PARP function to support the innate immune response in fighting the viral infection.

Potential therapeutic strategies for COVID-19

Potential contribution of IFN therapy

A growing body of evidence supports IFNs as a potential therapy for the SARS-CoV-2 pandemic. Multiple clinical trials are currently underway to evaluate the impact of IFN alone or in combination with other anti-viral drugs on SARS-CoV-2 infection (Table 1). The potential beneficial effect of IFNs, however, should be assessed in terms of the timing. For instance, IFN- β -1b was administered early after infection, highlighting that the timing of IFN-I is a crucial factor [82]. This observation is in line with previous clinical studies pointing out that IFN-I should be administered at an early stage of MERS-CoV infection [83, 84]. To avoid the ineffectiveness or potential adverse effects of late administration of IFN-I including malaise, fatigue, and fever [85], new therapeutic strategies are being evolved in the clinical setting. For instance, an aerosol formulation of recombinant IFN has been used to deliver the cytokine inside the lungs. In fact, studies have shown that the pulmonary concentration is higher following inhalation than with systemic administration, minimizing therefore the adverse effects of IFN [86, 87]. Additionally, Zhou et al. performed a clinical study on 77 SARS-CoV-2 patients in Wuhan. In this study, patients who received nebulized IFN- α -2b alone or in combination with Umifenovir, an anti-viral agent, showed a marked decrease in viral load in the upper respiratory tract [88].

IFN-III could also be a promising therapy for multiple different pathogens including respiratory syncytial virus, influenza B virus, influenza A virus, and SARS-CoV [89]. For instance, IFN-III was shown to be more effective than IFN-I in decreasing viral

Table 1. A list of COVID-19 clinical studies that are assessing the efficacy and safety of IFN treatment.

Identifier number	Study design	Enrollment	Drug of interest
ChiCTR2000029387	Single study, randomized, open labeled, prospective clinical trial	101 Patients	• RBV + IFN-α • LPV/r + IFN-αRBV + LVP/r + IFN-α
NCT04276688	Prospective randomized open-label controlled clinical trial	127 Patients	• LPV/r • RBV • IFN-β-1b
NCT04315948	Multi center, adaptive, randomized, open clinical study	3100 Patients	• Remdesivir • LPV/r • IFN-β-1a • Hydroxychloroquine • Standard care
IRCT20100228003449N28	Randomized clinical trial	79 Patients	• IFN-β-1a • Hydroxychloroquine + LPV/r • Hydroxychloroquine + ATZ/r
NCT04492475	Randomized, controlled clinical trial	1000 Patients	• Remdesivir + IFN-β-1a
ChiCTR2000030262	Open label, non-randomized clinical trial	33 Patients	• IFN-κ + TFF2
IRCT20100228003449N28	Randomized clinical trial	92 Patients	• IFN-β-1a

LPV/r, Lopinavir/Ritonavir; RBV, Ribavirin; ATZ/r, Atazanavir/Ritonavir.

replication in intestinal epithelial cells [90]. Of note, it has been observed that SRAS-CoV-2 morbidity is linked to increased IFN-I and IFN-III expression levels in the lung [91]. Additionally, in mice exposed to synthetic viral RNA, IFN-III released by dendritic cells in the lung has been correlated with enhanced lung epithelium damage, consequently exacerbating disease severity [91]. Complicating this picture, a study performed by Hadjadj et al., indicated diminished type I IFN levels in peripheral blood immune cells from severe COVID-19 patients [92]. Taken together, these findings suggest that in contrast to local, the systemic production of IFNs in the setting of COVID-19 may exert beneficial effects.

Potential contribution of NAD⁺ boosting therapy

NAD⁺ boosting therapy has recently become a potential treatment for SARS-CoV-2 infection. A randomized double-blinded case-control trial (ID# NCT04407390) is currently evaluating the efficacy of the NAD⁺ precursor nicotinamide riboside (NR) (1 g/day) for COVID-19 patients. Additionally, a phase II clinical study was conducted to assess the impact of NR, in combination with hydroxychloroquine, on mild to moderate COVID-19 patients. The findings revealed a 30% decrease in recovery time following NR administration when compared to patients receiving standard care [11]. PARP inhibitors have also been shown to exert protective effects in the context of SARS-CoV-2 infection by decreasing the cytokine storm mediated by excessive release of pro-inflammatory cytokines (IL-6 and TNF- α) and attenuating lung fibrosis [93].

Besides NAD⁺ boosting therapy, silence information regulator-1 (SIRT-1), implicated in mitochondria biogenesis, has been proposed to exert beneficial effects post-SARS-CoV-2 infection. It has been indicated that SIRT-1 decreases viral replication and the hyper-inflammatory response mediated by IL-1 β , IL-6, and TNF- α [94]. Given the above, NAD⁺ boosting and mitochondria-targeted therapies may be critical players in suppressing the cytokine storm and potential treatments against SARS-CoV-2 viral attack. Further experimental investigations and clinical data, however, are needed to draw a firm conclusion about the efficacy and safety of NAD⁺ boosting and mitochondria-targeted therapies in the context of SARS-CoV-2 infection.

Conclusion

Mounting evidence suggests that an imbalanced innate immune response and mitochondria alterations play a crucial role in COVID-19 progression and

development. A particularly insidious aspect of SARS-CoV-2 infection is the inhibition of the innate immune response that is associated with release of IFNs, together with enhanced mitochondrial dysfunction and NAD⁺ depletion. Additional evidence concerning how SARS-CoV-2 inhibits the innate immune system and enhances mitochondrial dysfunction is needed. Defining the mechanisms of IFN evasion and preventing NAD⁺ depletion may open up novel therapeutic approaches. While an effective treatment for COVID-19 is still elusive, a better understanding of the molecular pathways that are altered following SARS-CoV-2 infection should expedite the development of therapeutic and preventive treatments for this newly emerged virus.

Author's contributions

NJH conceived the manuscript and initiated the literature search and writing. All authors contributed to the further writing and content of the manuscript. Both GWB and FAZ assisted with the editing.

Declaration of Interest

The authors have no conflicts of interest to declare.

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Research Article

IL-33 induces type-2-cytokine phenotype but exacerbates cardiac remodeling post-myocardial infarction with eosinophil recruitment, worsened systolic dysfunction, and ventricular wall rupture

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Myocardial infarction (MI) is the leading cause of mortality worldwide. Interleukin (IL)-33 (IL-33) is a cytokine present in most cardiac cells and is secreted on necrosis where it acts as a functional ligand for the ST2 receptor. Although IL-33/ST2 axis is protective against various forms of cardiovascular diseases, some studies suggest potential detrimental roles for IL-33 signaling. The aim of the present study was to examine the effect of IL-33 administration on cardiac function post-MI in mice. MI was induced by coronary artery ligation. Mice were treated with IL-33 (1 µg/day) or vehicle for 4 and 7 days. Functional and molecular changes of the left ventricle (LV) were assessed. Single cell suspensions were obtained from bone marrow, heart, spleen, and peripheral blood to assess the immune cells using flow cytometry at 1, 3, and 7 days post-MI in IL-33 or vehicle-treated animals. The results of the present study suggest that IL-33 is effective in activating a type 2 cytokine milieu in the damaged heart, consistent with reduced early inflammatory and pro-fibrotic response. However, IL-33 administration was associated with worsened cardiac function and adverse cardiac remodeling in the MI mouse model. IL-33 administration increased infarct size, LV hypertrophy, cardiomyocyte death, and overall mortality rate due to cardiac rupture. Moreover, IL-33-treated MI mice displayed a significant myocardial eosinophil infiltration at 7 days post-MI when compared with vehicle-treated MI mice. The present study reveals that although IL-33 administration is associated with a reparative phenotype following MI, it worsens cardiac remodeling and promotes heart failure.

Introduction

Cardiovascular diseases, primarily acute myocardial infarction (MI) and stroke, represent the leading cause of mortality in the Western world and are on the rise in developing countries. Despite improved outcomes due to advances in medical and interventional treatments, a considerable number of MI patients still develop progressive left ventricular dysfunction [1]. The healing process post-MI implicates a variety of cellular and molecular mechanisms that induce the replacement of necrotic myocardial tissue with a collagen-based scar [2,3]. Understanding the progression and resolution of the inflammatory processes occurring following an MI may reveal new strategies aimed at preserving cardiac function and preventing

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ischemic cardiomyopathy, therefore complementing available neurohumoral blockade and interventional approaches.

Interleukin (IL)-33 (IL-33) is a constitutively expressed cytokine belonging to the IL-1 family, known to activate innate and adaptive immunities [4]. Following tissue injury, IL-33 is released as an alarmin from damaged cells, and plays a role in the activation of immune cells and in promoting tissue repair [5,6]. The two known receptor isoforms for IL-33 are the long ST2 (ST2L) and the soluble ST2 (sST2) [7–9]. The long or transmembrane isoform (ST2L) is expressed on a variety of immune cells and is responsible for IL-33 signaling effects [10–16]. sST2, however, acts as a decoy receptor thereby preventing IL-33 signaling [17]. sST2 has gained much attention as a cardiovascular biomarker mainly predicting congestive heart failure and decreased survival in patients with acute MI [8,18–20].

IL-33 has been associated with a number of inflammatory, immune, and allergic disorders including arthritis, atherosclerosis, inflammatory bowel disease, obesity, and asthma [21]. IL-33 is also implicated in the activation of type 2 innate lymphoid cells (ILC2), which are a member of the newly discovered innate lymphoid cell (ILC) family and are involved in tissue remodeling and lipid metabolism in adipose tissues [14,22–24]. Following their activation, ILC2 produce type 2 cytokines such as IL-5 and IL-13 [25–27], which participate in eosinophil homeostasis, recruitment, and proliferation [25,28]. ILC2 are found in the heart during inflammation settings such as myocardial ischemia and eosinophilic pericarditis [29,30].

In the heart, IL-33 is released from cardiomyocytes, coronary artery smooth muscle cells, and cardiac fibroblasts [4,20,29]. IL-33 is reported to have protective effects on cardiac function in many cardiovascular settings including cardiac overload [31,32], abdominal aortic aneurysm [33] and atherosclerosis [34,35], as well as MI, and is portrayed as a potential novel therapeutic cytokine [36–38]. However, a study performed on atherosclerotic-prone apolipoprotein E (ApoE)-deficient mice reported that the IL-33/ST2 pathway is not implicated in this pathophysiology [39]. Another, recent study involving patients with end-stage heart failure showed that the expression of the IL-33/ST2 pathway in the failing human heart is associated with increased pro-fibrotic signaling proteins and overall cardiac fibrosis, suggesting a role of this pathway in pro-fibrotic myocardial remodeling [40]. Moreover, an exogenous administration of IL-33 has been shown to promote eosinophilic pericarditis and adversely affect heart function even in healthy mice [41]. The aim of the present study was to assess the impact of IL-33 on the myocardial pathological changes, including ventricular remodeling, myocardial dysfunction, fibrosis, immune cell infiltration, and survival following MI.

Materials and methods

Experimental design

Animals were housed in sterilized cages and maintained on a 12-h (light:dark) cycle with access to autoclaved rodent chow and water *ad libitum*. Operating procedures were performed in the animal care facility of the American University of Beirut Medical Center (AUBMC). All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the AUB (IACUC # 18-2-RN560). Adult wild-type (WT) male C57/BL6J mice (12–14 weeks of age) were randomized into four different groups: sham+vehicle, sham+IL-33, MI+vehicle, and MI+IL33. In the sham and MI groups treated with IL-33, a single daily injection of mouse recombinant IL-33 (BioLegend, San Diego, CA, U.S.A.) (1 µg suspended in 200 µl PBS) was administered intraperitoneally (i.p.) right after sham operation or left anterior descending (LAD) ligation (starting day 0) and continued for 4 or 7 days thereafter. IL-33 dose and route of administration were based on previous studies [36,37]. In the sham and MI groups treated with vehicle, a single daily injection of PBS (200 µl/day) was i.p. administered right after sham operation or LAD ligation (starting day 0) and continued for 4 or 7 days thereafter. For flow cytometric analyses, mice were given a single (i.p.) daily injection of IL-33 or its vehicle right after sham-operation or LAD ligation (day 0) and continued for 1, 3, or 7 days thereafter.

Induction of MI

Mice were subjected to a permanent ligation (MI group) ($n=167$) or no ligation (sham group) ($n=58$) of the LAD coronary artery as previously described [42]. In brief, mice were first administered a dose of 0.05 mg/kg buprenorphine (Reckitt Benckiser Pharmaceuticals, Richmond, VA) for pre-emptive and post-operative analgesia. Mice were then anesthetized with 2% isoflurane, intubated orotracheally, and ventilated with a MiniVent ventilator (type 845) (HSE Harvard, Germany). After shaving the chest wall, a small thoracotomy was performed between the left third and fourth intercostal space. The pericardial sac was cut open, the left ventricle (LV) visualized and the LAD isolated with a 7-0 prolene suture (Ethicon, Norderstedt, Germany), 2 mm below the left atrioventricular border. Successful ligation was confirmed by visual discoloration of the ventricle distal to the occlusion site and confirmed by ST elevation on ECG using Indus MouseMonitor[®] system. The same procedure was performed for sham-operated mice, but

without LAD ligation. Ribs, muscles, and skin were closed in layers using 6-0 prolene sutures (Ethicon, Norderstedt, Germany). The mouse was then weaned from isoflurane anesthesia, flushed with oxygen, extubated, and allowed to warm under an infrared lamp until full consciousness.

Echocardiography

To evaluate the progression of cardiac function or dysfunction, serial echocardiographic measurements were performed at baseline, day 1 post-surgery (LAD ligation or sham operation), and on the day of killing (4 or 7 days). All measurements and assessments were performed using the Visual Sonics Echo system (Vevo 2100, VisualSonics, Inc., Toronto, Canada) and the 22–55 MHz (MS550D) linear transducer (VisualSonics). Mortality rate was recorded for the entire study period. Mice were placed in the echocardiography room for at least 30 min before the examination. Mice were maintained unconscious using 2% isoflurane and placed in a supine position on a heating platform. The chest was shaved and ultrasonic gel was applied to the thoracic area to allow maximal visibility of the heart chambers. The ultrasonic probe was placed on the chest along the long-axis of the LV and adjusted to obtain clear two-dimensional B-mode and M-mode parasternal long axis images. Five minutes were allowed for each animal to stabilize in that position and before acquiring any measurement. Heart rate was maintained constant throughout the procedure (450–550 beats/min). For each parameter, three consecutive cardiac cycles were analyzed and averaged.

