Aus dem
CharitéCentrum für Innere Medizin mit Gastroenterologie und Nephrologie (CC13)
Medizinische Klinik für Geriatrie und Altersmedizin
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**Habilitationsschrift**

# Effects of age- and sex on inflammatory pathways, mitochondrial homeostasis, and metabolic sensing in cardiomyopathies

zur Erlangung der Lehrbefähigung
für das Fach Experimentelle Medizin

vorgelegt dem Fakultätsrat der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

von

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Eingereicht: August 2023
Dekan: Herr Prof. Dr. Joachim Spranger
1. Gutachter/in: N. N.
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**Contents**

## 1. Abbreviations

| | |
|---|---|
| ADP | Adenosine diphosphate |
| AMPK | AMP-activated protein kinase |
| AR | androgen receptor |
| ArgI | arginase I |
| ATP | Adenosine triphosphate |
| BMM | bone marrow derived macrophages |
| CAD | coronary artery disease |
| CD | cluster of differentiation |
| CFA | Complete Freund's Adjuvant |
| Col1A1 | collagen 1 A1 |
| Col3A1 | collagen 3 A1 |
| CVB | coxsackievirus B |
| CVD | cardiovascular disease |
| DCM | dilated cardiomyopathy |
| DCMI | inflammatory dilated cardiomyopathy |
| E2 | estradiol |
| ER | estrogen receptor |
| ERK | extracellular signal-regulated kinase |
| EAM | experimental autoimmune myocarditis |
| ECM | extracellular matrix |
| EF | ejection fraction |
| FoxO | Forkhead box protein O |
| FoxP3 | transcription factor forkhead box p3 |
| HF | heart failure |
| HFpEF | heart failure with preserved ejection fraction |
| HFrEF | heart failure with reduced ejection fraction |
| ICM | ischemic cardiomyopathy |
| IL | interleukin |
| iNOS | inducible nitric oxide synthase |
| LPS | lipopolysaccharide |
| LV | left ventricle |
| M1 | classically activated macrophages |

| | |
|---|---|
| M2 | alternative activated macrophages |
| MAPK | mitogen-activated protein kinases |
| mTOR | mammalian Target of Rapamycin |
| MyD88 | myeloid differentiation factor-88 |
| NAD | nicotinamide adenine dinucleotide |
| NFκB | nuclear factor kappa B |
| OXPHOS | oxidative phosphorylation system |
| p38 | p38 mitogen-activated protein kinase |
| PBMC | peripheral blood mononuclear cells |
| PGC-1α | peroxisome proliferator-activated receptor-gamma coactivator-1apha |
| PPCM | peripartum cardiomyopathy |
| ROS | reactive oxygen species |
| SASP | senescence-associated secretory phenotype |
| Sirt | sirtuin |
| SOD2 | superoxide dismutase 2 |
| TGF-β | transforming growth factor beta |
| Th cell | T-helper cell |
| TLR4 | toll like receptor 4 |
| TNF-α | tumor necrosis factor alpha |
| Treg | regulatory T cell |

## 2. Introduction

Cardiovascular diseases (CVD) are the most common cause of death worldwide.[1,2] Particularly, heart failure is one of the most common CVD, affecting more than 10% of all patients over the age of 70.[3,4] An increasing prevalence of heart failure (HF)[5,6] is closely linked to immense social and economic costs.[7] HF is associated with cardiomyocyte hypertrophy and apoptosis, inflammation, interstitial fibrosis as well as with structural and functional disruptions of the myocardium that occur in a sex-specific manner.[8,9] HF normally occurs as a consequence of ischemic or non-ischemic cardiomyopathy and has different prevalence, risk factors and outcomes in women and men.[10]

### 2.1 Cardiomyopathy (definition, classification, epidemiology, risk factors)

Cardiomyopathy refers to diseases of the myocardium that are accompanied by structural modifications of the walls of the heart chambers (e.g., enlarged, thickened, or stiffened), affecting the pump function.[11] The most common cardiomyopathies are ischemic cardiomyopathy (ICM) due to coronary artery disease (CAD) or nonischemic dilated cardiomyopathy (DCM; inflammatory or idiopathic), hypertrophic cardiomyopathy, arrhythmogenic right ventricular dysplasia, restrictive cardiomyopathy, transthyretin amyloid cardiomyopathy but also Takotsubo cardiomyopathy, chemotherapy-induced cardiomyopathy or peripartum cardiomyopathy (PPCM).[12-14] This habilitation thesis will only focus on inflammatory cardiomyopathy (myocarditis-related DCM) and idiopathic DCM.

Myocarditis is a pathological inflammatory process of the myocardium with high mortality rate, which is associated with myocyte necrosis, myocardial immune cell infiltration, extensive scarring, and left ventricular remodeling causing structural and functional abnormalities.[15-19] Viral infection with, e.g., parvovirus B19, coxsackievirus B (CVB), or human herpesvirus 6, is a main cause of acute myocarditis.[15,16,18,20] In addition, autoimmune, toxic or metabolic mechanisms may also contribute to the development of myocarditis. [15,16,18,20] The long-term viral persistence within the cardiac tissue as consequence of an unsuccessful viral clearance (several months to years) promotes a virus-associated chronic inflammatory cardiomyopathy (myocardial inflammation with established DCM (DCMI)),[21,22] which at the end-stage of myocarditis leads to chronic heart failure, followed by sudden death.[17-19] Importantly,

immunohistological evidence of myocarditis can be detected in about 40% of the patients with DCM[15,23] and in the tissue myocardial inflammation is found, implying a co-occurrence of myocarditis and DCM.[24] Several studies demonstrated that viral and post-viral myocarditis are a major hallmark of acute or chronic DCM.[18,21] DCMI is associated with chronic inflammation, followed by a profound cardiac remodeling due to an impaired activation of the immune system[18,25] (Figure 1).

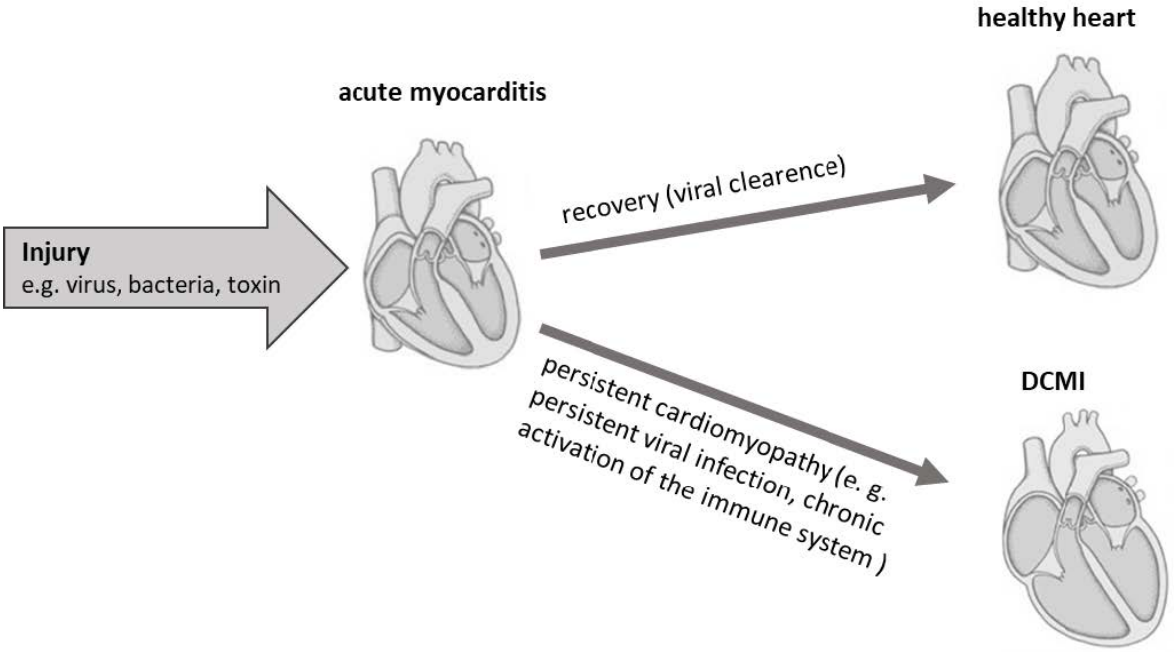


**Figure 1: Schematic depiction of the development of pathogen-associated chronic inflammatory cardiomyopathy.** Virus, bacteria, or toxin injury promotes the infiltration of immune cells in the heart. The activation of the adaptive immune system promotes viral clearance and downregulation of the immune response. Nevertheless, in many patients, an ongoing injury with a persistent viral infection or a chronic activation of the immune system is observed. The chronic inflammatory response leads to persistent cardiomyopathy and, in the end-stage, heart failure.[18]

Particularly, DCM is characterized by left ventricular or biventricular chamber dilation and systolic dysfunction, leading to an impaired myocardial contractility, which is accompanied by a normal left ventricular wall thickness.[26-29] Inflammatory or idiopathic DCM is the third most common cause of heart failure and sudden death after hypertension and coronary artery disease and is closely associated with heart transplantation.[26] In addition, DCM is associated with interstitial fibrosis, cardiomyocyte degeneration, and myofilament loss.[26] DCM can be divided in primary (i.e., genetic, familiar non genetic or acquired) or secondary (infiltrative or autoimmune) DCM.[29]

Approximately 20-48% of DCM cases are familiar DCM.[29] In contrast, acquired DCM might be triggered by myocarditis, Takotsubo (stress-related) or tachycardia.[29]

### 2.2 Role of aging on metabolic pathways in cardiomyopathies

Aging is defined as the deterioration of the physical condition and is one of the main risk factors for several chronic diseases, e.g., type 2 diabetes, cancer, dementia, and CVD.[7,30] In addition, multimorbidity, disability, frailty (declined physical activity, fatigue, and weight loss), and mortality are closely related to aging.[7,31] Aging is also an important risk factor for the development of cardiomyopathy.[32] Aging-associated comorbidities, systemic inflammation, obesity, diabetes, or vascular and cardiac senescence are important causes of cardiomyopathy in elderly individuals.[33] However, the underlying cellular mechanisms are complex and consist of the multifactorial process including inflammation, oxidative stress, mitochondrial dysfunction, accumulation of senescent cells and a decline in autophagy.[34-38] Metabolic pathways, e.g. mTOR, AMPK and sirtuins profoundly influences age-related cardiomyopathy.[39,40] $NAD^+$-dependent deacetylase sirtuins are highly conserved metabolic sensors, which are located in the nucleus (Sirt1, Sirt6 and Sirt7) or in mitochondria in mammals (Sirt3, Sirt4 and Sirt5).[41,42] Sirtuins are responsible for the deacetylation of histones, several transcription factors and proteins.[43] Importantly, they play a pivotal role in aging processes.[44] Especially Sirt1 seems to play a protective role against age-related changes.[39] Moreover, the expression and activity of Sirt1, as well as of it partner protein AMPK, decrease dramatically with aging in animals and humans.[39,40] In addition, activation of Sirt1 appears to be directly involved in the extension of lifespan in mice.[45-47] Moreover, Sirt1 seems to have cardioprotective effects attributed in part to its anti-apoptotic action on cardiomyocytes.[48,49] In accordance, Sirt1-related caloric restriction dramatically diminishes the exacerbated expression of pro-inflammatory mediators and reduces the CVD risk in several animal models and in humans.[7,50,51] Furthermore, the increased ROS formation and reduced antioxidant expression observed during aging processes seems to be strongly associated with the age-related decline of Sirt1, this may be explained by the fact that it is involved in the regulation of oxidative stress and antioxidants expression via FoxO signaling.[52] Apart from Sirt1, the expression and activity of Sirt3, which is localized in mitochondria, also decreases dramatically with aging,[53,54] which may alter mitochondrial function and biogenesis and promote chronic

inflammation.[55-58] The hyperacetylation of mitochondrial proteins of the OXPHOS system has profoundly effects on the mitochondrial oxidative metabolism.[59] Especially in the heart, Sirt3 deficiency and the resulting hyperacetylation of mitochondrial proteins (e.g., SOD2 and OXPHOS) affects the mitochondrial redox potential and metabolism, leading to an impaired contractile function.[60,61] In agreement with these results, several studies have proven a reduced cardiac antioxidative defense in older healthy and diseased individuals with DCM.[62]

Apart from impaired mitochondrial homeostasis, chronic low-grade systemic inflammation is an additional hallmark of aging and aging processes[63-65] and is presumed to accelerate biological aging and to favor age-dependent diseases, such as cancer, type 2 diabetes, and CVD.[66-69]

Taken together, the published data suggest an age-related decrease in the expression and activity of sirtuins and other proteins involved in metabolic pathways which contribute to impaired cardiac function and cardiomyopathy.

### 2.3 Role of aging-associated inflammation in cardiomyopathies

Inflammation, particularly low-grade chronic inflammation is one of the hallmarks of aging.[70] Age-dependent inflammatory processes play a prominent role in the development and complications of several CVD,[71] including atherosclerosis, heart failure, myocarditis, DCM and PPCM.[15,72-75] Importantly, chronic or prolonged stress on the heart (e.g., due pressure overload) promotes an imbalance between pro-inflammatory and anti-inflammatory cytokines, leading to a pro-inflammatory shift.[76] Moreover, several studies propose that activation of the p38 mitogen-activated protein kinase (MAPK), ERK and NFκB leads to the release of pro-inflammatory cytokines and are closely associated with the development of cardiac hypertrophy.[76-78]

Among others, lymphocytes play a key role in heart diseases, e. g., myocarditis and DCM.[79-81] Particularly, T helper cell 1 (Th1)- and Th2-related cytokines (pro-inflammatory and anti-inflammatory, respectively) are directly involved in the progression of viral-related and autoimmune myocarditis.[18,82-86] In addition to Th1 and Th2 cells, pro-inflammatory interleukin 17 (IL-17)-producing Th17 cells and $CD4^+CD25^+FOXp3^+$ regulatory T cells (Treg) play an important role in viral and autoimmune myocarditis.[18,83,87-89] Importantly, Treg cells are negative regulators of the inflammatory process in CVB-related myocarditis.[83,89] Aside from lymphocytes,

macrophages are also closely associated with myocardial inflammation.[90] Macrophages can be divided in classically activated M1 macrophages, which have a pro-inflammatory signature, or alternatively activated M2 macrophages, which are involved in anti-inflammatory/immune regulatory actions, e.g., wound healing and fibrosis formation[91,92] (Figure 2).

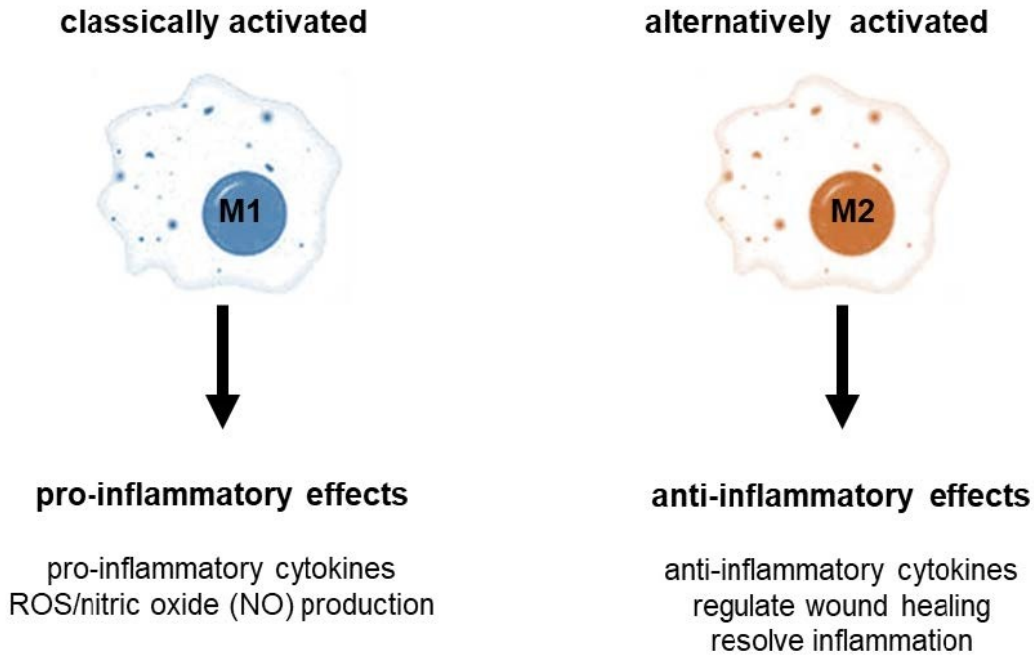


**Figure 2: Schematic depiction of macrophage polarization.** Classically activated M1 macrophages secrete pro-inflammatory cytokines (e.g., iNOS, TNF-α, IL-1β, IL-12 and IL-18) and enhance ROS/NO production, while alternatively activated M2 macrophages secrete anti-inflammatory cytokines (e.g., arginase 1 and IL-10), regulate wound healing and resolve inflammation.[93]

Furthermore, the aberrant expression of pro-inflammatory cytokines during inflammatory processes leads to the switch of the macrophage's phenotype (M2 to M1) over time, which under certain conditions may promote the perpetuation of inflammation.[94] Thus, M2-derived M1-macrophages may play a pathogenic role, e.g., in chronic inflammatory diseases.[95] Importantly, the mRNA expression of pro-inflammatory toll like receptor 4 (TLR4), which is activated by LPS, is dramatically increased in cardiac tissue from patients with myocarditis and DCM when compared to healthy individuals.[96-99] In accordance with this, a TLR4 activation is closely associated with the progression of autoimmune myocarditis in BALB/c mice.[100]

Taken together, immune cells, particularly T cells and macrophages, play an important role in the orchestration of the pro-inflammatory/anti-inflammatory response in myocardial diseases, e.g., myocarditis, DCMI and DCM.

## 2.4 Role of sex in cardiovascular physiology and pathophysiology

Sex-related differences (e.g., increased LV mass in men) are described in the normal heart physiology and function.[10,101] Sex differences in cardiovascular diseases are also well documented.[8,102-104] Myocardial infarction, atherosclerosis, myocarditis, DCM, and heart failure with reduced ejection fraction (HFrEF) have a higher prevalence and increased severity in men,[10,105-107] while hypertension, and several comorbidities, e.g., type 2 diabetes and obesity, are more common in older women.[106,108-110] Postmenopausal women show an increased incidence of heart failure,[10,111,112] suggesting that older women lose cardioprotection due to 17β-estradiol (E2) deficiency.[113] Indeed, the cardioprotective effect of E2 prevents the development of cardiovascular diseases in younger female animals and humans.[113,114] It is important to note that sex differences seem to evoke a different pathophysiology in heart failure between women and men.[8,115-117] While more women develop heart failure with preserved ejection fraction (HFpEF), more men develop HFrEF.[115,118-120] Patients with HFpEF have a lower mortality rate than patients with HFrEF, but remain symptomatic, have a lower quality of life, and are more likely to be (re)hospitalized.[121-123]

Sex differences are also well documented for myocarditis.[98,124-126] Several animal and human studies have described that myocarditis is more common in male animals or individuals and has a more severe disease progression than in females,[10,127,128] although the underlying causes are not yet completely understood.[19] As described above, viral persistence may lead to the development of DCMI/DCM,[21,22] which occurs in many patients.[15,23] Moreover, DCM is more frequent in males, as this group is more prone to impaired cardiac remodeling and more vulnerable to cardiac stress.[3,129] Of note, sex differences in the pathomechanisms of myocarditis seem to be related to differences in the immune system.[10,98,124,125,130]

In summary, sex differences are present in CVD and profoundly influence the incidence and severity of the disease.

## 2.5 Role of sex in cardiac inflammation

The development of autoimmune myocarditis is closely related to an exacerbated expression of pro-inflammatory cytokines e.g., TNF-α, IL-1β, and IL-6, and enhanced ROS formation.[131,132] In addition, several reports described anti-myosin autoantibodies, myocardial fibrosis, alterations in ECM, and cardiac remodeling in CVB3-associated chronic myocarditis in mice.[131,133-135]

Several studies demonstrated sex differences in the inflammatory response in animal models of acute or chronic myocarditis, which is associated with a deleterious pro-inflammatory response in males.[125,126,136-138] It is noteworthy that, in spite of this, males and females show similar viral replication in the heart in myocarditis. Males show an exacerbated cardiac inflammation in response to CVB3 infection, while females do not develop myocarditis.[10,125,126,139]

Viral infections, e.g., CVB3, are the major cause of myocarditis,[18] involving cell-mediated immunity of Th1, Th2, Th17, and CD4+CD25+FOXp3+ Treg-cells.[17,83,89,140,141] Moreover, the increased levels of Th1- and Th2-related cytokines (e.g., tumor necrosis factor α (TNF-α), IL-1 β, and IL-16) observed in myocarditis are strongly associated with the development of DCM.[142] In addition, several animal studies described a Th1-mediated immune response in male BALB/c and C57Bl/6 mice after infection with CVB3, while female mice develop an IL-4 associated-Th2 and Treg (FOXp3+, Tim-3+, CTLA4+)-mediated immune response.[10,82,83,125,126,138,143] In accordance, macrophage polarization (M1 or M2) in CVB3-induced myocarditis is sex-specific, i.e., higher expression of pro-inflammatory M1 macrophages in male and anti-inflammatory/immune regulatory M2 phenotype (Tim-3+ alternative activated macrophages) in female mice[10,136,144,145] (Figure 3). Furthermore, male murine macrophages show an increased TLR4 in CVB3-myocarditis.[98,138,146] Male animals can also present M2 macrophages (expressing TLR4 and IL-1β), which are profoundly related to fibrotic remodeling processes in the heart.[133,147] In contrast, the lack of TLR4 promotes less severe myocarditis and a lower inflammatory state in the heart of male mice.[147]

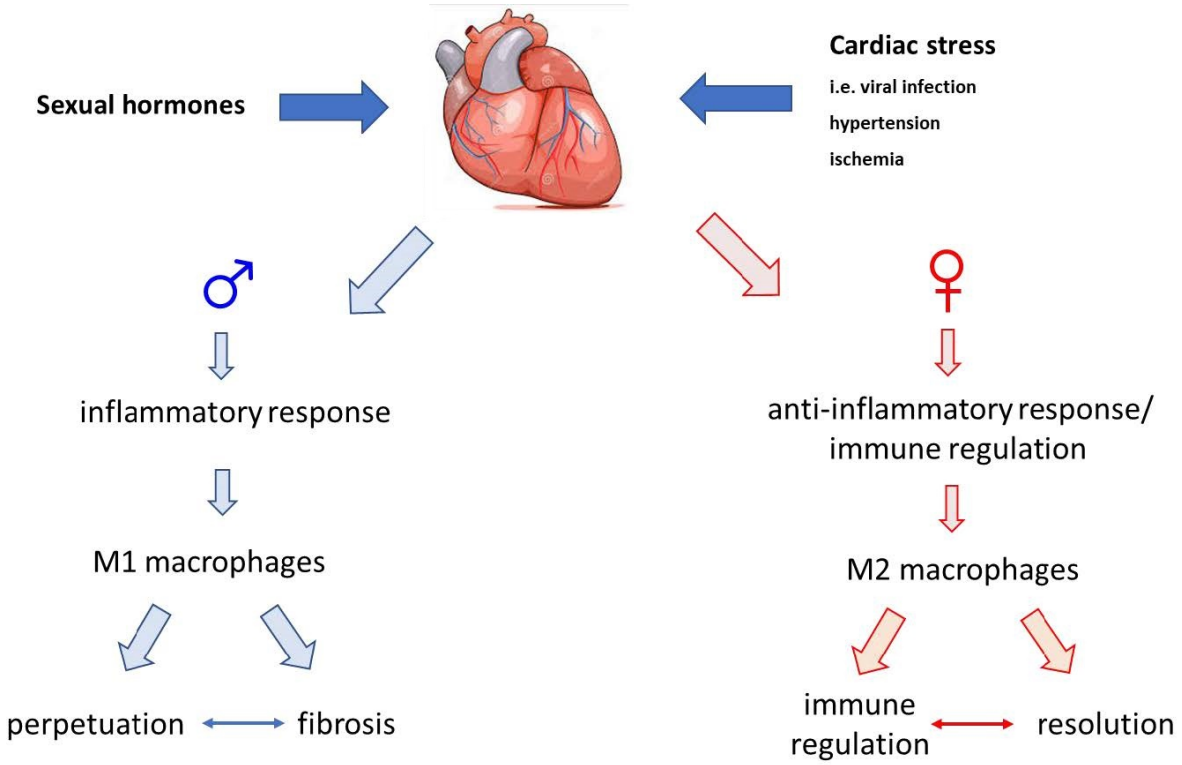


**Figure 3: Schematic depiction of the sex-specific macrophage polarization in the heart.** Sexual hormones influence the inflammatory response of the heart during cardiac stress. Male hearts develop a pro-inflammatory Th1 cell immune response, leading to a pro-inflammatory M1 macrophage polarization which promotes the perpetuation of the inflammatory state. On the other hand, cardiac stress activates the anti-inflammatory/immune regulatory Th2 immune response in female hearts. Th2 cells induces a M2 macrophage polarization, which is related to immune regulatory actions and the resolution of the inflammation.[10, 135]

Taken together, male hearts undergo a pro-inflammatory immune response, which is associated with perpetuation of the inflammation and cardiac remodeling. Whereas anti-inflammatory/immune regulatory effects are observed female hearts.

## 2.6 Role of sex hormones in cardiac inflammation

The immune system is profoundly affected by the sex hormones, e.g., E2 and testosterone, via sex steroid hormone receptors (e.g., ERα, ERβ and androgen receptor (AR).[10,148,149] In addition, sex hormones also regulate inflammation[148] and modulate tissue response to inflammation in a sex-dependent manner due to their effects on non-immune cells such as cardiomyocytes, endothelial cells, and fibroblasts (e.g., via p38 and ERK signaling).[150-152]

Importantly, males are more susceptible to develop cardiac autoimmunity and chronic inflammation,[137] which are closely associated with elevated testosterone levels, leading to cardiac remodeling due to collagen deposition, fibrosis formation and remodeling of the ECM in males.[127,153-155] In contrast to testosterone, E2 seems to have anti-inflammatory effects via activation of estrogen receptors (ER).[156-159] E2 exhibits anti-inflammatory actions on endothelial cells and immune cells in vitro,[150,156,158,160,161] and E2 depletion leads to the release of pro-inflammatory cytokines in humans.[162-164] In accordance with this, using transcriptomics technology, Kararigas *et al.* described an increased natural killer cell-mediated cytotoxicity and transendothelial migration pathways of leukocytes in the hearts of female mice lacking ERβ and exposed to pressure overload.[165] In addition, in LPS-activated human peripheral blood mononuclear cells (PBMC) and murine macrophages, E2 inhibits the expression of pro-inflammatory cytokines[166] and decreases the NF-κB activity.[167,168] Moreover, E2 seems to inhibit ROS-associated cardiac damage, apoptosis in cardiomyocytes, cardiac hypertrophy, and fibrosis formation.[8,169]

However, both pro- and anti-inflammatory effects of E2 have been described.[170,171] This discrepancy in E2 effects is due to the dose administered, which might cause opposite effects.[158] The pro-inflammatory effects of E2 are associated with a NFκB-mediated Th1 and/or Th17 immunity, whereas the anti-inflammatory effects of E2 are mediated via Th2-, Treg cells and regulatory macrophage activation.[98,172-174]

Furthermore, E2 has been found to induce a M2 phenotype through the inhibition of M1-related cytokines and the production of M2-related cytokines.[175] The E2 effect might be mediated via ERα, which is involved in the promotion of M2 macrophage polarization, leading to an anti-inflammatory phenotype.[176] Although sex hormone effects in the modulation of the immune system are well documented, the specific molecular and cellular mechanisms are poorly understood.

### 2.7 Impact of sex on cardiac metabolism

Mitochondria play a pivotal role in heart physiology and pathophysiology, as they are highly represented in cardiomyocytes (7000 -10000 mitochondria per cell).[98] Importantly, mitochondrial genes and energy metabolism-related genes are downregulated in male hearts when compared to female hearts.[98,109] Although female hearts contain less mitochondria, they have, e.g., higher ADP/oxygen ratio, and a

better antioxidant capacity, and show higher mitochondrial efficiency in comparison to male hearts.[9,98,177-181] Thus, the preserved mitochondrial homeostasis in younger female hearts seems to be associated with E2 and its ER actions.[182,183] In addition, it has been shown that E2 is closely involved in the regulation of ATP production, ROS formation, antioxidant defense, mitochondrial membrane potential and calcium handling.[184] On the other hand, testosterone seems to improve mitochondrial function via PGC-1α, which is associated with cardiac metabolism, in cardiomyocytes in aging men.[185] In addition, the activity of mitochondrial proteins and oxygen consumption are increased in cardiomyocytes overexpressing the androgen receptor in aging men.[185]

Interestingly, mitochondrial ROS plays a crucial role in immune responses and regulates cell function in health.[186] However, exacerbated ROS levels are harmful to the body, as they promote irreversible DNA damage and are involved in the development of various diseases.[98,187,188] In addition, high levels of ROS promote mitochondrial dysfunction and cellular senescence by the induction of a senescence-associated secretory phenotype (SASP).[189-191] Mitochondrial ROS and the NLRP3 inflammasome are closely associated; the inflammasome activates mitochondrial ROS and vice versa.[192-194] Of note, ROS is involved in cardiomyocyte damage, apoptosis, and inflammatory processes in myocarditis.[195-197] The regulation of mitochondrial ROS levels is strongly related to sex hormones, including E2.[98,198,199]

Aside from mitochondrial function, sex differences in autophagy are described in animal and human hearts during myocarditis.[200] Autophagy activity is increased in female vascular smooth muscle cells in comparison to male cells after a stressful stimulus.[8,201] Importantly, the observed sex differences in the autophagy activity seem to be promoted by sexual hormones, since E2 via ERs profoundly influences the autophagy activity.[8]

In summary, sex hormones modulate mitochondrial function and ROS formation and thus directly influence cardiac health.

## 2.8 Objectives

Chronic inflammation and mitochondrial dysfunction are important hallmarks of aging and key factors leading to cardiomyopathy. Although sex differences in the immune system and mitochondrial homeostasis have been documented, the underlying cellular mechanisms responsible for these differences remain obscure.

As the prevalence of age-related CVD is increasing and adequate prevention and therapy are lacking, the development of effective personalized therapeutic tools is needed to protect the cardiac function in older male and female individuals.

Therefore, the current study was focused on the sex- and age-related differences in inflammatory processes and mitochondrial homeostasis in DCM and inflammatory DCM.

The aims of this study were:

1) To investigate the role of sex in inflammation and mitochondrial homeostasis during cardiac aging in healthy individuals.

2) To investigate how sex and age affect the inflammatory response and mitochondrial homeostasis in patients with cardiomyopathy.

3) To investigate the role of the sexual hormones in the polarization of macrophages and the resulting inflammatory response.

# 3 Own work

## 3.1 Sex differences in the aging human heart: decreased sirtuins, pro-inflammatory shift and reduced anti-oxidative defense (PMID: 30964749)

**Barcena de Arellano ML**, Pozdniakova S, Kühl AA, Baczko I, Ladilov Y, Regitz-Zagrosek V. Sex differences in the aging human heart: decreased sirtuins, pro-inflammatory shift and reduced anti-oxidative defense. Aging (Albany NY), 2019; 11(7): 1918-33.
https://doi.org/10.18632/aging.101881

Systemic inflammation and impaired mitochondrial biogenesis are hallmarks of aging and prominent risk factors for the development of cardiovascular diseases. $NAD^+$-dependent deacetylases, e.g., Sirt1 or Sirt3 are associated with anti-aging processes and an extended lifespan. Of note, an aging-related reduction of sirtuins plays a crucial role in inflammatory processes and mitochondrial homeostasis in the heart.

The aim of this part of the work was to elucidate age- and sex-related differences in the inflammatory state as well as in the expression of proteins involved in mitochondrial function and the oxidative defense in human healthy hearts. Our results indicate that the expression of cardiac Sirt1 was higher in younger women in comparison to younger men, while Sirt1 expression was significantly lower in older female but not older male hearts. In accordance with the Sirt1 results, the acetylation of nuclear Ku70, which is a direct Sirt1 target, was significantly elevated in older female hearts. In addition, the cardiac AMPK activity was significantly decreased in older individuals in a sex-independent manner.
Moreover, proteins involved in the regulation of mitochondrial function, e. g., TOM40 and Sirt3 as well as SOD2 (a protein involved in anti-oxidative response) were significantly increased in the heart of younger women compared to younger men, whereas these proteins were drastically reduced in older female individuals. The number of cardiac macrophages as well as the expression of pro-inflammatory cytokines was significantly increased in older female hearts. Consistently, the pro-inflammatory shift in the heart of older women was confirmed by the decreased

expression of the anti-inflammatory mediator IL-10 and an imbalance in the IL-12/IL-10 ratio in the heart of older women.

Our results provide evidence that cardiac aging leads to the significant downregulation of the Sirt1 expression accompanied by increased acetylation of nuclear Ku70 in older women but not in men. In younger female hearts, we observed an improved mitochondrial function, which was linked to the beneficial upregulation of mitochondrial and anti-oxidative proteins. Importantly, this effect was lost in older female hearts. Furthermore, a prominent pro-inflammatory shift in aged female hearts was accompanied by impaired expression of Sirt1 and mitochondrial proteins. This study provides a molecular basis for the increased incidence of cardiovascular diseases in older women.[202]

These findings provided evidence of age-related sex-specific differences in the expression of mitochondrial proteins and inflammatory markers and served as steppingstone to study age- and sex-related effects on mitochondrial homeostasis and inflammation in cardiomyopathy.

Research Paper

# Sex differences in the aging human heart: decreased sirtuins, pro-inflammatory shift and reduced anti-oxidative defense

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**Keywords:** cardiac aging, inflammatory response, sex differences, sirtuins, SOD2
**Received:** October 17, 2018 **Accepted:** March 20, 2019 **Published:** April 8, 2019



## ABSTRACT

**Aging is associated with increased inflammation and alterations in mitochondrial biogenesis, which promote the development of cardiovascular diseases. Emerging evidence suggests a role for sirtuins, which are $NAD^+$-dependent deacetylases, in the regulation of cardiovascular inflammation and mitochondrial biogenesis. Sirtuins are regulated by sex or sex hormones and are decreased during aging in animal models. We hypothesized that age-related alterations in cardiac Sirt1 and Sirt3 occur in the human heart and examined whether these changes are associated with a decrease in anti-oxidative defense, inflammatory state and mitochondrial biogenesis. Using human ventricular tissue from young (17-40 years old) and old (50-68 years old) individuals, we found significantly lower Sirt1 and Sirt3 expression in old female hearts than in young female hearts. Additionally, lower expression of the anti-oxidative protein SOD2 was observed in old female hearts than in young female hearts. Aging in female hearts was associated with a significant increase in the number of cardiac macrophages and pro-inflammatory cytokines, as well as NF-kB upregulation, indicating a pro-inflammatory shift. Aging-associated pathways in the male hearts were different, and no changes in Sirt1 and Sirt3 or cardiovascular inflammation were observed. In conclusion, the present study revealed a female sex-specific downregulation of Sirt1 and Sirt3 in aged hearts, as well as a decline in mitochondrial anti-oxidative defense and a pro-inflammatory shift in old female hearts but not in male hearts.**

## INTRODUCTION

Aging, including cardiac aging, is a natural and multifactorial process characterized by a series of mechanisms, including deregulated autophagy, oxidative stress, systemic inflammation and mitochondrial dysfunction [1-9]. Recent studies have emphasized the importance of $NAD^+$-dependent deacetylase sirtuins, mainly cytosolic- or nucleus-localized Sirt1 and mitochondrial-localized Sirt3, in the aging process [10]. Decreased sirtuin expression has been found in aged humans and animals [11, 12]. Additionally, recent reports have suggested a significant reduction in the cellular $NAD^+$ concentration and sirtuin activity in aged

animals [11]. Furthermore, AMPK, a key regulator of cellular metabolism that significantly contributes to sirtuin activity, e.g., via increasing $NAD^+$ synthesis [13], showed declined activity associated with aging [14].

The decline of Sirt1 and Sirt3 expression or activity may result in the following two detrimental consequences: first, it may impair mitochondrial biogenesis [15-17] and function [18, 19], and second, it may lead to excessive inflammatory response, particularly due to the reduced anti-inflammatory actions of Sirt1 [20, 21]. Several reports suggest that Sirt3 ameliorates mitochondrial stress by upregulating mitophagy and anti-oxidant machinery proteins, including manganese superoxide dismutase (SOD) and catalase [22, 23].

Both mitochondrial dysfunction and chronic inflammation are well-known hallmarks of aging [1, 24, 25]. Furthermore, in both cases, elevated ROS formation, another aging hallmark [26], is to be expected. Though the age-associated reduction in sirtuin expression and activity as well as its contribution to mitochondrial dysfunction and pro-inflammatory shift are well-documented, the underlying cellular mechanisms are far from understood.

Recent studies applying *in vivo* and *in vitro* models have suggested a role for the female sex hormone estradiol (E2) in the expression and activity of AMPK and sirtuins [27, 28]. Apart from being the downstream target of AMPK, Sirt1 deacetylates the upstream activator of AMPK, LKB1 kinase, thus providing the positive feedback loop between AMPK and Sirt1 [29, 30]. Of note, E2 blood concentrations are reduced in aged women, i.e., after menopause. In fact, postmenopausal may women have lower E2 concentrations in their blood compared with age-matched men [31]. The loss of E2 is accompanied by the release of pro-inflammatory cytokines, leading to activation of inflammatory pathways in aging [32]. Furthermore, loss of ovarian hormones due to reproductive aging leads to the decline in anti-oxidative defense, mitochondrial biogenesis and function in females [33, 34].

Though several reports have demonstrated sex differences in the expression of AMPK and sirtuins in mouse brain and kidney [35], alterations in the human heart in aging remain unknown. Therefore, in the present study, we aimed to investigate the age-related alterations in Sirt1, AMPK and Sirt3 signaling in cardiac tissue from men and women along with markers for mitochondrial biogenesis, anti-oxidative defense and the inflammatory state. We found a female sex-specific downregulation of Sirt1 and Sirt3 expression in aged hearts, which is accompanied by the downregulation of SOD2, a key mitochondrial anti-oxidative enzyme, and by increased expression of inflammatory mediators.

## RESULTS

### Age-related Sirt1 and AMPK alterations in women

The expression of Sirt1 and AMPK is downregulated in aging [11]; however, sex differences in this process remain unknown. To examine this issue, we analyzed the expression and activity of Sirt1 and AMPK in human cardiac tissue. Sirt1 expression was significantly reduced in old compared to young women ($p< 0.05$) (Figure 1A).

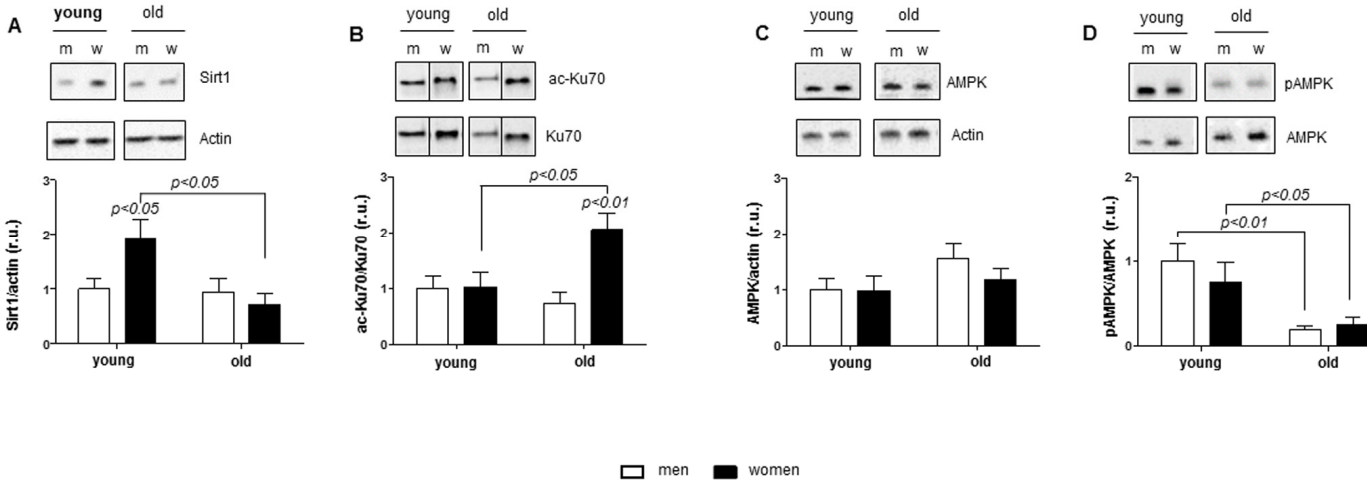


**Figure 1. Age-related alterations in Sirt1 and AMPK expression.** Western blot expression analysis of (**A**) Sirt1, (**B**) acetylated Ku70, (**C**) total AMPK and (**D**) phosphorylated AMPK (Thr172) performed with human cardiac tissue lysates from young and old men (m) or women (w). pAMPK was normalized to AMPK. Proteins were normalized to actin. Data are shown as the mean ± SEM (n= 6-8/group). Representative imaging of western blot analysis; the lanes were run on the same gel. All data were normalized to the corresponding control and expressed in relative units (r.u.).

To test whether the Sirt1 downregulation is associated with altered protein acetylation, acetylation of the nuclear protein Ku70, a direct target of Sirt1, was analyzed. Consistently, Ku70 acetylation in aged female hearts was significantly elevated compared to young women ($p< 0.05$) (Figure 1B). In contrast, neither Sirt1 expression nor Ku70 acetylation were altered in male hearts with aging.

Analysis of AMPK expression and the phosphorylation rate, i.e., pAMPK/AMPK, revealed a significant decrease in AMPK phosphorylation with aging in both sexes (Figure 1D) in the presence of unchanged total AMPK contents (Figure 1C).

**Age-related expression of mitochondrial and anti-oxidative enzymes in women and men**

A key role has been suggested for Sirt1 and AMPK in the regulation of mitochondrial biogenesis and function [13, 37-39]. In the next step, we tested whether the female sex-specific downregulation of Sirt1 in aged female hearts is accompanied by an alteration in mitochondrial biogenesis markers. By analyzing the expression of two key transcription factors involved in mitochondrial biogenesis, i.e., mitochondrial TFAM and nuclear PGC1-α, we found no age- or sex-related alterations (Figure 2A-B). Interestingly, expression of TOM40, a key protein controlling mitochondrial protein import, was markedly upregulated in aged male hearts (Figure 2C). In agreement, no sex- or age-related differences were found in the expression of mitochondria-encoded (*cox1* and *nd4*) or nuclear-encoded (*atp5b*, *cox5b* and *nsufs1*) mitochondrial genes (Supplementary Figure 1).

In contrast, analysis of the expression of two key mitochondrial proteins that control the acetylation of mitochondrial proteins (Sirt3) and the mitochondrial redox state (SOD2) revealed their significantly lower levels in old female vs. young female hearts (Figure 2D-E). Similar to SOD2, the expression of catalase, a key peroxisomal and mitochondrial anti-oxidative enzyme, was significantly lower in old compared to young female hearts (Figure 2F).

In contrast to females, expression of SOD2 and catalase was significantly elevated in male hearts. Thus, heart

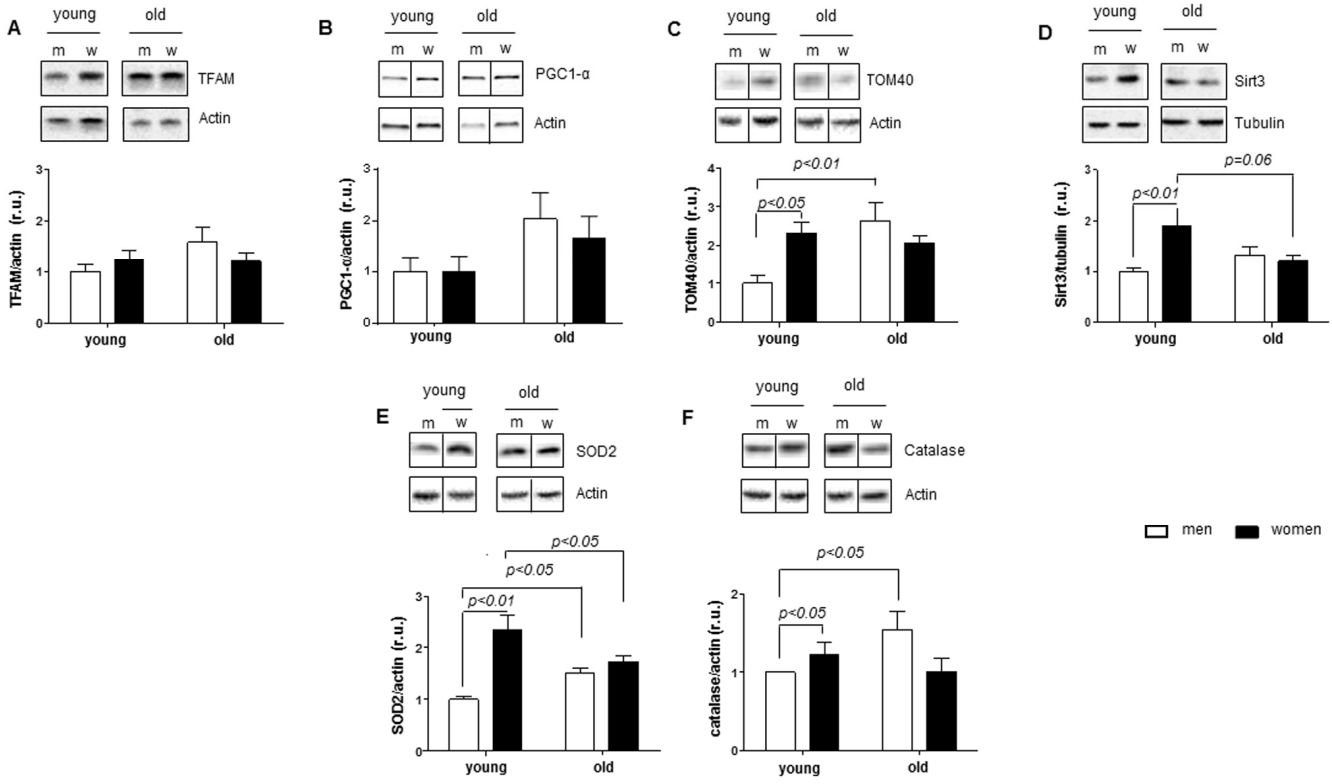


**Figure 2. Age-related alterations in expression of mitochondrial and anti-oxidative enzymes.** Expression of proteins related to mitochondrial function in non-diseased hearts in young and old men (m) and women (w). Western blot analysis and statistical analysis of (**A**) TFAM, (**B**) PGC1-α, (**C**) TOM40, (**D**) Sirt3, (**E**) SOD2 and (**F**) catalase protein expression. Proteins were normalized to tubulin or actin. Data are shown as the mean ± SEM (n= 6-9/group). Representative imaging of western blot analysis; the lanes were run on the same gel. All data were normalized to the corresponding control and expressed in relative units (r.u.).

aging is associated with a reduction in anti-oxidative defense in females (SOD2), whereas this defense is increased (SOD2 and catalase) in male hearts (Figure 2E-F).

**Aged-related pro-inflammatory state in women**

An enhanced systemic pro-inflammatory state is a characteristic feature of aging [40]. Thus, we aimed to examine whether alterations in Sirt1 expression and anti-oxidative defense may be accompanied by pro-inflammatory reactions in aged female hearts.

By analyzing the number of cardiac macrophages, a significant increase in the cardiac macrophage number was observed in old female hearts, but not in male hearts (Figure 3A-E). The amount of CD206-immune reactive macrophages was higher in young women when compared to old women or to young men (Figure 3F-L).

To support this finding, expression analysis of pro- and anti-inflammatory factors was performed. NF-κB p50 was significantly elevated in aged female hearts, whereas it was markedly reduced in aged male hearts (Figure 4A). In contrast, IKBα was reduced in aged female hearts when compared to young female hearts (Figure 4B).

It has been suggested that FOXO1 phosphorylation suppresses inflammatory responses via NF-κB [41].

FOXO1 inactivation increases with aging in male animals [41], suggesting that an increase in FOXO1 phosphorylation might contribute to the reduced level of inflammation in old animals.

FOXO1 was decreased in old male individuals when compared to young male hearts ($p= 0.06$), but not in female hearts ($p< 0.05$) (Figure 4C). FOXO1 phosphorylation was altered in cardiac male tissue during aging ($p< 0.01$), while unchanged in female hearts during aging (Figure 4D). The phosphorylation of FOXO1 ($p> 0.05$), was similar in female and male cardiac tissues in young individuals (Figure 4D).

Similar to NF-kB, the mRNA of pro-inflammatory cytokine IL-18 was upregulated in old female hearts but not in male hearts (Figure 4E). The TNF mRNA was unchanged in male and females hearts during aging ($p> 0.05$) (data not shown).

Further analysis of the IL12/IL10 ratio revealed a significantly lower ratio in young female hearts (ratio= 0.2, $p< 0.05$) compared to young male hearts (1.2). This sex difference was lost in aged hearts, i.e., 1.1 in old female hearts and 1.3 in old male hearts, which also confirmed the pro-inflammatory shift in aged female hearts (Figure 4F).

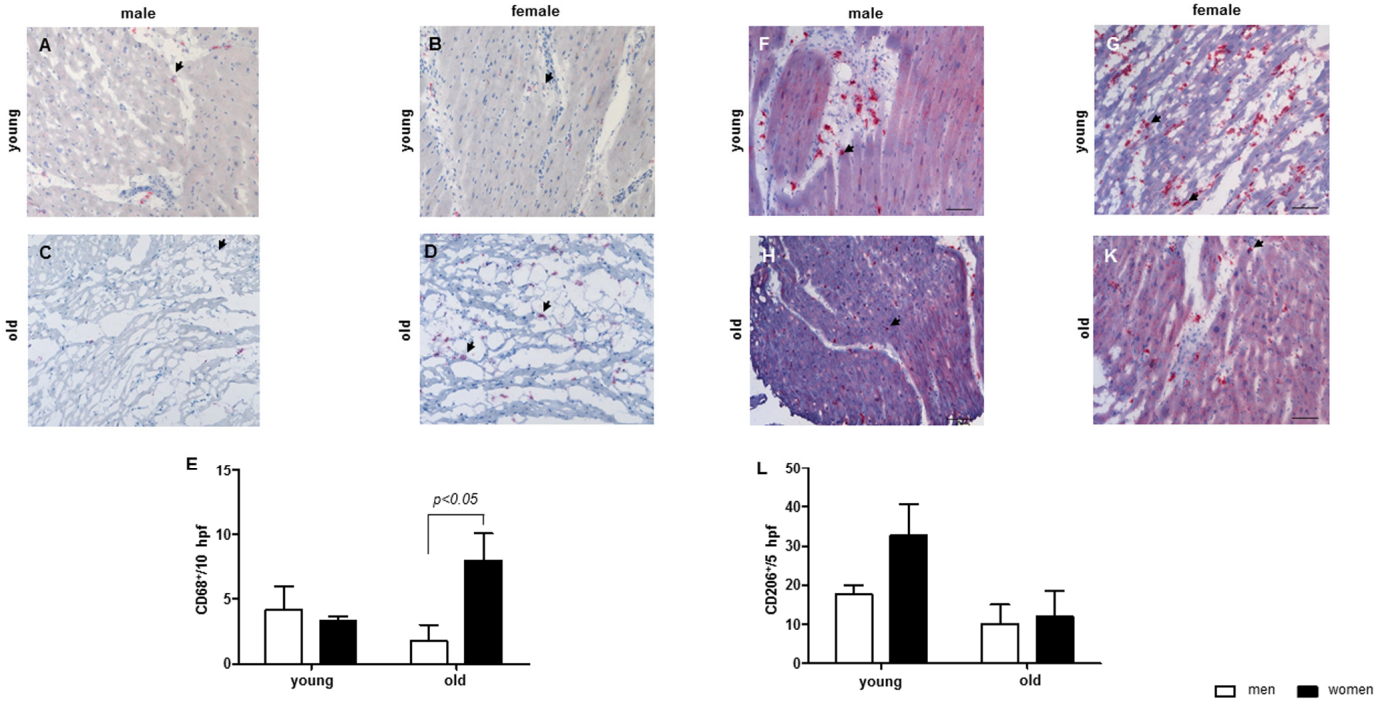


**Figure 3. Aging effect on the number of macrophages in cardiac tissue.** Representative images of cardiac cryosections stained with antibodies against (**A**-**D**) CD68, followed by (**E**) statistical analysis of CD68-positive cells per high power field (hpf). (**F**-**K**) CD206, followed by (**L**) statistical analysis of CD206-positive cells per high power field (hpf). The analyses were performed with myocard from young and old men (m) or women (w). Data are shown as the mean ± SEM (n= 6-8/group). Arrows show CD68- or CD206-positive cells.

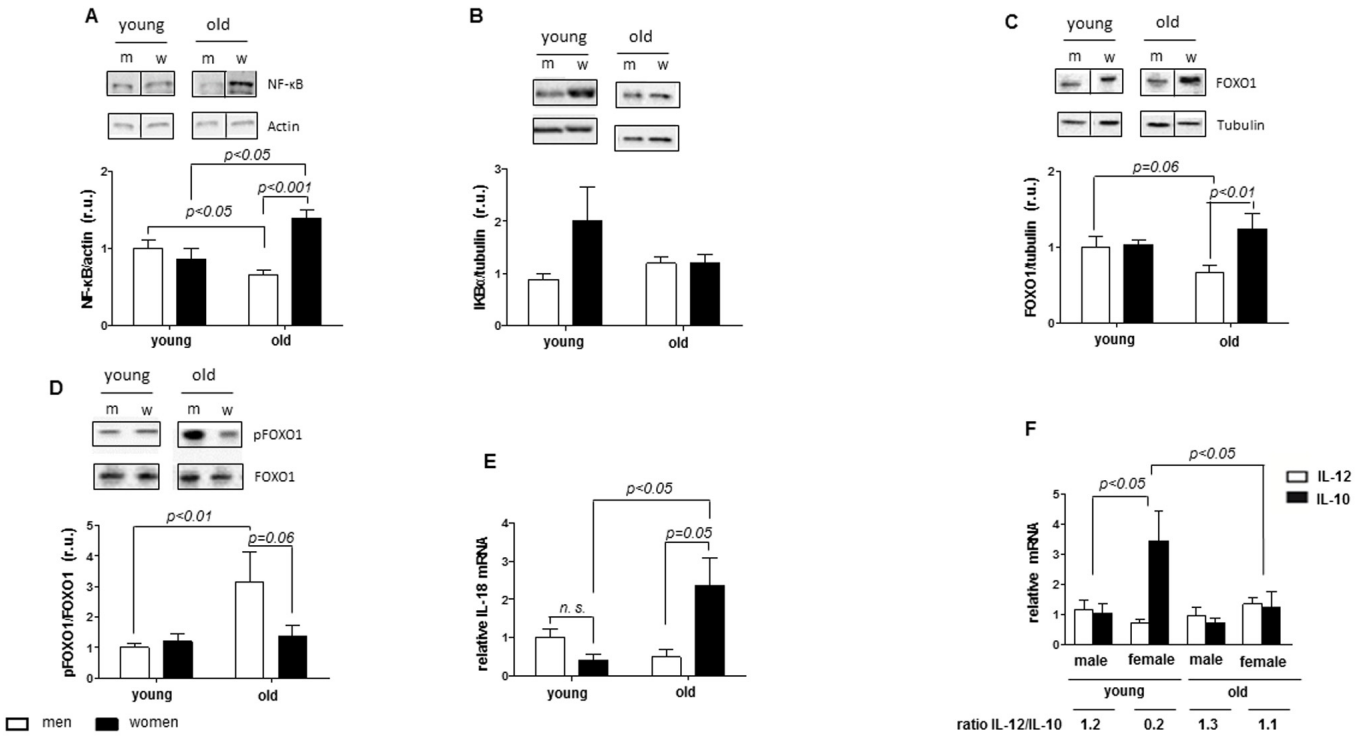


**Figure 4. Female-specific aged-induced pro-inflammatory state.** Protein and relative mRNA expression of pro-inflammatory and anti-inflammatory mediators in non-diseased hearts in young and old men (m) and women (w). Western blot analysis of (**A**) NF-κB p50, (**B**) FOXO1 and (**C**) phosphorylated FOXO1 and real-time PCR analysis of (**D**) IL-18, (**E**) IL-12 and (**F**) IL-10 mRNA expression. Data are shown as the mean ± SEM (n= 6-9/group). pFOXO1 was normalized to total FOXO1. Representative imaging of western blot analysis; the lanes were run on the same gel. All data were normalized to the corresponding control and expressed in relative units (r.u.).

## DISCUSSION

The aim of the study was to examine age-related changes in Sirt1, AMPK and Sirt3 expression in women and men in relation to mitochondrial biogenesis, anti-oxidative defense and inflammation in human hearts. The main findings are as follows: (i) Aging leads to a significant downregulation in Sirt1 expression and the corresponding elevated acetylation of nuclear proteins in female but not male hearts; (ii) The higher expression of some mitochondrial and anti-oxidative proteins in young females is lost with aging either due to their downregulation in aged females, e.g., Sirt3 and SOD2, or due to upregulation in aged males (TOM40, SOD2 and catalase); and (iii) Aging leads to a significant pro-inflammatory shift in female but not male hearts.

AMPK and Sirt1 are partner proteins orchestrating a wide variety of intracellular processes including cellular resistance to oxidative stress, general metabolism, inflammation, and mitochondrial biogenesis and function [42]. Aging is accompanied by the down-regulation of AMPK and Sirt1 activity in animals and humans [43]. Whether these alterations are sex-dependent remains unknown. We found a sex-independent downregulation of the AMPK phosphorylation rate, a widely used indicator of AMPK activity. Furthermore, the female-specific downregulation of Sirt1 expression was observed in aged hearts. Consistently, acetylation of Ku70, a nuclear protein and a direct Sirt1 client, was significantly increased only in old female but not male hearts, demonstrating reduced Sirt1 activity. Although we did not investigate whether the nuclear or cytosolic Sirt1 content is specifically reduced with aging in female hearts, previous reports demonstrated that Sirt1 physically interacts with and deacetylates nuclear Ku70 [44].

Mitochondrial dysfunction is a characteristic fingerprint of aging [45]. Notably, downregulation of Sirt1 activity has been previously attributed to disturbances in mitochondrial biogenesis [19]. To examine the age-related effects on mitochondrial biogenesis in male and female hearts, expression analysis of key transcription factors (PGC1alpha and TFAM) as well as several nuclear- and mitochondria-encoded mitochondrial proteins was performed by western blot and PCR. Surprisingly, we found no sex- or age-related

differences in the expression of mitochondrial genes or PGC1alpha and TFAM expression, which excludes the sex-dependent regulation of mitochondrial biogenesis in aging human hearts, even though such changes were reported in lower organisms or in the liver and kidneys from 22-month-old mice. Species and organ differences may account for these discrepancies [35, 46].

Previous reports argue for an activating effect of Sirt1-dependent deacetylation on PGC1alpha [47, 48]. It is tempting to speculate that female-specific Sirt1 down-regulation and enhanced acetylation of nuclear proteins (e.g., Ku70) may also be accompanied by PGC1alpha hyperacetylation and inactivation. Indeed, considering that PGC1alpha directly interacts with the SOD2 promoter regions and regulates its expression [49], the observed downregulation of SOD2 in aged female but not male hearts argues for a potential reduction in PGC1alpha activity.

Several studies have demonstrated favorable expression of anti-oxidative enzymes in female vs. male hearts, which is lost in postmenopausal women [50]. In agreement with these findings, the expression of two key anti-oxidative enzymes, i.e., SOD2 and catalase, in our study was significantly higher in young female vs. male hearts, whereas this sex difference is lost in aged hearts. Interestingly, this age-related alteration in the sex difference in the anti-oxidative enzymes is due to their opposite regulation in female and male hearts. Indeed, SOD2 expression in aged female hearts was down-regulated, whereas expression of both enzymes was upregulated in aged male hearts. This finding leads to an intriguing idea that there are mechanisms activated in aged male hearts leading to improved anti-oxidative defense. Though the underlying cellular mechanisms upregulating the anti-oxidative enzymes SOD2 and catalase in aged male hearts still have to be elucidated, previous reports demonstrated an elevation in blood estrogen concentrations in males with age [31]. Given that estrogen upregulates anti-oxidative enzymes including SOD2 [51, 52], one may assume that the known age-related changes in the estrogen concentration in blood from males (increase) and females (decrease) may contribute to the sex difference in SOD2 and catalase expression observed in our study.

Similar to SOD2, a sex-dependent alteration in the expression of Sirt3, a major regulator of the mitochondrial acetylome, was observed in our study. Global analysis of the mitochondrial protein acetylome performed by Herbert et al. [53] revealed a several-fold increase in the acetylation of multiple lysine residues in the liver of Sirt3-deficient mice. The net contribution of Sirt3 activity comprises the regulation of mitochondrial dynamics [54] and function [55], i.e., OXPHOS activity, ATP synthesis and fatty acid oxidation. Therefore, downregulation of Sirt3 expression in aged female hearts may lead to increased mitochondrial protein acetylation of i.e. SOD2 and disturbed mitochondrial functions, which may further exacerbate the reduced anti-oxidative defense in the mitochondria, i.e., reduced SOD2 expression.

Aside from the transcriptional regulation of Sirt3 expression, a recent study by Kwon et al. [12] suggested an unexpected mechanism for the post-translational regulation of Sirt3 activity and stability via Sirt1-mediated deacetylation. Indeed, the authors found the presence of Sirt1 in the mitochondria and its interaction with Sirt3. The Sirt1-driven deacetylation of Sirt3 significantly increases its stability and enzymatic activity. Of note, the authors observed a hyper-acetylation, and therefore instability of Sirt3 in aged mice. Based on our finding, i.e., reduced Sirt1 and Sirt3 expression in aged female hearts, one may suppose a female-specific downregulation of this novel Sirt1-Sirt3 axis. Altogether, the current study provides convincing evidence of female sex-specific downregulation of Sirt1 and Sirt3 accompanied by disturbed expression in some mitochondrial proteins and anti-oxidative defense.

In addition to regulating mitochondrial function and biogenesis, emerging data also suggest the role of Sirt1 in acute and chronic inflammatory response, e.g., via inhibition of transcription factor NF-kB [20, 21]. Since, chronic inflammation is a characteristic feature of aging [40, 56], we were wondering whether female sex-specific Sirt1 downregulation may be accompanied by enhanced inflammation in aging female hearts. Indeed, the present study revealed a significant pro-inflammatory shift in aged female but not male hearts demonstrated by an increase in the cardiac macrophage content as well as upregulation of NF-kB, IL-12 and IL-18 expression, specifically in hearts from old women. In contrast, the anti-inflammatory protection was significantly decreased in women with age, which was confirmed by the elevation of the IL-12/IL-10 ratio. Similar increase in the IL-12/IL-10 ratio in the brain of old female mice has been found by Zhang et al. .[57] Interestingly, it seems that NF-κB is only involved in the inflammatory response in female but not involved in male hearts based on the data of NF- κB, IKBα and the pro-inflammatory cytokines.

In agreement with pro-inflammatory shift in heart of aged women, an increased number of CD68-positive macrophages along with increased pro-inflammatory cytokine expression was found. However, the macrophages found in the cardiac tissue from young women seem to be anti-inflammatory macrophages, since these macrophages were CD206 positive. A shift

in the IL-12/IL-10 ratio to the site of IL-10 might indicate that the cardiac macrophages in the cardiac tissue of young women are regulatory macrophages, since regulatory macrophages produce high amounts of IL-10 and low amounts of IL-12 [64]. This shift is lost in aged women. Altogether, the findings from this study argue for a female sex-specific pro-inflammatory shift in aged hearts. Although the underlying mechanisms of this shift remain unclear, the female sex-specific downregulation of Sirt1 and SOD2 expression suggest a potential contribution.

Sexual hormones modulate the immune system via hormone receptors and regulate inflammation.[58] Although both the pro- and anti-inflammatory actions of estrogens have been described [59], the majority of studies argue for the anti-inflammatory effects of estrogen receptor activation. Particularly, E2 exhibits anti-inflammatory actions on endothelial and immune cells *in vitro* [60, 61]. E2 loss leads to the expression of pro-inflammatory cytokines in humans [32]. In human activated peripheral blood mononuclear cells (PBMCs), E2 inhibits the expression of pro-inflammatory cytokines [62] and decreases NF-κB activity [63].

One limitation of the study is that our cohorts are relatively small and the human material archived high heterogeneity. Due to the small sample number, the statistical significance might not accurately show age or sex differences.

In conclusion, the present study revealed a female sex-specific downregulation of Sirt1 and Sirt3 in aged human hearts accompanied by a decline in the mitochondrial anti-oxidative defense and a pro-inflammatory shift.

## MATERIALS AND METHODS

### Human left ventricular samples

Human non-diseased whole lateral left ventricular (LV) wall tissue was collected from organ donors (men= 16 and women= 15). The whole tissue was frozen immediately after collection in liquid nitrogen and stored at -80°C. The donors were between 17 and 68 years of age. We divided the LV samples into 4 groups: young (17-40 years; male: n= 7 and female: n= 7) and old (50-68 years; male: n= 9 and female: n=8) individuals.

We obtained the informed consent from all donors or their legal guardians. Sample collection and the experimental protocols were approved by the Scientific Board at the Hungarian Ministry of Health (ETT-TUKEB: 4991-0/2010-1018EKU). All research was performed in accordance with the German and Hungarian guidelines.

### RNA extraction and quantitative real-time PCR

Total RNA isolation from cardiac human tissue as well as quantitative real-time PCR were performed as previously described [36]. The mRNA contents of target genes were normalized to the expression of ribosomal protein large P0 (RPLP0) or hypoxanthine phosphoribosyl transferase (HPRT). The purity of the isolated RNA was analyzed with the Bioanalyzer "caliper LabChip" (Agilent Technologies, Rattingen, Germany).

### Protein extraction and immunoblotting

LV samples were homogenized in RIPA buffer (50 mmol/l Tris-HCl, pH 7.4, 150 mmol/l NaCl, 1 mmol/l EDTA, 1% NP-40, and 0.25% Na-deoxycholate) supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany), and the phosphatase inhibitors sodium orthovanadate (1 mmol/l $Na_3VO_4$) and sodium fluoride (1 mmol/l NaF). Proteins were quantified using the BCA Assay (Thermo Scientific Pierce Protein Biology, Schwerte, Germany). Equal amounts of total proteins were separated on SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. The membranes were immunoblotted overnight with the following primary antibodies: Sirt3 (1:1000, Cell Signaling, USA), Ku70 (1:200, Santa Cruz, USA), ac-Ku70 (1:2000, Lys 331, Abcam, UK), TFAM (1:200, Santa Cruz, USA), SOD2 (1:1000, Santa Cruz, USA), catalase (1:1000, Cell Signaling, USA), Sirt1 (1:1000, Cell Signaling, USA), AMPK (1:2000, Cell Signaling, USA), p-AMPK (1:2000, Thr172, Cell Signaling, USA), FOXO1 (1:1000, Cell Signaling, USA), p-FOXO1 (1:1000, Ser 256, Cell Signaling (USA), TOM40 (1:1000, Abcam, UK) and NFκB p50 (1:1000, Santa Cruz, USA). Equal sample loading was confirmed by analysis of actin (1:1000, Santa Cruz, USA) and tubulin (1:50.000, Sigma-Aldrich, USA). Immunoreactive proteins were detected using ECL Plus (GE Healthcare, Buckinghamshire, UK) and quantified with ImageLab (version 5.2.1 build 11, Bio-Rad Laboratories (USA)).

### Immunohistochemistry

For immunohistochemistry, 5 µm cryo-sections of human left ventricle were fixed in formalin for 1 hour at room temperature and subjected to a heat-induced epitope retrieval step prior to incubation with anti-CD68 antibody (clone PGM-1, Agilent Technologies, Santa Clara, CA, USA). The detection was performed by the LSAB method applying the Dako REAL™ Detection

System (Agilent Technologies, Santa Clara, CA, USA). Nuclei were counterstained with hematoxylin and mounted on slides with glycerol gelatin (both Merck KGaA, Darmstadt, Germany). Negative controls were performed by omitting the primary antibody. Images were acquired using an AxioImager Z1 microscope (Carl Zeiss MicroImaging, Inc.). Positive cells were quantified in 5 high power fields (hpf) (field of vision in x400 original magnification). All evaluations were performed in a blinded manner.

### Statistical analysis

The data are given as the mean ± SEM. The GraphPad Prism 5 (GraphPad Software, 2003, San Diego, USA) was used for the statistical analysis. The data were evaluated using the non-parametric test (Mann-Whitney test, for two independent groups) or two-way analysis of variance (to test two independent variables). The Bonferroni post-test was used as a post hoc test. Statistical significance was accepted when $p < 0.05$.

## AUTHOR CONTRIBUTIONS

M.B.d.A. conceived the project, analyzed the data, prepared the figures and wrote the main manuscript text. S.P. analyzed the data and wrote the main manuscript text. A.K. performed and analyzed the immunohistochemical experiments and revised the manuscript, I.B. provided the human tissue and revised the manuscript, Y.L. analyzed the data and wrote the main part of the manuscript and V.R.Z. generated research funds and coordinated the project. All authors commented on the manuscript.

## ACKNOWLEDGEMENTS

We thank Jenny Jansen, Natalie Haritonow, Arne Kühne and Pavelas Karkacas for their technical assistance.

## CONFLICTS OF INTEREST

The authors declare no competing interests.

## FUNDING

This work was funded by the DZHK (German Centre for Cardiovascular Research; 81Z2100201) (including salaries and consumables). Non-diseased cardiac tissue collection and management was supported by the Hungarian National Research, Development and Innovation Office (GINOP-2.3.2-15-2016-00040) and Ministry of Human Capacities (20391-3/2018/FEKUSTRAT to IB.

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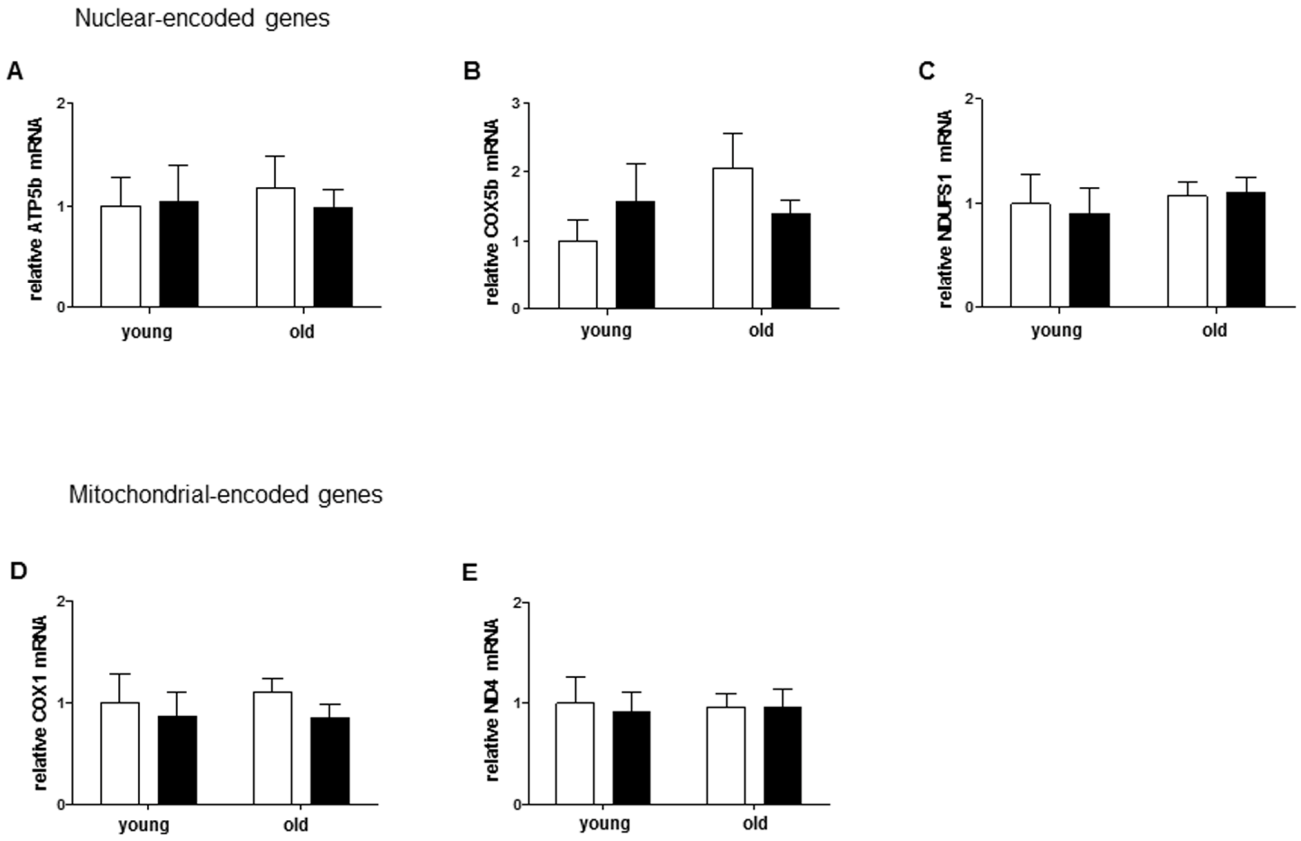
## SUPPLEMENTARY MATERIAL

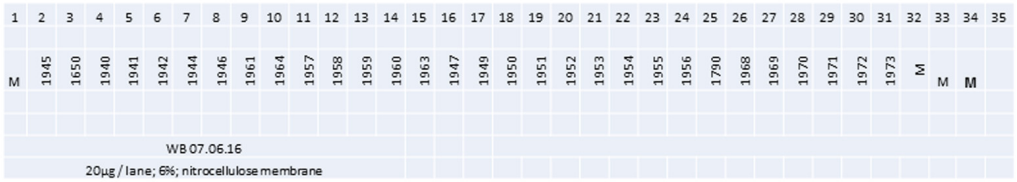
## Supplementary Figure

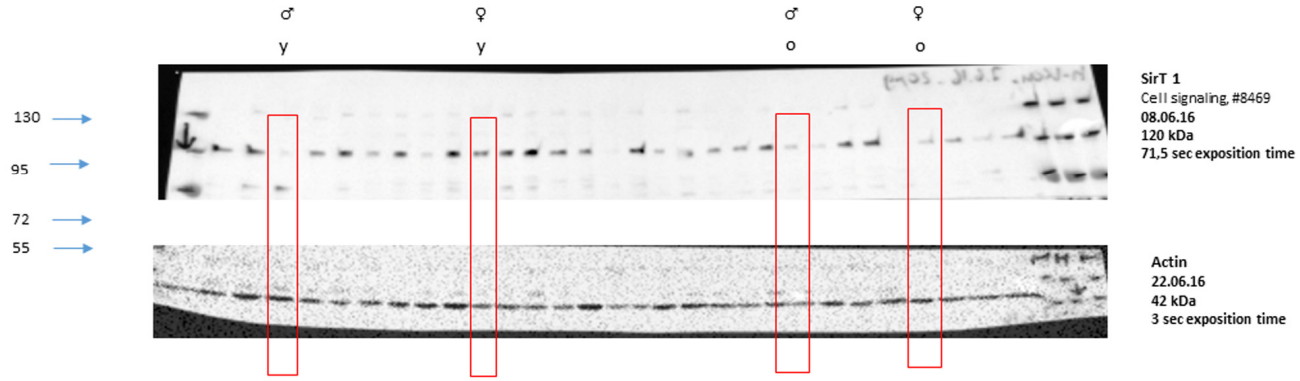



**Supplementary Figure 1. No sex- or age-dependent differences in mitochondrial biogenesis.** Relative mRNA expression of nuclear- and mitochondrial-encoded genes in non-diseased hearts in young and old men and women. Real-time analysis for the nuclear-encoded genes (**A**) ATP5b, (**B**) COX5b and (**C**) NDUFS1 and mitochondrial-encoded genes (**D**) COX1 and (**E**) ND4. Data are shown as the mean ± SEM (n= 6-9/group).