Necropsy and tissue harvest

Mice were administered 100 µl Heparin (Heparin Sodium 1000 I.U./ml) 30 min prior to killing. Following deep anesthesia with 4% isoflurane, blood was collected by cardiac puncture, centrifuged at 2200 rpm for 10 min, and the plasma flash-frozen in liquid nitrogen and stored at -80°C for future use. Mice were then subjected to cervical dislocation. Hearts were immediately injected with cardioplegic solution to arrest the LV chamber in diastole. After excision, hearts were rinsed with physiological saline, weighed, and the left and right atria were resected along with the large vessels. The mid-section was then kept in 4% formalin, and the base and apex were snap-frozen in liquid nitrogen and stored at -80°C for further experiments. Lungs were collected, weighed, and placed in a 37°C oven overnight to assess pulmonary congestion (Lungs Wet – Lungs Dry) $\times 100/\text{Lungs Wet}$). Spleens were collected, weighed, and stored in liquid nitrogen. Tibia length was also measured and used for normalization. For FACS tissue harvesting, single cell suspensions were obtained from mouse peripheral blood, bone marrow, spleen, and heart. Briefly, mice were given 100 µl of Heparin (1000 I.U./ml) 30 min prior to necropsy. Mice were then subjected to isoflurane anesthesia, blood was collected by cardiac puncture, and hearts perfused through the LV with 5 ml of ice-cold PBS. Hearts and spleens were excised and placed in ice cold PBS (1% FBS) (FACS buffer). Femurs and tibias were also harvested, cleaned of all connective tissue, and bone marrow was then collected by flushing through the bones with ice-cold FACS buffer. Heart specimens were cut in fragments of 2–3 mm³. Fragments were suspended in 5-ml DMEM (10% FBS) containing an enzymatic cocktail (Collagenase D (450 U/ml), Roche/Sigma, cat# 11088858001; Dispase (1 U/ml), STEMCELL Technologies, cat# 07923; and DNase I grade II (60 U/ml), Roche/Sigma, cat# 10104159001) and mechanically dissociated using the Miltenyi GentleMACS for 15 s. Dissociated tissue was digested for 30 min at 37°C with gentle rotation. Whole blood was first subjected to immunofluorescence staining, then lysed using the red blood cell lysis buffer (Qiagen, cat# 158904). Spleens, bone marrow suspensions, and digested heart tissue were then triturated, and cells filtered through a 40-µm nylon mesh (BD Falcon). Cell suspensions were centrifuged (400 $\times$ g, 4°C for 10 min) and subjected to red blood cell lysis for 3 min. The lysis reaction was stopped using 20 ml FACS buffer and the mix was centrifuged (400 $\times$ g, 4°C for 5 min). Total leukocyte numbers were determined using Trypan Blue. For each panel, 100 µl of cell suspension containing 1×10^6 cells was used from each organ.

Determination of infarct size

The LV was separated from the right, and sliced transversally into three sections (1–2 mm in thickness): apex, mid-section, and base. Sections, including the right ventricle, were incubated with 2% of 2,3,5 triphenyltetrazolium chloride (TTC) for 10 min at 37°C in the dark. Sections were photographed with a high-resolution digital camera (EOS rebelT3i DSLR, Canon, Tokyo, Japan). Infarct scar area, defined as regions unstained by TTC, were quantified using ImageJ 1.52 software and calculated as the ratio of infarct area to total LV area. All measurements were performed by an investigator who was blinded to the identity of the animals.

Survival

Starting with recovery from surgery, survival rate was assessed based on the animals that survived until the day of killing. An autopsy was performed on each animal found dead. Mortality was assessed using Kaplan–Meier survival curve analysis.

Histology

Formalin-fixed heart mid-sections were embedded in paraffin blocks, cut into transverse 5- μ m sections and placed on microscope slides. After dewaxing and hydration steps using xylene and ethanol (100, 95, 70%), sections were stained with Hematoxylin and Eosin (H&E) and Masson's Trichrome (MTC) to assess cardiomyocyte hypertrophy and areas of myocardial fibrosis, respectively. Imaging was performed on an Olympus CX41 microscope, and quantification was assessed in a blinded fashion using ImageJ 1.52 software. A minimum of ten fields per section and three to four animals were analyzed per experimental group. The area of myocardial fibrosis was quantitatively measured from the photomicrographs of MTC stained slides by ImageJ 1.52 software. The fibrosis was identified using the differences in color (blue fibrotic area opposed to red myocardium) of MTC-stained slides. The results are presented as the ratio of fibrotic area to the whole area of the myocardium using 10 $\times$ magnification. For cardiomyocyte hypertrophy, a 20 $\times$ objective was used, and digital images taken from the subendocardial and subepicardial regions of the LV wall were analyzed. The cross-sectional area (CSA) of individual cardiomyocytes was measured by tracing boundaries of each cardiomyocyte on each image. CSAs were defined from an average of 100 cardiomyocytes per mouse.

Western blot analysis

Mouse tissues were ground with liquid nitrogen and proteins were extracted in RIPA buffer containing protease inhibitors (Roche, Basel, Switzerland), and quantified using Thermo Scientific NanoDrop 1000 UV-Vis spectrophotometer (Thermo Scientific, Waltham, MA) according to the manufacturer's recommended protocol. Concentrations were estimated based on an average obtained from triplicate measurements of the same sample. The Lowry colorimetric assay and Nanodrop 1000 methods were previously compared in our lab and both gave the same range of protein quantification. An equal amount of protein (100 μ g) from each sample was heated at 95°C for 10 min and electrophoresed under reducing conditions on a 15% SDS polyacrylamide gel and transferred on to a nitrocellulose membrane. Membranes were blocked with 5% nonfat milk 1 h at room temperature, and incubated with the primary antibody against transforming growth factor β (TGF- β) (1/500, cat# ab92486, Abcam); α smooth muscle actin (α -SMA) (1/200, Abcam, cat# ab5694); IL-1 β (1/500, Abcam, cat# ab9722); tumor necrosis factor- α (TNF- α) (1:1000, Cell Signaling Technology, cat# 11948); and IL-4 (1/1000, Abcam, cat# ab9728); IL-10 (1/1000; Abcam, cat#ab9969) and IL-13 (1/500; Abcam; cat#ab106732) overnight on a rocker at 4°C. After four washes with Tris-buffered saline-Tween (TBST) (0.02%), membranes were incubated for 1 h with species-appropriate peroxidase-conjugated secondary antibody (1/40000) at room temperature. Finally, membranes were washed twice with TBST (0.02%) and TBS, and immunoreactive bands were then visualized with enhanced chemiluminescence (Clarity Western ECL substrate, Bio-Rad) to detect protein bands using the ChemiDoc MP imaging system. Due to the instability of housekeeping proteins such as β -actin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and β -tubulin in animals models of myocardial ischemic infarction [43], total protein levels were used as loading controls. The band densities of each protein of interest were normalized to the density of the total protein bands appearing in the same lane and on the same membrane. All data were then calibrated as fold change to the average of sham-operated vehicle-treated group. Western blot bands and total protein representative figures are shown in (Supplementary Figure S4).

Ribonucleic acid extraction and reverse transcription quantitative polymerase chain reaction

Total ribonucleic acid (RNA) was isolated from whole mouse heart using TRIzol isolation reagent. Reverse transcription was performed using the QuantiTect Reverse Transcription Kit (Qiagen), and reverse transcription quantitative polymerase chain reaction (RT-qPCR) was completed using the QuantiFast SYBR Green PCR Kit (Qiagen) according to the manufacturer's instructions. Real-Time PCR was performed on a CFX-96 RT-PCR system (Bio-Rad). Cycle quantification (C_q) values were assessed during the exponential amplification phase. The quantified data were analyzed using the $2^{-\Delta\Delta C_q}$ method and the relative expression levels of target genes, defined as fold change, and were normalized to the corresponding fold change of the sham+vehicle group, which was defined as 1.0. The primers used for RT-qPCR are listed in Table 1. mRNA levels were standardized with the housekeeping gene hypoxanthine-phosphoribosyl transferase (*HPRT*), and all experiments were performed using $n=3$ for sham+vehicle and $n=5$ or 6 for all other groups.

Table 1 Primers used for RT-PCR analysis

Mouse	mRNA forward primer (5'–3')	Reverse primer (5'–3')
HPRT	GTTGGGCTTACCTCACTGCT	TAATCACGACGCTGGGACTG
sST2	ACGCTCGACTTATCCTGTGG	CAGGTCAATTGTTGGACACG
BAX	ATCCAAGACCAGGGTGGCT	CCTTCCCCCATTCATCCAG
BCL2	AGTACCTGAACCGGCATCTG	TATGCACCCAGAGTGATGCAG
IL-5	ATGAGGCTTCCTGTCCCTACT	TACCCCCACGGACAGTTTGA
MMP2	AGATGCAGAAGTTCTTTGGGCTGC	AGTTGTAGTTGGCCACATCTGGGT
MMP9	ACCACAGCCAATATGACCAGGAT	AAGAGTACTGCTTGCCAGGAAGA
CTGF	ACCCAATATGATGCGAGCC	GGTAACTCGGGTGGAGATGC

Assessment of cytokine levels by enzyme-linked immunosorbent assay

The concentrations of IL-5 (Stem Cell Technologies, cat# 02024); granulocyte macrophage-colony stimulating factor (GM-CSF, Invitrogen, cat# BMS612); and IL-33 (Abcam, cat# ab213475) were determined in mouse plasma using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions. All samples were analyzed in duplicates.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling assay

Myocardial tissue sections obtained from sham-operated and LAD-ligated mice (treated with IL-33 or its vehicle (PBS)) at two different time points (4 and 7 days) were analyzed. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining was performed on 5- μ m-thick paraffin-embedded heart sections. Tissue sections were deparaffinized and rehydrated, then cardiomyocyte DNA fragmentation was evaluated using the Click-iT Plus TUNEL Assay for In Situ Apoptosis Detection (Thermo Fisher). The operation was performed according to the protocol provided by the manufacturer. Images were captured by the Zeiss Axio light microscope. Apoptotic nuclei were stained red with Alexa Fluor 647 and counterstained with DAPI (4,6-diamino-2phenylindole (blue)) to count the total number of normal nuclei per field. The total number of both TUNEL-positive myocytes and normal cardiomyocyte nuclei was counted using ImageJ (version 1.52) software.

Single cell suspension and flow cytometry

Mice were killed on days 1, 3, and 7 post-MI. A subset of five to six mice for the MI group and three to four mice for the sham-operated group per time point was used for flow cytometric analysis. Immunofluorescence staining was performed on single cell suspensions obtained from mouse peripheral blood, bone marrow, spleen, and heart as previously described [29,44]. Immune cell populations were defined based on previously published data [45,46]. For each panel, 100 μ l of cell suspension was used from each organ and stained with 50 μ l of the antibody mix. Each panel was used in the presence of 0.5 μ g blocking antibody, anti-CD16/CD32 (clone 2.4G2, BD Pharmingen). Dead cells were excluded by the use of a viability dye (DAPI, Thermo Fisher). Samples were acquired on a BD FACS ARIA SORP cell sorter flow cytometer (BD Bioscience) and the raw data analyzed using FlowJo software (v10.5.3, Tree Star). All antibodies were purchased from Biolegend, eBioscience, or BD Biosciences. Data were analyzed on cells within the live gate and reported as percentage of cell number in CD45⁺ cells (neutrophils and eosinophils) and as percentage of cell number in live cells (classical monocytes, macrophages, and ILC2). Flow cytometry panels showing antibodies and their corresponding fluorochromes are summarized in Table 2. Representative pseudocolor flow cytometric gatings of the population subsets are shown in (Supplementary Figures S5–S8).

Flow cytometric analysis

Samples were acquired on a BD FACS Aria flow cytometer equipped with four lasers, and capable of detecting up to 17 fluorochromes. Data were analyzed using the FlowJo software 10.5.3 (Treestar, Ashland, OR). Voltages were set and the compensation matrix was computed based on unstained and single stained controls. Fluorescence minus one (FMO) controls were used to set gates. From each sample, a total of $0.5\text{--}1 \times 10^6$ events were collected and doublets were excluded from the analysis using FSC (Area) versus SSC (Height). Dead cells were excluded using DAPI staining. All data percentages were calculated from total live cells or from CD45⁺ cells.

Table 2 Flow cytometry panels showing antibodies and their corresponding fluorochromes and the targeted cell populations

Panel number	Antibodies	Populations
1	CD11b-eFluor450 (M1/70), Ly6C-FITC (AL21), F480-APC (BM8), Ly6G-PE (1A8)	- Classical monocytes (CD11b+Ly6C(hi)Ly6G-F480-) - Macrophages (CD11b+Ly6C(lo)F480+)
2	CD45-APC-eFluor 780 (30-F11), CD11b-eFluor450 (M1/70), CD11c-PECy7 (HL3), Ly6G-PE (1A8), SiglecF-FITC (E50-2440)	- Neutrophils (CD45+CD11b+Ly6G(hi)) - Eosinophils (CD45+ CD11c- SiglecF+)
3	Lineage *CD3 ξ (145-2C11), CD4 (RM4-5), CD19 (1D3), CD11b (M170), CD11c (N418), Gr1 (RB6-8C5), NK1.1 (PK136), Fc ϵ RI (MAR-1), Ter119 (TED119)] all on FITC background, ICOS-APC (C398.4A), CD25-PE (PC61.5)	ILC2 (Lin-ICOS+CD25+)

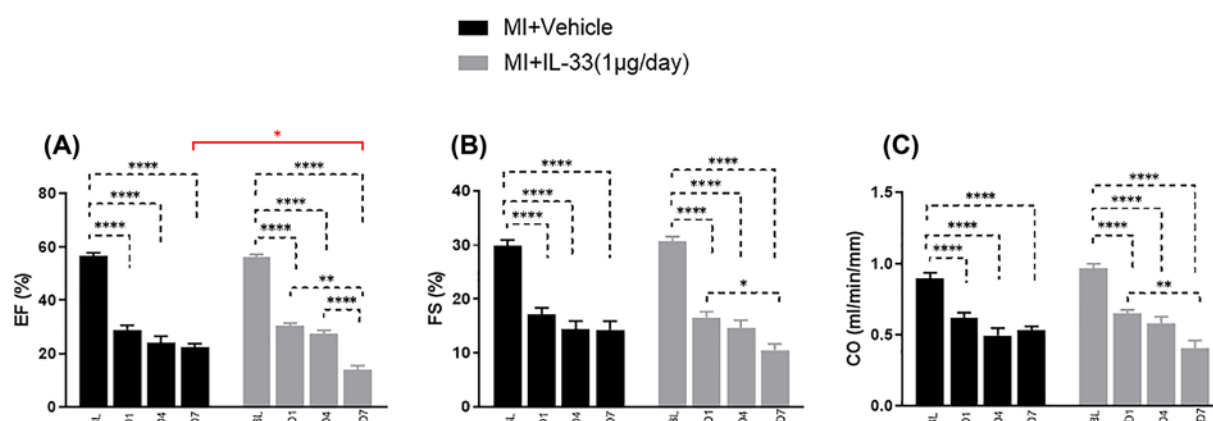


Figure 1. Functional cardiac parameters 4 and 7 days post-MI in vehicle-treated and IL-33 treated mice (A) EF, (B) FS, (C) CO. Bar graph data are mean $\pm$ SEM. * P <0.05, ** P <0.01, **** P <0.0001.

Statistical analysis

All analyses were performed using GraphPad Prism 8 (GraphPad Software, La Jolla, CA). Data are presented as means or fold changes $\pm$ standard error of the mean (SEM). Differences among experimental groups were analyzed for statistical significance using one-way analysis of variance (ANOVA) if there was one independent variable or two-way ANOVA if there were two independent variables. To adjust for multiple comparisons, we performed Tukey's post-hoc test, which maintains overall type I error (α) at 5%. P -value <0.05 was considered statistically significant. Mortality rates were evaluated by the Kaplan–Meier method and survival curves were compared using the log rank test.