# Original Blots

**Original Blot 1.**

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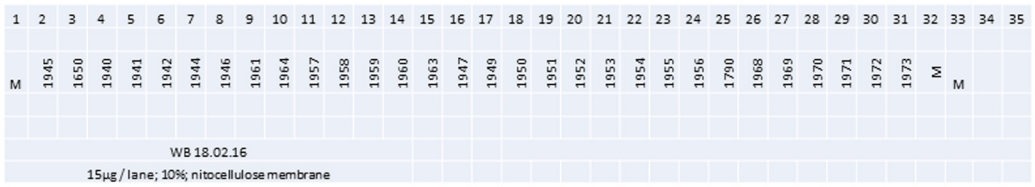
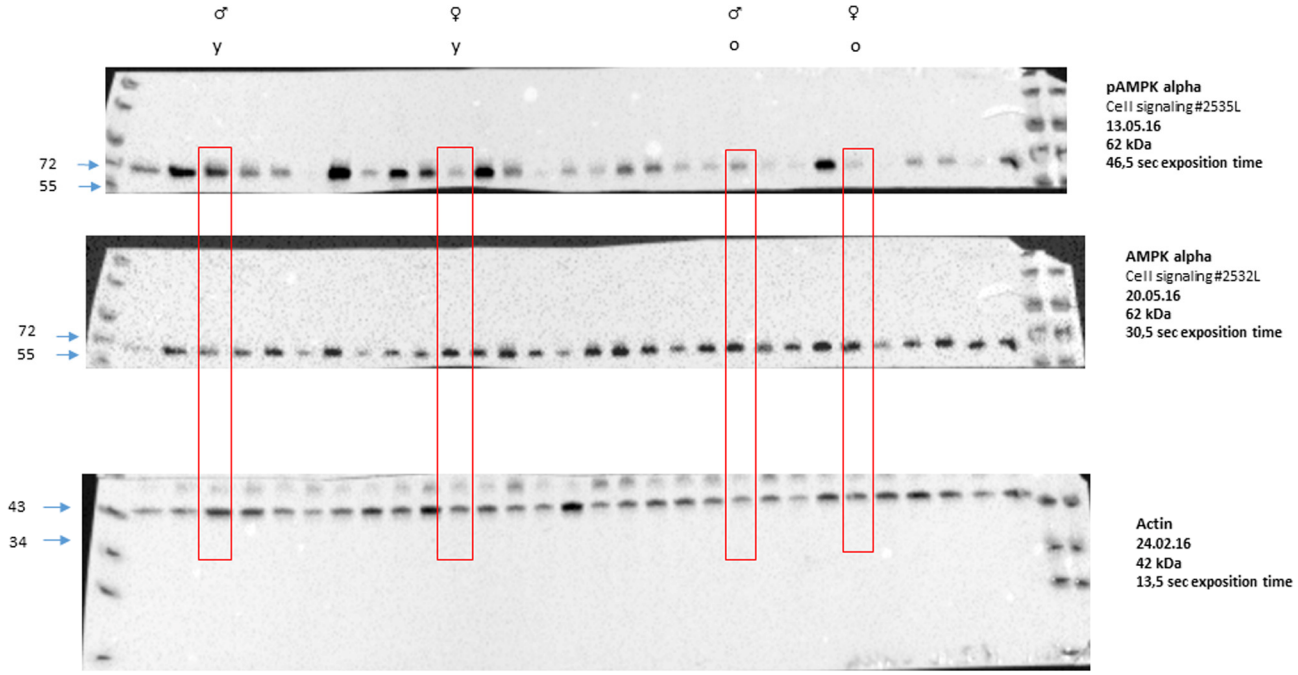


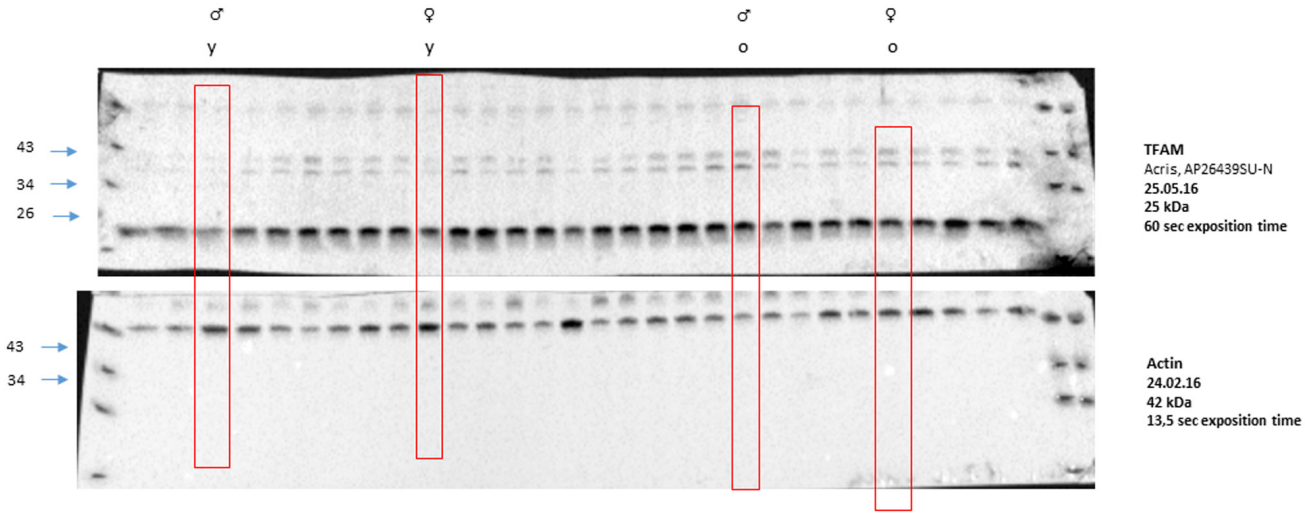



**Original Blot 3.**

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| M | 1945 | 1650 | 1940 | 1941 | 1942 | 1944 | 1946 | 1961 | 1964 | 1957 | 1958 | 1959 | 1960 | 1963 | 1947 | 1949 | 1950 | 1951 | 1952 | 1953 | 1954 | 1955 | 1956 | 1790 | 1968 | 1969 | 1970 | 1971 | 1972 | 1973 | M | M | | |

WB 18.02.16

15µg / lane; 10%; nitocellulose membrane




**Original Blot 4.**

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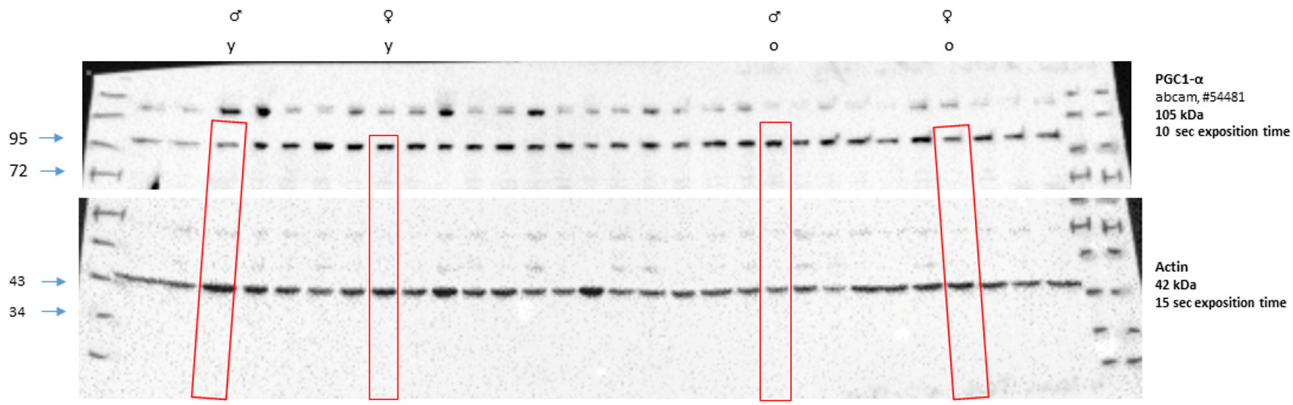
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| M | 1945 | 1650 | 1940 | 1941 | 1942 | 1944 | 1946 | 1961 | 1964 | 1957 | 1958 | 1959 | 1960 | 1963 | 1947 | 1949 | 1950 | 1951 | 1952 | 1953 | 1954 | 1955 | 1956 | 1790 | 1968 | 1969 | 1970 | 1971 | 1972 | 1973 | M | M |



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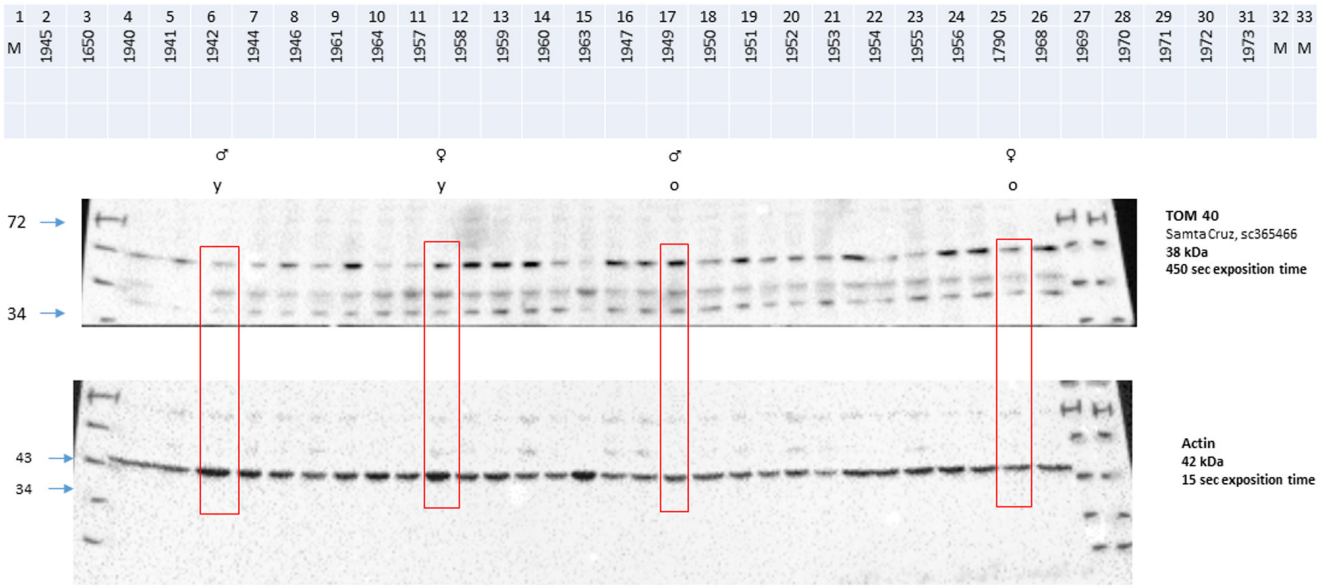


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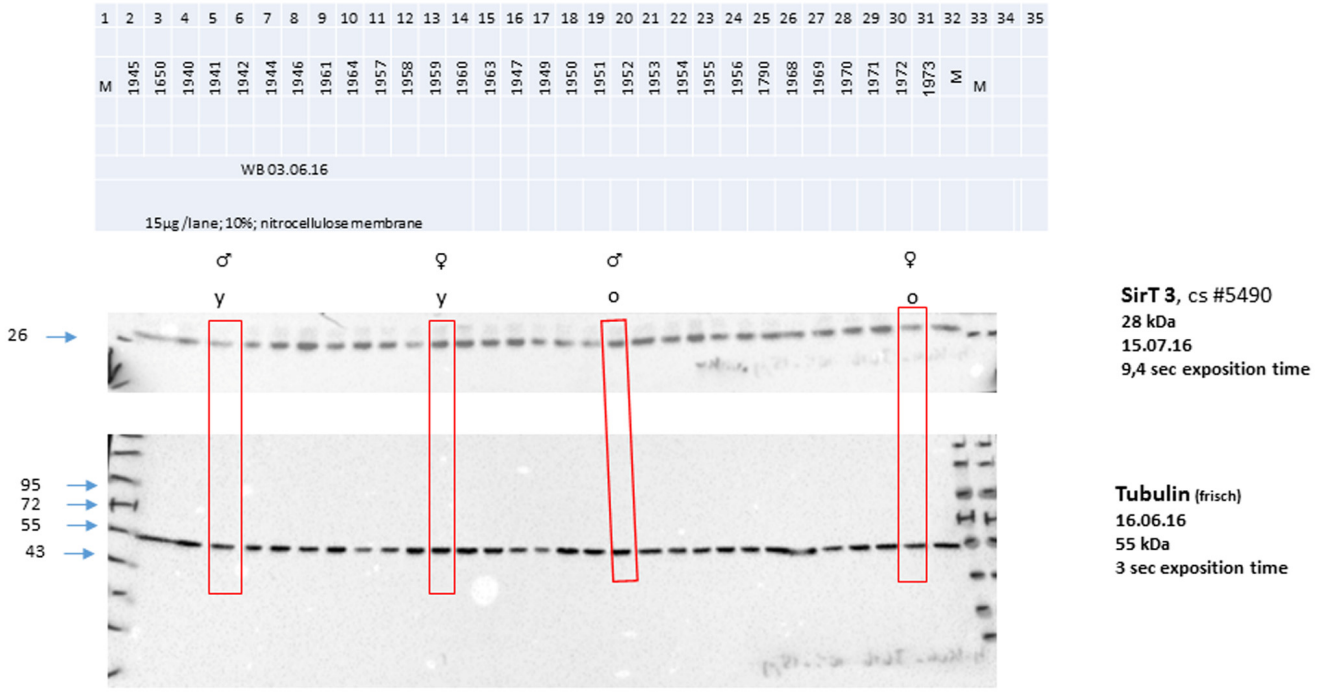

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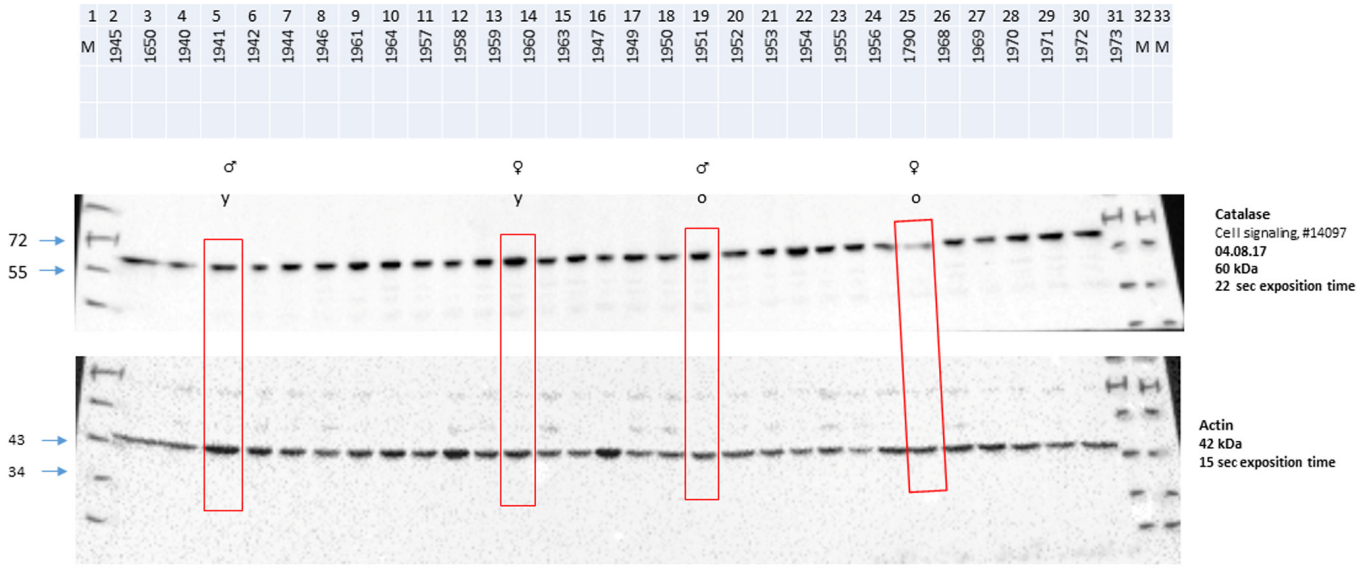


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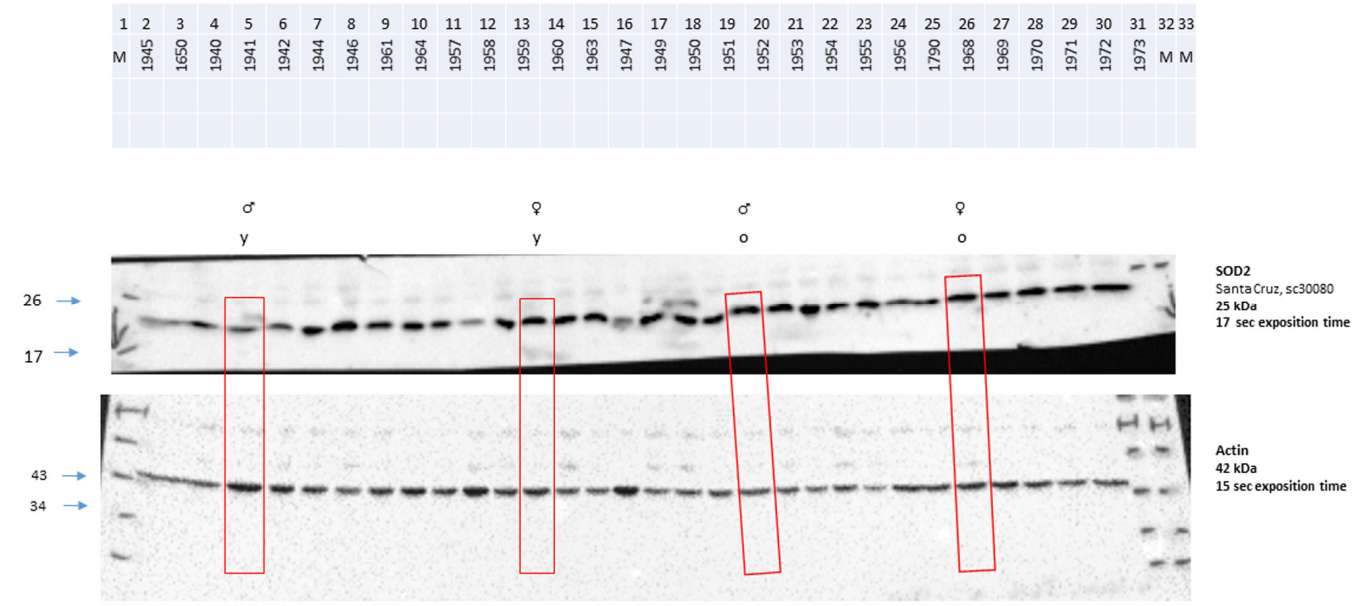

### 3.2 Dilated cardiomyopathy impairs mitochondrial biogenesis and promotes inflammation in an age- and sex-dependent manner (PMID: 33303703)

**Barcena ML**, Pozdniakova S, Haritonow N, Breiter P, Kühl AA, Milting H, Baczko I, Ladilov Y, Regitz-Zagrosek V. Dilated cardiomyopathy impairs mitochondrial biogenesis and promotes inflammation in an age- and sex-dependent manner. Aging (Albany NY), 2020; 12(23): 24117-33.
https://doi.org/10.18632/aging.202283

Myocardial damage in DCM is associated with inflammation and impaired mitochondrial function or biogenesis. The aim of this work was to elucidate age-related sex differences in the expression of genes and proteins involved in mitochondrial function and biogenesis and inflammation in cardiac tissue of patients with DCM. The metabolic regulator Sirt1 was significantly decreased in cardiac tissue from older male and female DCM patients. Another key metabolic regulator, AMPK was activated in older male DCM hearts, while younger male DCM hearts showed a decreased activation. The expression of several mitochondrial proteins (TOM40, TIM23, and Sirt3), as well as mRNA (*cox1*, *mt-nd4*) were markedly reduced in aged DCM patients, either in a sex dependent or independent manner. Of note, the expression of a key anti-oxidative mitochondrial enzyme SOD2 was significantly reduced solely in male aged DCM patients. In addition, older male DCM patients showed a stronger inflammatory response in the heart than females.

In conclusion, our study demonstrated a decreased mitochondrial biogenesis and alterations in the anti-oxidative defense in hearts from older DCM patients in a sex-dependent and independent manner. Furthermore, the diminished mitochondrial biogenesis seems to be associated with an increased inflammatory response in DCM.[203]

Since, we found age and sex differences in mitochondrial biogenesis and pro-inflammatory state in idiopathic DCM, we were wondering if similar mechanisms occur in other cardiomyopathies, e.g., DCMI.

Research Paper

# Dilated cardiomyopathy impairs mitochondrial biogenesis and promotes inflammation in an age- and sex-dependent manner

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**Keywords:** dilated cardiomyopathy, inflammation, mitochondrial proteins, sex differences, aging
**Received:** July 28, 2020 **Accepted:** September 29, 2020 **Published:** December 2, 2020



## ABSTRACT

**Dilated cardiomyopathy (DCM) belongs to the myocardial diseases associated with a severe impairment of cardiac function, but the question of how sex and age affect this pathology has not been fully explored. Impaired energy homeostasis, mitochondrial dysfunction, and systemic inflammation are well-described phenomena associated with aging. In this study, we investigated if DCM affects these phenomena in a sex- and age-related manner.**
**We analyzed the expression of mitochondrial and antioxidant proteins and the inflammatory state in DCM heart tissue from younger and older women and men.**
**A significant downregulation of Sirt1 expression was detected in older DCM patients. Sex-related differences were observed in the phosphorylation of AMPK that only appeared in older males with DCM, possibly due to an alternative Sirt1 regulation mechanism. Furthermore, reduced expression of several mitochondrial proteins (TOM40, TIM23, Sirt3, and SOD2) and genes (*cox1*, *nd4*) was only detected in old DCM patients, suggesting that age has a greater effect than DCM on these alterations. Finally, an increased expression of inflammatory markers in older, failing hearts, with a stronger pro-inflammatory response in men, was observed. Together, these findings indicate that age- and sex-related increased inflammation and disturbance of mitochondrial homeostasis occurs in male individuals with DCM.**

## INTRODUCTION

Dilated cardiomyopathy (DCM) is a non-ischemic heart muscle disease with structural and functional myocardial abnormalities characterized by dilation of the ventricular chamber and impaired contraction [1]. Myocardial damage triggers inflammation followed by the recruitment of immune cells to the injured site and the release of cytokines [2, 3].

Furthermore, aging is considered to be a risk factor for cardiovascular diseases and to have a negative impact

on ventricular function [4–7]. On the molecular level, aging is accompanied by a disturbance in energy homeostasis, mitochondrial dysfunction, and increased inflammation [4]. Importantly, sex differences in the mitochondrial function in several pathologies, including cardiovascular diseases, have been demonstrated [8]. Mitochondrial biogenesis and respiration are regulated by PGC-1α, a transcriptional coactivator, and its downregulation has been shown to lead to hypertrophy and heart failure [9–11]. Furthermore, the reduced activity of PGC-1α itself, as well as its modulators, especially AMP-activated kinase (AMPK) among others, has been associated with aging [12]. A decline in AMPK activity, a crucial regulator of energy metabolic homeostasis, has been shown in older subjects, and increasing this AMPK activity may extend lifespans [13]. Sirtuins (Sirt), a conserved family of global metabolic regulators with $NAD^+$-dependent deacetylase activity [14], and Sirt1 (an evolutionarily conserved AMPK partner) in particular [15, 16], have been widely reported to protect against age-associated diseases and therefore, to increase health span and life span [17–21]. Consistent reduction of $NAD^+$ levels in older mice is accompanied by a decrease in Sirt1 activity, while its genetic or pharmacological restoration promotes longevity [18, 22, 23].

Among other factors, AMPK and Sirt1 play essential roles in mitochondrial biology. Particularly, AMPK and Sirt1 promote PGC-1α activity and, thus, mitochondrial biogenesis. Furthermore, AMPK controls mitochondrial clearance, i.e., mitophagy [24] and a decline in the AMPK activity leads to impaired mitophagy, accumulation of dysfunctional mitochondria, and ROS formation, which may trigger inflammation- and aging-related diseases [4, 25, 26]. Additionally, the release of mtDNA from damaged mitochondria into the cytosol may lead to an inflammatory response, via Toll-like receptors—and STING-dependent inflammasome activation [25]. Similarly, emerging data have suggested that Sirt1 may play an anti-inflammatory role [27–29].

In addition to Sirt1, Sirt3 is another sirtuin playing an essential role in mitochondrial biology. Sirt3 is the main mitochondrial-localized deacetylase that maintains the activity of numerous mitochondrial enzymes, e.g. SOD2, and thus, supports metabolic and redox balance in mitochondria [30]. Both we and others have shown a decline in Sirt3 expression in older human myocardium [31, 32]. It is important to note that an anti-inflammatory role of mitochondria-localized Sirt3 has been reported [33, 34].

Sex is another potential risk factor associated with cardiovascular diseases. Men have an increased incidence and severity of atherosclerosis, myocardial infarction, heart failure, and DCM [35–37], whereas women with DCM have better chances of survival than men [38]. Heart failure is associated with cardiomyocyte hypertrophy, apoptosis, inflammation, and interstitial fibrosis, which all occur in a sex-specific manner [39]. Estrogen (E2) seems to play a protective role, as a decline in its levels is associated with deleterious left ventricle remodeling and cardiac dysfunction [40].

Altogether, sex and age have significant impacts on cardiomyopathy, however, there is still a large gap in the research about signaling in heart diseases dependent on age or sex. Here, we investigated age- and sex-related alterations in the expression of metabolic regulators, i.e., AMPK and Sirt1, mitochondrial biogenesis, and inflammation parameters in patients with DCM. The analyses revealed a decreased Sirt1 and Sirt3 expression in older DCM patients in both sexes, whereas the activity of AMPK was increased only in men. Sex differences were also found in mitochondrial antioxidant capacity, e.g., SOD2. DCM in the hearts of older patients was associated with a reduced expression of mitochondrial proteins and increased inflammation, which were both sex dependent and independent.

## RESULTS

### DCM-related Sirt1 and AMPK alterations in older patients

Sirt1 and AMPK are key regulators of metabolic pathways. Previously, we observed a decreased expression of Sirt1 and AMPK in older individuals [32]; however, age- and sex-related differences in patients with DCM remained unexplored. Therefore, in the present study, the expression of Sirt1, AMPK, and pAMPK in control (non-diseased) and diseased human cardiac tissue from young and old male and females was analyzed.

In older individuals, Sirt1 expression was significantly decreased in the DCM group when compared to the control (Figure 1A). Both Sirt1 and AMPK share many common target molecules and interact with each other [15]. To test whether the Sirt1 downregulation is associated with the alteration of AMPK activity (indirectly highlighted by the phosphorylation rate), the pAMPK/AMPK ratio was analyzed. A significant increase in AMPK phosphorylation in older men with DCM, but not in women, in the presence of unchanged total AMPK content, was observed (Figure 1B, 1C). Surprisingly, the pAMPK/AMPK ratio was markedly reduced in younger individuals (Supplementary Figure 1C). The linear regression analysis revealed a significant dependence of the DCM-related alteration in

the AMPK phosphorylation on age, with the threshold appearing at 40 years (Figure 1D). Therefore, DCM seems to have an opposite effect on AMPK phosphorylation in younger (downregulation) and older (upregulation) patients (Figure 1D).

No significant changes in Sirt1 or in AMPK expression in younger individuals with DCM were found (Supplementary Figure 1A, 1B).

**DCM-related expression of mitochondrial and anti-oxidative enzymes in older patients**

Disturbance of mitochondrial homeostasis, e.g., biogenesis, is a hallmark and a trigger of heart failure [41]. Therefore, markers of mitochondrial biogenesis were analyzed in cardiac tissue.

PGC-1α is a key transcription co-activator involved in mitochondrial biogenesis and a direct target of Sirt1 [42]. It has been shown that downregulation of Sirt1 may impair PGC-1α activity [42], and, in turn, mitochondrial biogenesis [43].

In the present study, the protein level of PGC-1α was affected neither in an age- nor in a sex-specific manner (Figure 2A and Supplementary Figure 2A). Nevertheless, the expression of several mitochondrial proteins (TOM40, TIM23, and Sirt3), as well as mRNA (*cox1*, mt-*nd4*) was markedly reduced in older but not in younger DCM patients (Figures 2, 3, Supplementary Figure 2 and Supplementary Figure 3, respectively). Of note is the significant reduction of the expression of a key anti-oxidative mitochondrial enzyme SOD2 solely in older male DCM patients (Figure 2E). In contrast, the expression of cytosolic anti-oxidative enzyme catalase was significantly upregulated in older DCM individuals of both sexes (Figure 2F). Taken together, our data suggest a disturbance in mitochondrial biogenesis and anti-oxidative defense in hearts with DCM.

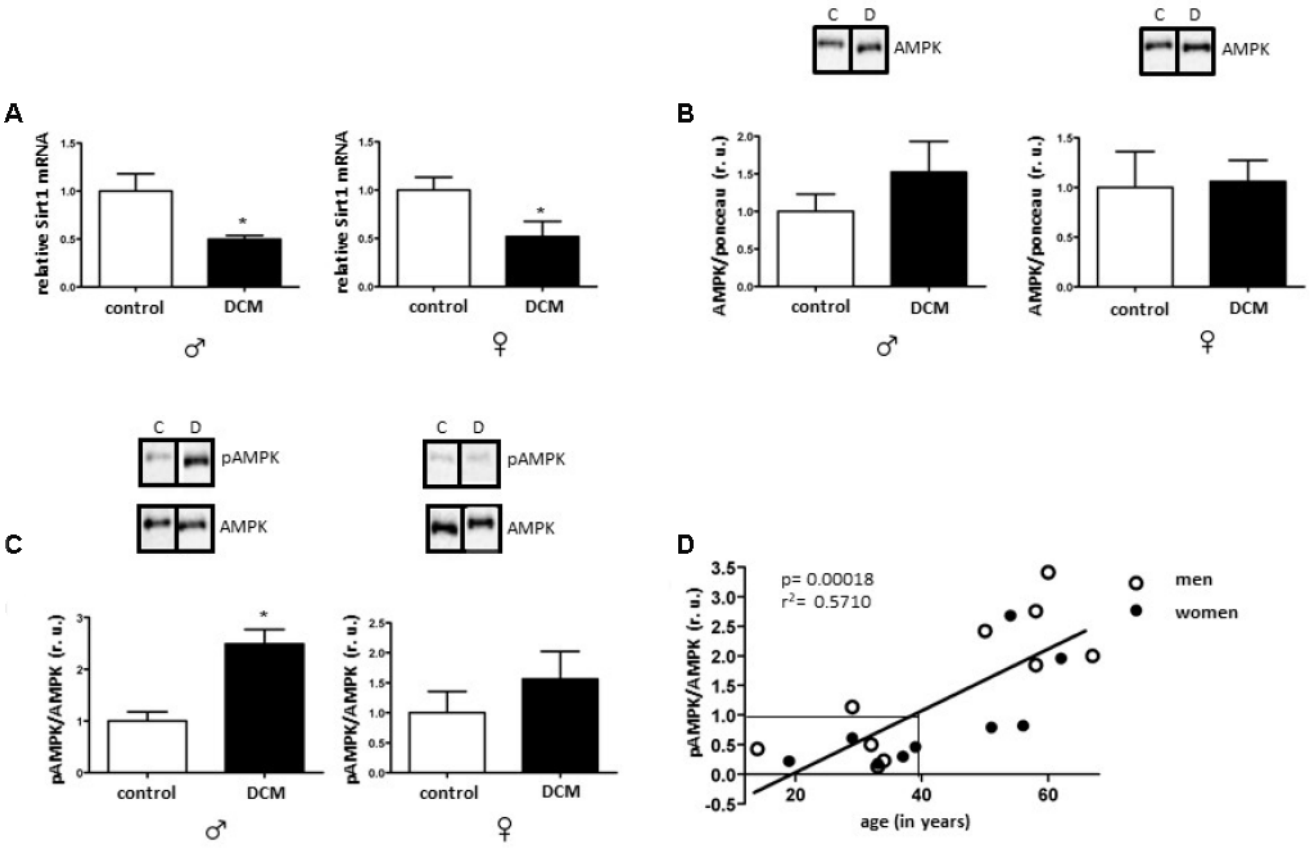


**Figure 1. Effects of DCM on Sirt1 expression and AMPK phosphorylation in older patients.** Expression analysis of Sirt1 mRNA (**A**), total AMPK (**B**) and phosphorylated AMPK (Thr172) (**C**) performed with human cardiac tissue lysates from old control (non-diseased) and DCM men (♂) and women (♀). Data are means ± SEM (n= 5). Representative imaging of western blot analysis; the lanes were run in two gels. All data were normalized to the corresponding control and expressed in relative units (r.u.). (**D**) Linear regression analysis between pAMPK/AMPK ratio (dependent variable) and age (explanatory variable) was performed with function lm() in R. pAMPK/AMPK values were obtained from old and young male and female hearts diagnosed with DCM.

**DCM-related pro-inflammatory state alterations in older patients**

The cardiac pro-inflammatory state is expanded during aging processes [44]. Downregulation of Sirt1 and AMPK, along with mitochondrial dysfunction, may significantly contribute to the initiation and perpetuation of inflammation in the heart [45, 46]. Thus, we examined the data for alterations in the Sirt1 and AMPK signaling and mitochondrial homeostasis that were accompanied by a pro-inflammatory response in older DCM patients.

The number of cardiac CD68 immune-reactive cells was markedly increased in older hearts with DCM when compared to the older control hearts (Figure 4A and Supplementary Figure 4). In accordance with this finding, the NF-κB expression was also significantly elevated in older male DCM hearts (Figure 4B). It has been suggested that FOXO1 is involved in the polarization of macrophages [47]. FOXO1 expression was significantly increased in older DCM patients (Figure 4C), further suggesting a DCM-related pro-inflammatory state.

In contrast to these findings, the mRNA of pro-inflammatory cytokines, e. g. TNF-α was not changed in older DCM hearts (data not shown), and the IL-1β was significantly reduced in older DCM hearts in comparison to control hearts (Figure 4D). In contrast, IL-12 mRNA expression was significantly elevated in older male hearts. (Figure 4E). In addition, the mRNA of the anti-inflammatory cytokine IL-10 was reduced in older male and female hearts (Figure 4F). Further analysis of the IL12/IL10 ratio revealed a significantly higher ratio in older male and female DCM hearts (ratio= 4.2 and 2.3) compared to control male or female hearts (1.0). (Figure 4G) Altogether, the pro-inflammatory response in DCM is stronger in older male hearts.

## DISCUSSION

In the current study, we investigated age- and sex-related alterations in metabolic, mitochondrial, and inflammatory markers in DCM patients. The main findings are as follows: 1) In older patients, DCM is associated with significant Sirt1 downregulation in both sexes, while pAMPK/AMPK ratio upregulation only

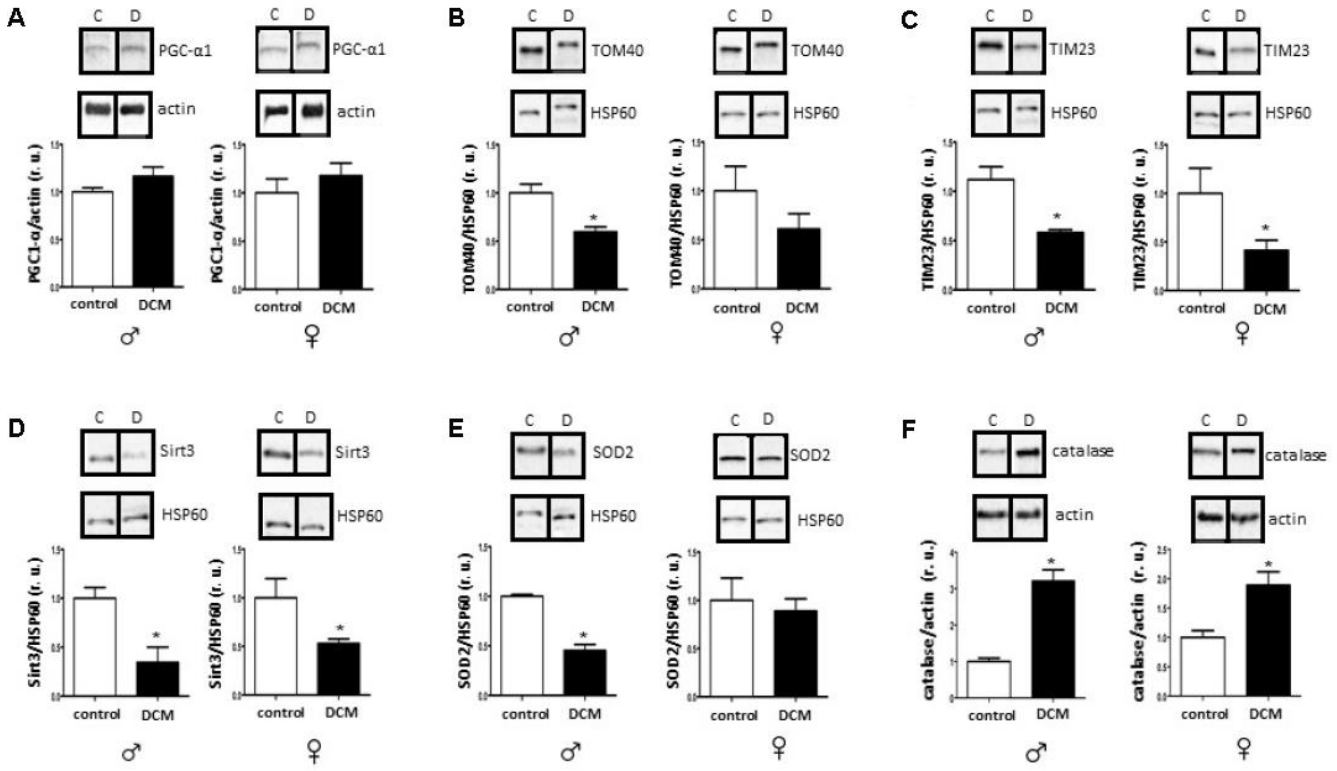


**Figure 2. DCM-related alterations in expression of mitochondrial proteins and anti-oxidative enzymes in older patients.** Western blot analysis of PGC1-α (**A**), TOM40 (**B**), TIM23 (**C**), Sirt3 (**D**), SOD2 (**E**) and catalase (**F**) expression performed with human cardiac tissue lysates from old control (non-diseased) or DCM men (♂) and women (♀). Representative imaging of western blot analysis; the lanes were run in two gels. All data were normalized to the corresponding control and expressed in relative units (r.u.). Data are means ± SEM (n= 5).

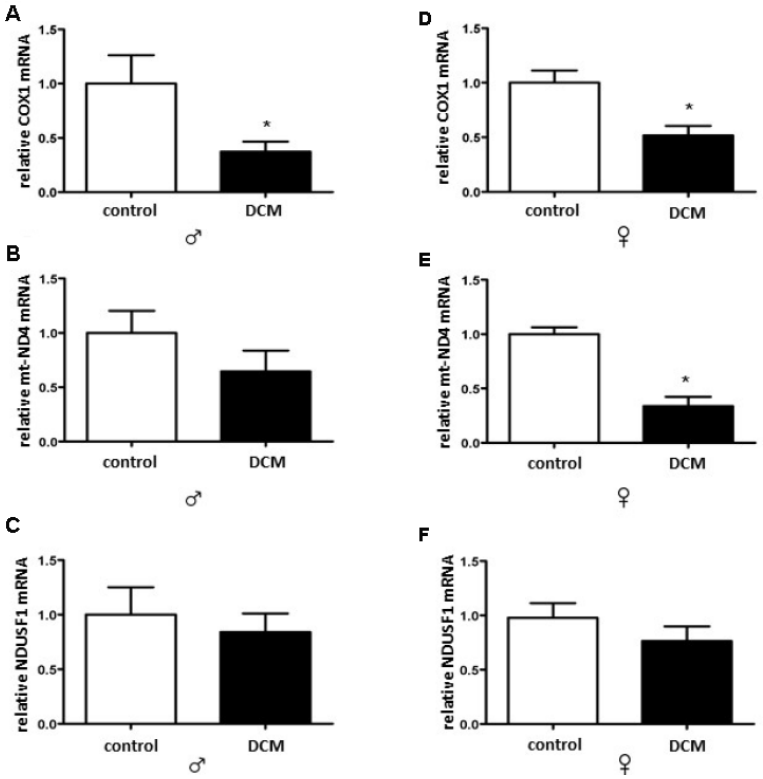


**Figure 3. DCM-related alterations in expression of mitochondrial genes in older patients.** Real-time PCR analysis for the mitochondrial-encoded genes *cox1* (**A**, **B**) and *mt-nda* (**C**, **D**) as well as the nuclear-encoded genes *ndusf1* (**E**, **F**), performed with human cardiac tissue lysates from old control (non-diseased) or DCM men (♂) and women (♀). Data are shown as the mean ± SEM (n= 5).

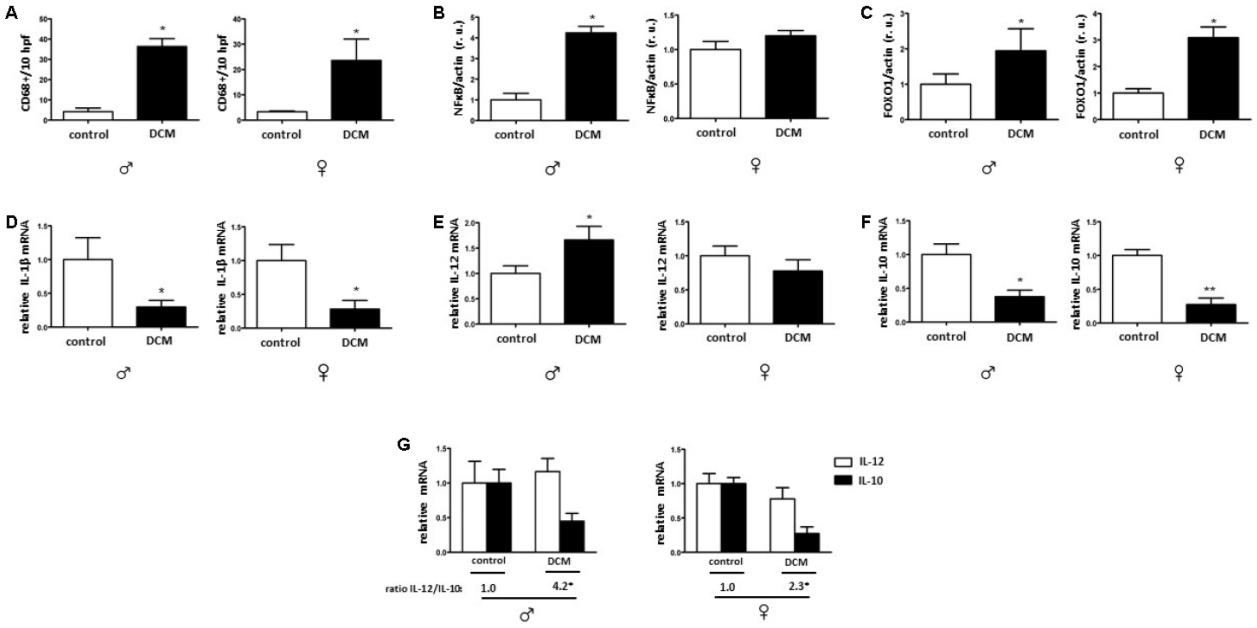


**Figure 4. DCM effects on the pro-inflammatory state.** Immunohistological analysis of CD-68 immuno-reactive cells (**A**). Western blot analysis of NF-κB (**B**), FOXO1 (**C**) as well as the real-time PCR analysis of IL-1β (**D**), IL-12 (**E**), and IL-10 (**F**) mRNA expression performed with human cardiac tissue lysates from old control (non-diseased) or DCM men (♂) and women (♀). (**G**) Ratio of IL-12/IL-10. Representative imaging of western blot analysis. All data were normalized to the corresponding control and expressed in relative units (r.u.). Data are shown as the mean ± SEM (n= 5).

appeared in males; 2) expression of several mitochondrial proteins (TOM40, TIM23, and Sirt3), including a main mitochondrial antioxidant SOD2, and mRNA (*cox1*, *nd4*) is reduced in older but not in younger DCM patients; 3) expression of the inflammatory markers in older hearts with DCM is increased, with a stronger pro-inflammatory response in older males with DCM. Taken together, these findings indicate a disturbance of metabolic sensing, impairment of mitochondrial biogenesis, and increased inflammation in older individuals with DCM in both sex-dependent and -independent manners.

The crucial role of Sirt1 in inflammation, DNA repair, apoptosis, and aging has already been investigated [48, 49]. Its reduced expression and activity have been associated with various pathologies, e.g., diabetes, Alzheimer's disease and coronary artery disease [50–52]. Sirt1 activation by resveratrol tends to decelerate aging processes and the onset of chronic diseases [53–55]. In the present study, the Sirt1 expression was significantly diminished in DCM hearts in older individuals in a sex-independent manner, while no alterations were observed in younger DCM patients. In accordance with our data, consistent reduction of Sirt1 expression was reported in older mice [56] as well as in control hearts of older women. Likewise, Sirt1-deficient mice showed a progressive DCM strongly associated with mitochondrial dysfunction [57]. Furthermore, reduced Sirt1 expression was reported in monocytes of older patients with cardiovascular diseases [52], suggesting that aging directly aggravates cardiac dysfunction via disruption of Sirt1 signaling, promoting mitochondrial dysfunction, apoptosis, and inflammation as a result.

AMPK is a key Sirt1 partner in regulating metabolic activity and inflammation that is activated under metabolic stress [58]. AMPK and SIRT1 may regulate each other and share many common targets [15]. An increasing number of reports emphasize the supporting role of AMPK activity in cardiac metabolic homeostasis [59]. From the other side, persistent AMPK overactivation might be deleterious in some cases, with a chronic AMPK activation appearing to aggravate the pathological damage that strokes and myocardial ischemia cause [13, 60, 61], by, e.g., decreasing fatty acid oxidation [62]. Furthermore, sustained AMPK activation leads to excessive mitochondrial fragmentation and mitophagy, leading to the depletion of functional mitochondria [63]. Here, DCM in older patients was associated with increased phosphorylation of AMPK compared to the control group, whereas in young DCM patients AMPK phosphorylation was markedly reduced. Though diminished AMPK activity in hearts with DCM has been previously observed [61], no age dependence has been demonstrated. In fact, both we and others [13, 32] observed a marked reduction of the cardiac AMPK phosphorylation in older, control individuals. It is tempting to speculate that, with the reduced AMPK phosphorylation in older hearts, additional DCM-related metabolic impairment may trigger the compensatory activation of the AMPK phosphorylation. Fitting in with this view, decreased ATP levels are commonly observed in DCM patients [64].