Results

IL-33 treatment worsens cardiac dysfunction 7 days post-MI

Induction of MI decreased ejection fraction (EF), fractional shortening (FS), and cardiac output (CO) significantly at D1 in all MI groups (**** P <0.0001 vs. Baseline; Figure 1A–C) indicating successful ligation of the LAD coronary artery. There was a progressive decline in all hemodynamic parameters (EF, FS, and CO) between days 1 and 4 post-MI, but not to a significant extent (Figure 1A–C). Comparing D1 and D7 post-MI, a significant decrease in EF (** P <0.01), FS (* P <0.05), and CO (** P <0.01), was observed in the IL-33 treated MI group, but not the vehicle-treated MI group, indicating a worsened adverse LV remodeling with IL-33 treatment (Figure 1A–C). Of note, EF decreased significantly at D7 post-MI in the IL-33 treated group compared with the vehicle-treated group at the same time point (Figure 1A, * P <0.05). LV end-systolic and diastolic volumes and diameters (LVESV, LVEDV, LVESD, and LV end-diastolic diameter (LVEDD)) (normalized to tibia length) increased progressively and significantly as early as day 1 post-MI in all groups regardless of the treatment (Figure 2A–F). However, the MI group treated with IL-33 showed substantially higher increases in LVESV, LVEDV, LVESD, and LVEDD at 7 days post-MI compared with the vehicle-treated MI group, indicating worsened cardiac dysfunction (Figure 2A–F). Moreover, IL-33 treated MI group experienced a significant decrease in EF (Figure 1A, **** P <0.0001), and increase in LVESV, LVEDV, LVESD, and LVEDD (Figure 2A–D, *** P <0.001 to **** P <0.0001) between days 4 and 7 post-MI that was

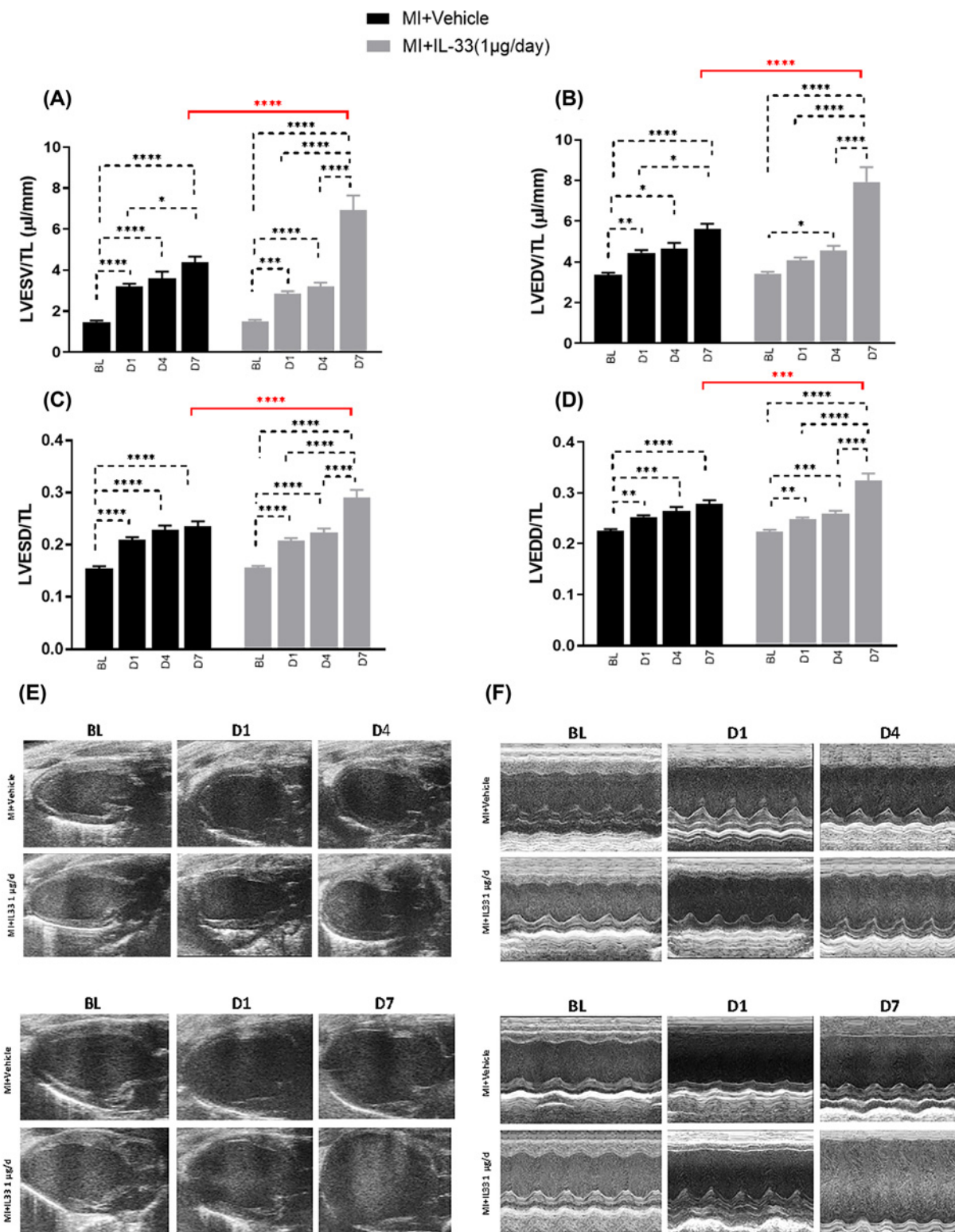


Figure 2. Increased left ventricular volume and diameter with IL-33 treatment 7 days post-MI: analysis of LV end-systolic and diastolic volume and diameter

(A) LVESV; (B) LVEDV; (C) LVESD; (D) LVEDD. Representative B-mode and M-mode (E,F) echocardiographic images obtained in mice before undergoing LAD ligation (baseline (BL)), at day 1 post-MI (D1), and at day 4 or day 7 post MI (D4, D7) for mice treated with IL-33 or its vehicle (PBS). Echocardiographic analyses revealed impaired contractile function with IL-33 treatment 7 days post-MI when compared with MI+vehicle at 7days. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

not observed in the vehicle-treated MI group. No significant changes in hemodynamic parameters were observed in any sham-operated groups (data not shown).

IL-33 treatment worsens cardiac remodeling 7 days post-MI

LV weight/tibia length, an index of hypertrophy, was measured post-mortem (4 and 7 days post-MI). A significant increase in LV hypertrophy was observed in the IL-33 treated MI group when compared with the vehicle-treated MI group at 7 days post-MI (6.30 ± 0.24 vs 5.56 ± 0.16 mg/mm; $*P < 0.05$) (Figure 3A). Moreover, MI mice treated with IL-33 showed a significant increase in pulmonary congestion in comparison with the vehicle-treated MI group at 7 days post-MI as evidenced by wet-to-dry water percentage (78.66 ± 0.57 vs $76.98 \pm 0.21\%$; $*P < 0.05$) (Figure 3B). Infarct size and expansion was comparable between the MI groups treated with IL-33 or vehicle at 4 days post-MI (30.35 ± 1.76 vs $28.17 \pm 3.78\%$). However, infarct expansion was more pronounced at 7 days in both MI groups, but to a greater extent in the IL-33 treated group (59.03 ± 1.69 vs $40.58 \pm 3.51\%$; $*P < 0.05$), indicating altered LV remodeling with IL-33 treatment following MI (Figure 3C,D).

IL-33 treatment increases mortality rate post-MI

Kaplan–Meier survival curve analysis showed a tendency toward decreased survival between days 1 and 4 in MI mice treated with IL-33 compared with vehicle-treated group (Figure 3E) ($n=22$ with 81.82% survival vs $n=17$ with 94.12% survival). However, a significant increase in mortality rate was observed by 7 days post-MI in the IL-33 treated group in comparison with vehicle-treated MI group (Figure 3F) ($n=46$ with 50.0% survival vs $n=38$ with 73.68% survival); most of the mice died between days 3 and 5 post-MI and upon autopsy, cardiac rupture was observed. None of the mice died from sham operation in both groups (IL-33 treated: $n=29$; vehicle-treated: $n=29$).

IL-33 treatment increases hypertrophy and fibrosis 7 days post-MI

Histological assessment revealed enlarged cardiomyocyte CSA and increased fibrosis in both MI groups (MI+vehicle and MI+IL-33) compared with the sham-operated groups at 7 days post-MI. Treatment with IL-33, however, further increased the degree of hypertrophy (399.5 ± 13.2 vs 284.5 ± 21.08 μm^2 ; $*P < 0.05$) (Figure 4A) and fibrosis (30.88 ± 1.85 vs $14.35 \pm 1.99\%$; $***P < 0.0001$) (Figure 4B) 7 days post-MI compared with the vehicle-treated MI group.

IL-33 treatment increases cardiac BAX/BCL2 mRNA levels and DNA fragmentation

mRNA expression levels of the apoptosis-regulating genes *BAX* and *BCL2* are shown in Figure 5 at 4 and 7 days post-MI: *BAX* (Figure 5A,D); *BCL2* (Figure 5B,E). *BAX/BCL-2* ratio increased in both MI groups at 4 days post-MI, but only significantly in the IL-33 treated MI group compared with IL-33 treated sham group (1.61 ± 0.13 vs 0.90 ± 0.10 fold change; $*P < 0.05$) (Figure 5C). At 7 days post-MI, mRNA expression levels of the *BAX/BCL2* ratio returned to baseline in the vehicle-treated MI group, but remained significantly higher in IL-33 treated MI group compared with the vehicle-treated MI group and IL-33 treated sham group, respectively (1.82 ± 0.38 vs 0.99 ± 0.06 fold change; $*P < 0.05$ and 1.82 ± 0.38 vs 0.68 ± 0.18 fold change; $**P < 0.01$) (Figure 5F). To explore whether IL-33 affects cardiomyocyte cell death, a TUNEL assay was performed on myocardial sections harvested 4 and 7 days post-MI. IL-33 significantly increased cardiomyocyte DNA fragmentation in the IL-33 treated MI group compared with all other groups at 4 days post-MI ($****P < 0.0001$) (Figure 5G,H). At 7 days, TUNEL-stained nuclei were significantly elevated in both MI groups, but to a significantly higher extent in the IL-33 treated MI group in comparison with the MI group treated with vehicle ($***P < 0.001$) (Figure 5G,I). These results indicate that the administration of IL-33 in the presence of MI increases the susceptibility to cardiomyocyte death which may contribute to increased infarct size.

IL-33 treatment affects cardiac and plasma inflammatory cytokines post-MI

Protein expression of representative pro-inflammatory cytokines was quantified by Western blot and GM-CSF plasma levels were quantified by ELISA. Four days after MI, cardiac active IL-1 β protein levels increased significantly in the vehicle-treated MI group when compared with the vehicle-treated sham-operated group (4.64 ± 1.28 vs 1.00 ± 0.18 fold change; $*P < 0.05$) (Figure 6A). Cardiac active IL-1 β tended to increase in the sham and MI groups treated with IL-33 without reaching significance (Figure 6A). At 7 days post-MI, cardiac active IL-1 β protein levels returned to baseline in all groups except for the vehicle-treated MI group, which became significantly more elevated than the IL-33 treated MI group (3.38 ± 1.03 vs 0.69 ± 0.19 fold change; $*P < 0.05$) (Figure 6B).

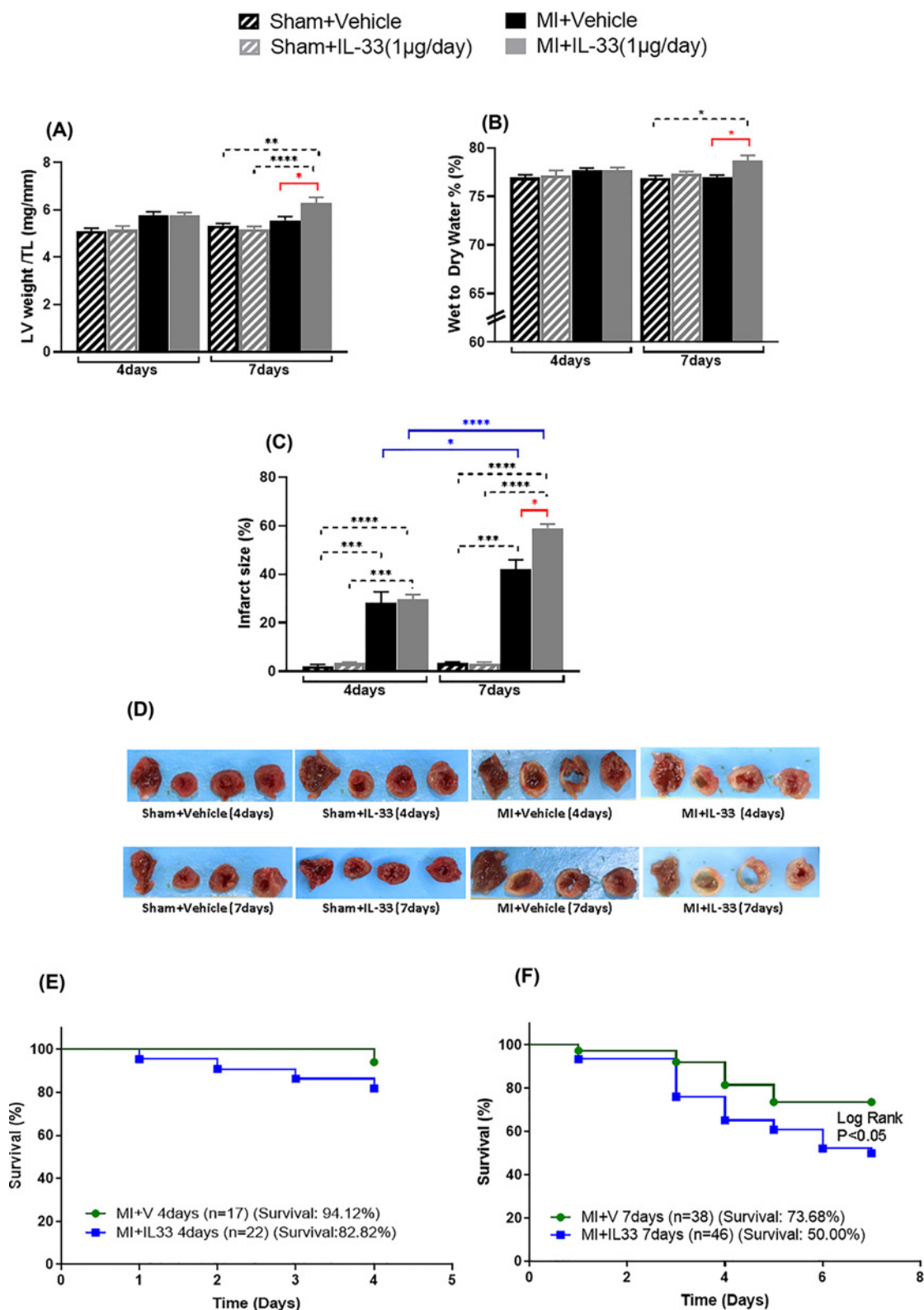


Figure 3. Increased adverse left ventricular remodeling and death with IL-33 treatment post-MI

(A) Variation of LV weight normalized to tibia length at 4 and 7 days post-LAD ligation or sham-operation. (B) Percentage of retained water in the lungs represented as degree of pulmonary congestion at 4 and 7 days. (C,D) Infarct size and representative images at 4 and 7 days post-MI, evaluated by TTC staining. (E,F) Kaplan–Meier survival curves for mice subjected to MI at 4 and 7 days. Bar graph data are mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

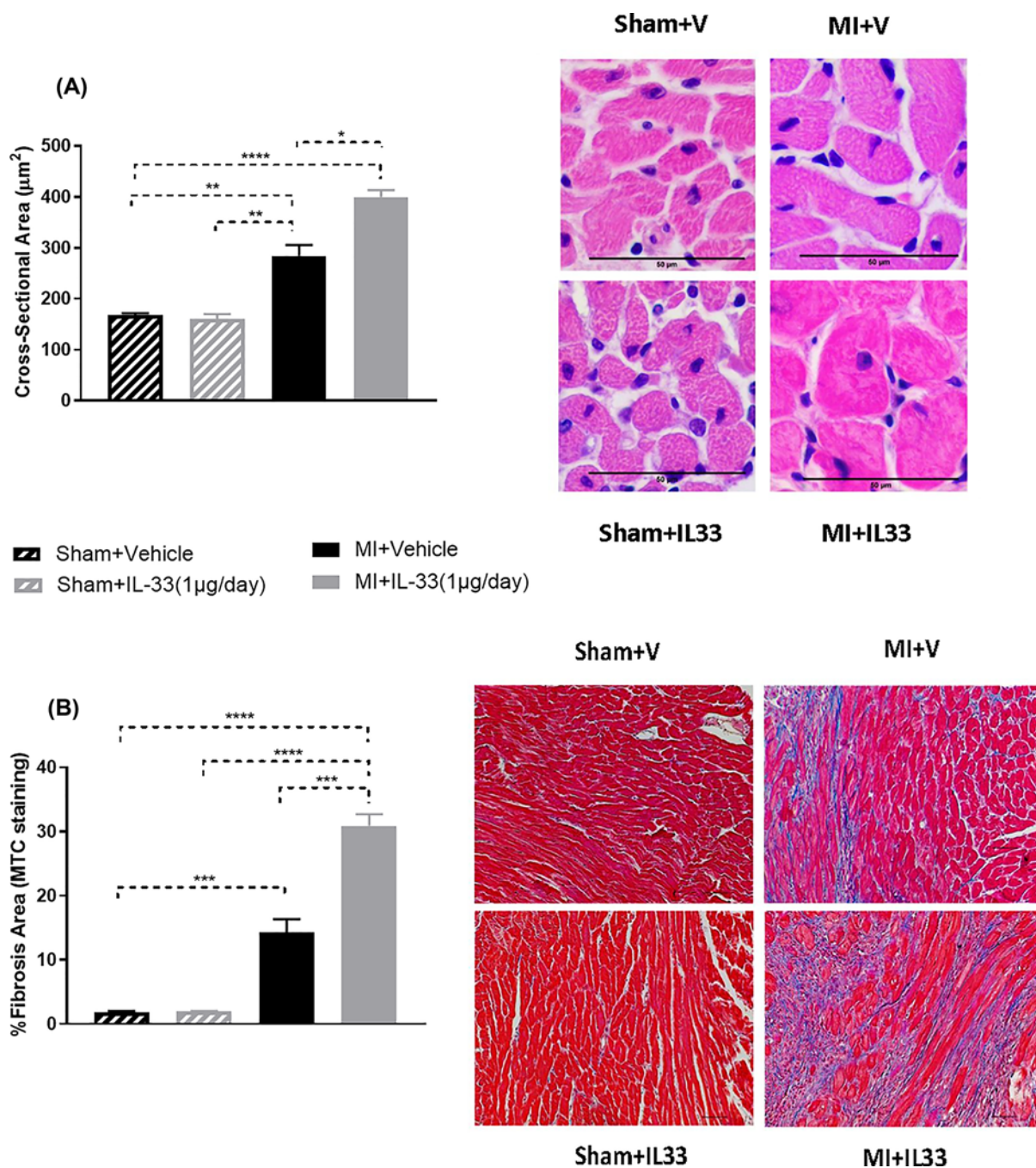


Figure 4. Administration of IL-33 in mice significantly enhances cardiac remodeling (fibrosis and hypertrophy) *in vivo* at 7 days

(A) Quantification of myocyte CSA and representative H&E stained cross-sections at the mid-ventricle level of LAD-ligated mice treated with IL-33 or vehicle for 7 days. (B) Quantification of fibrosis and representative MTC stained cross-sections at the mid-ventricle level of LAD ligated mice treated with IL-33 or vehicle for 7 days. IL-33 treatment exacerbates cardiac remodeling (fibrosis, and hypertrophy) post-MI when compared with the MI mice treated with vehicle ($n=4$ per group). * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$.

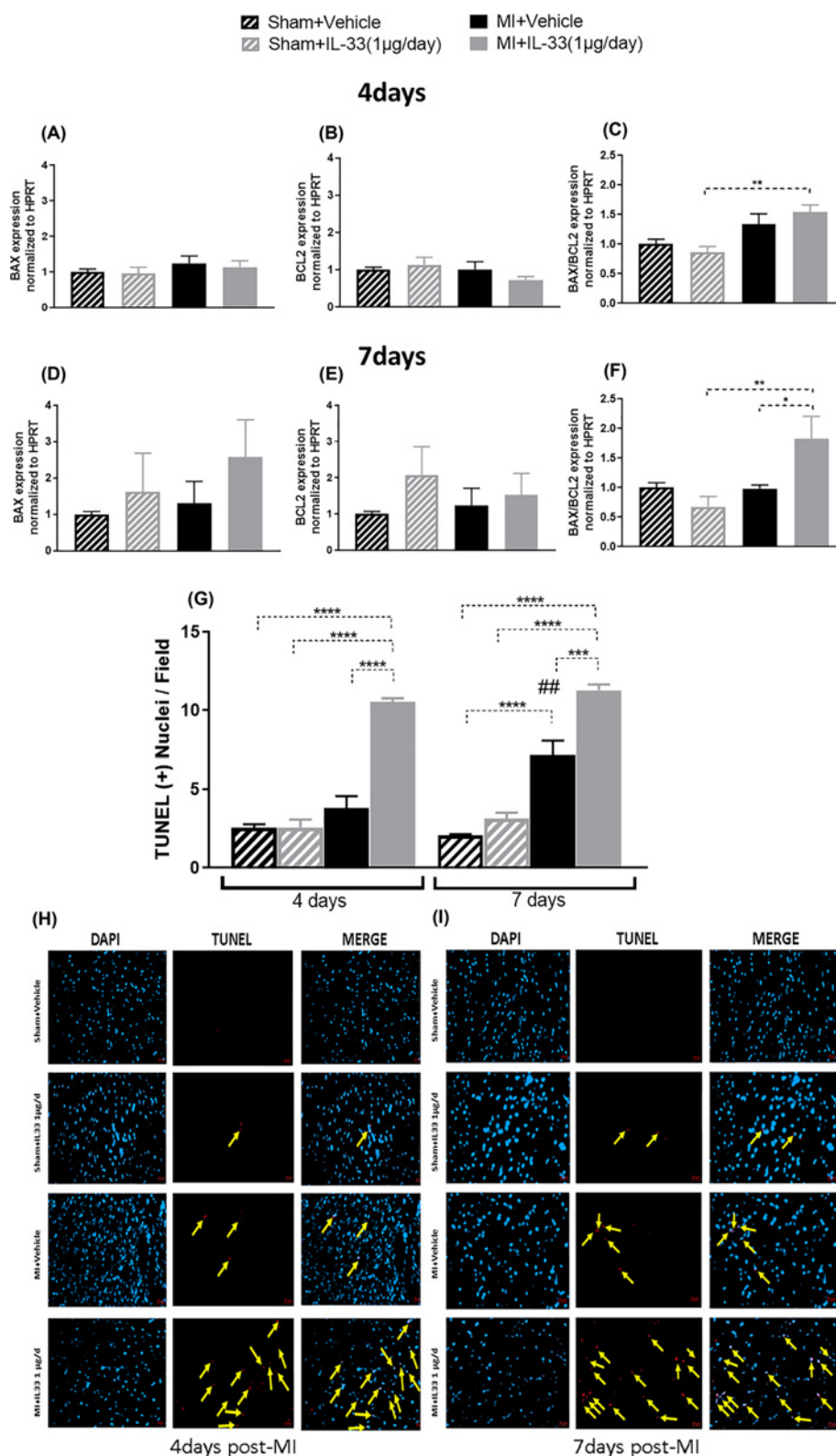


Figure 5. IL-33 administration increases cardiomyocyte apoptosis at 4 and 7 days post-MI

Normalized BAX and BCL2 gene expression and BAX/BCL2 ratio by quantitative RT-PCR at 4 (A–C) and 7 days (D–F) post-MI ($n=4–6$ per group) showing increased BAX/BCL2 ratio at both time points. Number of TUNEL positive cardiomyocytes at 4 and 7 days post MI with representative figures (G–I). DNA fragmentation increases with IL-33 treatment at 4 and 7 days post-MI. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$, ## $P<0.01$ (MI+vehicle 7d vs MI+vehicle 4d) ($n=3–4$ per group).

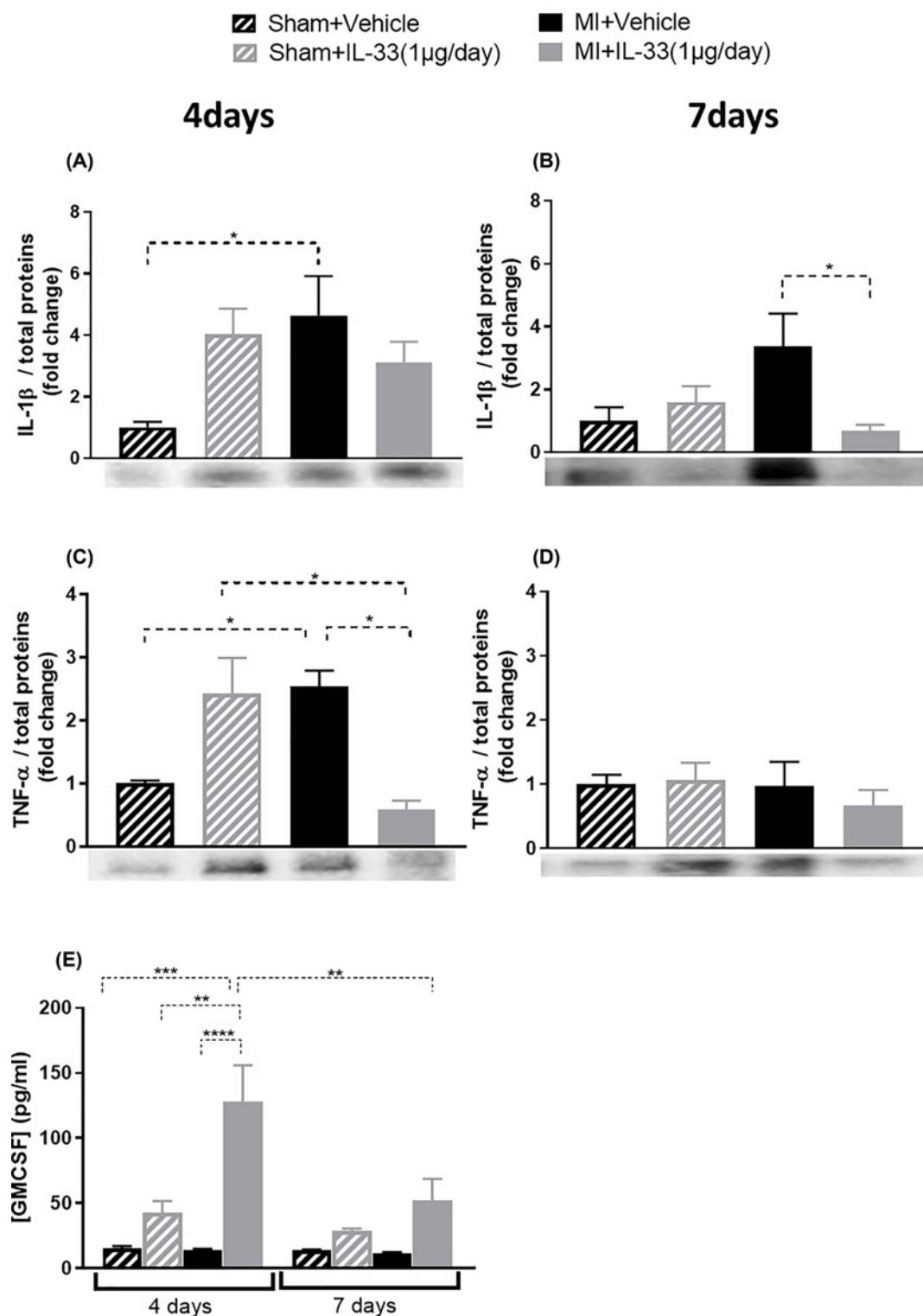


Figure 6. IL-33 treatment affects inflammatory cytokine protein expression

(A,B) IL-1β protein expression with representative bands at 4 and 7 days showing an increase in IL-1β cytokine expression in the MI group treated with vehicle at 4 days ($P < 0.05$) and a significant decrease in the MI group treated with IL-33 in comparison with the vehicle-treated group at 7 days. (C,D) TNF-α protein expression with representative bands at 4 and 7 days showing decreased TNF-α cytokine expression in the MI group treated with IL-33 for 4 days ($P < 0.05$) ($n = 4$ or 5 per group). (E) ELISA results for GM-CSF plasma protein concentrations at 4 and 7 days post sham or MI operation. Increased plasma concentrations of GM-CSF at 4 days with IL-33 treatment ($n = 4$ or 5 per group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Cardiac TNF- α expression increased significantly in the vehicle-treated MI group compared with the relative sham-operated group at 4 days (2.55 ± 0.24 vs 1.00 ± 0.05 fold change; $*P < 0.05$) (Figure 6C). By itself, IL-33 increased cardiac TNF- α levels at 4 days (2.43 ± 0.56 (sham+IL-33) vs 1.00 ± 0.05 (sham+vehicle) fold change). However, TNF- α protein levels were attenuated in the IL-33 treated MI group 4 days post-MI when compared with the MI group treated with vehicle (0.59 ± 0.14 vs 2.55 ± 0.24 fold change; $*P < 0.05$) (Figure 6C). At 7 days post-MI, TNF- α protein levels in all groups were at baseline with no significant differences among them (Figure 6D).

Plasma GM-CSF levels were significantly elevated in the IL-33 treated MI group at 4 days post-MI compared with all other groups ($**P < 0.01$ to $****P < 0.0001$) (Figure 6E). Plasma GM-CSF decreased at 7 days post-MI 51.97 ± 16.78 (pg/ml) (MI+IL-33 D7) vs 128.1 ± 27.75 (pg/ml) (MI+IL-33 D4); $**P < 0.01$, but remained insignificantly more elevated than all other groups at the same time point. The sham-operated group treated with IL-33 showed a slight but insignificant increase in GM-CSF plasma concentrations compared with all vehicle-treated groups at both 4 and 7 days post-MI (Figure 6E).

IL-33 treatment increases MMP2 and MMP9 mRNA expression 7 days post-MI

MMP9 mRNA levels significantly increased in the IL-33 treated MI group compared with all other groups at 4 and 7 days post-MI ($*P < 0.05$, $**P < 0.01$ and $****P < 0.0001$) (Figure 7A,B). MMP2, however, did not increase in any group at 4 days (Figure 7C), but significantly increased at 7 days (Figure 7D) in the IL-33 treated MI group compared with the vehicle-treated sham group (4.46 ± 1.25 vs 1.00 ± 0.07 fold change; $*P < 0.05$). These results suggest that the administration of IL-33 in the presence of MI potentially enhances extracellular matrix (ECM) degradation.