The disturbance of metabolic homeostasis in older DCM hearts is further highlighted by the reduced mitochondrial biogenesis observed in the present study. Particularly, a reduced Sirt3 expression in older DCM hearts, a main mitochondria-localized deacetylase involved in the regulation of mitochondrial enzymes activity [65, 66], argues for a disturbed mitochondrial function. The pivotal role of Sirt3 in cardiovascular health/diseases is largely accepted and Sirt3 dysfunction is associated with numerous pathologies [67, 68].

Aside from impaired mitochondrial biogenesis and acetylation capacity, numerous mitochondrial abnormalities have been reported in patients with DCM elsewhere [69–71], which may additionally contribute to the mitochondrial dysfunction in older DCM hearts. Mitochondrial dysfunction is typically accompanied by impaired ATP synthesis with enhanced ROS formation [72]. Though we did not measure adenine nucleotide homeostasis in the present study, the enhanced activation of key metabolic sensors, i.e., AMPK, solely in older male DCM hearts suggests the elevation of the AMP/ATP or ADP/ATP ratio. Interestingly, a male-specific downregulation of the main mitochondrial antioxidant enzyme SOD2 was found in older DCM hearts. Altogether, the data suggest that DCM aggravates metabolic and oxidative stress in older hearts in both a sex-dependent and -independent manner.

The results of research conducted thus far suggests that the higher incidence of cardiac diseases, particularly of DCM, in males may be due to the fact that females are protected by higher levels of steroid hormones, 17β-estradiol among others. However, in older, post-menopausal females, the protective effect of 17β-estradiol is lost. In fact, older men have higher 17β-estradiol concentrations in their blood compared to older women [73]. Our recent report [32] showed that cardiac aging is defined by the decline in mitochondrial anti-oxidative defense and a pro-inflammatory shift in females but not in males.

An increased inflammatory response accompanied by decreased mitochondrial function [74, 75] is typical for older hearts.

NFκB promotes inflammation in myocardial tissue in age-related diseases [76]. In accordance with this function, our results showed enhanced NFκB expression in DCM hearts in older males. [27, 77]. Pro-inflammatory mediators are increased in aging under certain physiological conditions [78]. In the present study, the expression of pro-inflammatory factor IL-12 was elevated in older DCM hearts only in males, while a substantial decrease in IL-10 expression was observed in older DCM hearts in a sex-independent manner. A reduced IL-10 expression is a typical fingerprint observed in cardiac aging [32] as well as in aging macrophages [79]; furthermore, IL-10 deficiency promotes a detrimental course of inflammation [80]. The total amount of cardiac macrophages was elevated in DCM patients in this study and accompanied by a shift in the IL-12/IL-10 ratio to IL-12 side, which is a marker of pro-inflammatory macrophages [81].

Our results, in combination with those from other previous studies, suggest an increased inflammatory phenotype in older DCM hearts, which is even more pronounced in males.

In conclusion, the present study revealed that DCM is associated with the downregulation of key metabolic regulator Sirt1, diminished mitochondrial biogenesis and anti-oxidative defense, and an increased inflammatory response in older DCM hearts in both sex-dependent and -independent manners.

## MATERIALS AND METHODS

### Human samples

Human lateral left ventricular (LV) wall tissue from patients with idiopathic end-stage DCM was collected during organ transplantation (men=10 and women=10) and from healthy organ donors (men=16 and women=15). The informed consent from all donors or their legal guardians was obtained. The patients with DCM had ejection fractions (EF) <30%. The tissue was frozen in liquid nitrogen immediately after collection and stored at -80° C. The patients were between 19 and 70 years old, while healthy donors were between 17 and 68 years of age. The control (non-diseased) LV samples were divided into 4 groups of young (17-40 years; male: n=7 and female: n=7) and old (50-68 years; male: n=9 and female: n=8) individuals; DCM samples were divided into 4 groups of young (19-40 years; male: n=5 and female: n=5) and old (50-70 years; male: n=5 and female: n=5) individuals.

For DCM: Sample collection and the experimental protocols were approved by the scientific boards at the Heart and Diabetes Centre (HDZ) NRW (21/2013) and at the Charité – Universitätsmedizin Berlin (EA2/158/16). All research was performed in accordance with the guidelines from the relevant regulatory German authorities.

For healthy donors: The scientific board at the Hungarian Ministry of Health (ETT-TUKEB: 4991-0/2010-1018EKU) approved the sample collection and the experimental protocols. All research was performed in accordance with the German and Hungarian regulatory guidelines.

### RNA extraction and quantitative real-time PCR

Total RNA from cardiac human tissue was homogenized in RNA-Bee (Amsbio, Abbingdon, UK) and the Phenol/Chloroform (Roth, Karlsruhe, Germany) extraction protocol was used for the RNA isolation. The Caliper LabChip bioanalyzer (Agilent Technologies, Ratingen Germany) was used to analyze the purity of the isolated RNA. Quantitative real-time PCR were performed using the Brilliant SYBR Green qPCR master mix (Applied Biosystems, Foster City, CA, USA). The relative amount of target mRNA was determined using the comparative threshold (Ct) method as previously described [34]. The mRNA contents of target genes were normalized to the expression of hypoxanthine phosphoribosyl transferase (HPRT).

### Protein extraction and immunoblotting

LV samples from DCM and control hearts were homogenized in a Laemmli buffer (253mM Tris/HCL pH 6.8, 8% SDS, 40% glycerin, 200mM DTT, 0.4% bromophenol blue). Proteins were quantified using the BCA Assay (Thermo Scientific Pierce Protein Biology, Schwerte, Germany). Equal amounts of total proteins were separated on SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. The membranes were immunoblotted overnight with the following primary antibodies: AMPK (1:2000, Cell Signaling, USA), p-AMPK (1:2000, Thr172, Cell Signaling, USA), PGC-1α (1:1000 Abcam, UK), TOM40 (1:1000, Abcam, UK), TIM 23 (1:5000, BD, USA), Sirt3 (1:1000, Cell Signaling, USA), SOD2 (1:1000, Santa Cruz, USA), catalase (1:1000, Cell Signaling, USA), NFκBp65 (1:200, Santa Cruz, USA) and FOXO1 (1:1000, Cell Signaling, USA). Equal sample loading was confirmed by analysis of actin (1:1000, Santa Cruz, USA), HSP60 (1:1000, Cell Signaling, USA) or Ponceau S staining. Immunoreactive proteins were detected using ECL Plus (GE Healthcare, Buckinghamshire, UK) and quantified with ImageLab (version 5.2.1 build 11, Bio-Rad Laboratories (USA)).

### Immunohistochemistry

For immunohistochemistry, 5 µm cryo-sections of human LV were fixed in formalin for 1 hour at room temperature and subjected to a heat-induced epitope retrieval step prior to incubation with anti-CD68 antibody (clone PGM-1, Agilent Technologies, Santa Clara, CA, USA). The detection was performed by the LSAB method applying the Dako REAL™ Detection System (Agilent Technologies, Santa Clara, CA, USA). Nuclei were counterstained with hematoxylin and mounted on slides with glycerol gelatin (both Merck KGaA, Darmstadt, Germany). Negative controls were performed by omitting the primary antibody. Images were acquired using an AxioImager Z1 microscope (Carl Zeiss MicroImaging, Inc.). Positive cells were quantified in 10 high power fields (hpf) (field of vision in x400 original magnification). All evaluations were performed in a blinded manner.

### Statistical analysis

The data are given as the mean ± SEM. The GraphPad Prism 5 (GraphPad Software, 2003, San Diego, USA) was used for statistical analysis. The data were evaluated using the non-parametric test (Mann-Whitney test for two independent groups). A simple linear regression analysis was performed with function lm() in R. Statistical significance was accepted when $p < 0.05$.

## AUTHOR CONTRIBUTIONS

M.B. conceived the project, analyzed the data, prepared the figures, and wrote the main manuscript text. S.P. analyzed the data and wrote the main manuscript text. N.H. performed western blot experiments and analyzed the data. P.B. analyzed data and revised the manuscript. A.A.K. performed the immunohistochemical experiments, analyzed the data, and revised the manuscript. H.M. acquired the human DCM tissue and revised the manuscript. I.B. acquired the human tissue and revised the manuscript. Y.L. analyzed the data, and wrote and revised the manuscript. V.R.Z. generated research funds, initiated, and coordinated the project. All authors commented on the manuscript.

## ACKNOWLEDGMENTS

We would like to thank Michael Nnaji for revising the manuscript. We would also like to express our gratitude to Jenny Jansen and Arne Kühne for their technical assistance.

## CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

## FUNDING

This work was funded by the DZHK (German Centre for Cardiovascular Research; 81Z2100201) (including salaries and consumables). Control cardiac tissue collection and management was supported by the Hungarian National Research, Development and Innovation Office (GINOP-2.3.2-15-2016-00040) and the Ministry of Human Capacities Hungary (20391-3/2018/FEKUSTRAT) to IB. We acknowledge support from the German Research Foundation (DFG) and the Open Access Publication Fund of Charité – Universitätsmedizin Berlin.

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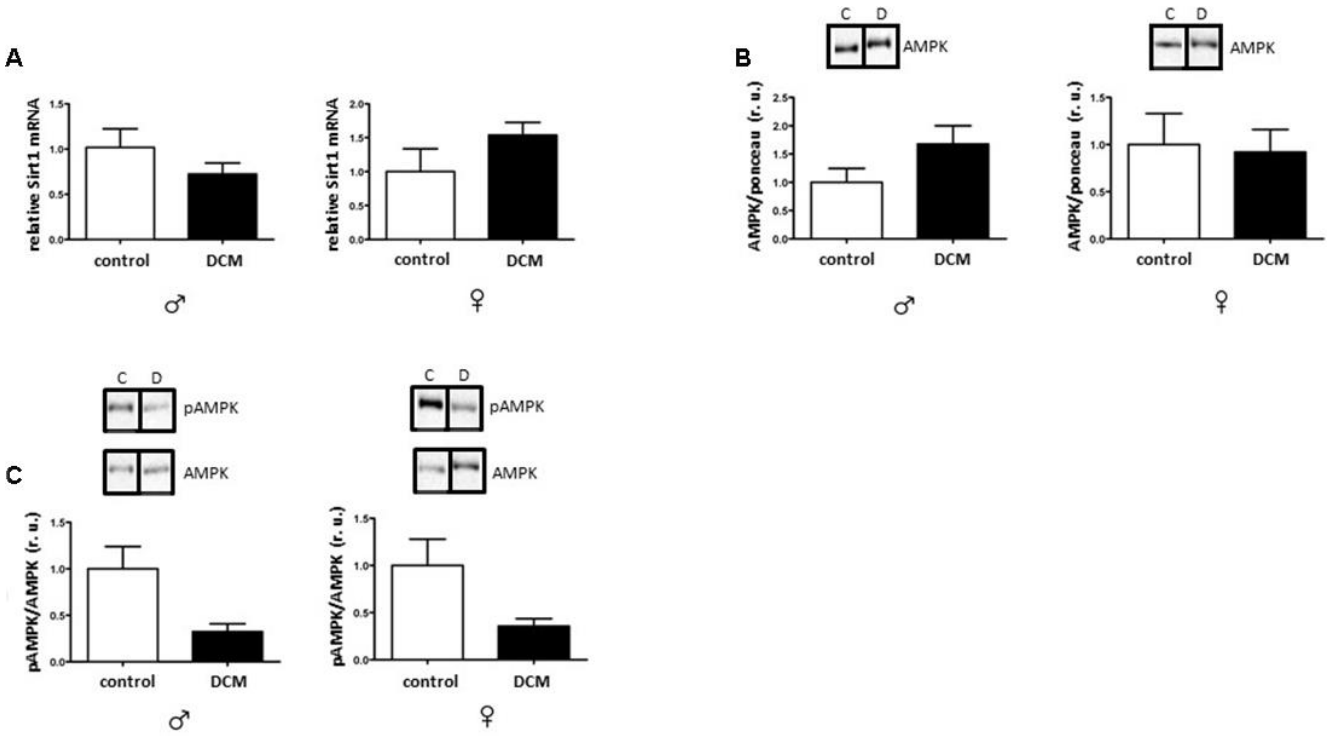
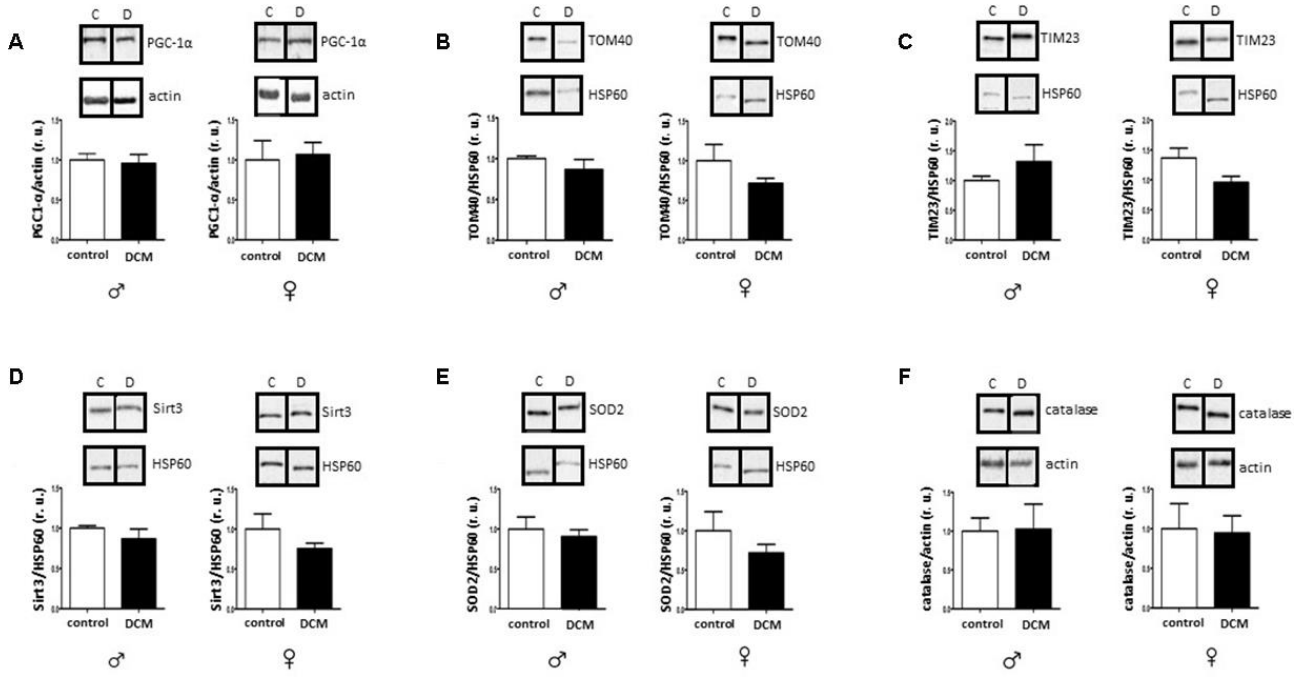
# SUPPLEMENTARY MATERIALS

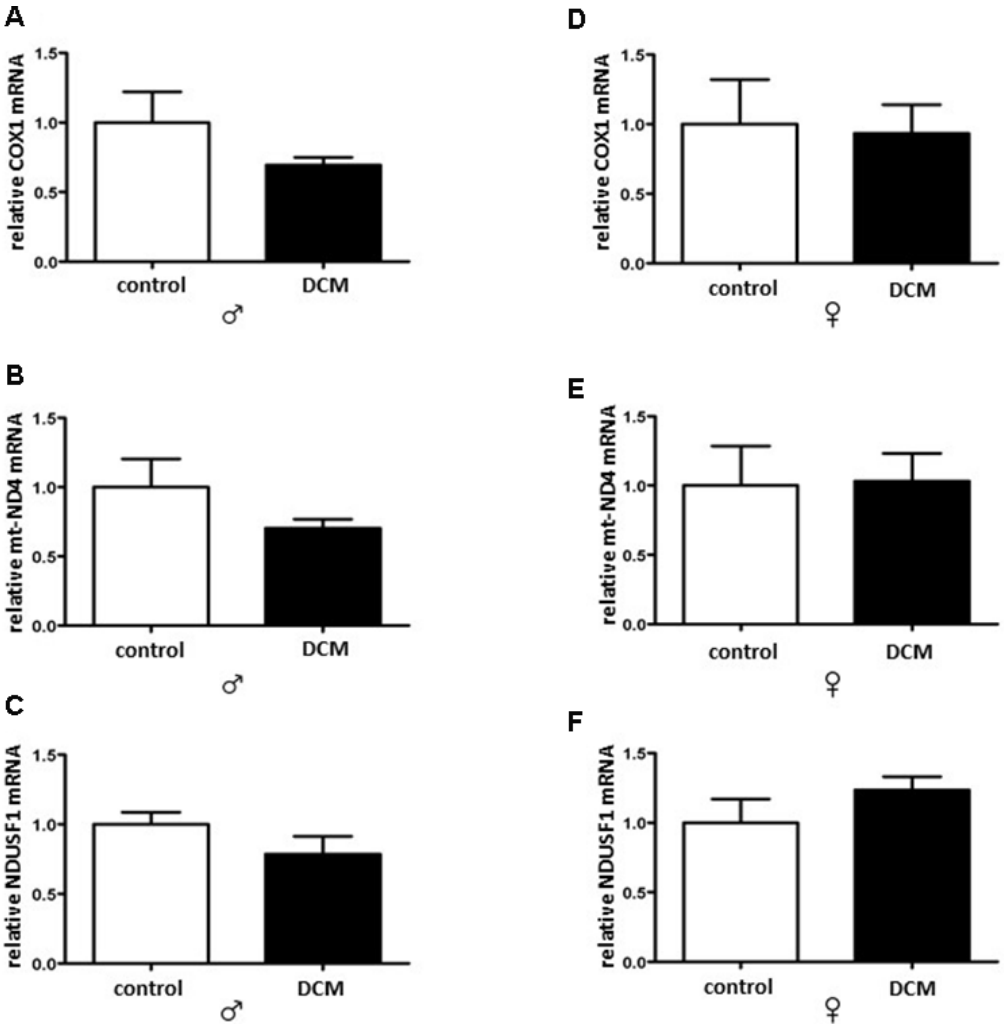
## Supplementary Figures



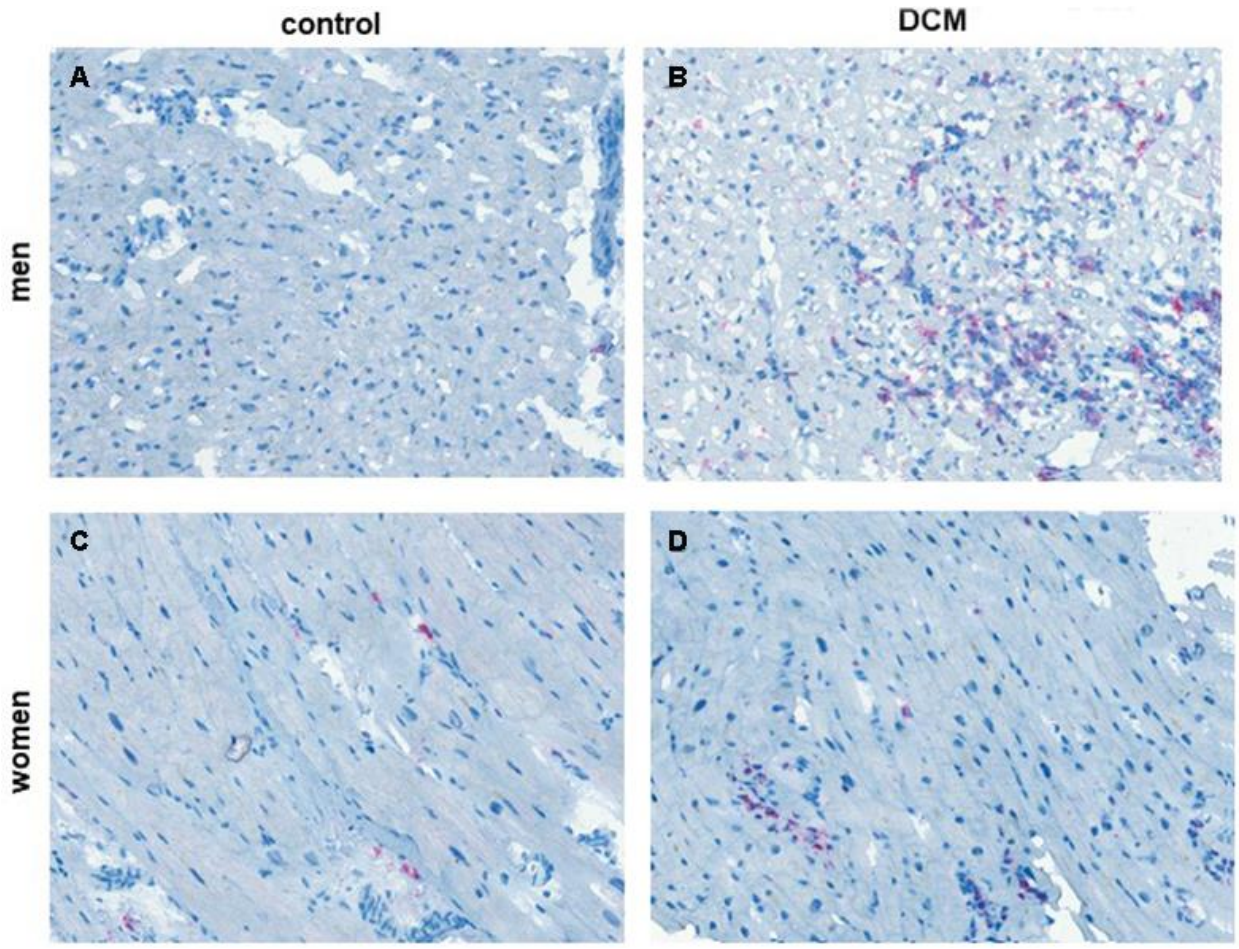

**Supplementary Figure 1. DCM effects on Sirt1 expression and AMPK phosphorylation in young patients.** Western blot expression analysis of Sirt1 (**A**), AMPK (**B**) and phosphorylated AMPK (Thr172) (**C**) performed with human cardiac tissue lysates from young control (non-diseased) or DCM men (♂) and women (♀). Proteins were normalized to actin or ponceau. Data are shown as the mean ± SEM (n= 5). Representative imaging of western blot analysis; the lanes were run in two gels. All data were normalized to the corresponding control and expressed in relative units (r.u.).




**Supplementary Figure 2. DCM effects on expression of mitochondrial proteins in young patients.** Western blot analysis and statistical analysis of PGC1-α (**A**), TOM40 (**B**), TIM23 (**C**), Sirt3 (**D**), SOD2 (**E**) and catalase (**F**) protein expression performed with human cardiac tissue lysates from young control (non-diseased) or DCM men (♂) and women (♀). Proteins were normalized to HSP60 or actin. Data are shown as the mean ± SEM (n= 5). Representative imaging of western blot analysis; the lanes were run in two gels. All data were normalized to the corresponding control and expressed in relative units (r.u.).




**Supplementary Figure 3. DCM effects on expression of mitochondrial genes in young patients.** Real-time PCR analysis for the mitochondrial-encoded genes *cox1* (**A**, **B**) and *mt-nda4* (**C**, **D**), as well as the nuclear-encoded genes *ndusf1* (**E**, **F**), performed with human cardiac tissue lysates from young control (non-diseased) or DCM men (♂) and women (♀). Data are shown as the mean ± SEM (n= 5).




**Supplementary Figure 4. DCM effects on the number of macrophages in cardiac tissue of DCM patients.** Representative images of cardiac cryosections stained with antibodies against CD68 (**A**–**D**). The analyses were performed with myocardium from old control (non-diseased) and DCM men and women. Magnification: 200x.

### 3.3 Sex and age differences in AMPK phosphorylation, mitochondrial homeostasis, and inflammation in hearts from inflammatory cardiomyopathy patients (PMID: 37365150)

**Barcena ML**, Tonini G, Haritonow N, Breiter P, Milting H, Baczko I, Müller-Werdan U, Ladilov Y, Regitz-Zagrosek V. Sex and age differences in AMPK phosphorylation, mitochondrial homeostasis, and inflammation in hearts from inflammatory cardiomyopathy patients. Aging Cell, 2023; 22(8): e13894. https://doi.org/10.1111/ACEL.13894

Myocarditis is associated with myocardial inflammation, impaired metabolism, and mitochondrial dysfunction, leading to heart failure. Importantly, age and sex differences seem to play a crucial role in the development of heart failure, however the effect of these factors remains poorly understood. The aim of this work was to investigate the role of biological sex and age in the alterations in metabolic sensing via Sirt1 and AMPK, mitochondrial biogenesis, and inflammatory response in myocarditis-related inflammatory cardiomyopathy (DCMI).

For this purpose, mitochondrial genes and proteins, autophagy, inflammatory and senescence markers were analyzed in cardiac tissue from younger and older patients with DCMI.

Myocardial AMPK expression and activity was significantly increased in older men with DCMI, whereas Sirt1 remained unchanged in all groups investigated. In addition, the expression of all investigated mitochondrial proteins/genes was preserved in older male patients, while the expression of mitochondrial proteins/genes was significantly reduced in older women with DCMI (e.g., TOM40 and TIM23 and the mitochondrial oxidative phosphorylation genes *cox1 and nd4*). Furthermore, the acetylation state of mitochondrial proteins was significantly reduced in older men with DCMI, suggesting an improved mitochondrial homeostasis in this group. The analysis of inflammatory markers revealed a downregulation of NFκB in older male myocarditis patients, whereas the pro-inflammatory marker IL-18 was increased in older women with myocarditis-related cardiomyopathy. In addition, older male hearts showed a disturbed autophagy and DCMI promoted cellular senescence in older patients.

In conclusion, our study demonstrated a decline in mitochondrial homeostasis in older women with DCMI, while a preserved mitochondrial biogenesis was observed in cardiac tissue of older male patients. Along with the impaired cardiac mitochondrial homeostasis in older women with myocarditis, the pro-inflammatory response was activated and more cellular senescence was observed in this group.[204]

These key findings lead to the continued and deepened investigation of sex differences in chronic inflammation in chronic myocarditis, particularly in autoimmune myocarditis.

Received: 16 January 2023 | Revised: 28 April 2023 | Accepted: 10 May 2023

DOI: 10.1111/acel.13894

Aging Cell WILEY

RESEARCH ARTICLE

# Sex and age differences in AMPK phosphorylation, mitochondrial homeostasis, and inflammation in hearts from inflammatory cardiomyopathy patients

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## Abstract

Linked to exacerbated inflammation, myocarditis is a cardiovascular disease, which may lead to dilated cardiomyopathy. Although sex and age differences in the development of chronic myocarditis have been postulated, underlying cellular mechanisms remain poorly understood. In the current study, we aimed to investigate sex and age differences in mitochondrial homeostasis, inflammation, and cellular senescence. Cardiac tissue samples from younger and older patients with inflammatory dilated cardiomyopathy (DCMI) were used. The expression of Sirt1, phosphorylated AMPK, PGC-1α, Sirt3, acetylated SOD2, catalase, and several mitochondrial genes was analyzed to assess mitochondrial homeostasis. The expression of NF-κB, TLR4, and interleukins was used to examine the inflammatory state in the heart. Finally, several senescence markers and telomere length were investigated. Cardiac AMPK expression and phosphorylation were significantly elevated in male DCMI patients, whereas Sirt1 expression remained unchanged in all groups investigated. AMPK upregulation was accompanied by a preserved expression of all mitochondrial proteins/genes investigated in older male DCMI patients, whereas the expression of TOM40, TIM23, and the mitochondrial oxidative phosphorylation genes was significantly reduced in older female patients. Mitochondrial homeostasis in older male patients was further supported by the reduced acetylation of mitochondrial proteins as indicated by acetylated SOD2. The inflammatory markers NF-κB and TLR4 were downregulated in older male DCMI patients, whereas the expression of IL-18 was increased in older female patients. This was accompanied by progressed senescence in older DCMI hearts. In

**Abbreviations:** AMPK, AMP-activated protein kinase; BNP, brain natriuretic peptide; CK-MB, creatinine kinase MB; CRP, C-reactive protein; DCM, dilated cardiomyopathy; DCMI, inflammatory dilated cardiomyopathy; EF, ejection fraction; HHV6V, human herpesvirus 6; IL, interleukin; LV, left ventricular; LVAD, left ventricular assist device; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PGC-1α, peroxisome proliferator-activated receptor-gamma coactivator-1 alpha; PVB19, parvovirus 19; ROS, reactive oxygen species; SASP, senescence-associated secretory phenotype; Sirt, sirtuin; SOD2, superoxide dismutase 2; TGF-β, transforming growth factor β; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor α; VEGF, vascular endothelial growth factor.

conclusion, older women experience more dramatic immunometabolic disorders on the cellular level than older men.

**KEYWORDS**
acetylation, AMPK, inflammatory dilated cardiomyopathy, mitochondrial biogenesis, senescence

## 1 | INTRODUCTION

Myocarditis is characterized by myocardial inflammation (Fung et al., 2016) and is associated with immune cell infiltration, extensive scarring, and left ventricular remodeling leading to dilated cardiomyopathy (DCM), followed by heart failure and sudden death (Pollack et al., 2015). Myocarditis predominantly develops after a viral infection with, for example, parvovirus B19, human herpesvirus 6, or coxsackie B virus (Kuhl et al., 2005). Viral persistence in the heart leads to a virus-associated inflammatory cardiomyopathy (Tschope et al., 2021), which is linked to chronic inflammation and profound cardiac remodeling due to an exacerbated activation of the immune system (Cooper Jr., 2009; Kindermann et al., 2008). In particular, macrophages and T-cells (e.g., Th1, Th2, Th17, and FoxP3+/CD4+ T-cells) are present during viral or toxic injury in myocarditis (Fung et al., 2016). Furthermore, the increased levels of Th1 and Th2 cytokines (e.g., interleukin (IL)-6, IL-1β, and tumor necrosis factor α (TNF-α)) are closely related to the development of DCM (Fairweather et al., 2004). Our own studies and those of others have shown that sex differences exist in the inflammatory response in acute and chronic myocarditis in several animal models, which is characterized by an increased pro-inflammatory response in male hearts (Barcena et al., 2021; Cihakova et al., 2004; Frisancho-Kiss et al., 2006, 2007, 2009; Roberts et al., 2013). 16%–30% of patients with DCM present myocardial inflammation, suggesting a co-occurrence of myocarditis and DCM (Blyszczuk, 2019).

Sex-related differences in cardiac remodeling seem to be a significant part of men's increased risk of developing DCM and experiencing heart failure (Cleland et al., 2003; Luchner et al., 2002). However, in older individuals, these sex differences are less prominent, as the decrease in estrogen in older women makes them more likely to experience cardiac remodeling and cardiac dysfunction than younger women (Li & Gupte, 2017). Aging, in general, is also known to be accompanied by chronic systemic and cardiac inflammation (Barcena et al., 2022), which may further exacerbate the adverse consequences of reduced sex hormone levels in older women. In fact, the pro-inflammatory shift in healthy older women is more prominent than in healthy male hearts (Barcena et al., 2022; Barcena de Arellano et al., 2019), while in cardiomyopathies, for example, DCM, the inflammatory pathways are also strongly activated in older male hearts (Barcena et al., 2020; Lopez-Otin et al., 2013; Regitz-Zagrosek & Kararigas, 2017).

The signaling pathways involved in the myocarditis-induced remodeling leading to DCM are still poorly understood. However, increased collagen deposition and pathological fibrosis are observed during chronic inflammatory processes in myocarditis (Wynn, 2008). Due to higher testosterone levels, male hearts show elevated cardiac collagen deposition, fibrosis formation, and remodeling of the extracellular matrix in various cardiovascular diseases (Cavasin et al., 2006; Cocker et al., 2009; Coronado et al., 2012; Haddad et al., 2008), including experimental autoimmune myocarditis (EAM) (Schmerler et al., 2014).

Disturbed mitochondrial homeostasis plays a central role in many underlying cellular mechanisms involved in the development of DCM of various aetiologies (Ramaccini et al., 2020). Mitochondrial homeostasis comprises mitochondrial biogenesis and clearance control, that is, mitophagy. Both processes are important for the support of mitochondrial function, which is important for cardiomyocytes' energy balance. Two main energy sensors—AMPK and Sirt1—contribute significantly to mitochondrial homeostasis. Indeed, AMPK and Sirt1 promote PGC-1α activity, which is a key transcription factor regulating the expression of mitochondrial proteins, and thus, mitochondrial biogenesis (Gureev et al., 2019). Furthermore, AMPK and Sirt1 also control cellular autophagy activity, and thus, mitophagy (Jang et al., 2018; Sacitharan et al., 2020), a key process in the elimination of dysfunctional or damaged mitochondria.

Aging is accompanied by a strong downregulation of AMPK and Sirt1 activity (Barcena de Arellano et al., 2019; PLOS ONE Editors, 2022) which leads to impaired mitophagy, an accumulation of dysfunctional mitochondria, and ROS formation and which may trigger inflammation and further exacerbate post-myocarditis remodeling. It is worth noting that the release of mitochondrial DNA from damaged mitochondria into the cytosol may lead to an inflammatory response via Toll-like receptors and STING-dependent inflammasome activation (Riley & Tait, 2020). Furthermore, AMPK and Sirt1 suppress NF-κB signaling both directly and indirectly, which can subsequently reduce the expression of pro-inflammatory factors (Salminen et al., 2011; Yeung et al., 2004). Our recent report (Barcena de Arellano et al., 2019) demonstrated the association between the age-related reduction in AMPK activity and inflammation in the human heart.

Aside from Sirt1, the downregulation of another key cellular sirtuin, mitochondrial Sirt3, was also observed in aging hearts (Zhang et al., 2021). Sirt3 is a key mitochondrial deacetylase supporting mitochondrial dynamics and function via the deacetylation of numerous mitochondrial enzymes, including superoxide dismutase 2 (SOD2), whose activity is suppressed by hyperacetylation (Xu

et al., 2020). Thus, the reduced expression of Sirt3 is accompanied by reduced ATP synthesis and elevated ROS formation.

In the present study, we aimed to investigate sex- and age-related effects on mitochondrial homeostasis, autophagy, inflammation, and senescence in heart biopsies of patients with inflammatory dilated cardiomyopathy (DCMI). We also analyzed the relevant energy-sensing pathways, that is, AMPK and Sirt1. The study documents a reduced expression of mitochondrial proteins and mitochondria-encoded genes in older female patients, which was accompanied by a pro-inflammatory shift. Another interesting finding is that the acetylation rate of mitochondrial proteins, for example, the acetylation of SOD2, was significantly lower in the myocardium of older male than older female cardiomyopathy patients, suggesting that mitochondrial homeostasis is preserved in older male patients.

## 2 | MATERIALS AND METHODS

### 2.1 | Human left ventricular DCMI samples

Human lateral left ventricular (LV) wall or left ventricular apex tissue samples from patients with DCMI caused by viral myocarditis was retrospectively collected during organ transplantation or left ventricular assist device (LVAD) (men = 10–20 and women = 10–15). At the time of sample collection, 61% of the patients underwent organ transplantation, while a LVAD was implanted in 39% of the patients. The tissue was frozen immediately after collection in liquid nitrogen and stored at −80°C. The donors had an ejection fraction (EF) of <35% (Table 1) and were between 23 and 70 years of age. We divided the DCMI samples into four groups: younger (23–40 years; male: $n = 5$–10 and female: $n = 3$–5) and older (50–70 years; male: $n = 5$–10 and female: $n = 5$–10) individuals.

We obtained informed consent from all donors. Sample collection and the experimental protocols were approved by the Scientific Board at the Heart and Diabetes Center (HDZ) NRW (21/2013) and at the Charité – Universitätsmedizin Berlin (EA2/158/16). All experiments with the samples were performed in accordance with German regulations and the ethical standards as laid down in the Declaration of Helsinki.

### 2.2 | Human left ventricular Non-Diseased samples

Human non-diseased lateral left ventricular (LV) wall tissue was retrospectively collected from organ donors (men = 19 and women = 19). The donors were between 17 and 68 years of age. The LV samples were divided into four groups: younger (17–40 years; male: $n = 9$ and female: $n = 9$) and older (50–68 years; male: $n = 10$ and female: $n = 10$) individuals. The hearts were immediately put on ice in a Custodiol® solution (15 mmoL/L sodium

**TABLE 1** Characterization of patients with myocarditis-related cardiomyopathy.

| | Men | | Women | |
|---|---|---|---|---|
| | Young | Old | Young | Old |
| Age | 28.50 ± 6.14 | 60.88 ± 7.22 | 34.66 ± 7.57 | 62.40 ± 3.21 |
| BMI | 25.61 ± 4.52 | 25.72 ± 2.03 | 30.16 ± 1.32 | 27.39 ± 1.59 |
| EF (%) | 21.87 ± 2.72[a] | 22.66 ± 1.12[b] | 30.85 ± 3.12 | 32.50 ± 3.68 |
| FS (%) | 19.37 ± 5.18 | 15.00 ± 1.81 | 18.85 ± 2.71 | 21.08 ± 4.00 |
| BNP (pg/mL) | 1407 ± 615 | 401 ± 100 | 228 ± 42 | 596 ± 145 |
| Troponin I (pg/mL) | 41.43 ± 9.75 | 43.90 ± 11.96[b] | 21.66 ± 5.11 | 13.89 ± 3.13 |
| CRP (mg/dL) | 0.86 ± 6.21 | 1.05 ± 0.28[b] | 2.13 ± 1.47 | 0.38 ± 0.07 |
| CK-MB (ng/dL) | 9.05 ± 2.55 | 1.83 ± 0.28 | 2.99 ± 1.06 | 41.63 ± 23.69 |
| CD45 (cardiac tissue) | 1.05 ± 0.15a | 0.81 ± 0.24 | 0.36 ± 0.02[c] | 1.39 ± 0.61 |
| CD11b (cardiac tissue) | 1.16 ± 0.20 | 0.89 ± 0.15 | 0.49 ± 0.03 | 0.89 ± 0.19 |
| PVB19 positive | 33% | 61% | 100% | 63% |
| HHV6B positive | 22% | 15% | 0% | 18% |
| PVB19 and HHV6B positive | 44% | 23% | 0% | 18% |
| Number of virus copies | 1323 ± 565 | 904 ± 428 | 1189 ± 206 | 732 ± 522 |

*Note*: Data are shown as the means ± SEM. $n = 3$–12. Echocardiography analyses were performed to analyze EF, FS, and PW-ED. Elisa analyses were performed to analyzed BNP, Troponin I, CRP, and CK-MB. qRT-PCR analyses were performed to analyze CD45 and CD11b expression. Viral infection was proved using PCR analysis.

Abbreviations: BMI, body mass index; BNP, brain natriuretic peptide; CK-MB, creatine kinase muscle-brain type; CRP, C-reactive protein; EF, ejection fraction; FS, fractional shorting; LV-PW, left ventricle posterior wall thickness; PVB19, parvovirus b19; HHV6B, human herpesvirus 6.

[a] $p < 0.05$ for men versus women (young).

[b] $p < 0.05$ for men versus women (old).

[c] $p < 0.05$ for young versus old (women).

chloride, 9 mmoL/L potassium chloride, 1 mmoL/L potassium hydrogen 2-ketoglutarate, 4 mmoL/L magnesium chloride 6 $H_2O$, 18 mmoL/L histidine HCl $H_2O$, 180 mmoL/L histidine, 2 mmoL/L tryptophan, 30 mmoL/L mannitol, 30 mmoL/L calcium chloride 2 $H_2O$, 50 mEG Anion: Cl-, 300 mosmol/kg osmolality) for cardioplegia and multi-organ protection until dissection. After dissection, the samples were immediately frozen in liquid nitrogen and stored at −80°C.

The donors were healthy individuals. The cause of death was head or brain injury due to accidents (e.g., basilar skull fracture or brain contusion). The organs of the donors were used for multi-transplantation, but the hearts could not be transplanted for logistical reasons.

Informed consent from all donors or their legal guardians was obtained. The Scientific Board at the Hungarian Ministry of Health (ETT-TUKEB: 4991–0/2010-1018EKU) approved the sample collection and the experimental protocols. All research was performed in accordance with the German and Hungarian regulatory guidelines and the ethical standards as laid down in the Declaration of Helsinki.

## 2.3 | Measurement of clinical parameters

Cardiac function (ejection fraction and fractional shortening) was analyzed via echocardiography at the Heart and Diabetes Center (HDZ) NRW up to 3 months before LVAD implantation or heart transplantation. In addition, standard biochemical parameters, for example, brain natriuretic peptide (BNP), troponin I, creatinine kinase MB (CK-MB), and C-reactive protein (CRP), were routinely measured in the clinical laboratory at the HDZ NRW up to 3 months before operation.

## 2.4 | RNA extraction and quantitative Real-Time PCR

Both total RNA isolation from cardiac human tissue as well as a quantitative real-time PCR were performed as previously described (Barcena et al., 2020). The mRNA contents of target genes were normalized to the expression of hypoxanthine phosphoribosyl transferase (HPRT) and ribosomal protein lateral stalk subunit P0 (RPLP0). The purity of the isolated RNA was analyzed with the Caliper LabChip bioanalyzer (Agilent Technologies).

## 2.5 | Protein extraction and immunoblotting

Cardiac samples from both DCMI and non-diseased hearts were homogenized in Laemmli buffer (253 mM Tris/HCL pH 6.8, 8% SDS, 40% glycerin, 200 mM DDT, 0.4% bromophenol blue). Proteins were quantified using the BCA Assay (Thermo Scientific Pierce Protein Biology). Equal amounts of total proteins were separated on SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. The membranes were immunoblotted overnight with the following primary antibodies: Sirt1 (1:1000, #8469, Cell Signaling), AMPK (1:2000, #2532L, Cell Signaling), p-AMPK (1:2000, Thr172, #2535L, Cell Signaling), PGC-1α (1:1000, #54481, Abcam), TOM40 (1:1000, #sc365466, Santa Cruz), TIM23 (1:5000, #611222, BD), Sirt3 (1:1000, #5490, Cell Signaling), SOD2 (1:1000, #13194, Cell Signaling), ac-SOD2 (1:1000, #ab13533,Abcam), catalase (1:1000, #14097S, Cell Signaling), ATG5 (1:1000, #12994, Cell Signaling), p62/SQSTM1 (1:1000, #MAB8028, R&D systems), LC3 (1:1000, #4599, Cell Signaling), LAMP2 (1:1000; #NB300-591, Novus), NFκBp65 (1:200, #sc-8008, Santa Cruz), lamin B1 (1:1000, #13435, Cell Signaling), p53 (1:500, #sc-6243, Santa Cruz), MMP3 (1:1000, #14351, Cell Signaling) and phospho-histone-H2A.X (Ser139) (1:1000, #9718, Cell Signaling). Equal sample loading was confirmed by an analysis of GAPDH (1:1000, #8469, Cell Signaling), actin (1:1000, #sc1616-R, Santa Cruz), or HSP60 (1:1000, #4870, Cell Signaling). Immunoreactive proteins were detected using ECL Plus (GE Healthcare) and quantified with ImageLab (version 5.2.1 build 11, Bio-Rad Laboratories (USA)). Original blots are shown in a supplemental file.

## 2.6 | Mitochondrial mass analysis

To analyze the amounts of nuclear and mitochondrial DNA (mt-DNA), a quantitative real-time PCR was performed. The measurement of mitochondrial content was conducted with the ratio of mt-DNA to nuclear DNA (Jayarajan et al., 2019). Mt-DNA-specific mt-RNR2 primers (forward 5'-CCACATCTGCCGAGACGTAA-3' and reverse 5'-TAGTCCTCGTCCCACATGGA-3') and nuclear DNA-specific β-globin primers (forward 5'-AAGTACCACTAAGCCCCCTTTC-3' and reverse 5'-GGGAACACAAAAGACCTCTTCTGG-3') for SYBR Green were used.

## 2.7 | Telomere length measurement

Telomere length was measured in cardiac samples from both DCMI and non-diseased hearts using the Absolute Human Telomere Length quantification qPCR assay kit (ScienCell Research Laboratories) according to the manufacturer's protocol via quantitative real-time PCR (O'Callaghan & Fenech, 2011).

## 2.8 | Masson's trichrome staining

5 µm cryosections of the human left ventricle were stained with Masson's trichrome (Dako, Germany), according to the manufacturer's instructions to quantify fibrotic tissue (magnification ×200). Images were acquired with the Axiophot microscope (Zeiss). The overall fibrosis was determined via semiquantitative, visual evaluation. All sections were blindly evaluated by three different investigators.

### 2.9 | Immunofluorescence

5 µm cryosections of the human left ventricle were stained with antibodies against IL-18 (1:500, PA5-79479, ThermoFisher Scientific). The secondary antibody anti-rabbit FITC (1:100) (Dianova) was applied according to the manufacturer's protocol. Nuclei were stained using DAPI (1:50000) (Sigma) and cells were mounted with Fluoromount G (Southern Biotech). Negative controls were performed by omitting the primary antibodies. Images were acquired using a Leica TCS SPE II confocal unit with Leica DMI 6000 microscope (Leica Microsystems GmbH). All evaluations were performed in a blinded manner.

### 2.10 | Statistical analysis

The data are given as the means ± SEM. The GraphPad Prism 7 (GraphPad Software) was used for the statistical analysis. The data were evaluated using the non-parametric test (Mann–Whitney test, for two independent groups). Statistical significance was accepted when $p < 0.05$.

## 3 | RESULTS

### 3.1 | Characterization of patients with DCMI

To characterize the patients with DCMI, body mass index (BMI), cardiac functional parameters, and inflammatory state were analyzed. No differences in BMI between the groups were observed ($p > 0.05$) (Table 1). In 68% of the samples, parvovirus B19 (PVB19) was detected. 18% of the samples were positive for human herpesvirus 6B (HHV6B) and 14% for both PVB19 and HHV6B (Table 1). In 70% of the cardiac biopsies, a virus replication between 100 and 3799 copies was detected, while 30% of the cardiac tissue had fewer than 100 virus copies (Table 1). Interestingly, patients infected only with HHV6B had fewer virus copies than the other groups (data not shown). Of note, younger patients with DCMI had a higher number of virus copies in comparison to older patients in a sex-independent manner; however, the difference was not significant ($p > 0.05$) (data not shown). Regarding medication, 36% of the patients were treated with ACE inhibitors, 22% with an AT1-R blocker, and 69% with a beta blocker. In addition, 81% of the patients with DCMI took diuretics.

In patients with DCMI, cardiac function (ejection fraction and fractional shortening) as well as cardiac parameters (BNP, troponin I, CK-MB, and CRP) were analyzed (Table 1). The ejection fraction was significantly lower in men compared to women in an age-independent manner ($p < 0.05$). Furthermore, troponin I and CRP were significantly increased in older men in comparison with older women with DCMI ($p < 0.05$) (Table 1).

Pathological characterization of the cardiac tissue from patients with DCMI revealed immune cell infiltrates in a sex- and age-independent manner (data not shown). Interestingly, CD45 mRNA was significantly lower in the myocardium of younger women with DCMI when compared to older women and younger men ($p < 0.05$) (Table 1), while the mRNA values of cardiac CD11b did not differ between the groups ($p > 0.05$) (Table 1). In addition, cardiac biopsies from patients with DCMI showed increased pathological fibrosis formation in younger and older men and older women, but not in younger women (Figure S1).

### 3.2 | AMPK and pAMPK expression elevated in male patients with DCMI

Sirt1 and AMPK play a key role in metabolic regulation in the heart and their expression is downregulated in aged myocardium (Barcena et al., 2022; Barcena de Arellano et al., 2019). However, the existence of any age- and sex-related differences in DCMI has yet to be determined. In the current study, DCMI did not affect Sirt1 expression ($p > 0.05$), whereas a marked upregulation of AMPK was observed in male individuals with DCMI in comparison with non-diseased male control in an age-independent manner ($p < 0.05$) (Figure 1a–d). Furthermore, the phosphorylation of AMPK, an indicator of AMPK activity, was also significantly upregulated in younger and older men with DCMI in comparison with non-diseased male control ($p < 0.05$) (Figure 1e,f). No alterations in the expression of AMPK or pAMPK were found in women with DCMI when compared to a non-diseased control group ($p > 0.05$) (Figure 1c–f).

### 3.3 | Expression of mitochondrial proteins is reduced in older women with DCMI

As AMPK upregulation may promote mitochondrial biogenesis, we examined whether DCMI affects the expression of mitochondrial proteins and genes. Applying western blot or PCR assays, we found a downregulation of key mitochondrial import machinery proteins (Tom40 and Tim23) and oxidative phosphorylation (OXPHOS) genes (*cox1* and *nd4*) in older women with DCMI when compared to the non-diseased control ($p < 0.05$) (Figure 2d,f, Figure 3b,d). Surprisingly, the expression of PGC-1$\alpha$, a key transcription factor controlling the expression of mitochondrial genes, was rather elevated in this patient group ($p < 0.05$) (Figure 2b). These myocarditis effects observed in women were not detected in older male DCMI hearts ($p > 0.05$). Consistent with the increased PGC-1$\alpha$ expression in older women with DCMI, mRNA level of NFR1, a downstream transcription factor of PGC-1$\alpha$, in this group was also significantly upregulated ($p < 0.05$) (Figure 2g). The expression of other PGC-1$\alpha$ target ERR$\alpha$ was not affected in the hearts of older patients with DCMI ($p > 0.05$) (Figure 2h). Thus, DCMI is accompanied by a reduced expression of mitochondrial proteins and genes specifically in older female patients.

We further examined whether the reduced expression of the mitochondrial proteins may affect mitochondrial mass in DCMI. Analysis of mitochondrial mass—measured as a ratio of

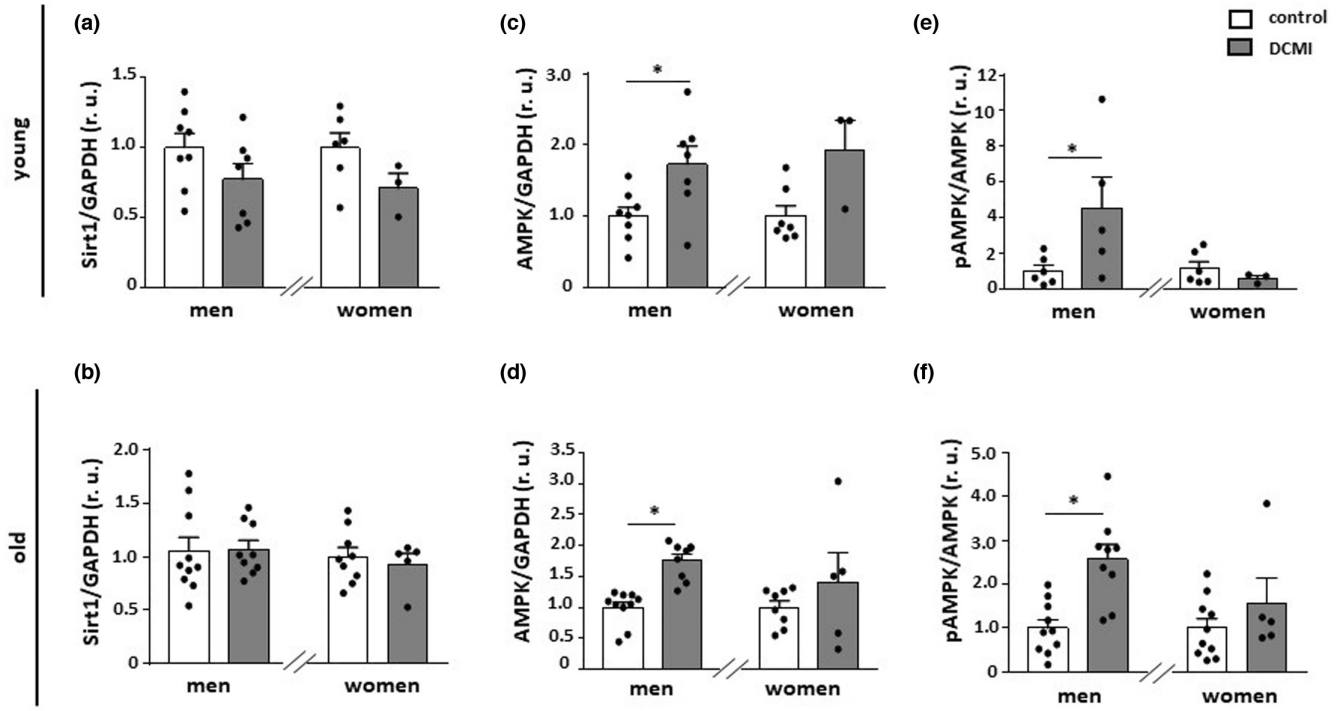


FIGURE 1 Alterations in the expression of Sirt1 and AMPK. Statistics from western blot analysis of (a and b) Sirt1, (c and d) total AMPK, and (e and f) phosphorylated AMPK (Thr172) performed with lysates of cardiac tissue from diseased and non-diseased, younger, and older men and women. Data are shown as the means ± SEM ($n$ = 3-10/group). Mann–Whitney Test; *$p < 0.05$ versus corresponding control. All data were normalized to the corresponding control and expressed in relative units (r.u.).

mitochondrial DNA/nuclear DNA—revealed, as expected, a significant reduction of mitochondrial mass in older, but not younger, female patients ($p < 0.05$ and $p > 0.05$, respectively) (Figure 3g,h). Surprisingly, a similar significant reduction of mitochondrial mass was also observed in older male patients ($p < 0.05$). We hypothesized that, in this patient group, mitochondrial turnover may be accelerated due to selective autophagy. When examining typical autophagy markers, we found an upregulation of ATG5 and SQSTM1 at the mRNA level in older male patients ($p < 0.01$) (Figure 4a–d), which indicates enhanced autophagy. Of note, ATG5 and SQSTM1 were significantly upregulated in older women at the protein level ($p < 0.01$), while the transcription of these proteins was unchanged ($p > 0.05$), suggesting that the autophagy was disrupted in older female patients (Figure 4). In contrast, the LC3II/LC3I ratio was not altered in patients with DCMI ($p > 0.05$) (Figure 4e). Similarly, the lysosomal membrane protein LAMP2 was not altered in DCMI ($p > 0.05$) (Figure 4f).

## 3.4 | DCMI affects mitochondrial protein acetylation in an age- and sex-dependent manner

Acetylation is a key regulator of the enzymatic activity of many proteins. In mitochondria, lysine acetylation is controlled by the deacetylase Sirt3, which is the only mitochondrial sirtuin with robust deacetylation activity (Onyango et al., 2002). In the present study, no effects of DCMI were found on Sirt3 expression in all groups investigated ($p > 0.05$) (Figure 5a,b).

To further investigate the acetylation of mitochondrial proteins, western blot analysis of acetylated SOD2, a widely used marker of mitochondrial protein acetylation state (Qiu et al., 2010), was applied. The acetylation of SOD2 was significantly reduced in older male patients with DCMI ($p < 0.01$), whereas it was increased in younger men ($p < 0.05$) (Figure 5c,d). No changes in total SOD2 expression were found in all groups investigated ($p > 0.05$) (Figure 5e,f).

To further investigate alterations in the antioxidant machinery, we analyzed the expression of catalase which, in addition to the SOD2, is a prominent cellular anti-oxidative enzyme located predominantly in peroxisomes (Karnati et al., 2013). In the present study, DCMI only significantly promoted the expression of catalase in older women ($p < 0.01$) (Figure 5h).

## 3.5 | Sex differences in the pro-inflammatory state in patients with DCMI

We have previously reported that the expression of pro-inflammatory mediators is promoted during aging processes in non-diseased human hearts (Barcena de Arellano et al., 2019) and in older patients with DCM (Barcena et al., 2020). Thus, we examined whether the expression of pro-inflammatory mediators is affected in DCMI. NFκB, a key regulator of cytokine expression, was significantly

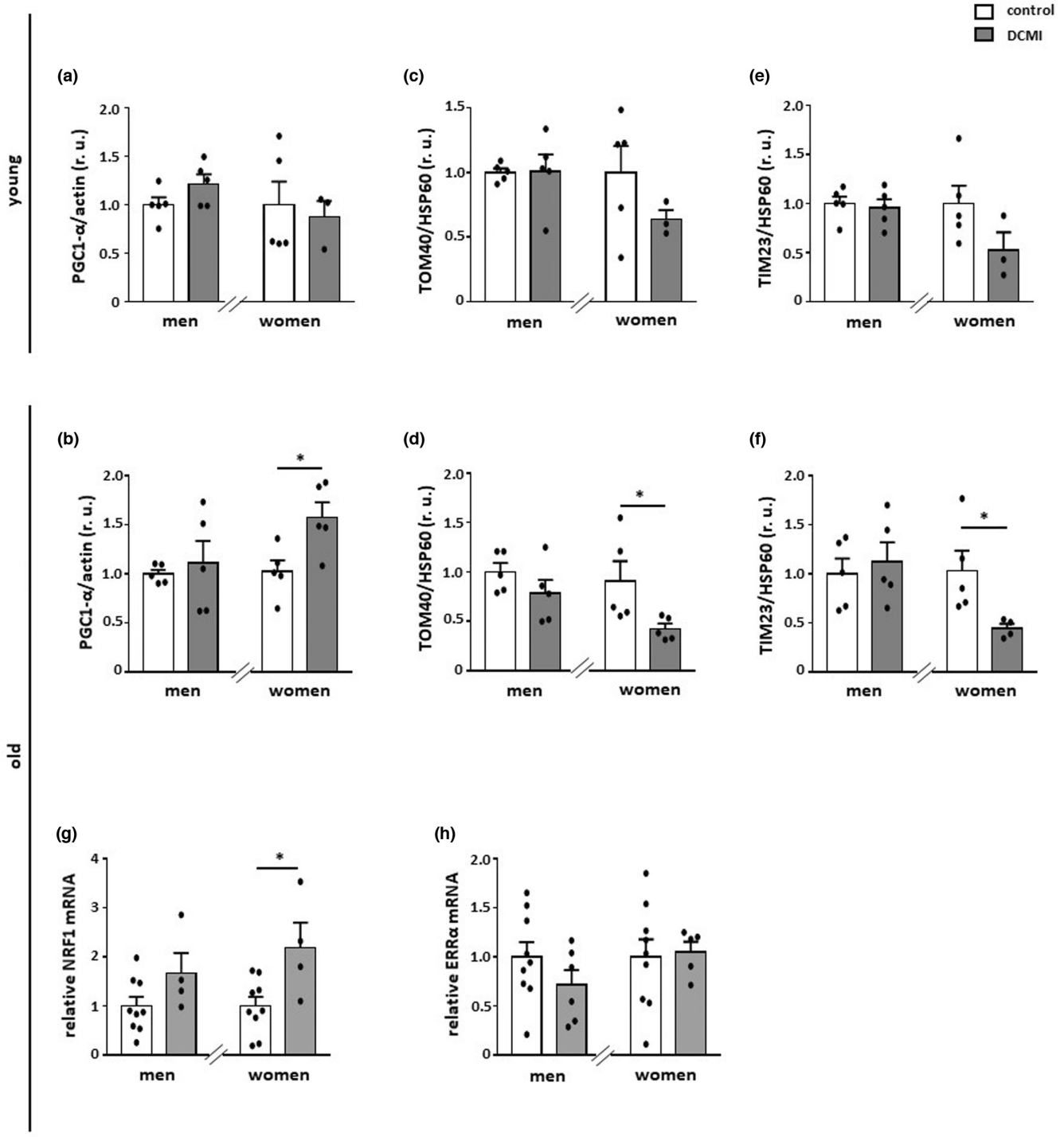


FIGURE 2 Alterations in the expression of PGC-1$\alpha$ and mitochondrial proteins. Statistics from western blot expression analysis of (a and b) PGC-1$\alpha$, (c and d) TOM40, and (e and f) TIM23, performed with lysates of cardiac tissue from diseased and non-diseased, younger and older men and women. (g and h) Relative mRNA expression of NRF1 and ERRα in diseased and non-diseased cardiac tissue in older men and women. Data are shown as the means ± SEM ($n = 3$-9/group). Mann–Whitney Test; $^*p < 0.05$ versus corresponding control. All data were normalized to the corresponding control and expressed in relative units (r.u.).

downregulated in older male DCMI patients ($p < 0.05$), while it did not change in female hearts ($p > 0.05$) (Figure 6a). Similarly, cardiac TLR4 mRNA expression was significantly decreased specifically in older male patients with DCMI ($p < 0.05$), (Figure 6b). The expression of the pro-inflammatory IL-12 changed in neither younger nor older individuals with DCMI in comparison to the corresponding controls ($p > 0.05$) (Figure 6c).

The mRNA level of the pro-inflammatory cytokine IL-18 was only dramatically increased in older female patients ($p < 0.05$) (Figure 6d), while immunofluorescence staining revealed an elevation of IL-18 in

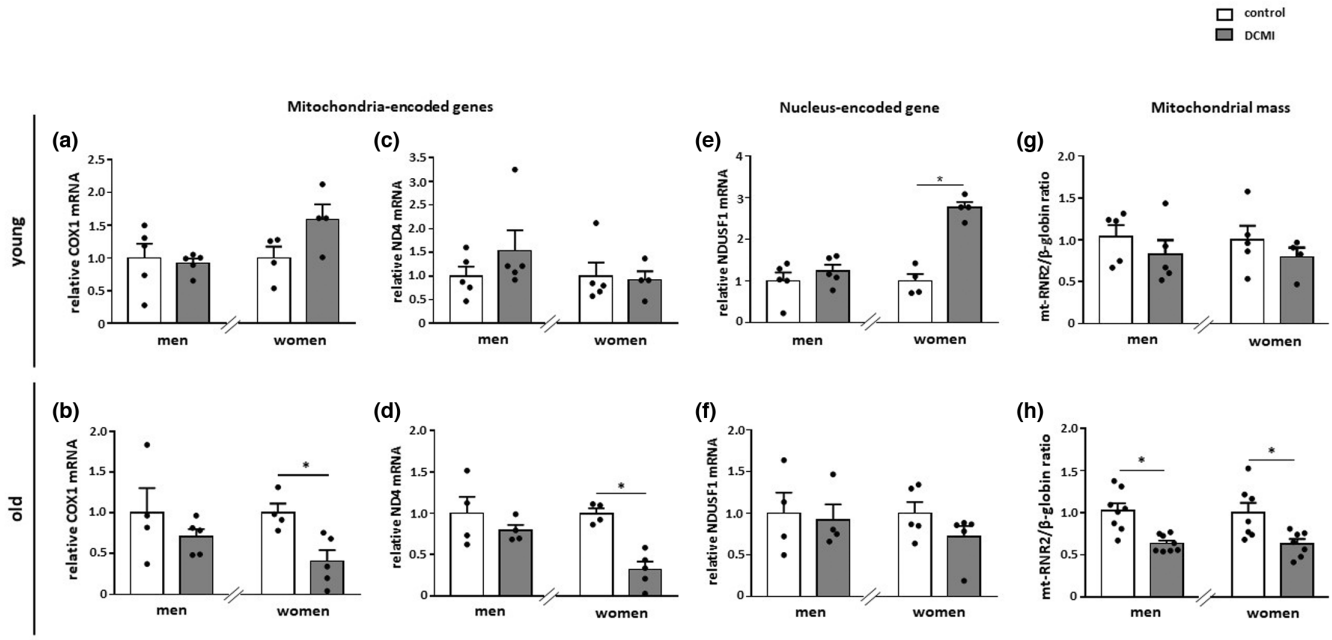


FIGURE 3 Alterations in the expression of mitochondrial genes and mitochondrial mass. (a–f) Relative mRNA expression of mitochondria- and nucleus-encoded genes in diseased and non-diseased cardiac tissue in younger and older men and women. (g-h) Mitochondrial mass was analyzed as the mt-RNR2/β-globin ratio. Data are shown as the means ± SEM ($n = 3$-8/group). Mann–Whitney Test; $^{*}p < 0.05$ versus corresponding control. All data were normalized to the corresponding control.

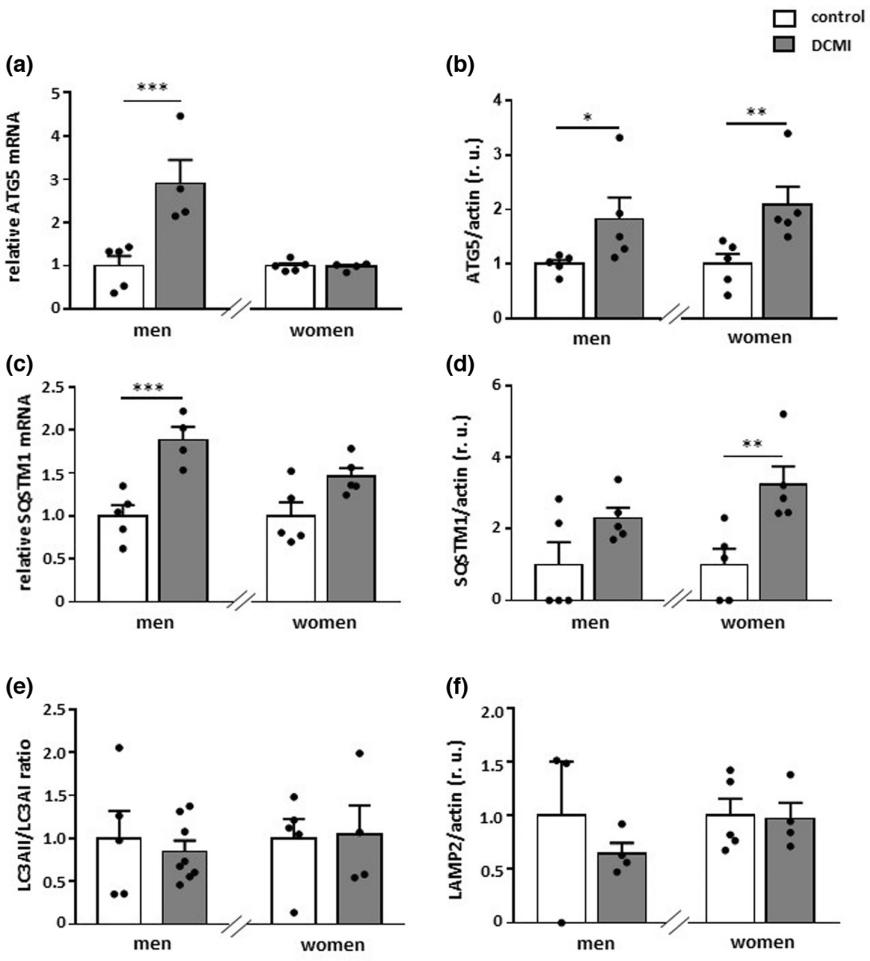


FIGURE 4 Alterations in the expression of autophagy markers. Statistics from expression analyses of (a and b) ATG5, (c and d) SQSTM1 at the mRNA and protein levels, (e) LC3II/LC3I ratio, and (f) LAMP2 performed with lysates of cardiac tissue from diseased and non-diseased older men and women. Data are shown as the means ± SEM ($n = 3$-8/group). Mann–Whitney Test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus corresponding control. All data were normalized to the corresponding control and expressed in relative units (r.u.).

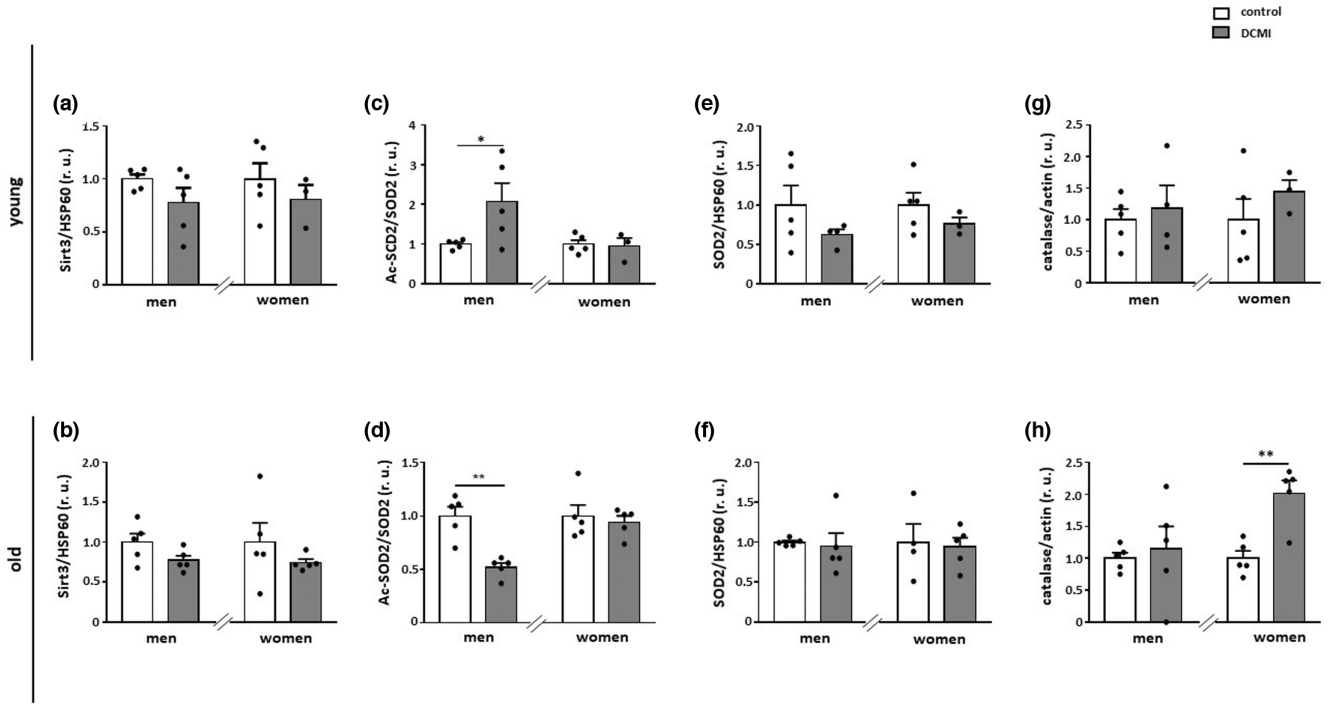


FIGURE 5 Alterations in mitochondrial acetylation state and expression of anti-oxidative enzymes. Statistics from western blot analyses of (a and b) Sirt3, (c and d) acetylated SOD2 (acSOD2), (e and f) total SOD2, and (g and h) catalase performed with lysates of cardiac tissue from diseased and non-diseased, younger and older men and women. Data are shown as the means ± SEM ($n = 3$-5/group). Mann–Whitney Test; $^{*}p < 0.05$, $^{**}p < 0.01$ versus corresponding control. All data were normalized to the corresponding control and expressed in relative units (r.u.).

older DCMI patients, which was significantly increased only in older female DCMI patients ($p < 0.05$) (Figure 6e,f). Analysis of the anti-inflammatory marker IL-10 expression revealed its significant reduction in younger DCMI hearts in a sex-independent manner ($p < 0.05$), whereas it was significantly decreased only in the male hearts among older individuals with DCMI ($p < 0.05$) (Figure 6g).

### 3.6 | DCMI induces cellular senescence

Chronic inflammation can promote cellular senescence (Barcena et al., 2022). As we observed an inflammatory impairment in the hearts of patients with DCMI, senescence markers were investigated in this group. Loss of nuclear protein lamin B1, which is involved in DNA repair and chromatin remodeling (Shimi et al., 2011), is an important biomarker of cellular senescence (Freund et al., 2012). The expression of nuclear lamin B1 in our study was significantly downregulated in the heart of older (Figure 7a), but not younger patients with DCMI (data not shown) in a sex-independent manner. Moreover, p53 was increased in older hearts of male and female DCMI patients ($p < 0.05$ and $p > 0.05$, respectively) (Figure 7b). In contrast, cardiac phospho-H2A.X and MMP3 expression was not affected in DCMI patients ($p > 0.05$) (Figure 7c,d and data not shown). At the mRNA level, we found a significant upregulation of VEGF in the hearts of older DCMI women ($p < 0.05$), while in older men it remained unchanged ($p > 0.05$) (Figure 7e). The senescence markers IL-6 and TGF-β were not affected by DCMI in the hearts of older men and women ($p > 0.05$) (Figure 7f,g). Finally, the analysis of the absolute telomere length revealed no effects of DCMI either in younger (data not shown) or in older patients (Figure 7h).

## 4 | DISCUSSION

In this study, we investigated the sex- and age-dependent effects of DCMI on cardiac markers of metabolism, mitochondrial homeostasis, autophagy, inflammation, and senescence. The main findings of this work are as follows: (1) The expression of total AMPK and pAMPK was elevated in male DCMI patients independent of age. (2) The expression of mitochondrial proteins was markedly downregulated in older female patients, while it remained stable in male patients. (3) This was accompanied by reduced acetylation of the mitochondrial matrix proteins, assessed by SOD2 acetylation, in older men with DCMI and elevated acetylation in younger men with DCMI. (4) Hearts from older female patients with DCMI showed a pro-inflammatory shift. (5) DCMI promoted cellular senescence in older patients.

Previous reports have emphasized the significant impact of disturbed metabolic homeostasis on the progress of cardiomyopathy of various origins (Asakura & Kitakaze, 2009; Fang et al., 2020; Jefferies & Towbin, 2010; Varga et al., 2015) In the present study, we used a cohort of patients in the end stage of DCMI. We first

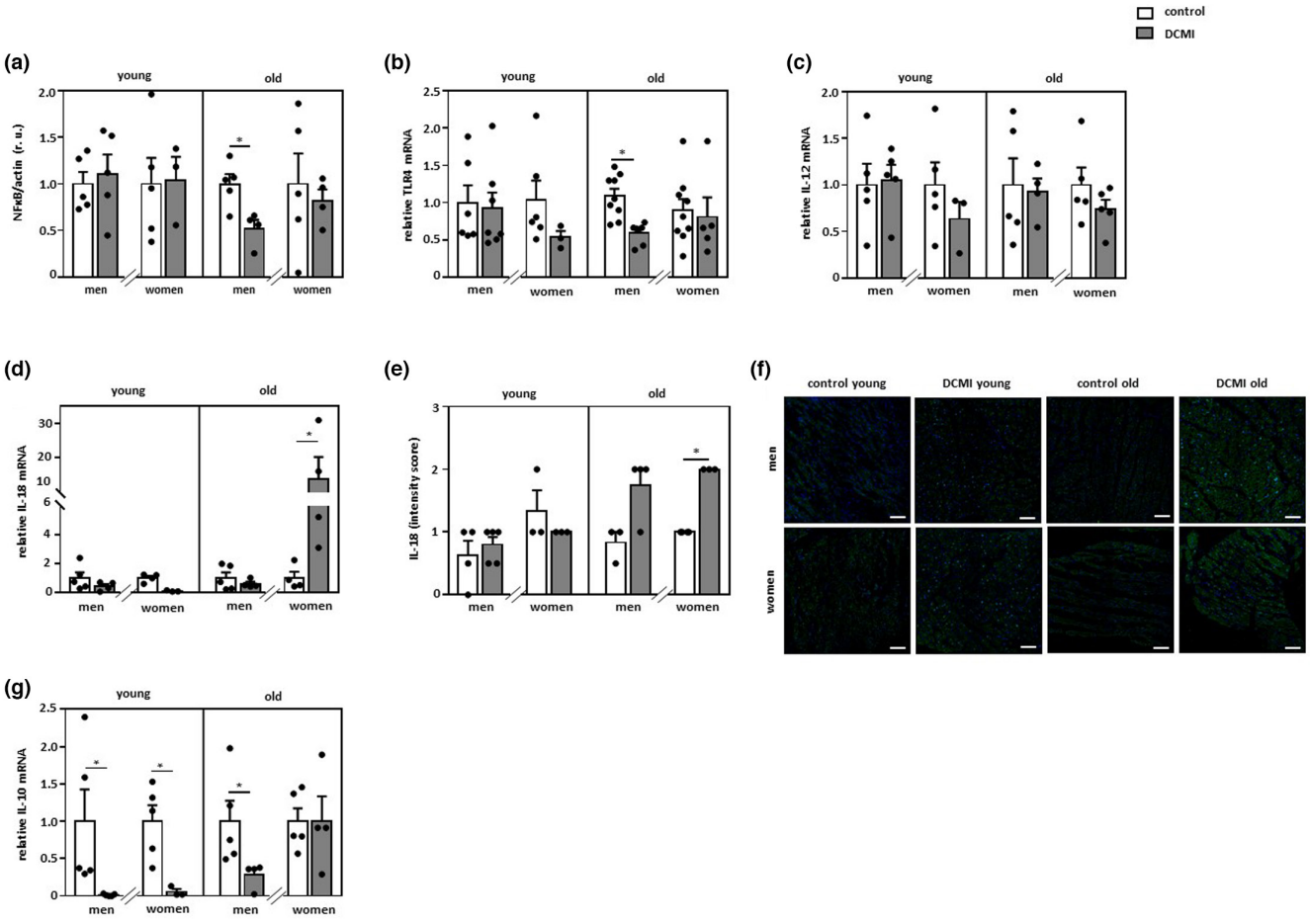


FIGURE 6 Alterations in pro- and anti-inflammatory markers. Statistics from western blot analyses of (a) NF-κB and real-time PCR analyses of (b) TLR4, (c) IL-12, (d) IL-18, and (g) IL-10 mRNA expression performed with lysates of cardiac tissue from diseased and non-diseased, younger and older men and women. Data are shown as the means ± SEM ($n = 3$-9/group). All data were normalized to the corresponding control and western blot data expressed in relative units (r.u.). (e and f) Representative images and statistics of IL-18 staining of cardiac tissue from younger and older men and women with DCMI ($n = 3$-5/group). Magnification 200×. Scale bar = 100 μm. Mann–Whitney Test; $^*p < 0.05$ versus corresponding control.

examined the expression of two main metabolic regulators in mammalian cells: Sirt1 and AMPK. Though cardiomyopathy did not affect Sirt1 expression, a significant upregulation of total AMPK was found in both older and younger male patients. This effect was accompanied by a marked upregulation of the phosphorylated form of AMPK, which is its active form. As AMPK is an important regulator of mitochondrial homeostasis, for example, biogenesis and clearance, we suspected such a pronounced upregulation of AMPK might affect mitochondrial biology.

Analyzing the expression of mitochondrial proteins (TOM40 and TIM23), as well as the expression of mitochondrial genes (*cox1* and *nd4*), we found no significant effects of cardiomyopathy in younger or older male patients. It is worth noting that in older, but not younger, female patients, cardiomyopathy led to a significant downregulation of all these mitochondrial markers, although the expression of TOM40 and TIM23 was not significantly but notably reduced in younger female patients. These data argue for a disturbance of mitochondrial biogenesis specifically in older female patients.

The downregulation of mitochondrial biogenesis is usually accompanied by a reduced number of mitochondria or mitochondrial mass. Indeed, in the older diseased women, the mitochondrial mass analyzed by the mitochondrial DNA/nuclear DNA ratio was significantly reduced in comparison with the control. Surprisingly, the mitochondrial mass was similarly reduced in older male patients, even though no alterations in mitochondrial protein expression were detected in this patient group. We hypothesized that, in older male patients, this mitochondrial mass reduction may be due to the enhanced mitochondrial clearance via autophagy, which may be caused by elevated AMPK activity (Wang et al., 2018). Indeed, AMPK may promote the expression and activation of autophagy proteins (Asakura & Kitakaze, 2009). Expression analysis of autophagy markers in the present study revealed an upregulation of at least two autophagy genes, that is, ATG5 and SQSTM1, in older men with DCMI, suggesting enhanced autophagy (Kuma et al., 2004; Pyo et al., 2013; Zhang et al., 2019). It is worth noting that these autophagy markers ATG5 and SQSTM1 were also upregulated in older female patients, but only at the protein level. Though the interpretation of these data

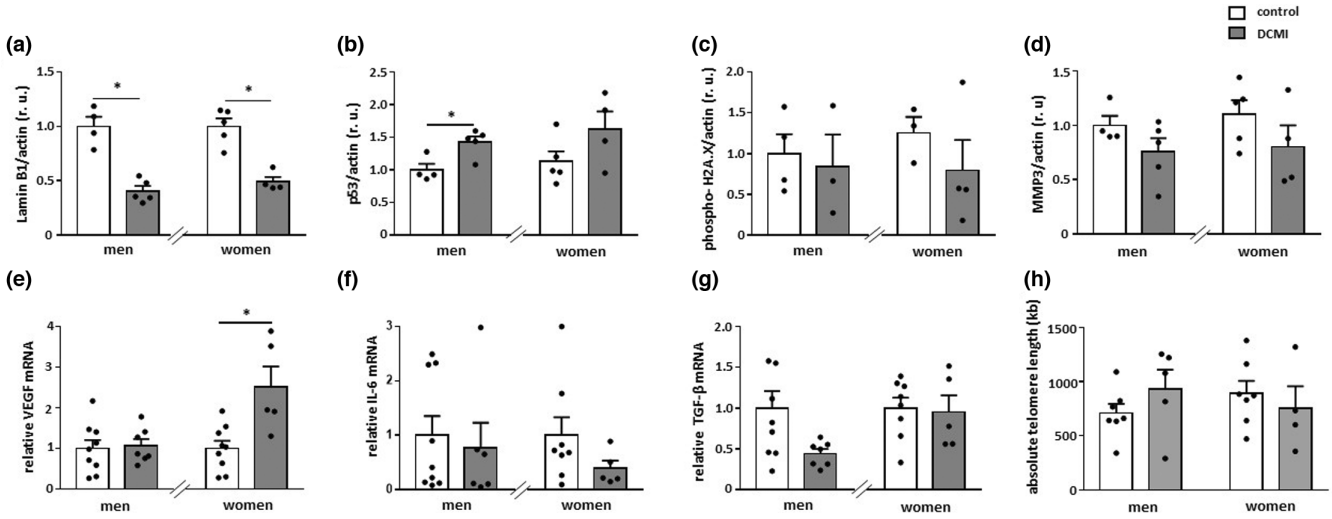


**FIGURE 7** Alterations in the expression of senescence markers. Statistics from western blot analyses of (a) lamin B1, (b) p53, (c) phospho-H2A.X, and (d) MMP3 and real-time PCR analyses of (e) VEGF, (f) IL-6, and (g) TGF-β mRNA expression performed with lysates of cardiac tissue from diseased and non-diseased, older men and women. Data are shown as the means ± SEM ($n = 3$-9/group). Mann–Whitney Test; $^*p < 0.05$ versus corresponding control. All data were normalized to the corresponding control and expressed in relative units (r.u.). (H) Absolute telomere length measurement performed with DNA of cardiac tissue from diseased and non-diseased, older men and women. Data are shown as the means ± SEM ($n = 4$-7/group).

is complex, based on previous animal studies (Triolo et al., 2022), one may posit that the accumulation of autophagy markers at the protein level in older female patients is due to disturbed autophagy.