IL-33 treatment promotes type 2 cytokines release

IL-33 administration in the MI group for 4 days resulted in a significant increase in IL-4, IL-13, and IL-10 cardiac protein expression ($*P < 0.05$ and $**P < 0.01$ vs. sham+vehicle) (Figure 8A,C,E). Moreover, IL-13 and IL-10 protein expression at 4 days was significantly more elevated in IL-33 treated MI group compared with the vehicle-treated MI group (4.61 ± 0.89 vs 1.83 ± 0.30 fold change; $**P < 0.01$) and (4.38 ± 1.56 vs 1.65 ± 1.56 fold change; $*P < 0.05$) (Figure 8C,E), respectively. At 7 days, IL-4, IL-13, and IL-10 protein expression returned to baseline in all groups (Figure 8B,D,F). Treatment with IL-33 increased IL-5 plasma concentrations in comparison with the vehicle-treated groups at 4 days in MI and sham groups (97.56 ± 4.94 (pg/ml) MI+IL-33 vs 0.44 ± 0.21 (pg/ml) MI+vehicle) ($****P < 0.0001$) and (95.10 ± 0.55 (pg/ml) sham+IL-33 vs 0.51 ± 0.29 (pg/ml) sham+vehicle) ($****P < 0.0001$), respectively (Figure 8G). Seven days post-MI, IL-5 plasma concentrations decreased in the IL-33 treated groups compared with their counterparts at 4 days (97.56 ± 4.94 (pg/ml) (MI+IL-33 D4) vs 23.92 ± 6.73 (pg/ml) (MI+IL-33 D7) ($****P < 0.0001$) and sham groups (95.10 ± 0.55 (pg/ml) (sham+IL-33 D4) vs 56.19 ± 6.74 (pg/ml) (sham+IL-33 D7) ($***P < 0.001$)), but remained significantly more elevated compared with the vehicle-treated MI and sham groups at 7 days post-MI (23.92 ± 6.73 (pg/ml) (MI+IL-33) vs 0.45 ± 0.06 (pg/ml) (MI+vehicle); ($*P < 0.05$)) and (56.19 ± 6.74 (pg/ml) (sham+IL-33) vs 1.55 ± 0.60 (pg/ml) (sham+vehicle); ($***P < 0.001$)) (Figure 8G). No plasma IL-5 levels were detected in vehicle-treated MI and sham groups.

IL-33 treatment modulates neutrophil and monocyte/macrophage production, mobilization, and recruitment

Neutrophils and monocytes were identified as CD45+CD11b+Ly6G(hi) and CD11b+Ly6C(hi)Ly6G-F480- cells, respectively. Compared with day 1, neutrophil and Ly6C(hi) monocyte production in the bone marrow peaked at 3 days in all experimental groups including the vehicle-treated sham-operated group (Figures 9A and 10A). At 7 days, bone marrow neutrophil and Ly6C(hi) levels decreased significantly in the IL-33 treated MI group compared with the levels observed at day 3 (neutrophils: $46.37 \pm 1.58\%$ (MI+IL33 D3) vs $29.85 \pm 1.94\%$ (MI+IL33 D7) $*P < 0.05$) (Ly6C(hi): $16.80 \pm 1.75\%$ (MI+IL-33 D3) vs $5.59 \pm 0.99\%$ (MI+IL-33 D7); $****P < 0.0001$) (Figures 9A and 10A). Moreover, bone marrow neutrophils at 7 days were significantly lower in the IL-33 treated MI group in comparison with the vehicle-treated MI group at the same time point (29.85 ± 1.94 vs $46.13 \pm 2.28\%$; $*P < 0.05$) (Figure 9A). In the spleen, compared with day 1, neutrophils peaked at 3 days post-MI in the IL-33 treated group (10.72 ± 1.47 vs $4.73 \pm 0.83\%$ D1; $*P < 0.05$) where levels were significantly higher than in the vehicle-treated MI group at the same time point (10.72 ± 1.47 vs $5.01 \pm 0.93\%$ (MI+vehicle D3); $*P < 0.05$, Figure 9B). Neutrophil circulation in the blood culminated at day 1 in all experimental groups and reached its lowest point at 7 days where levels in the IL-33 treated MI group were significantly lower than in the vehicle-treated group at the same time point (7.07 ± 2.95 vs $26.62 \pm 6.54\%$; $*P < 0.05$) (Figure 9C). At 7 days, Ly6C(hi) levels in the spleen and in the blood were significantly lower in

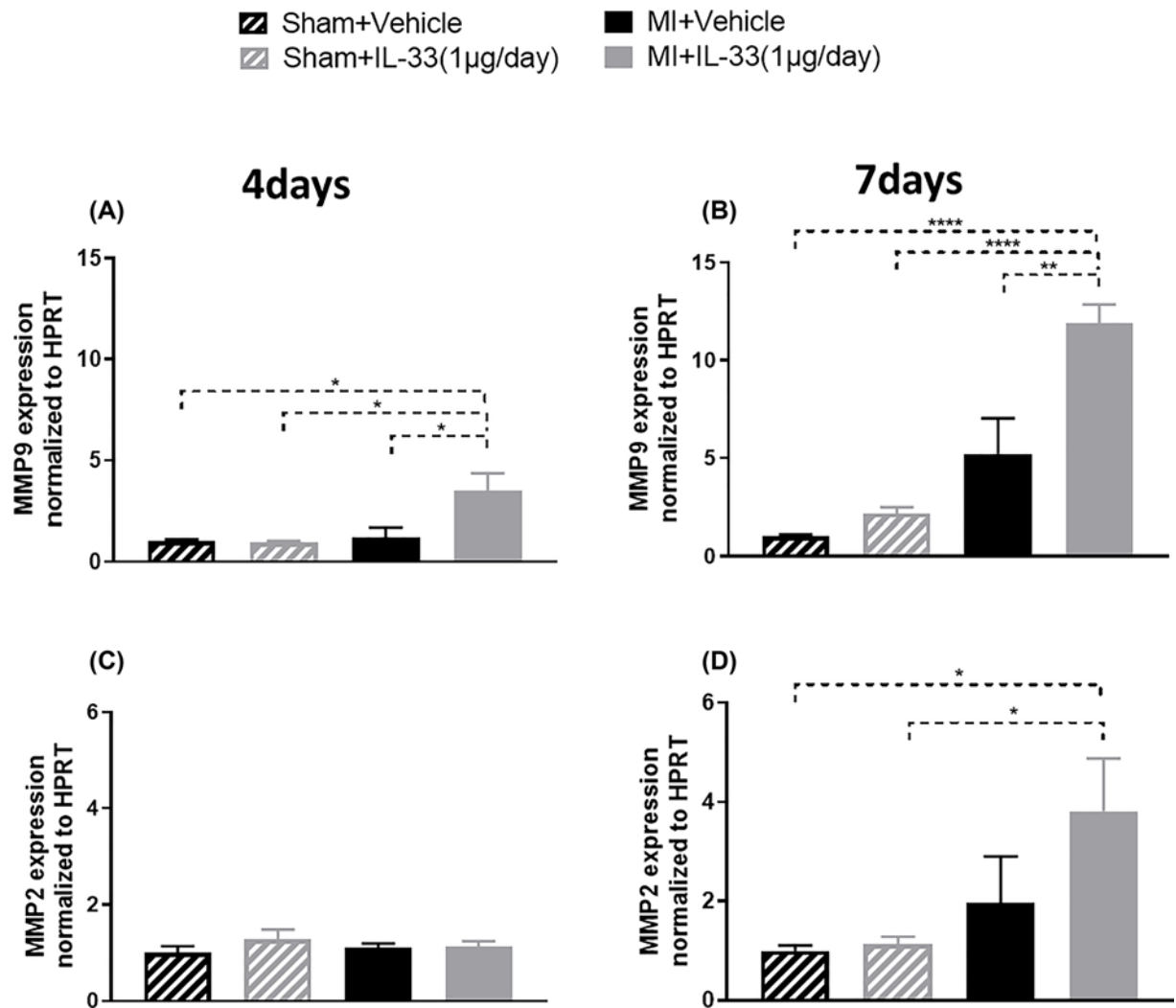


Figure 7. Up-regulation of matrix metalloproteinases with IL-33 treatment at 7 days

(A,B) Normalized MMP9 gene expression by quantitative RT-PCR at 4 and 7 days showing up-regulation of MMP9 with IL-33 treatment at 4 and 7 days ($P<0.05$). (C,D) Normalized MMP2 gene expression by quantitative RT-PCR at 4 and 7 days showing up-regulation of MMP2 with IL-33 treatment at 7 days post-MI ($P<0.05$) ($n=4-6$ per group). * $P<0.05$, ** $P<0.01$, **** $P<0.0001$.

the IL-33 treated MI group when compared with the levels observed in the vehicle-treated MI group (Figure 10B,C; * $P<0.05$). Of note, neutrophils and monocytes increased in the hearts of both experimental groups (MI+vehicle and MI+IL-33) on day 1 post-MI, and decreased significantly and concomitantly on day 7 post-MI (Figures 9D and 10D).

Macrophages were identified as CD11b+F480+Ly6C-. Compared with day 1, macrophages significantly peaked with IL-33 treatment in the bone marrow as early as 3 days post-MI in the IL-33 treated sham and MI groups, and remained elevated when compared with the vehicle-treated groups at 7 days post-MI (Figure 11A). In the spleen and blood, macrophages increased significantly with IL-33 treatment and culminated at 7 days post-MI (Figure 11B,C). In the heart, MI induced a significant increase in macrophage levels in the vehicle-treated group at 3 days post-MI when compared with day 1 (Figure 11D). However, a significant increase in macrophage levels was observed in the heart of the IL-33 treated MI group at 7 days when compared with the same group at day 1 and day 3 post-MI (Figure 11D). Furthermore, macrophage levels in the IL-33 treated MI group at 7 days were significantly more elevated than the vehicle-treated MI group at the same time point (Figure 11D).

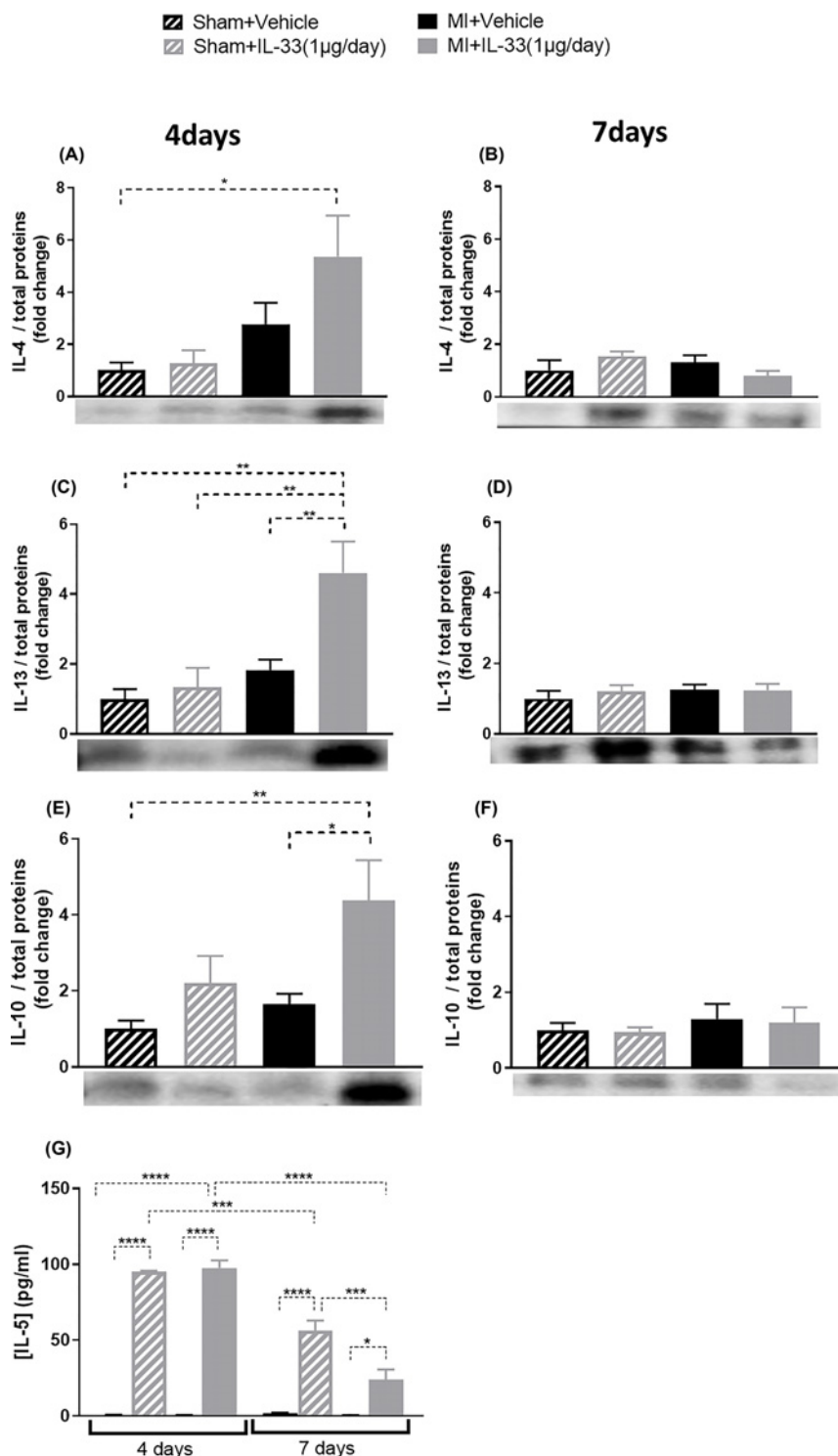


Figure 8. Increased type 2 cytokine production with IL-33 treatment

(A,B) IL-4 protein expression with representative bands at 4 and 7 days showing increased IL-4 cytokine expression in the MI group treated with IL-33 for 4 days ($P < 0.05$) ($n = 3$ –4 per group). (C,D) IL-13 protein expression with representative bands at 4 and 7 days showing increased IL-13 cytokine expression in the MI group treated with IL-33 for 4 days ($P < 0.05$). (E,F) IL-10 protein expression with representative bands at 4 and 7 days showing increased IL-10 cytokine expression in the MI group treated with IL-33 for 4 days ($P < 0.05$). (G) Increased plasma concentrations of IL-5 at 4 days with IL-33 treatment. Plasma concentrations of IL-5 were examined by ELISA at 4 and 7 days post-MI (sham animals $n = 3$ per group; MI animals $n = 5$ per group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