In agreement with our results, alterations in mitochondrial biogenesis have been reported in various forms of cardiomyopathy (Flarsheim et al., 1996; Rosca & Hoppel, 2013) however, the sex and age differences have been the subject of little investigation. Our recent report (Barcena et al., 2020) characterized impaired mitochondrial biogenesis in older patients with dilated end-stage cardiomyopathy, which was sex-independent. Therefore, age- and sex-dependent impairment of mitochondrial biogenesis may vary among different cardiomyopathy forms.

Posttranslational modification is a key regulator of mitochondrial enzymes' activity, and therefore mitochondrial function. Acetylation of mitochondrial proteins is increased in a failing heart (Horton et al., 2016; Parodi-Rullan et al., 2018), which may lead to the decreased activity of various mitochondrial enzymes, such as SOD2, succinate dehydrogenase, pyruvate dehydrogenase, and ATP synthase (Horton et al., 2016; Zhang et al., 2018). To explore the acetylation state in mitochondrial matrix proteins, we applied a widely used marker, that is, SOD2 acetylation. Though no effect of cardiomyopathy was found in the female patients, significant and age-dependent alterations of SOD2 acetylation were found in male patients: it was elevated in younger and reduced in older men. This reduced SOD2 acetylation appears to contradict previous reports proposing the existence of mitochondrial protein hyperacetylation in failing hearts (Parodi-Rullan et al., 2018). One reason for the contradiction may be the preserved Sirt3 expression seen in our study, as other studies have reported a strong Sirt3 downregulation in end-stage cardiomyopathy (Song et al., 2021; Sundaresan et al., 2015). Furthermore, the differences in acetyltransferase activity or NAD+ availability may be responsible for the contradiction. Thus, older male patients with DCMI may be the only group to benefit from the enhanced deacetylation of mitochondrial proteins and, in particular, SOD2, which is a key antioxidative mitochondrial enzyme.

In addition to mitochondrial dysfunction, heart failure is also associated with an increased pro-inflammatory response (Hoffmann et al., 2019; Yue & Yao, 2016), and there are interactions between mitochondrial dysfunction and inflammation in hearts. For example, pro-inflammatory mediators including IL-1β, IL-6, and TNF-α promote a reduction in the NAD+/NADH ratio, leading to an impairment in mitochondrial biogenesis (Hahn et al., 2014), while mitochondrial dysfunction may induce cardiac inflammation via the release of mitochondrial DNA or formation of ROS (Liao et al., 2013; Oka et al., 2012).

In keeping with the beneficial mitochondrial biology in older male patients observed in this study, the expression of the master regulators of the inflammatory response, that is, NF-κB and TLR4 expression, was significantly reduced in this group. When further analyzing the expression of various pro-inflammatory cytokines—IL-1β (data not shown), IL-12, and IL-18—we found significant upregulation only in IL-18 mRNA expression in older women with DCMI. The alteration in the IL-18 expression seems to be age-dependent as this factor was not affected in younger patients with DCMI. In agreement with this finding, several studies have also reported an IL-18 increase in older individuals (Dinarello, 2009; Ferrucci et al., 2005; Franceschi et al., 2007). Importantly, TLR4 is a promoter of IL-18 expression in cardiomyocytes (Liu et al., 2015) however, our results demonstrated a TLR4-independent upregulation of the IL-18 expression in the heart of older female patients.

Cardiomyopathy is accompanied by a decline in anti-inflammatory cytokines (Barcena et al., 2022; Kaur et al., 2022). In one of our

recent studies, we observed the sex-independent downregulation of IL-10 in older patients with end-stage dilated cardiomyopathy (Barcena et al., 2020). In the present study, we also observed a marked downregulation of cardiac IL-10 in younger male and female patients. However, in older patients, a significant IL-10 downregulation was found only in men.

Several forms of cardiomyopathy are associated with cardiac senescence (Mehdizadeh et al., 2022). In the present study, changes in the expression of senescence markers, like loss of lamin B1, were observed only in elderly DCMI patients in a sex-independent manner. It is well accepted that the increased expression of pro-inflammatory cytokines and chemokines promotes the development of a senescence-associated secretory phenotype (SASP) (Coppe et al., 2010; Furman et al., 2019). In accordance, in the present study expression analysis of several SASP molecules revealed elevation of VEGF, specifically in older female patients, suggesting the role of the pro-inflammatory shift in this patient group.

Regarding fibrosis in cardiac remodeling, the chronic activation of the inflammatory markers promotes the upregulation of collagen deposition and pathological fibrosis formation in many diseases including myocarditis (Wynn, 2008). Several studies have shown that severe fibrosis formation is more common in male hearts with myocarditis (Asakura & Kitakaze, 2009; Barcena et al., 2021; Cavasin et al., 2006; Cocker et al., 2009; Coronado et al., 2012; Haddad et al., 2008) however, in our study, we also observed significant fibrosis formation in the hearts of older female patients with DCMI.

In conclusion, DCMI in older women is associated with the reduced expression of mitochondrial proteins and elevated IL-18 and VEGF expression. These cardiomyopathy effects are absent in older male patients, which may be due to the significant elevation of AMPK expression and activity. In addition, mitochondrial homeostasis is further supported by reduced acetylation of mitochondrial proteins in older male patients.

## 5 | LIMITATION OF THE STUDY

In this study, a small patient cohort was investigated, as the availability of human myocardial samples from both diseased and healthy individuals is limited. In addition, myocardial samples were obtained from apex tissue (patients with LVAD, 39%) or from the left lateral wall (patients who underwent heart transplantation, 61%). Nevertheless, the key effects observed in the study, for example, alterations in AMPK phosphorylation and expression of mitochondrial proteins, as well as SOD2 acetylation in older diseased versus healthy individuals, have sufficiently high robustness (at least two-fold change) to ensure the validity of the conclusion.

One important limitation of the study is the medication of patients before samples have been obtained. Indeed, all patients were treated with numerous drugs, for example, ACE inhibitors, AT1 receptor blockers, beta-blockers, or diuretics, which might have mitochondrial effects (for review see (Betiu et al., 2022)). Particularly, treatment with beta-blockers may stimulate AMPK activity (Hu et al., 2019) and mitochondrial biogenesis (Yao et al., 2016). Similarly, AT1 receptor blockers and ACE inhibitors may upregulate Sirt1 and PGC-1α, and promote mitochondrial biogenesis (Liu et al., 2021; Picca et al., 2018). Furthermore, beta-blockers and ACE inhibitors might have immunoregulatory actions, leading to the modulation of several cytokines (Ohtsuka et al., 2001; Platten et al., 2009) Therefore, the results of the study should be taken with caution.

Another limitation of the study is the restriction of the statistical analyses to the comparison of DCMI versus controls. We avoided ANOVA analysis due to the small patient cohorts. Furthermore, the primary aim of the present study was to evaluate the effects of DCMI rather than the effects of sex or aging. Nevertheless, we also consider sex and age differences in the effects of DCMI. Furthermore, it would be interesting to analyze age as a continuous variable rather than categorical (young vs. old), to further confirm the age-related differences.

In addition, interleukins in the present study were mostly analyzed at the mRNA level. This is a limitation, especially for interleukins synthesized as inactive pro-interleukins, such as pro-IL-1β and pro-IL-18, which need to be cleaved enzymatically by caspase-1 for activation.

Finally, humans are light-sensitive organisms and are affected by circadian clocks that control daily changes in the expression of numerous genes throughout the body, including the heart (Durgan & Young, 2010). Because the human samples were obtained at different times, this limitation of the study should be considered.

### AUTHOR CONTRIBUTIONS

M.B. conceived the project, analyzed the data, prepared the figures, and wrote the main manuscript text. G.T. performed the real-time PCR experiments, western blot experiments, IHC staining, and analyzed the data. N.H. performed the real-time PCR, western blot experiments, and analyzed the data. P.B. performed the western blot experiments and analyzed the data. H.M. procured the human myocarditis tissue and revised the manuscript. I.B. procured the human tissue and revised the manuscript, U.M.W. revised the manuscript. Y.L. analyzed the data and wrote the main part of the manuscript, and V.R.Z. acquired research funds and coordinated the project. All authors commented on the manuscript.

### ACKNOWLEDGEMENTS

We thank Ms Summer Banks for the revision of the manuscript.

### FUNDING INFORMATION

This work was supported by the DZHK (German Centre for Cardiovascular Research), by the BMBF (German Ministry of Education and Research), and by the Ministry of Science, Research and Culture of the State of Brandenburg. Non-diseased cardiac tissue collection and management were supported by the Hungarian National Research, Development, and Innovation Office (K-128851 and TKP2021-EGA-32) to IB.

## CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

## DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Barcena, M. L., Tonini, G., Haritonow, N., Breiter, P., Milting, H., Baczko, I., Müller-Werdan, U., Ladilov, Y., & Regitz-Zagrosek, V. (2023). Sex and age differences in AMPK phosphorylation, mitochondrial homeostasis, and inflammation in hearts from inflammatory cardiomyopathy patients. *Aging Cell*, *00*, e13894. https://doi.org/10.1111/acel.13894

### 3.4 Sex-Specific Differences of the Inflammatory State in Experimental Autoimmune Myocarditis (PMID: 34122450)

**Barcena ML**, Jeuthe S[#], Niehues MH[#], Pozdniakova S, Haritonow N, Kühl AA, Messroghli DR, Regitz-Zagrosek V. Sex-Specific Differences of the Inflammatory State in Experimental Autoimmune Myocarditis. Front Immunol, 2021; 12(23): 686384.

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https://doi.org/10.3389/fimmu.2021.686384

Sex differences in the prevalence of autoimmune diseases are well documented. In fact, autoimmune diseases are more prevalent in women than in men. This phenomenon is associated with the immune stimulatory effects of estrogen. Nevertheless, some autoimmune diseases, e. g., myocarditis are more prevalent in men, which is characterized by acute myocardial inflammation.

The aim of this research project was to analyse sex differences in the cardiac function as well as the inflammatory state in rats with induced autoimmune myocarditis (experimental autoimmune myocarditis; EAM). Moreover, we proposed that a macrophage/fibroblast cross-talk might be involved in fibrosis formation in this animal model. Our results indicated that male, but not female EAM rats have an impaired cardiac function due to decreased cardiac stroke volume and ejection fraction. The cardiac dysfunction was accompanied with an increased number of myocardial immune cell infiltrates, which is more prominent in males. Male and female EAM rats showed a similar number of CD68+ cells in the heart, however, female EAM rats showed more anti-inflammatory CD68+ ArgI+ cells.
In addition, male EAM rats showed an increased Col3A1 expression and pathological fibrosis formation when compared to female EAM rats or to the control group. We also observed significantly increased expression of key pro-inflammatory factors, e.g., TLR4, c-fos and IL-6 in male EAM hearts, while female EAM rats showed a weaker inflammatory response. iNOS and IL-1β were solely increased in male rats. In accordance with these results, the anti-inflammatory factor IL-10 was significantly increased in female EAM hearts compared with male EAM animals.

In conclusion, this study confirms sex differences in the inflammatory response and pathological fibrosis formation in EAM, which seems to be directly associated with the decreased cardiac function in male rats.[205]

Since we found sex differences in macrophage polarization in our rat model for EAM, we will further deepen the role of sex on macrophage polarization in an *in vitro* model.

frontiers in Immunology

ORIGINAL RESEARCH
published: 28 May 2021
doi: 10.3389/fimmu.2021.686384

# Sex-Specific Differences of the Inflammatory State in Experimental Autoimmune Myocarditis

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OPEN ACCESS

**Edited by:**
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**Specialty section:**
*This article was submitted to Inflammation, a section of the journal Frontiers in Immunology*

**Received:** *26 March 2021*
**Accepted:** *14 May 2021*
**Published:** *28 May 2021*

**Citation:**
*Barcena ML, Jeuthe S, Niehues MH, Pozdniakova S, Haritonow N, Kühl AA, Messroghli DR and Regitz-Zagrosek V (2021) Sex-Specific Differences of the Inflammatory State in Experimental Autoimmune Myocarditis. Front. Immunol. 12:686384. doi: 10.3389/fimmu.2021.686384*

Increasing evidence suggests male sex as a potential risk factor for a higher incidence of cardiac fibrosis, stronger cardiac inflammation, and dilated cardiomyopathy (DCM) in human myocarditis. Chronic activation of the immune response in myocarditis may trigger autoimmunity. The experimental autoimmune myocarditis (EAM) model has been well established for the study of autoimmune myocarditis, however the role of sex in this pathology has not been fully explored. In this study, we investigated sex differences in the inflammatory response in the EAM model. We analyzed the cardiac function, as well as the inflammatory stage and fibrosis formation in the heart of EAM male and female rats. 21 days after induction of EAM, male EAM rats showed a decreased ejection fraction, stroke volume and cardiac output, while females did not. A significantly elevated number of infiltrates was detected in myocardium in both sexes, indicating the activation of macrophages following EAM induction. The level of anti-inflammatory macrophages (CD68+ ArgI+) was only significantly increased in female hearts. The expression of Col3A1 and fibrosis formation were more prominent in males. Furthermore, prominent pro-inflammatory factors were increased only in male rats. These findings indicate sex-specific alterations in the inflammatory stage of EAM, with a pro-inflammatory phenotype appearing in males and an anti-inflammatory phenotype in females, which both significantly affect cardiac function in autoimmune myocarditis.

**Keywords: sex differences, inflammation, experimental autoimmune myocarditis, cytokines, cardiac dysfunction**

## INTRODUCTION

Myocarditis is a cardiovascular disease that is associated with myocardial inflammation and infiltration of immune cells into the heart muscle (1). Of those immune cells, it is predominantly macrophages and T-cells that infiltrate the cardiac tissue during viral or toxic injury in myocarditis (1–3). Impaired regulation of the autoimmune response against auto-myocardial proteins can lead to chronic inflammation followed by fibrosis, dilated cardiomyopathy (DCM), and heart failure at the end stage of myocarditis (4, 5). Mice infected with coxsackievirus B3 (CVB3) develop a chronic myocarditis, associated with the presence of anti-myosin autoantibodies, myocardial fibrosis, and cardiac remodeling (6–8), leading to alterations in the extracellular matrix (ECM) (9). In addition, mice or rats immunized with cardiac myosin and Complete Freund's Adjuvant (CFA) exhibit experimental autoimmune myocarditis (EAM) (10, 11). Pro-inflammatory cytokines e.g., interleukin (IL)-6, IL-1β and tumor necrosis factor α (TNF-α) together with enhanced reactive oxygen species (ROS) production play a crucial role in the development of autoimmune myocarditis (7, 12). In the EAM model, male animals show an increased fibrotic remodeling of cardiac tissue, which is linked to DCM development (13). Moreover, male animals develop cardiac autoimmunity and chronic inflammation more often than females (14).

Sex differences in cardiovascular diseases leading to heart failure have been well documented (15, 16). Interestingly, men show higher prevalence and severity of cardiovascular diseases than premenopausal women (17–19). However, the risk of negative cardiovascular events increases in women after menopause (20). The male sex is more susceptible to the development of DCM or heart failure due to impaired cardiac remodeling and the cardiac response to stress (21, 22). Furthermore, female mice show less acute inflammation compared to male mice in a viral myocarditis model, although the rate of viral replication is not significantly different between the sexes (23, 24). Several pathological conditions in the heart are associated with increased testosterone levels, promoting increased collagen deposition, fibrosis formation, and remodeling of the ECM (25–28). Fibroblasts are responsible for preserving ECM balance (29–31). In cardiac tissue, the most prominent collagen fibers are collagen type I and collagen type III (32). Sex-related differences, regulated by sex hormones such as estrogen and testosterone, are also observed in the immune system (33, 34). In turn, the immune system also regulates sex hormone production and secretion (33). Sex hormones have an effect on cardiomyocytes, endothelial cells and fibroblasts and dramatically modulate the tissue response to inflammation in a sex-dependent manner (35, 36), e.g., *via* p38 and ERK signaling (37). It is interesting to note that male animals have a higher number of classically activated M1 macrophages, whereas females develop a population of alternatively activated TIM3-positive M2 macrophages (38, 39). Moreover, male mice can present a M2 macrophage subpopulation, which expresses the M1 macrophage marker toll-like receptor (TLR4) and IL-1β. It has been proposed that this M2 macrophage population is strongly involved in fibrotic remodeling of cardiac tissue (6, 40). Furthermore, estrogen decreases TNF-α expression in peripheral blood mononuclear cells (PBMC) (33, 41, 42) and increased TNF-α secretion was detected in premenopausal women who underwent oophorectomy (43). In contrast, testosterone induces a TH1-type immune response in both humans and rodents (23, 24, 44–46). Macrophages activate fibroblasts *via* TGF-β, platelet-derived growth factor (PDGF), and TNF-α (47, 48). Activated fibroblasts produce ECM and favor fibrosis formation after cardiac damage (49, 50). Even though these sex differences in molecular and cellular mechanisms in the immune system are well documented, their interplay in specific diseases is not yet fully understood.

In this study, we investigated sex-related alterations in the inflammatory state in EAM accompanied by fibrosis formation and decreased cardiac function. The functional analyses revealed an impaired cardiac function in male but not female animals. Sex differences were also found in macrophage polarization and fibrosis formation. EAM is associated with an increased expression of inflammatory markers in male hearts.

## MATERIAL AND METHODS

### Animals

Lewis rats were housed in cages with controlled temperature and humidity on a 12h light/12h dark cycle. They were kept in groups of four or five with free access to food and water. Male and female rats (age: male: 42-56 days and female: 50-80 days; body weight 230-260g, n=16) (Janvier, Le Genest-St-Isle, France) were immunized as previously described with a myosin dose of 0.25 mg to the rear food pads on day 0 (51). 21 days later the animals were euthanized; their hearts, spleens, tibias, lungs, livers, and kidneys were extracted and snap frozen in liquid nitrogen and stored at -80°C. Non-immunized Lewis male and female rats were used as the control (n=10). All procedures and experimental protocols were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health and were approved by the relevant local authorities (Landesamt für Gesundheit und Soziales).

### Cardiac Magnetic Resonance Imaging

Cardiac function was evaluated by electrocardiographically triggered cardiac magnetic resonance imaging (CMR) as described in an earlier study (13). Left ventricular ejection fraction, end-diastolic volume, end-systolic volume, and cardiac output were measured before, 14 days, and 21 days after immunization.

### Analysis of Heart Weight to Body Weight Ratio

Body weight (BW) was measured before performing CMR. After euthanasia, the hearts without atria were weighed, and the relative heart weight (HW) to body weight (BW) ratio (HW/BW) was calculated as described in (13).

### Analysis of Muscle Hypertrophy and Immune Cell Infiltrate in Heart Tissue

Using the H&E staining, heart muscle hypertrophy score and the amount of immune cell infiltrates was counted in myocardium from male and female immunized and non-immunized rats (n=12).

## Immunohistochemistry

Paraffin-embedded cardiac tissue sections were incubated with anti-arginase 1 (clone N-20, 1:100, Santa Cruz, USA) primary antibody followed by incubation with secondary antibody biotinylated rabbit anti-goat (1: 400, Dianova, Germany). Biotin was detected with alkaline phosphatase-labelled streptavidin (Agilent, USA) and visualized using RED (Agilent, USA) as a chromogen. Proteins and enzymes were inactivated with heat and alkaline pH prior to incubation with anti-CD68 (1:250, Amsbio #1518), followed by incubation with Alexa488-labelled secondary antibody (1:400, donkey anti-rabbit, Invitrogen, Germany). DAPI (Sigma, Germany) was used to stain nuclei and sections mounted with Fluoromount-G (Southern Biotech, USA). Negative controls were performed by omitting the primary antibodies. Images were acquired with an AxioImager Z1 (Zeiss MicroImaging GmbH, Germany). All evaluations were performed in a blinded manner.

5 μm paraffin-sections of rat LV myocardium were stained with picrosirius red to obtain collagen content (52).

## RNA Extraction and Quantitative Real-Time PCR

The total RNA from cardiac rat tissue was isolated with RNA-Bee (Amsbio, UK) and a quantitative real-time PCR was performed with Brilliant SYBR Green qPCR master mix (Applied Biosystems, USA). The relative amount of target mRNA was determined using the comparative threshold (Ct) method as previously described (53). The mRNA contents of target genes were normalized to the expression of hypoxanthine phosphoribosyl transferase (HPRT).

## Protein Extraction and Immunoblotting

LV myocardium from male and female EAM rats was homogenized in Laemmli buffer (253mM Tris/HCL pH 6.8, 8% SDS, 40% glycerin, 200mM DTT, 0.4% bromophenol blue) (54). Proteins were quantified with the BCA Assay (Thermo Scientific Pierce Protein Biology, Germany). Equal amounts of total proteins were separated on SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. The membranes were immunoblotted overnight with the following primary antibodies: Col3A1 (1:400, Santa Cruz, USA), ERK (1:1000, Santa Cruz, USA), p-ERK (1:2000, Santa Cruz, USA), p38 (1:500, Santa Cruz, USA) and p-p38 (1:500, Santa Cruz, USA). Equal sample loading was confirmed through an analysis of actin (1:1500, Santa Cruz, USA). Immunoreactive proteins were detected with ECL Plus (GE Healthcare, UK) and quantified with ImageLab (Bio-Rad Laboratories, USA).

## Statistical Analysis

All data are given as mean ± SEM. The data were evaluated with the non-parametric Mann-Whitney test for two independent groups or with two-way ANOVA analysis. Statistical analyses were performed with GraphPad Prism 5 (GraphPad Software, USA). Statistical significance was accepted when $p < 0.05$.

# RESULTS

## Impaired Cardiac Function in Male EAM Rats

Male rats showed a decline in stroke volume 21 days after immunization with cardiac myosin and CFA ($p< 0.05$), while no significant changes in female rats were detected ($p> 0.05$) (**Figure 1A**). Ejection fraction and cardiac output were significantly decreased in EAM male rats at 21 days after immunization in a sex-dependent manner ($p< 0.05$) (**Figures 1B, C**).

While male EAM rats had higher body and heart weights than female EAM rats ($p< 0.05$) (**Supplementary Figures 1A, B**), the relative heart weight to body weight ratio did not vary between sexes in the EAM rats ($p> 0.05$) (**Supplementary Figure 1C**).

The spleen, liver, and kidneys were significantly heavier in male EAM rats when compared to females, while the weight of the lungs was similar in both sexes ($p< 0.05$ and $p> 0.05$, respectively) (**Supplementary Figures 1D–G**).

## CD68+ ArgI+ Macrophages Are Increased in Myocardial Tissue in Female EAM Rats

EAM rats did not show a higher immunohistochemical score for heart muscle hypertrophy when compared to healthy rats ($p> 0.05$) (**Figure 2A**). Despite an increased number of infiltrates detected both in male and female myocardial tissue after immunization ($p< 0.01$) (**Figure 2B**), female EAM rats showed significantly fewer immune cell infiltrates than male EAM rats ($p< 0.05$) (**Figure 2B**).

The number of cardiac CD68+ immune-reactive macrophages was similar in male and female EAM rats ($p> 0.05$) (**Figure 2C**). However, female EAM hearts had an increased number of cardiac anti-inflammatory CD68+ ArgI+ macrophages ($p< 0.05$) (**Figure 2D**), indicating an enhanced infiltration of M2 associated macrophages in females.

## Male EAM Rats Show More Fibrosis in Myocardial Tissue

To explore sex differences in collagen expression and fibrosis formation in EAM rats, the RNA and protein expression of collagen (Col3A1, Col1A1, Col4 and Col6), matrix metallopeptidase (MMP9), tissue metallopeptidase inhibitor 1 (TIMP1) and the pro-fibrotic factor, TGF-β were examined.

RNA and protein Col3A1 expression was significantly increased in the heart of EAM rats when compared to healthy controls in a sex-dependent manner ($p< 0.05$ and $p> 0.05$, respectively) (**Figures 3A, B**). Female EAM hearts had significantly less Col3A1 than males ($p< 0.01$ and $p< 0.05$) (**Figures 3A, B**). In accordance with these data, immunized male rats showed significantly higher amounts of fibrosis in comparison to female EAM hearts or non-immunized male hearts ($p< 0.05$) (**Figure 3C**).

Col1A1 mRNA expression was also significantly up-regulated in hearts from male but not female immunized rats ($p< 0.05$) (**Supplementary Figure 2A**). No changes in Col4 and Col6 expression were detected in EAM hearts ($p> 0.05$) (**Supplementary Figures 2B, C**). MMP-9 mRNA expression was up-regulated in female EAM hearts in comparison to male immunized rats ($p< 0.05$) (**Supplementary Figure 2D**). In addition, TIMP-1 expression was significantly up-regulated in female EAM hearts ($p< 0.05$ vs male EAM hearts) (**Supplementary Figure 2E**). Moreover, immunized female rats showed a significantly decreased TGF-β mRNA expression when compared with male immunized rats ($p< 0.01$) (**Supplementary Figure 2F**).

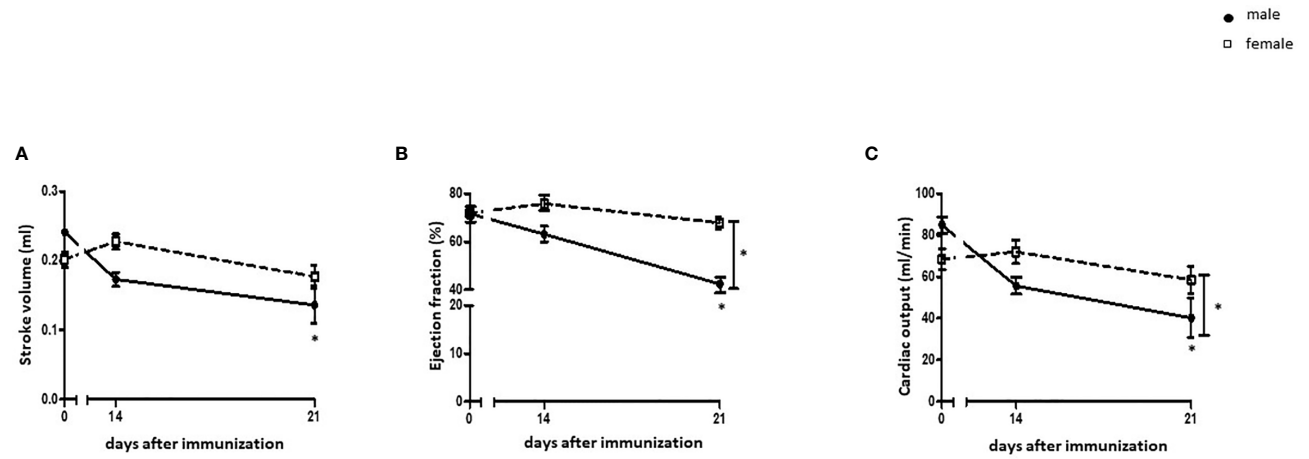


**FIGURE 1** | Cardiac function in EAM rats. Cardiac function parameters from the left ventricle were measured by CMR. **(A)** Stroke volume (SV) **(A)**, ejection fraction (EF) **(B)**, and cardiac output **(C)** were assessed before immunization, 14, and 21 days after immunization with cardiac myosin and CFA in male and female rats (n= 9-21). Data are shown as mean ± SEM. *p < 0.05.

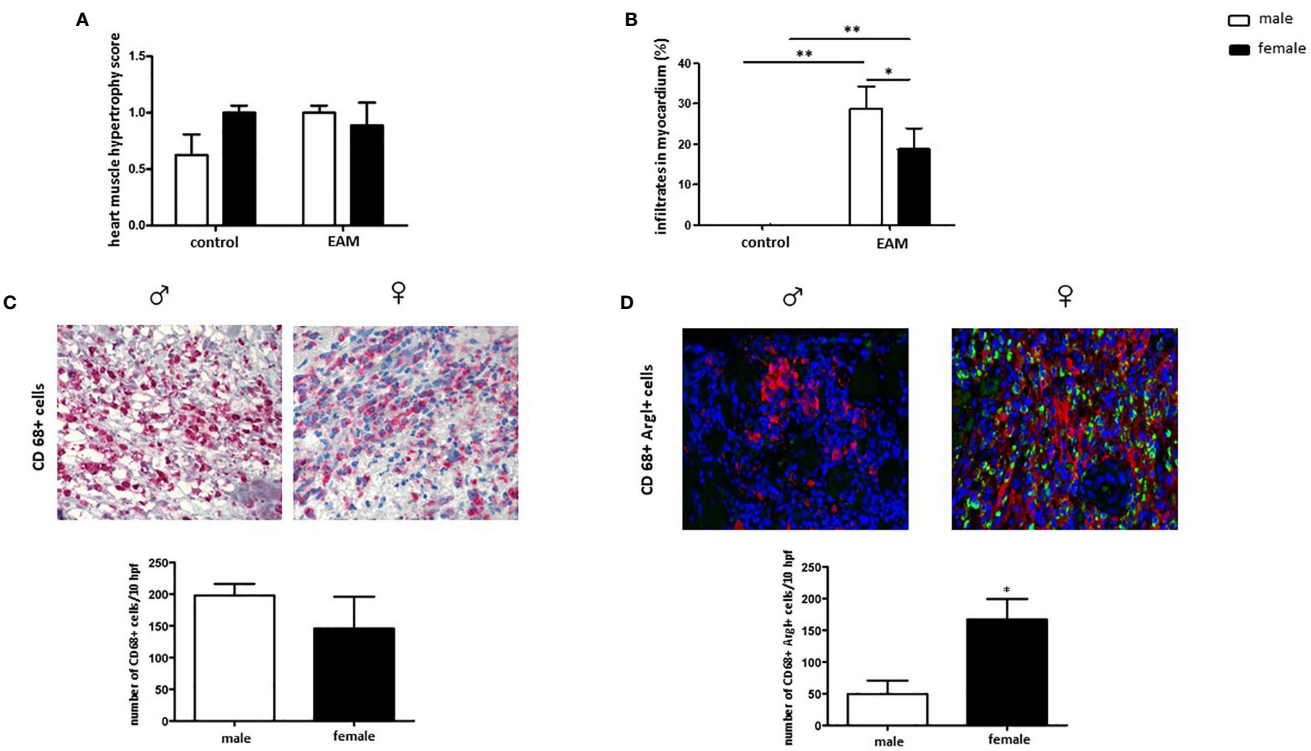


**FIGURE 2** | Increased number of cardiac CD68+ ArgI+ macrophages in female EAM rats. Immunohistochemical analysis of heart muscle hypertrophy **(A)**, myocardial immune infiltrates **(B)**, CD68+ immune-reactive cells **(C)**, and CD68+ ArgI+ cells **(D)** in myocardial tissue in male and female EAM animals. Data are shown as mean ± SEM (n= 4-12). *p < 0.05, **p < 0.01. Representative images of cardiac cryosections stained with antibodies against CD68 **(C)** and CD68 and ArgI **(D)** in myocardial tissue in male (♂) and female (♀) EAM animals (n= 4-12). Magnification 200x.

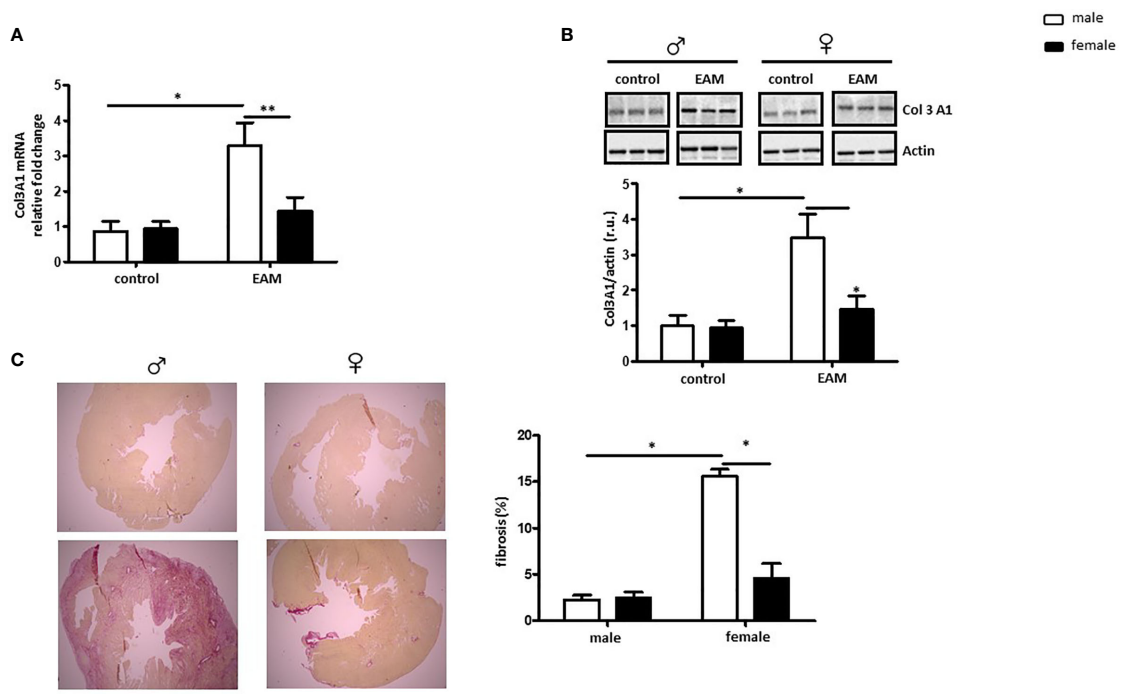


**FIGURE 3** | Male EAM rats develop more fibrosis in myocardial tissue. Analysis of Col3A1 mRNA **(A)** and protein expression **(B)** in cardiac tissue from control or EAM, male (♂) and female (♀). Data are shown as the mean ± SEM (n= 4-12). *p < 0.05, **p < 0.01. Representative imaging of western blot analysis; the lanes were run in the same gel. All data were normalized to the corresponding control and expressed in relative units (r.u.). **(C)** Representative Sirius red–dyed staining of cardiac tissue of 6 µm from male (♂) and female (♀) animals and corresponding statistics showing enhanced fibrosis in EAM rats (n= 4-12). Magnification 100x.

## Sex Differences in the Inflammatory Response in the EAM Model

Sex differences in the inflammatory response in myocarditis have been documented in both human and animal models (3). Both ERK and p38 activation are modulated *via* ER activation (55) and play a crucial role in the polarization of pro-inflammatory macrophages (56). Thus, we examined whether ERK and/or p38 activation (phosphorylation rate) are impaired in EAM hearts in a sex-dependent manner.

ERK phosphorylation was significantly increased in both male and female EAM rats ($p< 0.05$), while the amount of total ERK was unaffected ($p> 0.05$) (**Figure 4A**). In accordance with these findings, the pp38/p38 ratio was significantly increased in EAM rats in both sexes ($p< 0.05$) (**Figure 4B**). No significant changes in the p38 expression in EAM rats were found ($p> 0.05$) (**Figure 4B**).

The mRNA expression of the pro-inflammatory marker TLR4 was significantly increased in both male and female hearts from EAM rats when compared to healthy hearts ($p< 0.05$) (**Figure 5A**), however TLR4 mRNA was significantly up-regulated in male EAM hearts in comparison to female EAM hearts ($p< 0.05$) (**Figure 5A**). Furthermore, the pro-inflammatory markers c-fos, IL-6, iNOS, and IL-1β were only up-regulated in hearts from male but not female EAM rats ($p< 0.05$) (**Figures 5B–E**). In accordance with these data, IL-10 mRNA expression was significantly up-regulated in immunized female rats in comparison to immunized male rats ($p< 0.01$) (**Figure 5F**). The expressions of TNF-α, NFκB, c-jun, and STAT1 were unchanged in both sexes after immunization ($p> 0.05$) (**Supplementary Figures 3A–D**).

# DISCUSSION

In the current study, we investigated sex-dependent alterations in inflammation, collagen deposition and fibrosis formation in EAM rats. The main findings are: 1) Cardiac function was preserved in female rats after immunization, while the cardiac function was impaired in male EAM rats; 2) the number of cardiac anti-inflammatory CD68+ ArgI+ macrophages was only increased in female EAM rats; 3) collagen deposition and pathological fibrosis was only enhanced in hearts from male immunized rats; 4) pro-inflammatory mediators were significantly altered only in male EAM hearts. To summarize, an impaired inflammatory response and an exaggerated collagen deposition affecting the cardiac function were revealed in male EAM rats, while females demonstrated a protective response to adjuvant-induced EAM.

To the best of our knowledge, this is the first study to demonstrate sex differences in the inflammatory stage and in fibrosis formation, with a decline in cardiac function in an EAM rat model.

In clinical setting, men are more likely to develop myocarditis and DCM than women (17, 18, 57–59). More pronounced

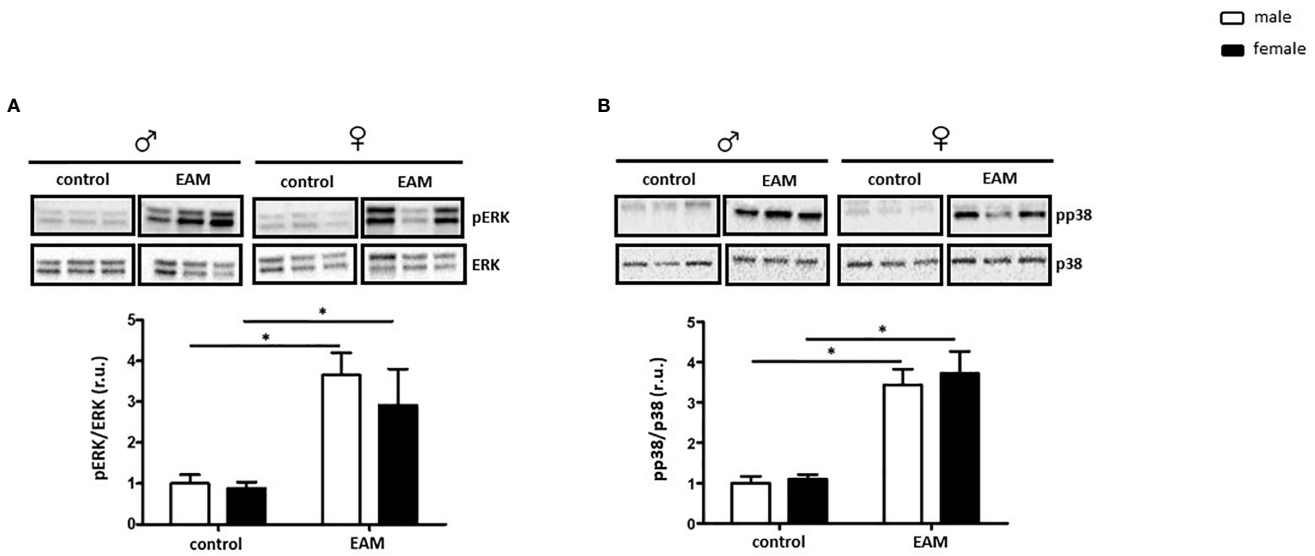


**FIGURE 4** | Sex-independent ERK and p38 activation in the EAM model. Western blot analysis of pERK/ERK ratio **(A)** and pp38/p38 ratio **(B)** in cardiac tissue lysates from control or EAM, male (♂) and female (♀). Data are shown as the mean ± SEM (n= 5-12). *$p < 0.05$. Representative imaging of western blot analysis; the lanes were run in the same gel. All data were normalized to the corresponding control and expressed in relative units (r.u.).