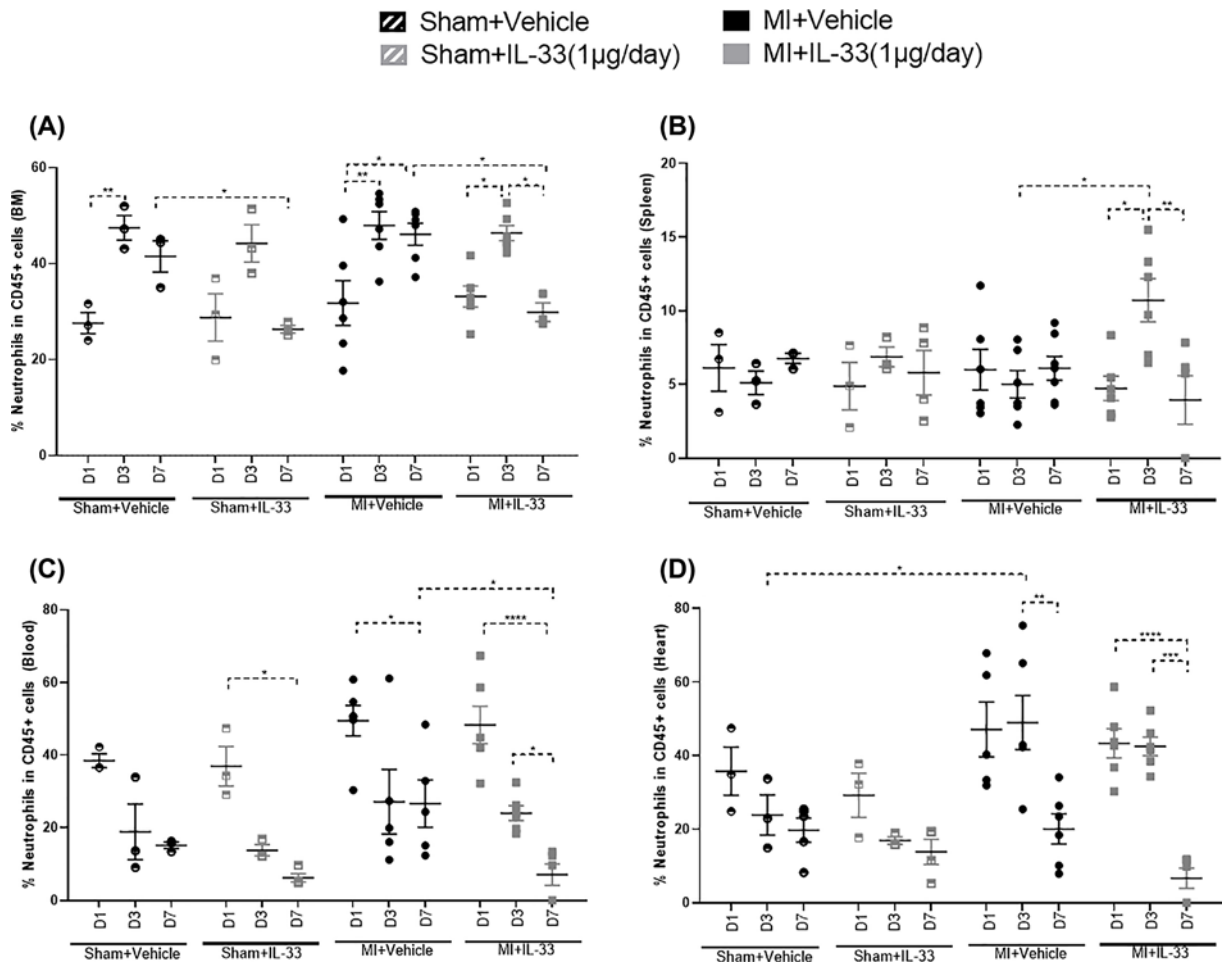


Figure 9. Comparable neutrophil resolution in the heart between IL-33-treated and vehicle-treated MI groups at 7 days post-MI

Time course variation (day 1 (D1), day 3 (D3) and day 7 (D7) post-surgery) of neutrophil percentage calculated out of CD45+ cells from, (A) the bone marrow (BM), neutrophil production in the bone marrow peaks at day 3 in all experimental groups when compared with day 1. (B) Spleen, neutrophil infiltration peaks at day 3 in the spleen in the MI group treated with IL-33. (C) Blood, neutrophils are at their highest levels at day 1 and decrease at day 7 in all experimental groups, and (D) heart, neutrophil infiltration drops significantly at day 7 in both MI groups. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$. Each dot represents one mouse ($n=3$ in sham-operated groups and $n=5-6$ in LAD-ligated groups).

IL-33 treatment elicits eosinophilia

Eosinophils were identified as CD45+SiglecF+CD11c-. IL-33 is known to directly facilitate the maturation of eosinophils by enhancing their survival, activation, and adhesion [47]. IL-33 treatment for 7 days induced a drastic increase in eosinophils in all organs (bone marrow, spleen, blood, and heart). Percentage of eosinophil out of CD45+ cells ranged between (8.81 ± 1.23 and $42.37 \pm 5.89\%$) in the IL-33-treated sham-operated group and (10.02 ± 1.74 and $62.26 \pm 5.56\%$) in the IL-33-treated MI group in all organs at 7 days (Figure 12A–D).

IL-33 treatment increases ILC2 levels at 7 days post-MI

ILC2 were identified as Lin-ICOS+CD25+. Although present in all organs in a very small percentage, ILC2 seemed to increase with IL-33 treatment. In the bone marrow, compared with day 1, a significant increase in ILC2s was observed at 3 days in the IL-33 treated sham-operated group ($0.14 \pm 0.01\%$ (D1) vs $0.46 \pm 0.11\%$ (D7); *** $P<0.001$) (Figure 13A). Moreover, treatment of the sham-operated group with IL-33 increased ILC2 production in the blood at 7 days when compared with the vehicle-treated sham-operated group at the same time point (0.014 ± 0.014 vs $0.30 \pm 0.06\%$; * $P<0.05$) (Figure 13C). In the spleen and in the heart, a tendency toward an increase in ILC2 percentage was

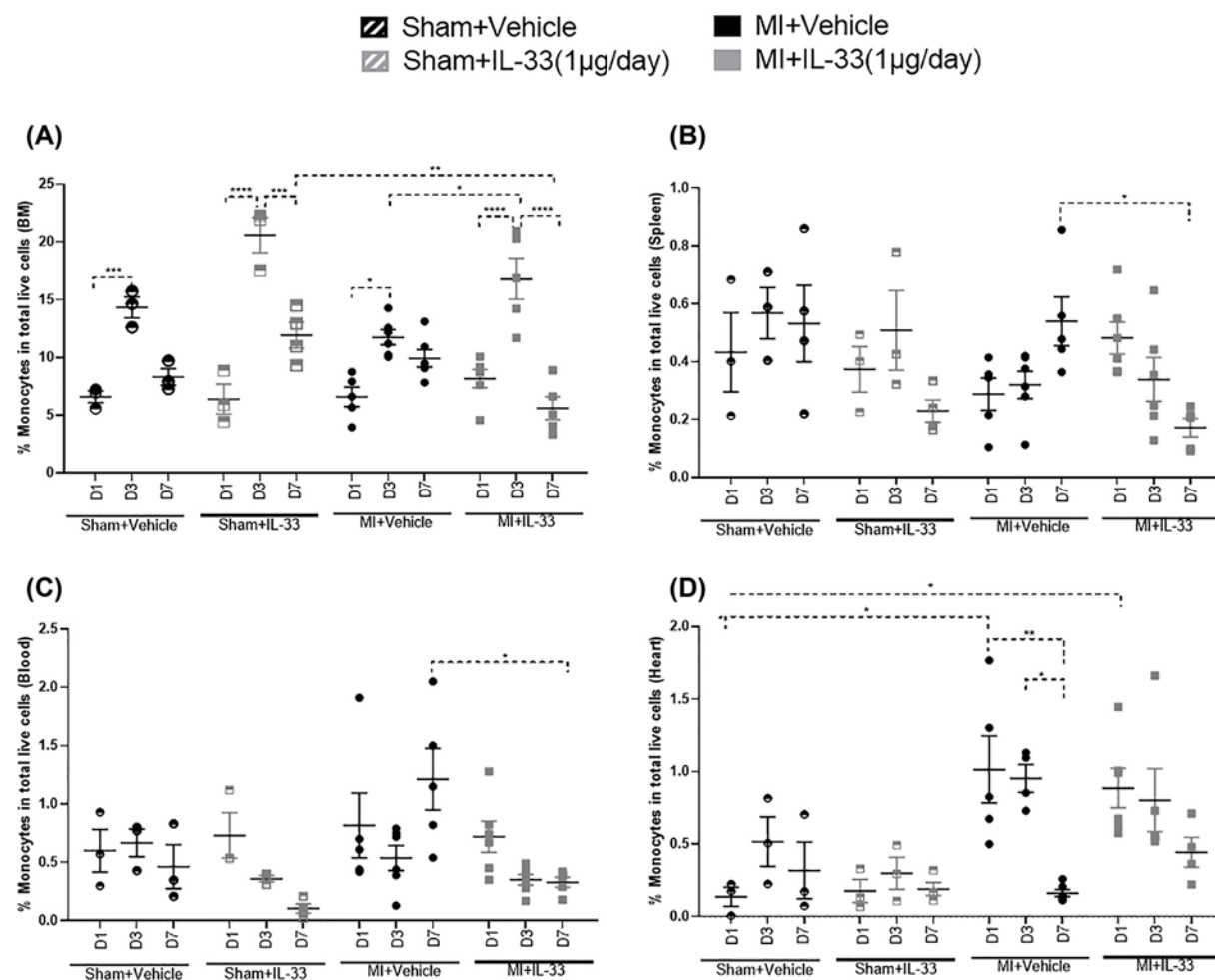


Figure 10. Dynamics of proinflammatory monocytes at different timepoints with and without IL-33 treatment

Time course variation (day 1 (D1), day 3 (D3) and day 7 (D7) post-surgery) of monocytes percentage calculated out of total live cells from (A) the bone marrow (BM), monocyte production in the bone marrow peaks at day 3 in all experimental groups when compared with day 1. (B) Spleen, a significant decrease in monocyte infiltration in the spleen is observed in the MI group treated with IL-33 when compared with the MI group treated with vehicle at day 7. (C) Blood, circulating monocytes in the blood decrease significantly at day 7 in the MI group treated with IL-33 when compared with the MI group treated with vehicle, and (D) heart, monocyte levels are at their highest in both MI groups at day 1 and decrease gradually over time. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Each dot represents one mouse ($n=3$ in sham-operated groups and $n=5-6$ in LAD-ligated groups).

observed in the IL-33-treated sham-operated group but without reaching significance. ILC2 significantly increased in the IL-33-treated MI group and ranged between (0.20 and 1.26)% of the total number of cells in all organs at 7 days (Figure 13A–D).

IL-33 treatment induces splenomegaly

In contrast with the normal spleen size and weight observed in the vehicle-treated sham and MI mice (ranging between 4.19 ± 0.42 and 4.81 ± 0.19 mg/mm), administration of IL-33 for 4 and 7 days induced a significant increase in spleen weight in a time-dependent manner regardless of the surgical intervention (Supplementary Figure S1). Increases in 1.73-folds and 2.6-folds in spleen weights were observed between the vehicle-treated and IL-33-treated sham-operated groups at 4 and 7 days, respectively. The variation in spleen weights in the IL-33-treated MI groups was comparable with that observed in the sham-operated groups where increases in 1.7-folds and 3-folds in spleen weights were seen between the vehicle-treated and IL-33-treated MI groups at 4 and 7 days respectively. These findings are consistent with previous data showing increase in spleen size and weight with IL-33 treatment [4,48].

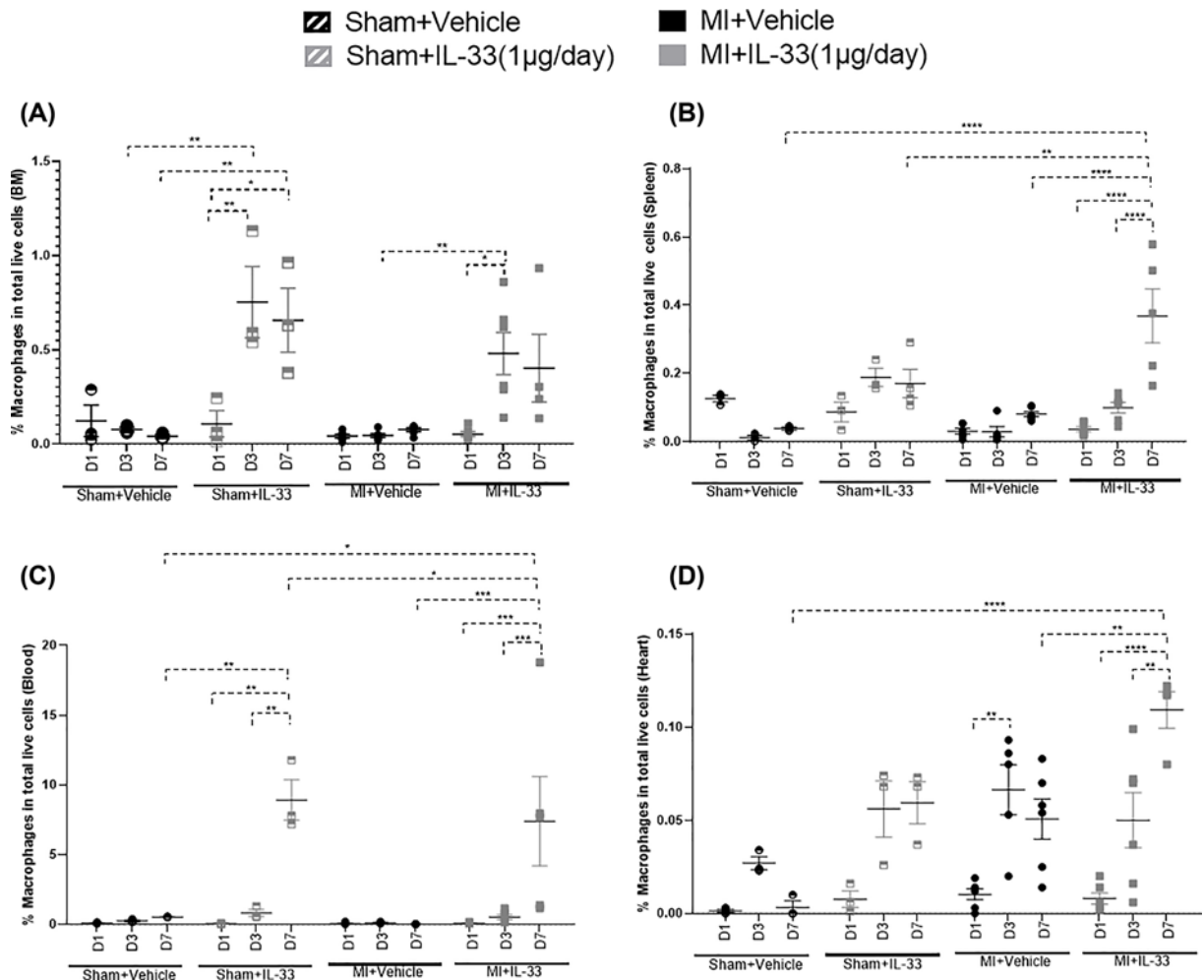


Figure 11. Increased macrophage levels with IL-33 treatment at 7 days post-MI

Time course variation (day 1 (D1), day 3 (D3) and day 7 (D7) post-surgery) of macrophage percentage calculated out of total live cells from, (A) the bone marrow (BM) macrophages in the bone marrow increase significantly with IL-33 treatment at day 3, (B) spleen, macrophage levels start increasing with IL-33 treatment as early as day 3 and become significantly more elevated in the MI group treated with IL-33 at day 7, (C) blood, a significant increase in macrophage circulation in the blood is observed with IL-33 treatment at day 7, and (D) heart, macrophage levels start increasing in the heart at day 3 and become significantly more pronounced in the MI group treated with IL-33 at day 7. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Each dot represents one mouse ($n = 3$ in sham-operated groups and $n = 5-6$ in LAD-ligated groups).

IL-33 treatment up-regulated cardiac sST2 expression at 7 days in the presence or absence of MI

mRNA levels of cardiac sST2 were measured at 4 and 7 days in all groups treated with IL-33 or vehicle. sST2 levels were not changed at 4 days in all sham and MI groups (Supplementary Figure S2A). However, at 7 days, sST2 mRNA levels increased significantly in the sham and MI groups treated with IL-33 compared with the sham group treated with vehicle ($P < 0.01$ and $P < 0.001$, respectively). MI increased sST2 levels in the vehicle-treated group 7 days post-MI with no significance when compared with the relative sham group. Administration of IL-33 significantly up-regulated sST2 cardiac expression in the MI group in comparison with vehicle-treated MI group ($P < 0.01$) (Supplementary Figure S2B). The receptor for IL-33 (ST2) is found primarily on many 'allergy associated' target cells, including, helper T cells (Th2), eosinophils, basophils, mast cells and ILC2 [49]. IL-33 participates in the synthesis and release of IL-5 from these target cells thereby possibly promoting eosinophilia [4].