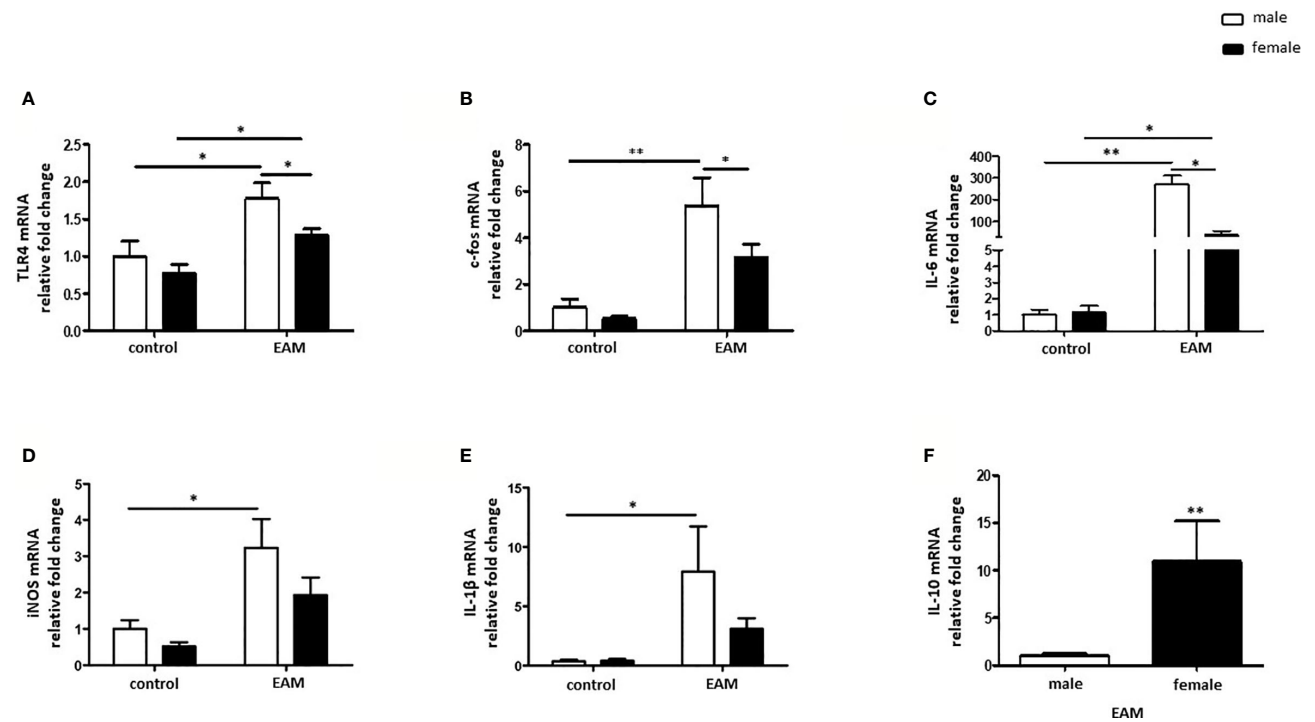


**FIGURE 5** | Sex differences in the inflammatory response in the EAM model. Real-time PCR analysis of TLR4 **(A)**, c-fos **(B)**, IL-6 **(C)**, iNOS **(D)**, IL-1β **(E)** and IL-10 **(F)** in rat cardiac tissue lysates from control or EAM, male (♂) and female (♀). Data are shown as the mean ± SEM (n= 5-12). *$p < 0.05$, **$p < 0.01$.

inflammation and fibrosis have been reported in male individuals with myocarditis than in female individuals (21, 22). A potential contribution of sex hormones may be a factor, as the association of DCM and heart failure with high testosterone levels has been previously reported (25, 60–62). These sex differences in humans correspond to sex differences in the mouse model. It is also interesting to note that female mice develop less inflammation after infection with CVB3 by similar viral replication (23, 24).

The EAM immunization protocol is used as a model of the chronic inflammatory phase of post-viral myocarditis (13), characterized by ongoing inflammation, fibrotic remodeling, appearance of anti-myosin antibodies, and development of DCM in the end-stage (63). Schmerler et al. have shown that male EAM rats had decreased ejection fraction and stroke volume (13). In keeping with those results, in our study the ejection fraction and the stroke volume showed a prominent decline in male EAM animals but no significant changes in females were detected, suggesting a preserved cardiac function in females.

Male EAM rats developed autoimmune myocarditis 21 days after immunization with cardiac myosin and CFA in the paw, accompanied by an increased amount of myocardial immune cell infiltrates and CD68+ immune reactive cells (13). In accordance with this study, we detected an increased number of immune cell infiltrates in the heart of male rats after immunization. In female EAM rats, although the infiltrates in the myocardial tissue were increased, it was significantly less than in male rats, indicating a weaker immune response in females.

Macrophages are the central regulator of the immune system in the heart in a normal state as well as during cardiac inflammation (64), and their crucial role in pro-fibrotic processes during chronic inflammation has been reported elsewhere (65). Though we found no sex differences in the number of immune reactive CD68+ macrophages in our EAM model, hearts from female EAM rats were infiltrated with an increased amount of anti-inflammatory CD68+ ArgI+ macrophages, suggesting that a predominant phenotype in females is alternative activated macrophages (M2) that favor an anti-inflammatory environment thus attenuating inflammation in female hearts in autoimmune myocarditis. However, M2 macrophages seem to be involved in the production of collagen and fibrosis formation (66), associated with an increased arginase activity (67). In agreement with this, sex differences in viral myocarditis and post-myocarditis complications, e.g., development of cardiac autoimmunity and DCM, are not caused by the virus itself, but rather by sex-related differences in the immune response (11). Moreover, Fairweather et al. have shown that, in a viral induced myocarditis model, the detrimental immune response in male individuals is driven by a predominant M1 response, while female animals show a stronger M2 response (38, 68). Our results suggest that macrophage polarization plays a crucial role in the development of sex differences in cardiac inflammation. The activation of the M2 response counteracts the detrimental effects of the pro-inflammatory macrophage polarization during acute inflammation, suggesting that a predominant M2 response is cardio protective (42). Our results indicate a pro-inflammatory M1-mediated and M2-mediated anti-inflammatory immune reaction in the heart of male and female rats, respectively.

Chronic activation of the inflammatory response leads to increased collagen deposition and pathological fibrosis is part of many diseases including myocarditis (69). Here it is important to remember that macrophages play a key role in the regulation of fibrosis (70) and activate fibroblasts *via* TGF-β, platelet-derived growth factor (PDGF) and TNF-α (47, 48). In our study, we detected an increased expression of Col3A1 and Col1A1 in the cardiac tissue from male EAM rats, while female EAM rats expressed similar amounts of Col3A1 and Col1A1 as healthy rats. In accordance, the anti-fibrotic factor, TIMP1 was up-regulated in immunized female rats, while the pro-fibrotic factor, TGF-β was decreased in females. Additionally, male EAM rats develop pathological fibrosis in the heart after immunization, while female EAM rats do not, suggesting that they undergo a different, fibrosis-independent, immune response. Indeed, severe fibrosis was previously reported in hearts from males with EAM (13), which may potentially be caused by increased testosterone levels (25–28, 71).

Enhanced ERK and p38 activity was detected in EAM rats in comparison to non-immunized rats of both sexes, arguing that other cascades are involved in activation of pro-inflammatory mediators in male EAM rats. In fact, a stronger M1 response and altered pro-inflammatory mediators were demonstrated solely in male animals. Male EAM rats showed an increased expression of TLR4, IL-6, c-fos, and iNOS when compared to healthy animals, while no significant changes were detected in female EAM rats, indicating that females did not develop a pro-inflammatory response after immunization. In accordance, Roberts et al. demonstrated sex-differences in the cardiac TLR4 expression in CVB3 infected mice, increasing the pathogenicity in male but not female infected mice (72). Of note, c-fos is a key transcription factor for the M1 spectrum, and iNOS is a signature M1 enzyme, reinforcing the observation of sex-dependent macrophages polarization in EAM (42, 73, 74).

Fairweather et al. have proposed a pivotal role for sex hormones in the sex-related differences in cardiac inflammation (75). While estrogen has cardio-protective properties in females, characterized by reducing cardiomyocyte apoptosis, counteracting fibrosis (76, 77), and deactivating cellular pathways that induce hypertrophy (17, 78, 79), testosterone increased cardiac inflammation in a myocarditis mice model (24) and encouraged a M1 response of macrophages in male individuals (25). Moreover, Koenig et al. also reported pro-inflammatory actions of androgens and anti-inflammatory actions of estrogen in CVB3 induced experimental myocarditis (80). Recent studies have also demonstrated that estrogen directly regulates macrophage polarization in different pathological tissue states (81–83), suggesting that E2 is directly involved in the polarization into M2 macrophages in female EAM rats. However, the spectrum of macrophage phenotypes to be researched is larger (42) and the role of sexual hormones should be investigated in the EAM model more in depth.

In conclusion, the present study revealed that autoimmune myocarditis is associated with an increased pro-inflammatory response in males, leading to fibrotic formation, while in females

the model is associated with a muted pro-inflammatory response, balanced immune-regulation, and preserved cardiac function.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The animal study was reviewed and approved by Landesamt für Gesundheit und Soziales, Berlin.

## AUTHOR CONTRIBUTIONS

MB conceived the project, analyzed the data, prepared the figures, and wrote the main manuscript text. MN performed the molecular experiments and analyzed the data. SJ generated the model, performed the functional experiments, obtained the tissue, and analyzed data. SP analyzed the data and wrote the main manuscript text. NH performed molecular experiments and analyzed the data. AK performed the immunohistochemical experiments, analyzed the data, and revised the manuscript. DM designed the functional experiments, provided the EAM tissue, and revised the manuscript. VR-Z generated research funds, initiated, and coordinated the project. SJ and VR-Z revised the manuscript. All authors contributed to the article and approved the submitted version.

## FUNDING

Parts of this work were funded by the DZHK (German Centre for Cardiovascular Research) and by the BMBF (German Ministry of Education and Research). We acknowledge support from the German Research Foundation (DFG) and the Open Access Publication Fund of Charité – Universitätsmedizin Berlin.

## ACKNOWLEDGMENTS

We would like to thank Jenny Jansen for their technical assistance.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.686384/full#supplementary-material

**Supplementary Figure 1 |** Sex differences in heart weight to body weight ratio. Body weight (BW) **(A)** and heart weight (HW) **(B)** and relative heart weight to body weight (HW/BW) **(C)** were measured 21 days after immunization with cardiac myosin and CFA. In addition, the weight from spleen **(D)**, lung **(E)**, liver **(F)**, and both kidneys **(G)** was assessed 21 days after immunization with cardiac myosin and CFA (n= 4-12). Data are shown as mean ± SEM. *p< 0.05, **p< 0.01.

**Supplementary Figure 2 |** Sex differences in the expression of pro- and anti-fibrotic factors in EAM. Real-time PCR analysis for Col1A1 **(A)**, Col4 **(B)**, Col6 **(C)**, MMP9 **(D)**, TIMP1 **(E)** and TGF-β **(F)** performed with rat cardiac tissue from control or EAM male (♂) and female (♀). Data are shown as the mean ± SEM (n= 4-12). *p< 0.05, **p< 0.01.

**Supplementary Figure 3 |** TNF-α and NFκB are not increased in EAM. Real-time PCR analysis for TNF-α **(A)**, NFκB **(B)**, c-jun **(C)** and STAT1 **(D)** performed with rat cardiac tissue from control or EAM, male (♂) and female (♀). Data are shown as the mean ± SEM (n= 5-12).

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

### 3.5 Male Macrophages and Fibroblasts from C57/BL6J Mice Are More Susceptible to Inflammatory Stimuli (PMID: 34867999)

**Barcena ML**, Niehues MH, Christiansen C, Estepa M, Haritonow N, Sadighi AH, Müller-Werdan U, Ladilov Y, Regitz-Zagrosek V. Male Macrophages and Fibroblasts from C57/BL6J Mice Are More Susceptible to Inflammatory Stimuli. Front Immunol, 2021; 12: 758767.
https://doi.org/10.3389/fimmu.2021.758767

Inflammatory cells, e. g., macrophages, seems to be directly involved in myocardial inflammation and remodeling. There is growing evidence for the role of sex hormones in macrophage differentiation, however, the mechanisms of sex-specific macrophage polarization are not well understood. This study aimed to analyze sex differences in the polarization of mouse bone marrow derived macrophages (BMM) under different stimuli (pro-inflammatory or anti-inflammatory). Moreover, we proposed that sex-related differences in macrophage polarization, promote differential activation of cardiac fibroblasts in male and females, leading to a pro-inflammatory milieu in males and an anti-inflammatory in females.

Our study showed that BMM are more susceptible to a pro-inflammatory stimulus. LPS treatment induced the expression of key pro-inflammatory mediators (e.g., TLR-4, c-fos, NFκB and IL-1β) and ROS formation in male and female BMM, however male macrophages showed a more prominent pro-inflammatory cytokine expression and ROS formation than females. Interestingly, male BMM showed a stronger response to anti-inflammatory stimuli than female BMM.

In accordance with these findings, TGF-β1 and TNF-α promoted different effects in male and female cardiac mouse fibroblasts. In addition, the treatment of male and female cardiac fibroblasts with pro-inflammatory conditioned medium from the same sex revealed a stronger activation of male cardiac fibroblasts in comparison to female fibroblasts.

In conclusion, our study showed sex differences in the plasticity of activated macrophages as well as in the activation of cardiac fibroblasts in mice, leading to sex-

specific phenotypes, which seem to be directly involved in cardiac tissue remodelling.[206]

frontiers in Immunology

ORIGINAL RESEARCH
published: 18 November 2021
doi: 10.3389/fimmu.2021.758767

# Male Macrophages and Fibroblasts from C57/BL6J Mice Are More Susceptible to Inflammatory Stimuli

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**Reviewed by:**
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**Specialty section:**
This article was submitted to Inflammation, a section of the journal Frontiers in Immunology

**Received:** 15 August 2021
**Accepted:** 22 October 2021
**Published:** 18 November 2021

**Citation:**
Barcena ML, Niehues MH, Christiansen C, Estepa M, Haritonow N, Sadighi AH, Müller-Werdan U, Ladilov Y and Regitz-Zagrosek V (2021) Male Macrophages and Fibroblasts from C57/BL6J Mice Are More Susceptible to Inflammatory Stimuli. Front. Immunol. 12:758767. doi: 10.3389/fimmu.2021.758767

Mounting evidence argues for the significant impact of sex in numerous cardiac pathologies, including myocarditis. Macrophage polarization and activation of cardiac fibroblasts play a key role in myocardial inflammation and remodeling. However, the role of sex in these processes is still poorly understood. In this study, we investigated sex-specific alterations in the polarization of murine bone marrow-derived macrophages (BMMs) and the polarization-related changes in fibroblast activation. Cultured male and female murine BMMs from C57/BL6J mice were polarized into M1 (LPS) and M2 (IL-4/IL-13) macrophages. Furthermore, male and female cardiac fibroblasts from C57/BL6J mice were activated with TNF-α, TGF-β, or conditioned medium from M1 BMMs. We found a significant overexpression of M1 markers (c-fos, NFκB, TNF-α, and IL-1β) and M2 markers (MCP-1 and YM1) in male but not female activated macrophages. In addition, the ROS levels were higher in M1 male BMMs, indicating a stronger polarization. Similarly, the pro-fibrotic markers TGF-β and IL-1β were expressed in activated cardiac male fibroblasts at a significantly higher level than in female fibroblasts. In conclusion, the present study provides strong evidence for the male-specific polarization of BMMs and activation of cardiac fibroblasts in an inflammatory environment. The data show an increased inflammatory response and tissue remodeling in male mice.

**Keywords: sex differences, inflammation, bone marrow macrophages, macrophage phenotype, activated fibroblasts**

## INTRODUCTION

In several cardiovascular diseases, inflammatory and pro-fibrotic responses play a detrimental role (1, 2). Inflammatory processes are tightly regulated by signals that initiate and maintain inflammation and promote resolution of the inflammation (3, 4). An imbalance in these mechanisms may promote cellular and tissue damage (3).

Macrophages are a crucial part of the cardiac immune response since they are the most abundant immune cells in the heart (5). It is worth noting that cardiac macrophages interact with other cells in the heart and directly or indirectly regulate different phases of cardiac diseases: acute inflammation, immune-regulation, and resolution, as well as cardiac remodeling (6). In addition, macrophages modulate the response to various stressful conditions in the heart (7). During cardiac stress, e.g., myocardial infarction or myocarditis, the population of tissue-resident macrophages expands by recruiting from the bloodstream or local proliferation (8–10). Macrophages can be polarized into M1 macrophages, which have a pro-inflammatory signature, or into M2 macrophages, which are involved in anti-inflammatory actions, wound healing, tissue remodeling, and immune regulatory actions (11, 12). An aberrant expression of pro-inflammatory cytokines during inflammatory processes leads to the macrophage phenotype switching into a pro-inflammatory phenotype, promoting the perpetuation of the inflammation (13). Key pro-inflammatory Th1-related cytokines, e. g., interferon gamma (IFN-γ) or toll-like receptor (TLR4) signaling, induce a M1 phenotype (14), which releases pro-inflammatory mediators like tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and reactive oxygen species (ROS) (15). Macrophage differentiation into the anti-inflammatory M2 phenotype is induced by exposure to IL-4 and IL-13 (16, 17). M2-macrophages express and release anti-inflammatory molecules including IL-10, transforming growth factor beta (TGF-β), and interleukin-1 receptor antagonist (IL-1ra) (18).

Macrophages play a significant role in the cardiac remodeling of the extracellular matrix (19) by activating cardiac fibroblasts *via* TGF-β, IL-1β, and TNF-α (20, 21). The depletion of monocytes and macrophages in the myocardium following cardiac stress decreases both fibroblast activation and collagen deposition (22).

Numerous factors may modulate macrophage polarization by cytokines. In particular, macrophage polarization may be affected by sex hormones, e.g., by the main female sex hormone, estradiol (E2). Although both pro- and anti-inflammatory actions of E2 have been described (23, 24), most studies argue for the anti-inflammatory effects of estrogen receptor (ER) activation in the heart (25, 26). Several reports propose the anti-inflammatory effects of E2 are caused by the inhibition of production and the release of pro-inflammatory cytokines with a M1 signature (27). Furthermore, the ERα seems to be involved in the promotion of M2 macrophage polarization, leading to an anti-inflammatory phenotype (25, 26, 28). In keeping with that profile, E2 loss leads to the expression of pro-inflammatory cytokines e.g., IL-1β, TNF-α, and IFN-γ in humans (29). The anti-inflammatory actions of E2 in male and female peripheral blood mononuclear cells are also observed after activation with lipopolysaccharide (LPS) (25, 30). Altogether, E2 seems to suppress pro-inflammatory and promote anti-inflammatory responses. The difference in E2 blood concentration in males and females may, therefore, be responsible for the sex difference in the inflammatory response.

In this study, we investigated sex-related alterations in the polarization of murine bone marrow macrophages (BMMs) and polarization-related changes in murine fibroblast activation. The analyses revealed that male BMMs are more susceptible to LPS treatment and promote a prominent M2 phenotype. Sex differences were also found in oxidative stress, i.e., less total ROS formation in female BMMs in a pro-inflammatory environment. Moreover, we demonstrated an activation of cardiac fibroblasts with the pro-inflammatory supernatant of cultures of M1 macrophages. Finally, 17β estradiol treatment improved the pro-inflammatory phenotype in male BMMs.

# MATERIAL AND METHODS

## Animals

Young age-matched male and female C57/BL6J mice (n= 18) (Forschungseinrichtungen für Experimentelle Medizin (FEM), Charité -Universitätsmedizin Berlin) were euthanized and heart, femur, and tibia were collected in ice-cold DPBS (Gibco, Germany) for further processing. A 12 h/12 h light and dark cycle was applied. Water and food were provided *ad libitum*. All experimental procedures were performed according to the established guidelines for the care and handling of laboratory animals and were approved by the Animal Care Committee of the Senate of Berlin, Germany (Approval number: T0333/08).

## Cell Culture

### Isolation and Cultivation of Murine Bone Marrow-Derived Macrophages

Bone marrow cells were collected by flushing the femur and tibia in DMEM (phenol red) (Gibco, Germany), 10% fetal bovine serum (FBS) (Biochrom, Germany), 1 mmol/l penicillin/streptomycin (Biochrom, Germany), and 1 mmol/l sodium pyruvate (Sigma, Germany) using a 20-gauge needle and were passed through a 70 μm cell strainer. Cells were cultivated for ten days (10% $CO_2$ and 37°C) in DMEM (phenol red), 0.05 mmol/l β-mercaptoethanol (Sigma-Aldrich, Germany), 1% non-essential amino acids (Thermo Scientific, Germany), 1 mM penicillin/streptomycin, 1 mM sodium pyruvate, 20% donor horse serum (Sigma-Aldrich, Germany), 10% FBS (Biochrom, Germany), and 20% L929-conditioned medium.

### Isolation and Cultivation of Murine Cardiac Fibroblasts

Hearts were cut into small pieces and digested 5 times by incubation in a collagenase/dispase buffer for two minutes at 37°C (31). The supernatant was carefully removed and diluted with ice cold growth medium (DMEM with phenol red, 10% fetal bovine serum (FBS), 1 mmol/l glutamine, 1 mmol/l penicillin/streptomycin, 1 mmol/l sodium pyruvate) and centrifuged at 1200 rpm for 5 min at 4°C. Cells were cultivated in fibroblast growth medium (DMEM, 10% FBS, 1 mmol/l penicillin/streptomycin, 1 mmol/l sodium pyruvate, and 1 mmol/l glutamine) until 80% confluence.

## Macrophage Polarization

BMMs were polarized into M1 macrophages with 10 ng/ml LPS (Sigma-Aldrich, Germany) and into M2 macrophages with 10 ng/ml recombinant mouse IL-4 (PeproTech, Germany) and 10 ng/ml recombinant mouse IL-13 (PeproTech, Germany) for 24 h.

## Fibroblast Activation

Fibroblasts were activated using 20 ng/ml TNF-α (32) (PeproTech, Germany), 10 ng/ml TGF-β (PeproTech, Germany) (33) or 10 ng/ml LPS (Sigma, Germany) for 24 h in fibroblast starvation medium (with 2.5% charcoal-stripped FCS, Biochrom, Germany).

## Activation of ERs

Cells were starved with a phenol free medium and 2.5% charcoal-stripped FCS (Biochrom, Germany) for 24 h prior to E2 treatment. After starvation, cells were treated with 10 nmol/l water soluble E2 (Sigma-Aldrich, Germany) or with 10 nmol/l dextrin (Sigma-Aldrich, Germany) as vehicle for 24 h.

## Treatment With Conditioned Medium From M1-BMMs

Mice cardiac fibroblasts were cultivated with mixture (1:1) of the fibroblast-starvation medium and conditioned medium from M1-BMMs for 24 h. To produce the conditioned medium from M1-BMMs, BMMs were treated with 10 ng/ml LPS (Sigma-Aldrich, Germany) for 24h. The cell culture medium was collected and centrifuged at 1200 rpm for 5 min at 4°C. The supernatant was stored at -80°C.

## Flow Cytometry

The purity of the BMM population was determined *via* flow cytometry analysis. $1x10^6$ cells were taken from the freshly harvested BMMs, processed and stained with the required antibodies according to the manufacturer's protocol. The fluorescently labeled monoclonal antibodies (mAbs) that specifically recognize proteins expressed by macrophages were used for phenotypical characterization. The used two-color panel included two surface antigens, F4/80 (1:100, Miltenyi Biotec, Germany) and CD11b (1:100, Miltenyi Biotec, Germany). In this two-color immunofluorescence protocol, the samples were single stained with each antibody, and then stained using both antibodies. Data were acquired with a MACS-Quant device (Miltenyi Biotec, Germany) using the MACSQUANTIFY™ software (**Figure 1A**).

## Immunofluorescence

BMMs and murine cardiac fibroblasts were cultivated in 8-chamber slides (Sigma-Aldrich, Germany). Cells were fixed with 4% Histofix (Roth, Germany) and permeabilized with 0.2% Triton X-100 (Sigma-Aldrich). BMMs were stained against F4/80 (1:100, Abcam, UK) or CD11b (1:100, Abcam, UK) (**Figures 1B–E**). Fibroblasts were stained with antibodies against vimentin (1:100), CD31 (negative control for endothelial cells) (1:100), and desmin (negative control for smooth muscle cells) (1:100) (**Figures 1F–K**). The secondary antibodies anti-mouse FITC (1:100) (Dianova, Germany) or anti-mouse Cy3 (Dianova, Germany) were applied according to the manufacturer's protocol. Nuclei were stained using DAPI (1:50000) (Sigma, Germany) and cells were mounted with Fluoromount G (Southern Biotech). Negative controls were performed by omitting the primary antibodies. Images were acquired using a BZ-9000E fluorescence microscope (Keyence, Germany). All evaluations were performed in a blinded manner.

## RNA Extraction and Quantitative Real-Time PCR

Total RNA from BMMs or murine cardiac fibroblasts was homogenized in RNA-Bee (Amsbio, UK). Quantitative real-time PCR was performed using the Brilliant SYBR Green qPCR master mix (Applied Biosystems, USA). The relative amount of target mRNA was determined using the comparative threshold (Ct) method as previously described (34). The mRNA content of target genes was normalized to the expression of hypoxanthine phosphoribosyl transferase (HPRT).

## Protein Extraction and Immunoblotting

BMMs were homogenized in a Laemmli buffer (253 mmol/l Tris/HCL pH 6.8, 8% SDS, 40% glycerin, 200 mmol/l Dithiothreitol, 0.4% bromophenol blue) (35). Proteins were quantified using the BCA Assay (Thermo Scientific Pierce Protein Biology, Germany). Equal amounts of total proteins were separated on SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. The membranes were immunoblotted overnight with the following primary antibodies: NFκB (1:1,000, Santa Cruz, USA), ERα (1:100, Santa Cruz, USA), ERβ (1:200, Santa Cruz, USA), GPR30 (1:500, Santa Cruz, USA), ERK (1:1,000, Santa Cruz, USA) and p-ERK (1:2000, Santa Cruz, USA). Equal sample loading was confirmed by an analysis of actin (1:1,500, Santa Cruz, USA). Immunoreactive proteins were detected using ECL Plus (GE Healthcare, Buckinghamshire, UK) and quantified with ImageLab [version 5.2.1 build 11, Bio-Rad Laboratories (USA)].

## Total ROS Measurements

BMMs were loaded with 0.01 mmol/l DCF (2′,7′-dichlorodihydrofluorescein diacetate, succinimidyl ester) for total ROS measurement for 30 min. Subsequently, the cells were washed twice with PBS containing calcium-chloride (1 mmol/l) and lysed with a 0.5% TritonX-100 buffer. The fluorescence intensity was analyzed by excitation at 485 ± 10 nm and emission at 530 ± 10 nm using a ViktorX Multilable Plate reader and subsequently normalized to protein level.

## Statistical Analysis

The data are given as the mean ± SEM. The data were evaluated using the non-parametric test (Mann-Whitney test for two independent groups) or two-way ANOVA analysis. Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, San Diego, USA). Statistical significance was accepted when $p < 0.05$.

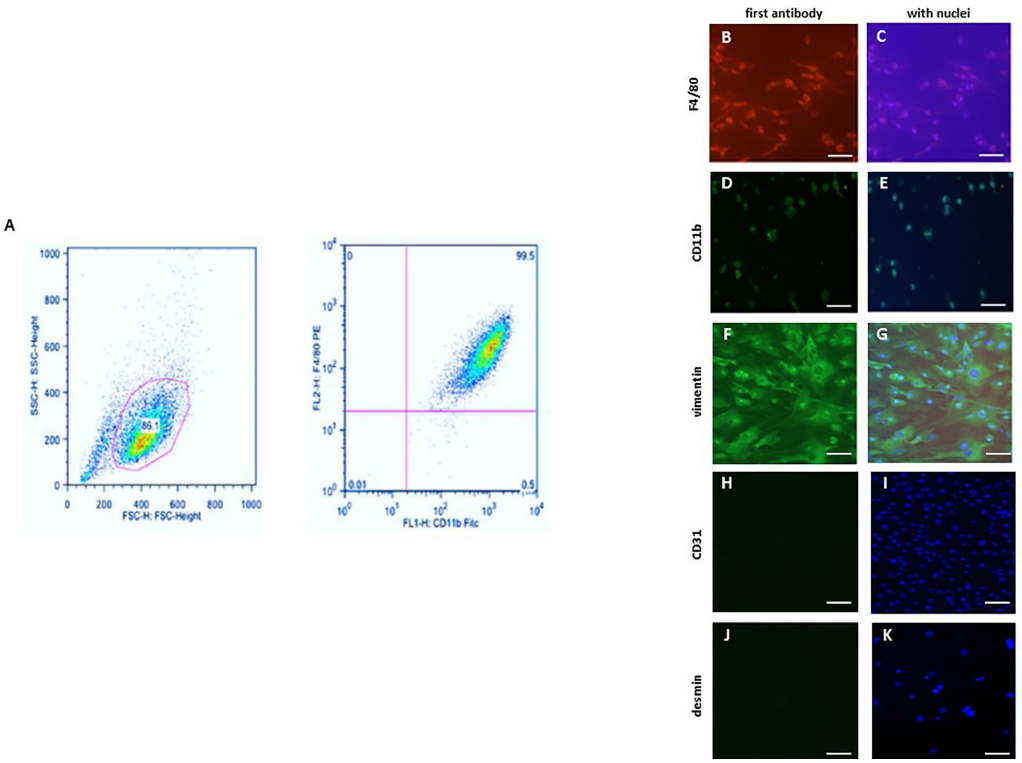


**FIGURE 1** | Characterization of bone marrow derived macrophages (BMMs) and cardiac fibroblasts. **(A)** Flow cytometric analysis of the F4/80 and CD11b expression in BMMs. Representative images of BMMs stained against **(B, C)** F4/80 and **(D, E)** CD11b. Representative images of cardiac fibroblasts stained against **(F, G)** vimentin, **(H, I)** CD31 (endothelial marker; negative control) and **(J, K)** desmin (smooth muscle marker; negative control). Nuclei were stained with DAPI **(C, E, G, I, K)** Magnification: 40x; scale bar: 50 µm. Data are representative of 3 independent experiments with similar results.

# RESULTS

## Male and Female BMMs Express Estrogen Receptors

To investigate the effect of E2 on the polarization of macrophages, we first analyzed the expression of the estrogen receptors in male and female BMMs. All three estrogen receptors (ERα, ERβ and GPR30) are expressed in male and female BMMs (**Figures 2A–E**). Since the stimulation of ER activates ERK1/2 (36), the effects of E2 on ERK1/2 phosphorylation in BMMs were shown. 24h E2 treatment increased ERK phosphorylation in both sexes (**Figure 2F**).

## LPS Elicits Stronger Pro-Inflammatory Response in Male Than in Female BMMs

To investigate sex differences in the M1 polarization of macrophages, male and female murine BMMs were treated with LPS. Mitogen-activated protein kinase p38 (p38) is a known downstream target of LPS and plays a crucial role in M1 macrophage polarization (12, 37). p38 was activated (indirectly highlighted by the phosphorylation rate) in male but not female BMMs after 24 h LPS treatment (**Figure 3A**). LPS treatment significantly increased c-fos and TLR4 expression at the RNA level in both sexes (**Figures 3B, C**). LPS treatment also significantly increased the NFκB mRNA in male and female macrophages, whereas the NFκB mRNA elevation in males was about four-fold higher than that in females (**Figure 3D**). Correspondingly, a significant increase of NFκB expression at the protein level was observed only in male macrophages after LPS treatment (**Figure 3E**). It is important to note that western blot assay also revealed a two-fold higher NFkB protein expression in males than females under basal conditions (**Figure 3E**), suggesting a pro-inflammatory phenotype in male BMMs under basal conditions. In accordance with these findings, TNF-α and IL-1β expression was about two times higher in male macrophages after LPS treatment (**Figures 3F, G**).

In contrast, LPS treatment downregulated the expression of the M2 marker, TIM-3, in male macrophages (**Figure 3H**). Both IL-10 as well as MCP-1, prominent M2 markers, were similarly upregulated in male and female macrophages after pro-inflammatory stimulus (**Figures 3I, J**).

## Male BMMs Show a More Prominent M2 Phenotype Than Females After IL4/IL13 Treatment

To evaluate the sex differences in the polarization of M2 macrophages, the expression of specific markers was investigated. IL-4/IL-13 co-treatment significantly increased the

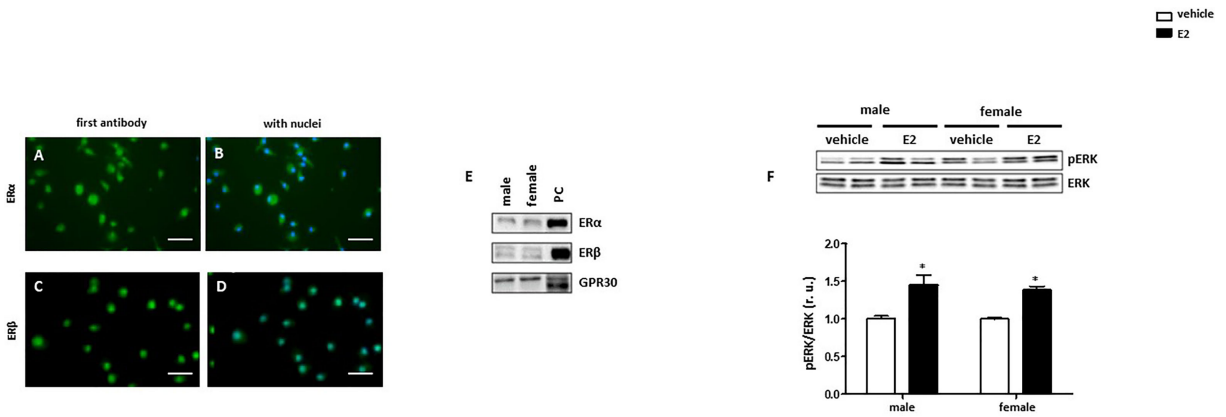


**FIGURE 2** | Male and female BMMs express active estrogen receptors. Representative images of BMMs cells stained against (green) **(A, B)** ERα and **(C, D)** ERβ. Nuclei were stained with DAPI (blue) **(B, D)**. Magnification: 40x, scale bar: 50 μm. Western blot analysis of **(E)** ERα, Erβ, and GPR30 and **(F)** pERK/ERK ratio in male and female BMMs with or without E2 treatment for 24 hrs. Data are representative of 3 independent experiments with similar results. Data are normalized to the male untreated group and expressed in relative units (r. u.). *p < 0.05, untreated *vs.* treated. PC = positive control (MCF7 lysate).

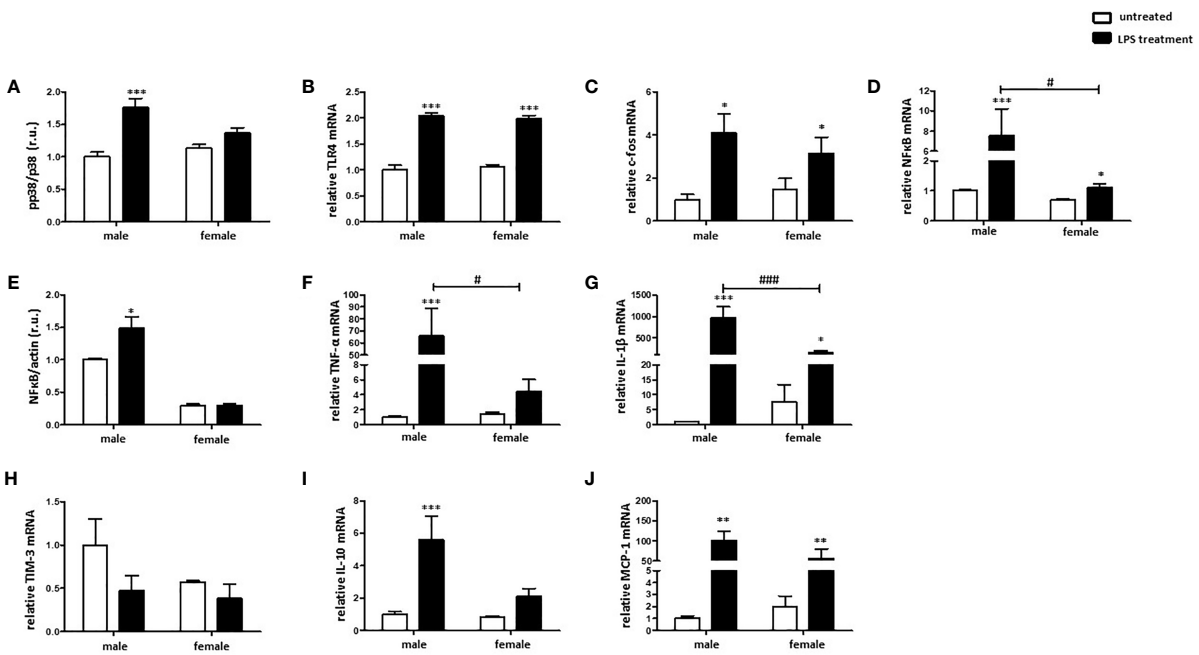


**FIGURE 3** | LPS elicits stronger pro-inflammatory response in male than in female BMMs. Expression analyses of **(A)** pp38/p38 ratio, **(B)** TLR4 mRNA, **(C)** c-fos mRNA, **(D)** NFκB mRNA, **(E)** NFκB protein, **(F)** TNF-α mRNA, **(G)** IL-1β mRNA, **(H)** TIM-3 mRNA, **(I)** IL-10 mRNA, and **(J)** MCP-1 mRNA performed with bone marrow macrophages lysates from male and female mice treated with 10 ng/ml LPS for 24 h. Data are shown as means ± SEM (n = 9; independent experiments with technical duplicates). Data are normalized to the male untreated group and expressed in relative units (r.u.). *p < 0.05, **p < 0.01, ***p < 0.001, untreated *vs.* treated; $^{\#}$p < 0.05, $^{\#\#\#}$p < 0.001, male *vs.* female.

MCP-1 mRNA expression in male and female BMMs (**Figure 4A**). In addition, YM1 mRNA expression was also upregulated in male and female M2 macrophages, whereas male macrophages showed about 2-fold stronger response than female cells (**Figure 4B**). The M2 marker RELM-α was also markedly upregulated in both male and female macrophages after IL-4/IL-13 co-treatment (**Figure 4C**). As might be expected, prominent pro-inflammatory markers, e. g., TNF-α was downregulated in male M2 macrophages (**Figures 4D**). Surprisingly, IL4/IL13 treatment had any effect on the expression of IL-1β neither in male nor in female macrophages (**Figure 4E**).

## TNF-α, but Not TGF-β, Activates a Pro-Fibrotic Phenotype in Mouse Cardiac Fibroblasts

Sex differences in collagen expression and fibrosis formation are well established (31). To investigate the role of sex in fibroblast activation, cultured male and female mouse cardiac fibroblasts were treated with TNF-α or TGF-β. 24 h treatment with TNF-α significantly increased the mRNA expression of the pro-fibrotic markers MCP-1 and IL-1β, in male and female cardiac fibroblasts and the mRNA expression of TGF-β in male cardiac fibroblasts (**Figures 5A–C**), while the TGF-β expression was not increased in female cardiac fibroblasts after TNF-α treatment (**Figure 5B**). Furthermore, male fibroblasts showed a higher elevation of TGF-β and IL-1β (about 2.0-fold) under TNF-α treatment than female fibroblasts (**Figures 5B, C**). In contrast, 24 h treatment with TGF-β did not affect the expression of the pro-fibrotic marker MCP-1 in female fibroblasts, while it increased it in male cells (**Figure 5E**). Neither TNF-α, nor TGF-β affected the expression of Col1A1, a key marker involved in fibrosis formation, in cardiac fibroblasts (**Figures 5D, F**). In addition, MCP-1, TGF-β and IL-1β were increased in male cardiac fibroblasts after LPS treatment, while in female fibroblasts only MCP-1 and IL-1β were increased (data not shown).

## Pro-Inflammatory Macrophage Environment Promotes a Pro-Inflammatory and Pro-Fibrotic Fibroblast Phenotype

To investigate sex differences in the macrophage-fibroblast interaction, male and female cardiac fibroblasts were cultivated with the corresponding male or female conditioned medium from pro-inflammatory M1 BMMs.

Cultivation of male and female cardiac fibroblasts with a pro-inflammatory male or female M1 conditioned medium, respectively, increased the expression of MCP-1, TNF-α, NFκB, and IL-1β at the mRNA level in both sexes (**Figures 6A–D**). It is worth noting that the responses were more prominent in male than in female cells. Male fibroblasts showed in the TNF-α and IL-1β expression an about two-fold stronger response than female cells.

## Female BMMs Are More Protected Against Oxidative Stress After Pro-Inflammatory Stimulus

To investigate the role of sex on ROS formation, total ROS level was measured in non-differentiated, as well as in M1 polarized male and female murine BMMs. M1 macrophages showed a two-fold higher ROS formation compared with untreated cells (**Figure 7**). Elevation of ROS was also more pronounced in male than in female M1 macrophages (**Figure 7**).

## E2 Treatment Promoted The Pro-Inflammatory Phenotype in Male BMMs

Both pro- and anti-inflammatory actions of E2 have been described (23, 24). To test the effects of E2, male and female M1 and M2 macrophages were post-treated with E2 for additional 24 h. The treatment significantly increased the expression of pro-inflammatory markers, e.g., c-fos, NFκB, and TNF-α, in male M1 macrophages, while it had no effects on female cells (**Figures 8A–C**). In M2 BMMs, E2 significantly decreased the expression of MCP-1 in male but not in female M2 macrophages (**Figure 8D**), while E2 had no effect on the expression of TNF-α (**Figure 8E**).