IL-33 plasma availability peaked at 4 days and decreased at 7 days while remaining significantly more elevated than in the MI group treated with vehicle (Supplementary Figure S2C). It is possible that sST2 levels are increased in the

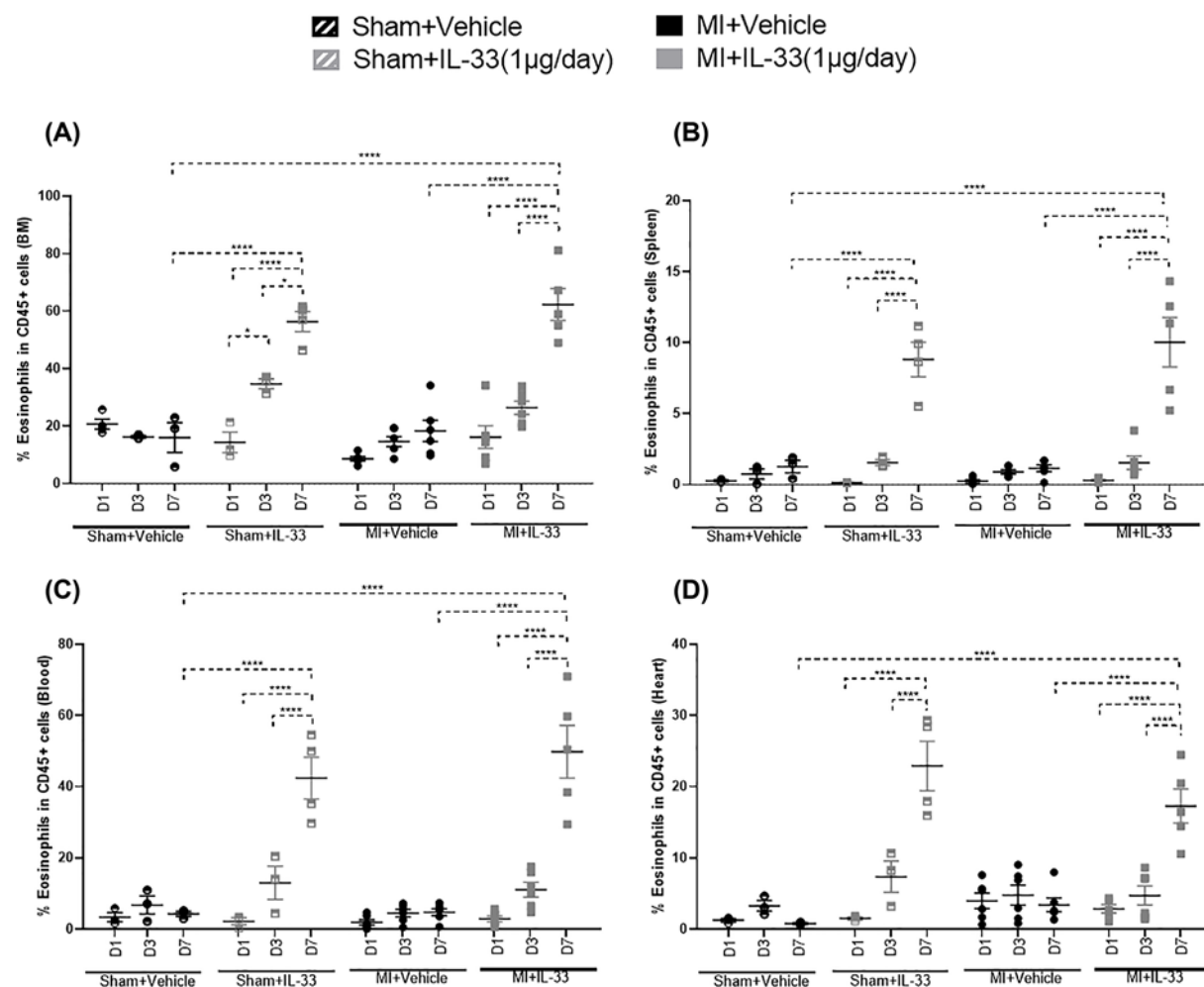


Figure 12. IL-33 induces eosinophilia at day 7 regardless of the surgical intervention

Time course variation (day 1 (D1), day 3 (D3) and day 7 (D7) post-surgery) of eosinophil percentage calculated out of CD45+ cells, (A) the bone marrow (BM), (B) spleen, (C) blood, and (D) heart. Eosinophil levels start increasing as early as day 3 in the sham and MI groups treated with IL-33 and become significantly more increased at day 7 in these two groups in all tissues. * $P<0.05$, **** $P<0.0001$. Each dot represents one mouse ($n=3-4$ in sham-operated groups and $n=5-6$ in LAD-ligated groups).

plasma at 7 days, therefore acting as a decoy receptor to IL-33 and decreasing its availability in the plasma at 7 days. The dynamic expression of plasma IL-33 was measured by ELISA at 4 and 7 days post-MI (Supplementary Figure S2B). Plasma IL-33 concentrations peaked at 4 days in the sham and MI groups treated with IL-33 (Supplementary Figure S2C), and decreased significantly at 7 days in both groups but remained significantly higher than the vehicle-treated sham and MI groups (Supplementary Figure S2C).

Decreased protein and gene expression of profibrotic markers with IL-33 post-MI

Cardiac protein expression of the fibrogenic cytokine TGF- β was not altered by IL-33 or MI at day 4. However, at 7 days, levels of TGF- β increased in the sham+IL-33 and MI+vehicle groups without reaching significance. Moreover, TGF- β protein expression decreased significantly in the MI group treated with IL-33 when compared with the MI group treated with vehicle at 7 days (0.84 ± 0.20 vs 2.29 ± 0.50 fold change; * $P<0.05$) (Supplementary Figure S3A,B). The transdifferentiation of fibroblasts into myofibroblasts is characterized by the up-regulation of α -SMA [50]. α -SMA cardiac expression also decreased at 7 days post-MI in the IL-33 treated MI group compared with the MI group treated with vehicle (0.68 ± 0.11 vs 1.63 ± 0.31 fold change; * $P<0.05$) (Supplementary Figure S3C,D). Gene expression of connective tissue growth factor (CTGF), a matrix-associated protein, significantly increased in the MI

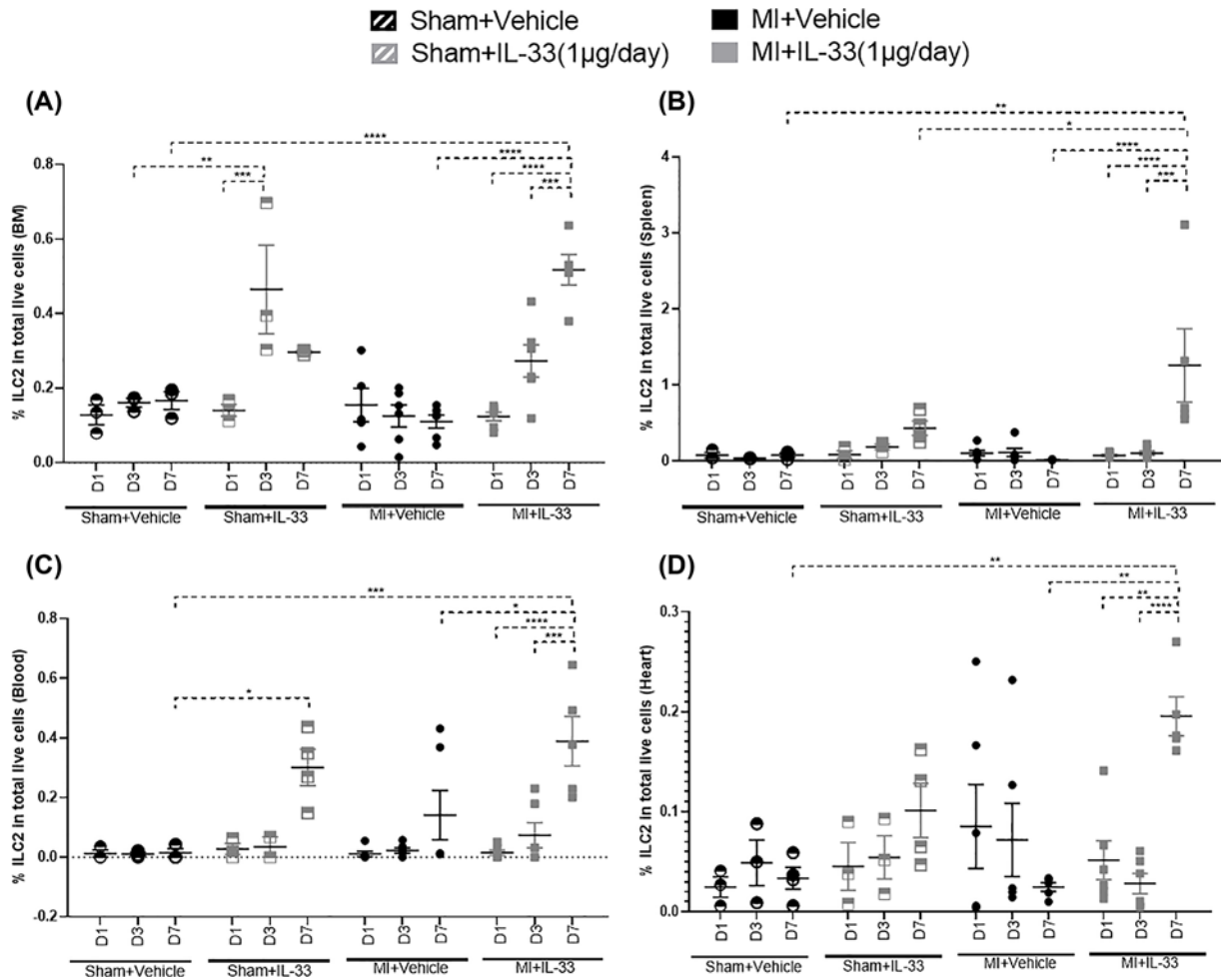


Figure 13. Treatment with IL-33 increases ILC2 levels at 7 days post-MI

Time course variation (day 1 (D1), day 3 (D3) and day 7 (D7) post-surgery) of ILC2 percentage calculated out of total live cells from, (A) the bone marrow (BM), ILC2 levels start increasing as early as day 3 with IL-33 treatment and become significantly more elevated in the MI group treated with IL-33 at day 7, (B) spleen, ILC2 levels peak in the spleen at day 7 in the MI group treated with IL-33, (C) blood, ILC2 circulation in the blood increases significantly at day 7 in the sham and MI groups treated with IL-33, and (D) heart, ILC2s increase significantly in the heart at day 7 in the MI group treated with IL-33. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$. Each dot represents one mouse. ($n=3$ in sham-operated groups and $n=5-6$ in LAD-ligated groups).

group treated with vehicle compared with the sham vehicle-treated group at 4 and 7 days post-MI (16.39 ± 2.69 vs 1.00 ± 0.03 fold change; *** $P<0.001$). In contrast, IL-33 treatment significantly decreased CTGF gene expression at 4 days post-MI compared with the vehicle-treated MI group (6.07 ± 1.22 vs 16.39 ± 2.69 fold change; ** $P<0.01$) (Supplementary Figure S3E). of note, CTGF gene expression remained more elevated in the vehicle-treated MI group at 7 days when compared with the sham-operated group (4.87 ± 0.72 vs 1.00 ± 0.21 fold change; * $P<0.05$) (Supplementary Figure S3F). These results indicate that administration of IL-33 in MI mice attenuates cardiac profibrotic markers in MI mice.

Discussion

Due to the limited capacity of the adult mammalian heart to regenerate, the healing process post-MI is phasic, relying mainly on an early sterile inflammatory response to remove the debris of dead cells followed by granulation and maturation phases to replace lost cells with fibrotic tissue and strengthen scar formation. Depending on the clinical state, and the presence or absence of comorbidities, alteration in transition between phases such as inflammation prolongation or increased fibrosis deposition can worsen cardiac remodeling post-MI. Providing a pharmacological

platform to promote appropriate transition between early remodeling phases post-MI through immunomodulatory mechanisms is gaining significant attention and constitutes the basis of the experiments presented in this manuscript.

In the present study, we assessed the reparative actions of the alarmin IL-33 on the infarcted myocardium. IL-33 was administered for 4 and 7 consecutive days following LAD coronary artery ligation or sham-operation. We observed that IL-33 was indeed effective in activating a type 2 immune response in the damaged heart, consistent with the anticipated wound healing response, as evidenced by early (day 4) increased levels of type 2 cytokines (IL-4, IL-5, and IL-13) and anti-inflammatory IL-10, and reduced levels of inflammatory TNF- α (at 4 and 7 days) and IL-1 β (7 days). Nonetheless, IL-33 was associated with a pronounced decrease in systolic function at day 7 with ventricular dilation and infarct expansion, along with increased apoptosis (increased BAX/BCL2 and TUNEL+ nuclei). The overall results of the present study reveal that IL-33 is associated with worsened cardiac function and increased adverse cardiac remodeling in the MI mouse model. Administration of IL-33 increased mortality due to cardiac rupture, induced pulmonary congestion and cardiomyocyte apoptosis, and promoted LV hypertrophy. Despite decreased systolic function, our FACS data did not show any major changes in monocyte and neutrophil cardiac infiltration pattern in the IL-33-treated MI group when compared with the vehicle-treated group at 7 days. These results are in agreement with previously published data showing no major increases in cardiac neutrophils and monocytes despite exacerbation in cardiac dysfunction and increased myocarditis with IL-33 treatment [41]. With IL-33 treatment, increased cardiac hypertrophy and fibrosis were observed. This may reflect a compensatory response to increased infarct expansion, although it is unsettled whether the fibrosis was productive or reflected decreased ECM turnover. Overall, IL-33 treatment was associated with a decrease in profibrotic markers in the infarcted heart (CTGF, TGF- β) and in activated myofibroblasts (α -SMA), as well as an increase in MMPs that degrade the ECM. Increase in MMPs is linked to the slippage of myocyte fascicles thereby promoting wall thinning in the infarcted area leading to cardiac rupture. Previous studies have shown that MMPs are not only involved in ECM degradation, but are also implicated with the progression to heart failure [51]. Targeted deletion of MMP2 or MMP9 decreases the incidence of cardiac rupture and attenuates LV enlargement following LAD ligation in mice [52–54]. In this study, we observe increased expression of MMP2 and MMP9 at 7 days that may have contributed to wall thinning and rupture. These metalloproteases regulate ECM turnover and were shown to be produced by eosinophils and contribute to airway remodeling [55,56].

We observed as well an increase in ILC2s with IL-33 treatment in the bone marrow, spleen, and blood, as well as the myocardium that was further enhanced at day 7 in the infarcted mice. Our current data are in agreement with recently published data stating that the activation of ILC2 by IL-33 plays a pathogenic role in the heart by inducing cardiac eosinophilic infiltration and exacerbating cardiac dysfunction [30]. Whether the cardiac ILC2s originated from a resident cardiac or pericardiac pool [29] or were recruited from the mediastinal cavity [30] as suggested by others was not established. Along with type 2 Th2, ILC2s are a major source of type 2 cytokines and mediators of the actions of IL-33 at barrier sites of the body [8], and in certain cases shown to amplify inflammation via activation of eosinophils [57,58].

As previously reported [4], IL-33 treatment induced splenomegaly, which we observed was associated with a delayed accumulation of eosinophils in the spleen. This finding is consistent with a recent report stating that IL-33 positively affects eosinophil development [47]. IL-33 has also been implicated in activating mature eosinophils. For instance, IL-33 was reported to play a role in either the survival or death of human eosinophils depending upon context [47] and to stimulate nuclear factor- κ B (NF- κ B)-mediated inflammatory gene expression in murine eosinophils by both IL-4-independent and IL-4-dependent/autocrine mechanisms [59]. In addition, IL-33 was reported to induce degranulation and superoxide anion production of human eosinophils as effectively as IL-5 [60]. Nevertheless, differential effects of IL-33 on collagen deposition and MMP2/9 levels in sham versus MI hearts suggest that some other factor(s) might be involved in activating eosinophils.

IL-33 induced a delayed increase in eosinophils in the heart in both the absence and presence of MI. Increased plasma concentrations of IL-5 and GM-CSF (which are potent cytokines associated with eosinophil development and increased viability [61]) at 4 days may have facilitated this recruitment. The IL-33-induced increase by day 4 in IL-4 and IL-13 in the infarcted myocardium may have contributed as well [62]. Previous studies have shown a direct correlation between increased serum GM-CSF levels and impairment of the healing process post-MI by acting at a distance in the BM and locally in the heart to produce and enhance immune cells accumulation [63]. In this study, elevated serum GM-CSF levels correlated with strong eosinophil recruitment and cardiac dysfunction following IL-33 treatment post-MI. Earlier studies indicated that eosinophils are rapidly activated within 2–3 days following MI, infiltrate into the myocardium, and contribute to cardiac rupture in some patients [64,65]. Other studies have linked eosinopenia to an acute MI in patients presenting with acute coronary syndrome [66,67]. A decrease in blood eosinophils might be indicative of their accumulation in the damaged myocardium [68]. This scenario is further substantiated by evidence showing a positive correlation between higher baseline serum levels of eosinophil

cationic protein (ECP) and follow-up occurrence of MACEs after PCI [69–71]. On the other hand, Shiyovich et al. [72] observed a U-shaped association between blood eosinophils within 72 h of admittance and greater risk of death in patients with acute MI, with a greater count associated with lower 1 year mortality rates. This observation was interpreted to mean that eosinophils might help control the inflammatory response.

Several studies have shown that increased sST2 expression can have a negative prognostic impact on the general cardiovascular risk profile [8,20]. For instance, sST2 mRNA was markedly increased after biomechanical strain in cultured cardiomyocytes and fibroblasts and serum sST2 levels were transiently increased as early as four hours following coronary artery ligation in mice [73] and 12 h in patients with acute MI [19]. Patients with cardiac fibrosis, hypertrophy, LV dilatation, and decreased ventricular contractility have elevated sST2 levels, which is considered an independent predictor of adverse outcomes in acute decompensated heart failure [74]. Moreover, in stable, well-treated congestive heart failure patients, elevated sST2 levels were associated with disease-specific end-points such as heart failure hospitalization and cardiovascular death [75]. Although in the present study we did not examine plasma sST2 levels, we did see an increase in cardiac sST2 mRNA levels at 7 days in our IL-33-treated MI mice, which corresponded with decreased IL-33 levels in the plasma at the same time point. Plasma IL-33 concentrations peaked at 4 days in the sham and MI groups treated with IL-33, and decreased significantly at 7 days in both groups but remained significantly higher than the vehicle-treated sham and MI groups. We therefore speculated whether sST2 levels are also increased in the plasma at 7 days, and are acting as a decoy receptor to IL-33 and decreasing consequently its availability in the plasma at 7 days.

Although the human and mouse IL-33 are only 55% identical, they both comprise 270 and 266 amino acids, respectively and have almost identical molecular weights (30 kDa human and 29.9 kDa murine) [5]. The IL-33 present in humans and mice have similar expression patterns and both orthologs are present in the nucleus and released extracellularly upon injury or necrosis [76]. Although previous investigators have used rat and human-derived IL-33 [4,32] and have shown variations in immune responses with IL-33 treatment, we used mouse recombinant IL-33 in our MI model. The administration of mouse IL-33 in the acute MI model in mice, can highly mimic the mechanisms occurring in human heart disease, and can therefore be a useful tool to elucidate the cardiovascular events arising following myocardial ischemia.

Previous studies have shown that the association of IL-33 with its transmembrane receptor (ST2L) could have protective effects on the heart. In the MI setting, Yin et al showed that IL-33 significantly decreases macrophage infiltration and alleviates inflammation in the heart, making IL-33 a potential therapeutic cytokine for cardiac dysfunction post-MI [37,38]. Despite similarities with IL-33 administration modality of this study, Yin et al. study differs in several aspects: (1) in the current study, hemodynamic parameters were collected at baseline, day 1, day 4, and day 7 post-MI. The results at day 1 clearly show that the hemodynamic parameters (EF, FS, CO, LV end-systolic volume (LVESV), LV end-diastolic volume (LVEDV), LV end-systolic diameter (LVESD), LVEDD) were in the same range in all MI groups, reflecting similar MI induction in all groups at day 1. Although no significant hemodynamic changes were observed at day 4, animals in the IL-33-treated MI group exhibited significantly decreased EF and increased LVESV, LVEDV, LVESD, LVEDD at day 7 post-MI in comparison with the vehicle-treated MI group. IL-33 administration and follow up was ethically limited to 7 days post-MI due to the very high mortality rate in the IL-33 treated MI animals (~50%). In the Yin et al. study, LV function was assessed at day 14 and day 28 post-MI and no data were shown at day 1 post-MI to prove that both MI groups (treated with vehicle and IL-33) underwent the same degree of MI. Additionally, IL-33 treated MI groups showed extraordinarily improved cardiac function in comparison with the vehicle-treated MI group at 14 and 28 days post-MI. (2) In the present study, DNA fragmentation was assessed at 4 and 7 days post-MI and was shown to be significantly increased with IL-33 treatment post-MI. Yin et al. observed DNA fragmentation at an earlier phase (3 days post-MI only) and was shown to be decreased with IL-33 treatment. (3) The flow cytometry data in this study showed that macrophages increased in the heart 3 days post-MI in the vehicle-treated group, without being affected in any other organ such as the bone marrow, spleen and blood. These results suggest a possible local monocyte to macrophage differentiation, a process that can take place as early as day 1 post-MI [77]. No significant difference in macrophage levels in the heart were observed between the IL-33 and vehicle-treated MI groups at 3 days post-MI. However at 7 days post-MI, a significant increase in macrophage infiltration in the heart was observed in the IL-33 treated MI group in comparison with the vehicle-treated group at the same time point. In contrast, in the Yin et al. study, immunofluorescent-staining showed a significantly decreased F4/80+ macrophage infiltration in the heart in the IL-33 treated MI animals at 3 days post-MI. Macrophage levels might have changed in the heart post-MI with IL-33 treatment if they were assessed at later stages.

The present study has few limitations that should be acknowledged. Despite increased infiltration of ILC2 and eosinophils following IL-33 administration, our study did not explore the role of ILC2-deficient and

eosinophil-deficient mice in the pathogenesis of MI. Moreover, the IL-33/ST2 axis is known to activate several signaling pathways including NF- κ B, mitogen activated protein kinases (MAPK) and phosphoinositide 3-kinase (PI3K) [4,78–83]. Inhibition of the PI3K signaling pathway is shown to reduce inflammation and improve outcomes in several pathological events [84–87], including MI [88]. The PI3K signaling pathway is also implicated in eosinophil chemotaxis and inhibition of the PI3K pathway was shown to impair release of eosinophils from bone marrow [89]. Future studies are warranted to explore the role of ILC2 and eosinophil KO mice and to see whether inhibition of the PI3K signaling pathway improves outcomes following IL-33 administration in the presence and absence of MI.

In conclusion, our findings indicate that IL-33 has opposing actions on the infarcted heart. On one hand, this alarmin creates a reparative phenotype in the infarcted heart through induction of a type 2 response, at the peak of inflammation-to-granulation transition, promoting a significant inflammatory resolution as early as day 4 post-MI, and inhibiting conventional prolonged inflammation by suppressing myocardial IL-1 β and TNF- α levels between 4 and 7 days post-MI. On the other hand, IL-33 worsened cardiac remodeling and function and increased cardiac rupture and death. The adverse cardiac outcomes following IL-33 treatment were mostly related to an impaired transition to the granulation phase characterized by a significant decrease in appropriate fibrotic markers and myofibroblasts formation, both being essential for the establishment of a mature scar at an early phase post-MI (Supplementary Figure S9). Granulation impairment seems to be concomitant with strong eosinophil infiltration. Of note, both neutrophil and Ly6C (hi) monocyte infiltration decreased significantly from day 3 to day 7 post-MI, and to comparable levels between the IL-33-treated and vehicle-treated MI groups, suggesting that these immune cells may not be implicated in the observed cardiac dysfunction. Whether eosinophils are ultimately and solely responsible for the adverse actions of IL-33 warrants further investigation.

Clinical perspectives

- The IL-33/ST2 axis is associated with a number of inflammatory, immune, and allergic disorders but is reported to have protective effects on cardiac function in many cardiovascular settings including cardiac overload, abdominal aortic aneurysm, and atherosclerosis. In the present study we examined the effect of IL-33 administration on myocardial pathological changes, including ventricular remodeling, myocardial dysfunction, fibrosis, immune cell infiltration, and survival following MI.
- Our results suggest that IL-33 is effective in activating a type 2 cytokine milieu in the damaged heart, consistent with reduced early inflammatory and pro-fibrotic response. However, IL-33 administration is associated with worsened cardiac function and adverse cardiac remodeling in the MI mouse model. IL-33 administration increased infarct size, LV hypertrophy, cardiomyocyte death, and overall mortality rate due to cardiac rupture. Moreover, IL-33-treated MI mice displayed a significant myocardial eosinophil infiltration at 7 days post-MI when compared with vehicle-treated MI mice.
- The present study reveals that although IL-33 administration is associated with a reparative phenotype following MI, it worsens cardiac remodeling and promotes heart failure.
- Designing monoclonal antibodies targeting extracellularly released cytokines and their corresponding receptors has been a great tool in revolutionizing drug therapy in a wide range of disease areas [90]. Since the discovery of IL-33 over a decade ago, it became well established that this cytokine participates in tissue homeostasis but can induce highly detrimental effects when activated inappropriately. Hence, several mechanisms have been developed to harness the activity and bioavailability of this versatile cytokine on multiple levels. Soluble receptor blockers of free IL-33 including the IL-33Trap and a form of the sST2 receptor have been developed to antagonize the unwanted biological functions mediated by IL-33 both *in vitro* and *in vivo* [91–93]. In allergic conditions involving hyperreactive immune responses, such as allergic asthma and atopic dermatitis, IL-33 is a disease sensitizer and mediates detrimental functions [15]. The use of recombinant sST2 or antibodies targeting IL-33 or ST2 to block the signaling of the IL-33/ST2 axis have proven beneficial outcomes in the management of allergic asthma and atopic dermatitis [94–98]. However, in a multitude of other processes, the IL-33/ST2 signaling pathway has pleiotropic immune functions and operates like a double-edged sword, presenting both tissue-reparative and pathological properties in many organs of the body. For

instance, in the kidneys, lungs, skin, gastrointestinal system, central nervous system, and cardiovascular system, IL-33 mediates both inflammatory and reparative responses [15,99]. In addition, the IL-33/ST2 axis is implicated in several diseases, including colitis, fibrosis, lupus, rheumatoid arthritis, multiple sclerosis, central nervous system disorders, and age-related macular degeneration [100]. Given the diverse roles of IL-33 in tissue injury and repair, determining the appropriate time to manipulate the IL-33/ST2 axis can be a great challenge in alleviating inflammation. In MI, where the reparative process is associated with a great imbalance in immune responses, neither the physiological nor the pathological functions of IL-33 are fully elucidated and await further exploration. Hence, the potential use of an anti-IL-33 antibody or the exogenous use of IL-33 as therapeutic strategies for the treatment of MI without activating unwanted immune responses remains a challenge. It awaits deeper understanding of the pharmacological dynamics to determine the ideal timing, route, and dosages in cardiac disease models.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contribution

F.A.Z. and Z.M. contributed to the study design and conception. R.G., N.J.H., A.K., C.T., E.A., A.B., R.F., H.I., A.J., G.W.B., Z.M., and F.A.Z. participated in the acquisition, analysis or interpretation of the data. R.G. performed and supervised all the experiments. F.A.Z. and R.G. drafted the manuscript. All authors judiciously revised the manuscript and gave their final approval for all the data presented, ensuring reliability and integrity.

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Abbreviations

ANOVA, analysis of variance; CO, cardiac output; CSA, cross-sectional area; CTGF, connective tissue growth factor; DAPI, 4,6-diamino-2-phenylindole; ECM, extracellular matrix; EF, ejection fraction; ELISA, enzyme-linked immunosorbent assay; FS, fractional shortening; FSC, forward scatter; IL, interleukin; ILC2, type 2 innate lymphoid cell; i.p., intraperitoneally; LAD, left anterior descending; LV, left ventricle; LVEDD, LV end-diastolic diameter; LVEDV, LV end-diastolic volume; LVESD, LV end-systolic diameter; LVESV, LV end-systolic volume; MI, myocardial infarction; MMP, matrix metalloproteinase; MTC, Masson's Trichrome; NF- κ B, nuclear factor- κ B; PI3K, phosphoinositide 3-kinase; RIPA, Radioimmunoprecipitation assay; RNA, ribonucleic acid; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SSC, side scatter; ST2, serum stimulation 2; ST2L, long or transmembrane isoform of ST2; sST2, soluble ST2; TBST, Tris-buffered saline-Tween; TGF- β , transforming growth factor β ; Th2, helper T cell; TNF- α , tumor necrosis factor- α ; TTC, 2,3,5 triphenyltetrazolium chloride; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; α -SMA, α smooth muscle actin.

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List of additional publications

1. Interactions between the renin angiotensin aldosterone system and COVID-19: pharmacological interventions

Nada J. Habeichi, Ghadir Amin, Gaelle Massoud, Reine Diab, Mathias Mericskay, George W. Booz, Fouad A. Zouein; ELSEVIER, edited by: Paul M. Pilowsky, **Book Chapter, page: 355-373.**
<https://doi.org/10.1016/B978-0-323-99618-1.00006-4>

2. Genomic, Proteomic, and Metabolic Comparisons of Small Animal Models of Heart Failure With Preserved Ejection Fraction: A Tale of Mice, Rats, and Cats

Alex N Smith, Raffaele Altara, Ghadir Amin, **Nada J Habeichi**, Daniel G Thomas, Seungho Jun, Abdullah Kaplan, George W Booz, Fouad A Zouein; Journal of the American Heart Association, **Review.**
PMID: 35904190? PMCID: PMC9375492? DOI: 10.1161/JAHA.122.026071

3. Macrophage responses associated with COVID-19: A pharmacological perspective

George W Booz, Raffaele Altara, Ali H Eid, Zena Wehbe, Souha Fares, Hassan Zaraket, **Nada J Habeichi**, Fouad A Zouein; ELSEVIER, **Review.**
PMID: 32919938. PMCID: PMC7483085. DOI: 10.1016/j.ejphar.2020.173547

4. Gender-biased kidney damage in mice following exposure to tobacco cigarette smoke: More protection in premenopausal females

Abdullah Kaplan, Emna Abidi, **Nada J Habeichi**, Rana Ghali, Hiam Alawasi, Christina Faki, Kazem Zibara, Firas Kobeissy, Ahmad Husari, George W Booz, Fouad A Zouein; Physiological Reports, **Original Paper.**
PMID: 31981316. PMCID: PMC6981307. DOI: 10.14814/phy2.14339

5. Spatiotemporal Dynamics of Immune Cells in Early Left Ventricular Remodeling After Acute Myocardial Infarction in Mice

Anthony T Bejjani, Sally A Saab, Dina H Muhieddine, **Nada J Habeichi**, George W Booz, Fouad A Zouein; Journal of Cardiovascular Pharmacology, **Review.**
PMID: 31764396. DOI: 10.1097/FJC.0000000000000777