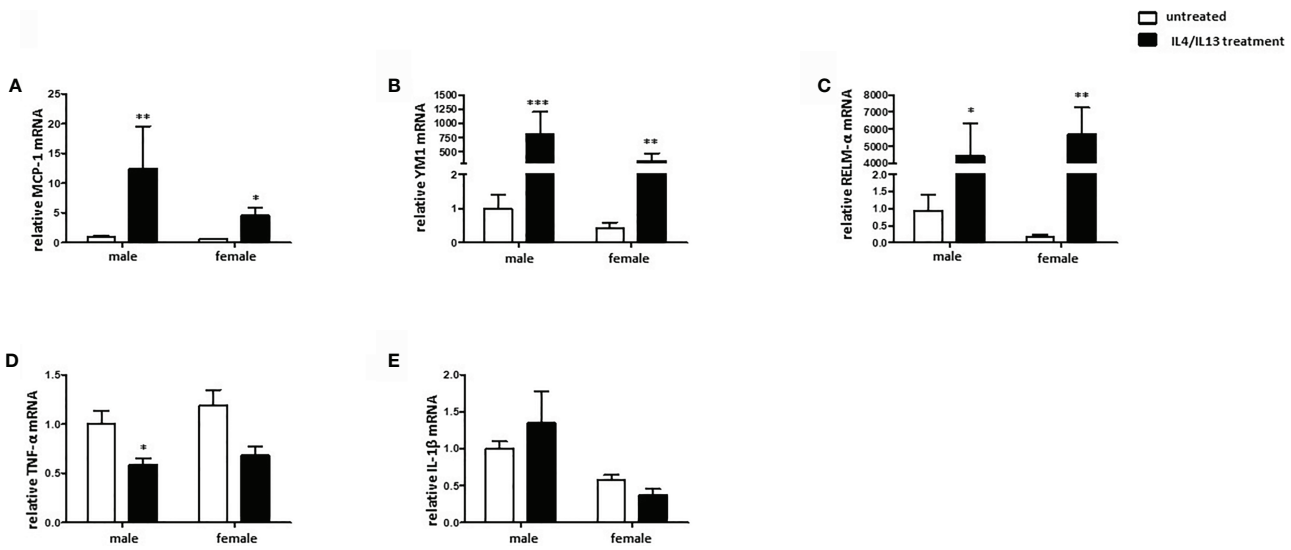


**FIGURE 4** | Male BMMs show a more prominent M2 phenotype compared with females. Real-time PCR analyses of **(A)** MCP-1, **(B)** YM1, **(C)** RELM-α, **(D)** TNF-α, and **(E)** IL-1β performed with bone macrophage lysates from male and female mice treated with 10 ng/ml IL-4 and 10 ng/ml IL-13 for 24h. Data are shown as means ± SEM (n = 9; independent experiments with technical duplicates). Data are normalized to the male untreated group. *p < 0.05, **p < 0.01, ***p < 0.001, untreated *vs.* treated.

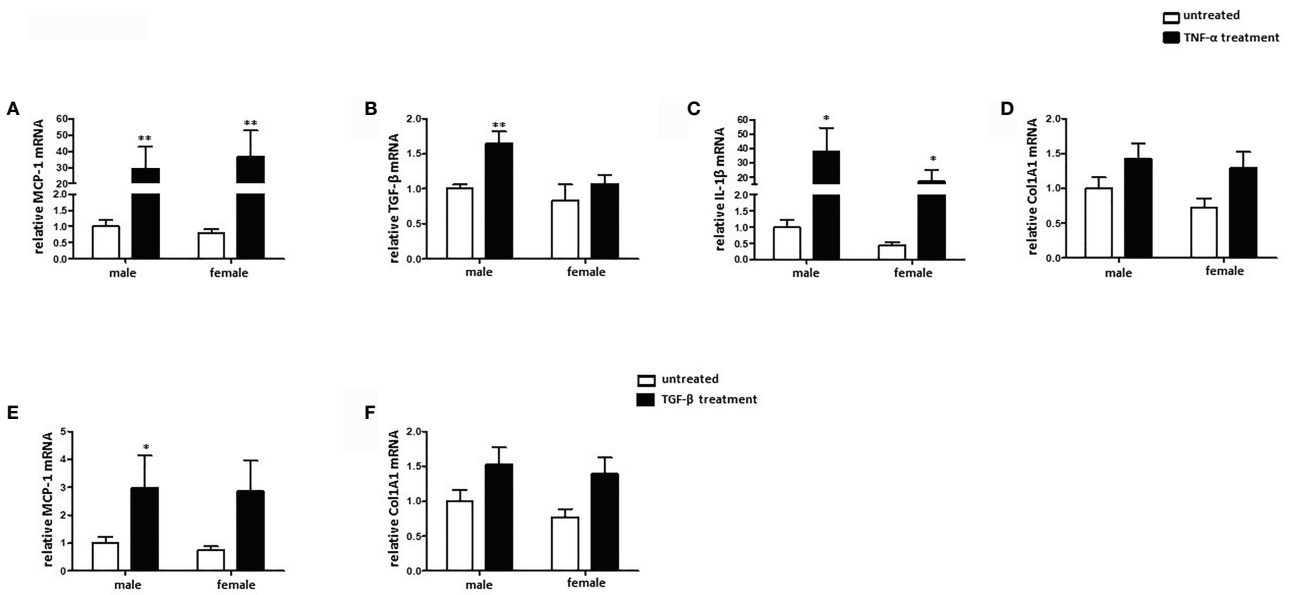


**FIGURE 5** | TNF-α, but not TGF-β, induces pro-fibrotic phenotype in mouse cardiac fibroblasts. Real-time PCR analyses of **(A)** MCP-1, **(B)** TGF-β, **(C)** IL-1β, and **(D)** Col1A1 performed with lysates from male and female mice cardiac fibroblasts treated with 20 ng/ml TNF-α for 24 h. Real-time PCR analyses of **(E)** MCP-1 and **(F)** Col1A1 performed with lysates from male and female mice cardiac fibroblasts treated with 10 ng/ml TGF-β for 24 h. Data are shown as means ± SEM (n = 6; independent experiments with technical duplicates). Data are normalized to the male untreated group. *p < 0.05, **p < 0.01, untreated *vs.* treated.

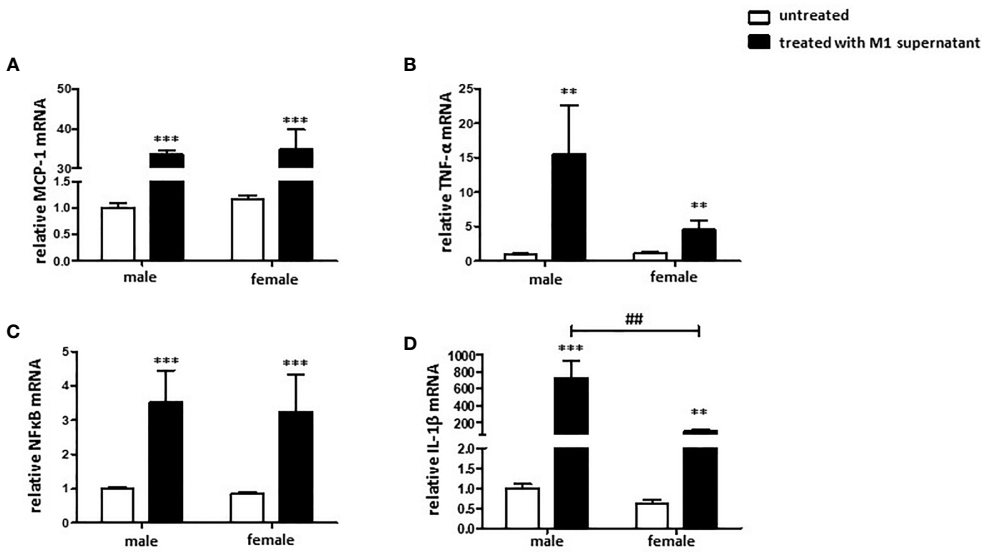


**FIGURE 6** | Pro-inflammatory environment promotes a pro-inflammatory and pro-fibrotic fibroblast phenotype. Real-time PCR analyses of **(A)** MCP-1, **(B)** TNF-α, **(C)** NFκB, and **(D)** IL-1β performed with lysates from male and female mice cardiac fibroblasts cultivated with conditioned medium from M1 polarized BMMs for 24 h. Data are shown as means ± SEM (n = 6; independent experiments with technical duplicates). Data are normalized to the male untreated group. **p < 0.01, ***p < 0.001, untreated *vs.* treated; ##p < 0.01, male *vs.* female.

## DISCUSSION

In the current study, we investigated sex-related alterations in the polarization of murine BMMs. The main findings are as follows: 1) Male BMMs show a stronger pro-inflammatory response to the LPS treatment than female BMMs; 2) Male BMMs show a more prominent M2 phenotype than females under IL4/IL13 treatment; 3) Treatment of cardiac fibroblasts with TNF-α or

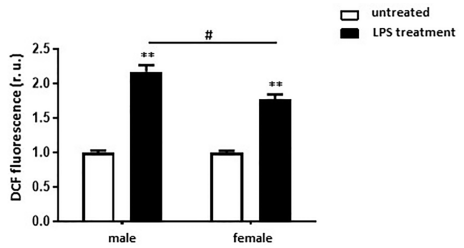


**FIGURE 7** | LPS treatment leads to stronger ROS formation in male than in female BMMs. Analysis of ROS formation (DCF fluorescence, r.u.) in untreated and M1 (10 ng/ml LPS for 24 h) male and female macrophages. Data are shown as means ± SEM (n = 6-7; independent experiments with technical duplicates). Data are normalized to the male untreated group. $^{**}p < 0.01$, untreated *vs.* treated; $^{\#}p < 0.05$, male *vs.* female.

conditioned medium from M1 macrophages promotes a stronger pro-inflammatory and pro-fibrotic response in male cells; 4) E2 treatment promotes the pro-inflammatory and suppresses the anti-inflammatory phenotype in male BMMs.

## Sex-Dependent M1 and M2 Polarization

Activation of p38 is known as a downstream signaling of LPS and is important in M1 macrophage polarization signaling (12, 37). Our study revealed that p38 was activated *via* LPS in male but not female murine BMMs, suggesting that sex influences the activation of p38. In accordance with our results, p38 activation was higher in male than female myocardium after ischemia-reperfusion injury in rats, leading to a lower myocardial inflammatory response in females (38). Importantly, p38 activation promotes the expression of transcription factors such as NFκB or other pro-inflammatory mediators, e.g., TNF-α and IL-1β (39), suggesting that the male-specific p38 phosphorylation observed in this study may be translated into the stronger pro-inflammatory response.

Indeed, the majority of the pro-inflammatory markers show stronger responses to LPS treatment in male than in female macrophages. Particularly, the key pro-inflammatory transcription factor NFκB (12) was strongly upregulated in M1 male BMMs after 24 h LPS treatment. In addition, typical M1 signature cytokines, i.e., TNF-α and IL-1β, were also strongly upregulated in male cells. These data suggest that LPS treatment elicits a stronger pro-inflammatory response in male than in female BMMs. In line with our findings, the pro-inflammatory response increased in male hearts in a model of experimental autoimmune myocarditis, while female hearts showed less inflammation and an increased number of M2 macrophages, leading to a stronger induction of cardiac inflammation and cardiac dysfunction in male rats (40). It is interesting to note that MCP-1 and YM1 were significantly more upregulated in male than in female M2 macrophages polarized with IL4/IL13, however the expression of RELM-α was similar in male and female BMMs. This sex-dependent difference in the expression of anti-inflammatory markers in M2 BMMs might be explained by the difference in polarization of different M2 macrophage subspecies, e.g., M2a, M2b, M2c, and M2d, which have distinct functions and are activated by different stimuli (15, 30, 41). In contrast, pro-inflammatory M1 markers such as TNF-α were strongly downregulated in male M2 BMMs, suggesting that male macrophages are more susceptible to M2-macrophage polarization.

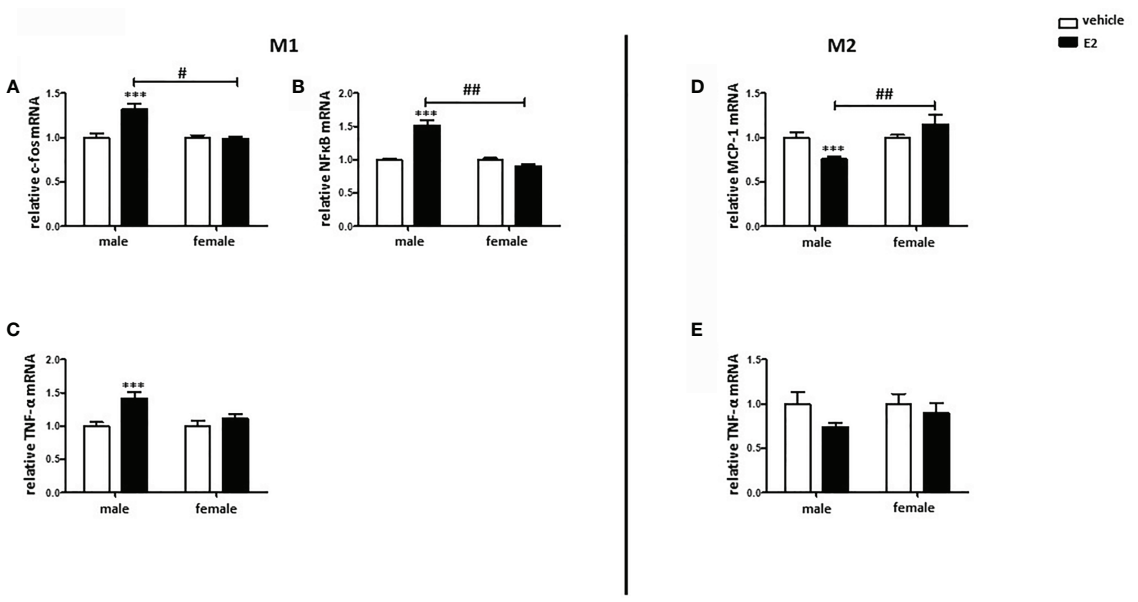


**FIGURE 8** | E2 treatment promoted the pro-inflammatory phenotype in male BMMs. Real-time PCR analyses of **(A)** c-fos, **(B)** NFκB, and **(C)** TNF-α in M1 macrophages, as well as **(D)** MCP-1 and **(E)** TNF-α in M2 macrophages with or without E2 treatment (10 nmol/l) for 24 h. Data are shown as means ± SEM (n=6; independent experiments with technical duplicates). Data are normalized to vehicle. $^{***}p < 0.001$, vehicle *vs.* E2 treated; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, male *vs.* female.

## Effects of M1 Polarization on ROS Formation

ROS formation plays a central role in many inflammatory diseases, as it is an important mediator of inflammation and cell injury (42). ROS formation was significantly increased after M1 polarization in both male and female BMMs, however the ROS level was significantly higher in male M1 macrophages than in female cells. In accordance with our results, Lagranha et al. reported that several mitochondrial-related sex differences are involved in the modulation of ROS homeostasis (43). Of note, sex hormones, especially E2, modulate mitochondrial ROS production (44, 45). Furthermore, it has been shown, in an atherosclerosis model using ovariectomized female mice, that E2 treatment decreased the expression of NADPH oxidase and the superoxide anion formation, while it increased the expression of two ROS-scavenging enzymes (Cu/ZnSOD and MnSOD), which suppose the E2 anti-oxidative effect (46).

## E2 Effects on BMM Polarization

E2 seems to play a crucial role in inflammatory processes (47, 48), and both pro-inflammatory as well as anti-inflammatory effects of E2 have been described (49, 50). In the present study, E2 treatment upregulated the expression of the pro-inflammatory markers, e.g., TNF-α, c-fos, and NFκB, in male M1 macrophages in a pro-inflammatory environment (LPS treatment), whereas it reduced the expression of the anti-inflammatory MCP-1 in male M2 macrophages exposed to an anti-inflammatory environment (IL4/IL13 treatment), suggesting that E2 promotes pro-inflammatory responses in male macrophages. In contrast, E2 had no effects on the expression of any markers investigated in female macrophages. In this regard, it was shown that E2 promotes sex-specific differences in the polarization of macrophages in an asthma animal model, as both male and female macrophages showed an increased expression of M2 genes induced by IL-4 after treatment with a specific ERα agonist, with stronger effects in females (51), thus profoundly impacting the immune system (52). In addition, Villa et al. proposed that E2 treatment decreases the M1 pro-inflammatory phenotype of macrophages, promoting the switch into M2c macrophages (53).

## Sex-Dependent Effects of Pro-Inflammatory Stimuli in Cardiac Fibroblasts

Activation of a pro-fibrotic program in cardiac fibroblasts may lead to pathological cardiac remodeling and heart failure (54). Activated fibroblasts express markers like MCP-1 and pro-fibrotic cytokines such as IL-1β (55–57). Moreover, Van Linthout describes TNF-α as crucial for fibroblast activation (21). In accordance with this, we demonstrated that TNF-α treatment significantly increased the expression of pro-fibrotic factors in a sex-independent manner. However, the TNF-α induced fibroblast activation was more prominent in male than in female fibroblasts and female fibroblasts expressed lower levels of pro-fibrotic factors such as TGF-β and IL-1β. Nevertheless, TNF-α did not change the expression of Col1A1 in neither male nor female fibroblasts. Col1A1, produced by fibroblasts *via* the TFG- β pathway, is fundamental for extracellular matrix synthesis and has been shown to play a key role in the development of diseases characterized by pathological fibrosis as well as the metastasis of various tumors (58, 59). Furthermore, TNF-α has been shown to decrease the Col1A1-expression in cultured fibroblasts (60), however our results did not support that finding.

In addition, TNF-α plays a crucial role in crosstalk between macrophages and fibroblasts (21). Depending on the macrophage subtype, macrophages can promote fibroblast activation or inhibition (41). Our study demonstrates an activation of cardiac fibroblasts with the pro-inflammatory supernatant of cultures of M1 macrophages, suggesting that a pro-inflammatory environment promotes a pro-inflammatory and pro-fibrotic phenotype in fibroblasts. Importantly, male fibroblasts showed more prominent effects from this treatment than female fibroblasts.

In addition, exposure to M1 supernatant strongly increased the ROS levels in male fibroblasts (unpublished data).

In conclusion, the present study revealed (i) a sex-dependent pro-inflammatory response to the M1 polarization stimuli in murine BMMs and (ii) a sex-dependent pro-inflammatory and pro-fibrotic response to the M1 macrophage environment in murine cardiac fibroblasts. The data suppose sex hormones and biological sex differences may play a pivotal role in the human immune system, which may dramatically affect cardiac inflammatory diseases, such as myocarditis.

## Limitations

We only investigated sex differences in macrophage polarization in C57/Bl6J mice. Since strain differences in the immune cell population have been reported (61, 62), we might consider strain differences in the macrophage polarization.

# DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

# ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care Committee of the Senate of Berlin, Germany, approval number: T0333/08.

# AUTHOR CONTRIBUTIONS

MB conceived the project, analyzed the data, prepared the figures, and wrote the main manuscript text. MN performed the molecular biological experiments and analyzed the data. CC performed the FACS analysis, characterization of ERs and ROS measurements,

and analyzed the data. ME prepared figures and wrote the main manuscript text. NH performed molecular biological experiments and analyzed the data. AS performed the ROS measurements and analyzed the data. UM-W revised the manuscript. YL analyzed the data and wrote the main manuscript text. VR-Z generated research funds and coordinated the project. All authors commented on the manuscript.

## FUNDING

This work was supported by the DZHK (German Centre for Cardiovascular Research) and by the BMBF (German Ministry of Education and Research). We acknowledge support from the German Research Foundation (DFG) and the Open access Publication Fund of Charité – Universitätsmedizin Berlin.

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# 4 Discussion

Increasing evidence demonstrates sex-specific differences in the prevalence, development, and pathophysiology of cardiovascular diseases. Among others, the biological sex plays a crucial role in acute and chronic myocarditis, inflammatory dilated cardiomyopathy, and dilated cardiomyopathy, leading to heart failure and sudden death. Premenopausal women show less incidence and better outcomes after viral infection of the myocardium, which is attributed to the cardioprotective effects of estradiol via estrogen receptor signaling. Postmenopausal women lose this cardiac protection which may significantly affect the development of cardiovascular diseases. Thus sex differences in the pathomechanisms of cardiomyopathies are changing with aging. In this habilitation thesis, the cellular mechanisms involved in sex differences in cardiac aging and cardiomyopathies have been investigated. Particularly, the role of inflammation, impaired mitochondrial homeostasis, autophagy, and cellular senescence were in the focus of the study.

## 4.1 Sex differences in cardiac aging

Cardiac aging is associated with impairment of metabolic processes and inflammation,[207,208] which significantly compromise the pumping function of the heart. The two metabolic sensors AMPK and Sirt1 play an essential role in cardiac aging by modulating a wide range of intracellular processes such as oxidative stress response, metabolism, inflammation, and mitochondrial biogenesis and function.[209] Noteworthy, the activity and expression of Sirt1 and AMPK in animals and humans is decline during aging processes.[40] We further investigated sex differences in these processes and demonstrated a sex-independent downregulation of the AMPK phosphorylation rate, suggesting a decreased AMPK activity (Manuscript 3.1,[202]). In contrast, a Sirt1 downregulation was observed specifically in older female non-diseased hearts (Manuscript 3.1,[202]). In addition, our data suggest a reduced Sirt1 activity in older female non-diseased hearts as demonstrated by the elevated acetylation of Ku70 (Manuscript 3.1,[202]), which is a direct target of Sirt1.[210] Since a decreased Sirt1 activity is associated with a compromised mitochondrial biogenesis,[211] we further investigated the aging effect on the key transcription factors, e.g., PGC-1α and TFAM. In our study, the PGC-1α and TFAM expression was not affected during aging processes in non-diseased hearts (Manuscript 3.1,[202]). In accordance with these results, the expression of mitochondrial genes was also unchanged, however a sex-related regulation of

mitochondrial biogenesis in aging processes was described in lower organisms or in the liver and kidneys from older mice.[212,213] Although the expression of PGC-1α was not affected by sex or age in our study, we speculate that the impaired Sirt1 activity observed in older female non-diseased hearts promotes inactivation of PGC-1α due hyperacetylation. In accordance, several studies demonstrated a Sirt1-dependent PGC-1α deacetylation.[214,215] In this regard, we observed a decreased SOD2 expression in older female non-diseased hearts (Manuscript 3.1,[202]), corroborating a possible reduced PGC-1α activity in this group, since PGC-1α directly regulates SOD2 expression.[216]

Moreover, the expression of anti-oxidative enzymes is improved in younger female hearts compared to age-matched men and this defense declined in postmenopausal women.[217] In this study, we demonstrated that the expression of SOD2 and catalase, two key anti-oxidative enzymes, was significantly increased in younger female hearts, whereas this favorable effect was lost in older female non-diseased hearts, leading to an impaired anti-oxidative defense (Manuscript 3.1,[202]). In contrast, SOD2 and catalase expression was increased in older male non-diseased hearts, suggesting an improved anti-oxidative defense in aged male hearts (Manuscript 3.1,[202]), however the underlying mechanisms are still unknown and need to be elucidated.

Similarly, to SOD2, Sirt3, a major mitochondrial-located deacetylase, was also downregulated in older women (Manuscript 3.1,[202]). Several studies demonstrated the positive effects of Sirt3 activity on the regulation of mitochondrial dynamics and function (e.g., OXPHOS activity, ATP synthesis and fatty acid oxidation).[218,219] The reduced Sirt3 expression can partially explain the reduced anti-oxidative defense in aged female non-diseased hearts (Manuscript 3.1,[202]). In accordance, Kwon *et al*. demonstrated an impaired Sirt3 activity in aged mice.[220]

Altogether, our study provides convincing evidence of female sex-specific downregulation of Sirt1 and Sirt3 accompanied by disturbed expression in some mitochondrial proteins and anti-oxidative defense.

Sirtuins, specially Sirt1, also play a crucial role in acute and chronic inflammation via NF-κB inhibition.[221,222] Importantly, in the present study, we demonstrated an increased number of cardiac macrophages, which was accompanied with an upregulation of NF-κB, IL-12, and IL-18 expression, in non-diseased hearts from older women, suggesting a pro-inflammatory shift in older female hearts (Manuscript 3.1,[202]). In contrast, the anti-inflammatory IL-12/IL-10 ratio was significantly decreased in older women with

age. Interestingly, Zhang *et al.* described similar effects in the brain of older female mice.[223] In addition, the present study showed an increased number of anti-inflammatory CD206-positive macrophages in younger female hearts, probably leading to an anti-inflammatory/immune regulatory phenotype in this group, which seems to be lost in older women (Manuscript 3.1,[202]). Altogether, the female-specific downregulation of Sirt1 and Sirt3 and the reduced anti-oxidative defense in older non-diseased hearts could promote the pro-inflammatory environment in aged women. In this line, *in vitro* and human studies demonstrated that E2 promotes anti-inflammatory effects on endothelial and immune cells.[150,158,166,167]

In conclusion, our study proposed a female sex-specific cardiac Sirt1 and Sirt3 downregulation in older individuals, which was accompanied by an impaired anti-oxidative defense and a pro-inflammatory shift.

## 4.2 Age and sex differences in end-stage cardiomyopathies

### 4.2.1 The role of age and sex in DCM

DCM is a cardiovascular condition, which is associated with impaired myocardial contractility, increased interstitial fibrosis, and cardiomyocyte degeneration.[26-29] In our last study, we demonstrated a decreased Sirt1 expression and activity in older female non-diseased hearts (Manuscript 3.1,[202]), therefore we aimed to investigate the sex-specific role of cardiac Sirt1 in DCM.

Sirt1 not only plays a prominent role in anti-aging processes or longevity,[224,225] but it is also closely associated with several chronic diseases, e.g., type 2 diabetes, Alzheimer's disease, and CVD.[226-228] In addition, Sirt1 regulates inflammation, DNA repair, and apoptosis.[229,230]

In this context, we could demonstrate a significantly reduced sex independent Sirt1 expression in older hearts from patients with DCM (Manuscript 3.2,[203]). In this line, Lu *et al*. reported decreased Sirt1 expression in older mice.[231] Another study showed that Sirt1 loss promotes the development of DCM in mice, which was accompanied by mitochondrial dysfunction.[232] In addition, in monocytes from older patients with coronary artery disease Sirt1 expression was significantly reduced.[228] Our data, together with studies from other groups, suggests an age-dependent Sirt1 signaling impairment, leading to mitochondrial dysfunction, apoptosis, and inflammation[233,234] (Manuscript 3.2,[203]).

Another important player in the regulation of metabolic activity and inflammation is AMPK.[235] AMPK and Sirt1 interact with each other and have common targets.[236] AMPK activation plays an essential role in cardiac metabolic homeostasis.[40] Of note, chronic overactivation of AMPK might exacerbate the pathological damage caused by stroke and ischemia by reducing fatty acid oxidation.[237-240] In our study, we found a decreased AMPK phosphorylation in younger patients with DCM, whereas AMPK phosphorylation was increased in older DCM patients (Manuscript 3.2,[203]). Dyck and Lopaschuk proposed a reduced cardiac AMPK activity in DCM patients,[238] however an age dependence was not analyzed. Of note, we and others demonstrated a declined AMPK phosphorylation in older apparently healthy hearts[240] (Manuscript 3.1,[202]). We speculate that the metabolic impairment observed in DCM together with aging processes, which may lead to ATP depletion, a main trigger for AMPK activation, is responsible for the compensatory activation of AMPK (Manuscript 3.2,[203]).

In addition, in this study we observed a reduced cardiac Sirt3 expression in older DCM patients (Manuscript 3.2,[203]), suggesting an impaired mitochondrial function in this group. In accordance, Sirt3 inactivity has been associated with impaired mitochondrial function/biogenesis in cardiovascular diseases, including DCM and metabolic syndrome due to hyperacetylation of key mitochondrial proteins.[241-245] Consistent with these findings, the expression of mitochondrial proteins, i.e., TOM40, TIM23 and SOD2, was significantly reduced in older hearts of male DCM patients, suggesting impaired biogenesis in this group (Manuscript 3.2,[203]).

Several studies proposed that the impaired mitochondrial function observed in older hearts is closely associated with an enhanced inflammatory response.[34,246] In addition, NFκB plays a key role in inflammatory processes in age-related cardiovascular diseases.[70] In this line, we demonstrated an increased NFκB expression in older male DCM hearts (Manuscript 3.2,[203]). Moreover, in older male DCM hearts IL-12 expression was increased, while the anti-inflammatory marker, IL-10 was decreased in a sex-independent manner (Manuscript 3.2,[203]). In accordance with this, in cardiac aging and in aged macrophages IL-10 expression is dramatically reduced, contributing to aggravation of the inflammatory state[223,247] (Manuscript 3.1,[202]). Furthermore, in this study, the number of pro-inflammatory cardiac macrophages seems to be increased in DCM patients (Manuscript 3.2,[203]). Altogether, our results suggest an enhanced cardiac inflammatory response in older DCM patients, being more prominent in men.

In conclusion, the present study demonstrated a compromised mitochondrial biogenesis and anti-oxidative defense that is associated with an increased pro-inflammatory phenotype in older DCM hearts in both sex-dependent and -independent manners.

### 4.3.2 Age and sex differences in myocarditis and inflammatory cardiomyopathy

In contrast to idiopathic DCM, inflammatory cardiomyopathy due to viral myocarditis, did not affect the Sirt1 expression in older hearts (Manuscript 3.2,[203], Manuscript 3.3,[204]). Nevertheless, similar to our results in DCM hearts, we observed a significant upregulation of the phosphorylated form of AMPK, but only in male hearts and in an age-independent manner (Manuscript 3.2,[203], Manuscript 3.3,[204]).

Analysis of mitochondrial biogenesis revealed a pronounced downregulation of mitochondrial proteins (i.e., TOM40 and TIM23) and mitochondrial genes (i.e., *cox1* and *nd4*) in older female DCMI hearts, but not in other groups (Manuscript 3.3,[204]), suggesting a disturbed mitochondrial homeostasis in older female DCMI patients. A compromised mitochondrial biogenesis was accompanied by the reduced mitochondrial mass (mitochondrial DNA/ nuclear DNA ratio), in the older women with DCMI (Manuscript 3.3,[204]). Surprisingly, the mitochondrial mass was also decreased in older male DCMI hearts, despite normal mitochondrial biogenesis. We supposed an enhanced autophagy-related mitochondrial clearance in older male DCMI hearts, which might be a cause for the reduced mitochondrial mass in this group. Indeed, activated autophagy, as defined by the upregulation of the autophagy markers ATG5 and SQSTM1 in older male DCMI hearts, was observed (Manuscript 3.3,[204]), which may be, at least partially, due to activated AMPK in this group.[248] In contrast, both autophagy markers were elevated at the protein, but not at the mRNA level in older female DCMI hearts, suggesting a disturbed autophagy[249] (Manuscript 3.3,[204]).

In this study, we also investigated the presence of posttranslational modifications (by SOD2 acetylation), which are involved in regulating the activity of mitochondrial enzymes, in DCMI (Manuscript 3.3,[204]). In this context, several groups demonstrated hyperacetylation of mitochondrial proteins in a failing heart.[61,250] However, our results revealed significant age-dependent alterations of SOD2 acetylation in male patients, leading to a reduced acetylation rate in older male hearts. We speculate that this unexpected result is caused by the preserved Sirt3 expression observed in our study

(Manuscript 3.3,[204]), while others reported a dramatic Sirt3 downregulation in end-stage cardiomyopathy.[251,252]

Impaired mitochondrial homeostasis in failing hearts is often accompanied by cardiac inflammation[253,254] (Manuscript 3.3,[204]). Similarly, the pro-inflammatory cytokine IL-18 was increased in older female DCMI hearts, suggesting a pro-inflammatory phenotype in this group. In contrast, the preserved mitochondrial homeostasis in older male DCMI hearts was accompanied by downregulation of prominent pro-inflammatory mediators (e.g., NF-κB and TLR4) (Manuscript 3.3,[204]).

Cardiac cellular senescence is observed in several forms of cardiomyopathies.[255] In accordance, in the present study, we observed promoted cellular senescence, e.g., reduced Lamin B1, in older male and female DCMI patients. In addition, the VEGF expression, a senescence-associated secretory phenotype (SASP) marker, was increased only in older female diseased hearts, reinforcing the evidence of a pro-inflammatory shift in this group (Manuscript 3.3,[204]).

A pro-inflammatory environment is associated with pathological fibrosis formation, leading to cardiac remodeling in several CVD including myocarditis.[256] It is well documented that severe pathological fibrosis formation is more prominent in males with myocarditis, whereas females are less affected,[127,153-155,248] nevertheless we also observed pathological fibrosis formation in the older female DCMI (Manuscript 3.3,[204]).

In conclusion, our study reveals a reduced expression of mitochondrial proteins associated with an elevated IL-18 and VEGF expression in DCMI in older women.

These cardiomyopathy effects are absent in older male patients, which may be due to the significant elevation of AMPK expression and activity. In addition, mitochondrial homeostasis is further supported by reduced acetylation of mitochondrial proteins in older male patients.

### 4.2.3 Sex differences in the cardiac function in EAM rats

Sex differences in the development and progression of myocarditis and DCM are well documented, having a higher incidence in men.[109,257-259] In addition, myocarditis is associated with enhanced inflammatory response and pathological fibrosis formation in men compared to women.[3,129] These sex differences are attributed to the sexual hormones, i.e., pro-inflammatory effects of testosterone and cardioprotective effects of

E2.[27,153,260,261] Similar to humans, female mice develop less inflammation and fibrosis formation after infection with CVB3.[126,138]

In the EAM model, we and others found that male rats have a decreased ejection fraction and stroke volume,[262] while female rats showed a preserved cardiac function (Manuscript 3.4,[205]).

Schmerler *et al*. also showed increased numbers of immune cell infiltrates and CD68 macrophages in the myocardium of male EAM rats 21 days after immunization.[262] In our study, we demonstrated an increased number of myocardial immune cell infiltrates in male and female immunized rats, however the amount of immune infiltrates was less prominent in female rats, suggesting a stronger immune response in male EAM animals (Manuscript 3.4,[205]).

Several studies described the pivotal role of macrophages in the healthy and diseased heart, being involved in inflammatory and pro-fibrotic processes during chronic inflammation.[263,264] In this study, we found a similar amount of cardiac CD68+ macrophages in male and female EAM rats, however, female EAM rats showed an increased number of anti-inflammatory CD68+ ArgI+ M2 macrophages (Manuscript 3.4,[205]). Our results suggest an anti-inflammatory/immune regulatory phenotype in female EAM rats due to M2-related attenuation of inflammation (Manuscript 3.4,[205]).

It is well documented that sex differences in the development of autoimmune myocarditis and DCM are closely associated with opposite modulation of the immune response and macrophage phenotype.[10,265,266] In this line, several studies demonstrated a dominant M1 phenotype in males in a model for viral myocarditis, whereas females showed a M2 phenotype.[98,136] We hypothesize that cardiac inflammation is profoundly modulated by the different macrophage phenotypes (Manuscript 3.4,[205]). Moser and Edwards postulated that M2 polarization depletes the pro-inflammatory effects of M1 macrophages during acute inflammation.[267] Our results suggest a pro-inflammatory M1 phenotype in males and an anti-inflammatory/immune regulatory M2 phenotype in the heart of female EAM rats, suggesting cardioprotective actions in females (Manuscript 3.4,[205]).

In myocarditis and other diseases, chronic inflammation is accompanied by enhanced collagen deposition and pathological fibrosis formation.[256] In our study, we observed an increased cardiac Col1A1 and Col3A1 expression in male EAM rats, whereas collagen expression was unchanged in female EAM rats. In agreement, the expression of TIMP-1, a prominent anti-fibrotic marker was elevated only in female EAM rats, while

TGF-β, which has pro-fibrotic actions, was decreased in this group (Manuscript 3.4,[205]). In accordance to the increased collagen expression in male EAM rats, males but not females develop pathological fibrosis in the myocardium. (Manuscript 3.4,[205]). Similarly, Schmerler *et al*. demonstrated severe cardiac fibrosis formation in male EAM rats.[262] Several studies proposed that increased testosterone levels may promote pathological fibrosis formation in males.[127,153-155,248]

Since a pro-inflammatory M1 phenotype and increased pathological fibrosis formation was only observed in male EAM rats, we further investigated sex differences in the inflammatory state in the heart of EAM rats. The analysis of the pro-inflammatory mediators (e. g., TLR4, IL-6, c-fos, and iNOS) revealed their significant elevation in the heart of male EAM rats, whereas in females these factors remain unchanged (Manuscript 3.4,[205]). In accordance, another study described sex-specific differences in the expression of TLR4 in the heart of CVB3 infected mice, demonstrating a male-specific pro-inflammatory phenotype.[139] Of note, sex differences in cardiac inflammation and macrophage polarization seem to be influenced by sexual hormones, E2 and testosterone being cardioprotective and detrimental, respectively.[19,138]

In conclusion, the present study demonstrated a pro-inflammatory and pro-fibrotic phenotype, leading to cardiac dysfunction in males with autoimmune myocarditis, while females showed an attenuated pro-inflammatory response and preserving cardiac function.

### 4.3 Sex differences in macrophage polarization and fibroblast activation

MAPK play a key role in inflammation and macrophage polarization. Particularly, p38 MAPK activation is involved in LPS-mediated signaling and in M1 polarization.[92,268] Analyzing sex differences in this process, we detected LPS-mediated p38 MAPK activation only in male murine BMM. We hypothesized that p38 MAPK activation is influenced by biological sex (Manuscript 3.5,[206]). In line with our findings, Wang *et al*. described an enhanced cardiac p38 activation in male rather than in female rats after ischemia-reperfusion injury, which was associated with reduced cardiac inflammation in females.[269] Moreover, p38 MAPK activation is not only involved in macrophage polarization, rather it also enhances the expression/release of several pro-inflammatory markers (e.g., NFκB, TNF-α and IL-1β).[270] In this regard, we expected that male macrophages develop an enlarged pro-inflammatory response.

As expected, male BMM were more susceptible to LPS treatment. In addition, prominent pro-inflammatory markers (e.g., NFκB, TNF-α), which are involved in M1 signature,[92] were solely increased in male macrophages (Manuscript 3.5,[206]). In this respect, we hypothesized that LPS treatment induces a more robust pro-inflammatory response in male than in female BMM. In accordance, in a previous study we observed a pro-inflammatory cardiac environment in male EAM rats, whereas female hearts seem to be protected against inflammation, probably due enhanced cardioprotective M2 phenotype (Manuscript 3.4,[205]). In contrast, the expression of M2 markers (i.e., MCP-1 and YM1) was strongly upregulated in male M2 polarized macrophages compared to female macrophages, whereas the expression of the anti-inflammatory marker, RELM-α was similar in males and females (Manuscript 3.5,[206]). We hypothesized that the discrepancy in the sex-specific M2 polarization arises from the difference in the M2 macrophage subtypes, e.g., M2a, M2b, M2c, and M2d.[267,271,272] On the other hand, in male M2 macrophages, TNF-α was profoundly downregulated in our study (Manuscript 3.5,[206]). Altogether, our results suggest that male murine BMM react more sensitive to M1 and M2-macrophage stimulus.

Another key player in inflammation and cell injury in several inflammatory diseases is ROS.[273] In our study, we observed an increased ROS formation after LPS treatment in male and female BMM, nevertheless male M1 macrophages produced more ROS than female macrophages (Manuscript 3.5,[206]). In line with these results, mitochondrial ROS homeostasis is modulated in a sex-dependent manner.[179] In addition, several studies demonstrated that E2 is profoundly involved in the regulation of mitochondrial ROS production.[184,274] Moreover, E2 is a key player in inflammatory processes in the heart and metabolic disease,[156,275] having pro-inflammatory or anti-inflammatory effects.[276,277] Our data demonstrated an E2-dependent upregulation of TNF-α, c-fos, and NFκB in male M1 BMM, suggesting pro-inflammatory effects of E2 in male macrophages (Manuscript 3.5,[206]). However, E2 treatment did not modulate the inflammatory response in female macrophages. According to our results, Keselman *et al*. proposed E2-mediated sex differences in the polarization of macrophages in an asthma animal model.[278]

An inflammatory environment might activate cardiac fibroblasts, promoting pathological cardiac remodeling and heart failure.[279] Several studies demonstrated that pro-fibrotic markers (e.g., MCP-1 and IL-1β) and prominent pro-inflammatory cytokines (e.g., TNF-α) are enhanced in activated fibroblasts.[256,280-282] In this line, our study

showed a strongly TNF-α-related elevation of pro-fibrotic factors in male and female murine cardiac fibroblasts (Manuscript 3.5,[206]). In accordance with the macrophage results, TNF-α-induced fibroblast activation was more pronounced in males (Manuscript 3.5,[206]), suggesting that female fibroblasts react less susceptible to a pro-inflammatory environment.

Van Linthout and Tschöpe[282] discussed a role of pro-inflammatory mediators (i.e., TNF-α) in the interplay between macrophages and fibroblasts. In accordance, our results showed that pro-inflammatory M1 conditioned medium promoted the activation of cardiac fibroblasts, however female cardiac fibroblasts showed a weaker expression of pro-inflammatory mediators (Manuscript 3.5,[206]). We hypothesize that a pro-inflammatory environment promotes a more pronounced pro-inflammatory and pro-fibrotic phenotype in male cardiac fibroblasts.

In conclusion, the present study proposed a sex-dependent pro-inflammatory response leading to the M1 polarization of BMM and a sex-dependent pro-inflammatory and pro-fibrotic response to pro-inflammatory environment, principally in male murine cardiac fibroblasts, which seem to be in part regulated by sex hormones and biological sex.

### 4.4 Conclusion

In summary, our data suggest that age and sex are closely involved in the interplay between inflammation, cardiac remodeling, and mitochondrial dysfunction in cardiomyopathies, and promote a chronic inflammatory stage and severe cardiac dysfunction (Figure 4).

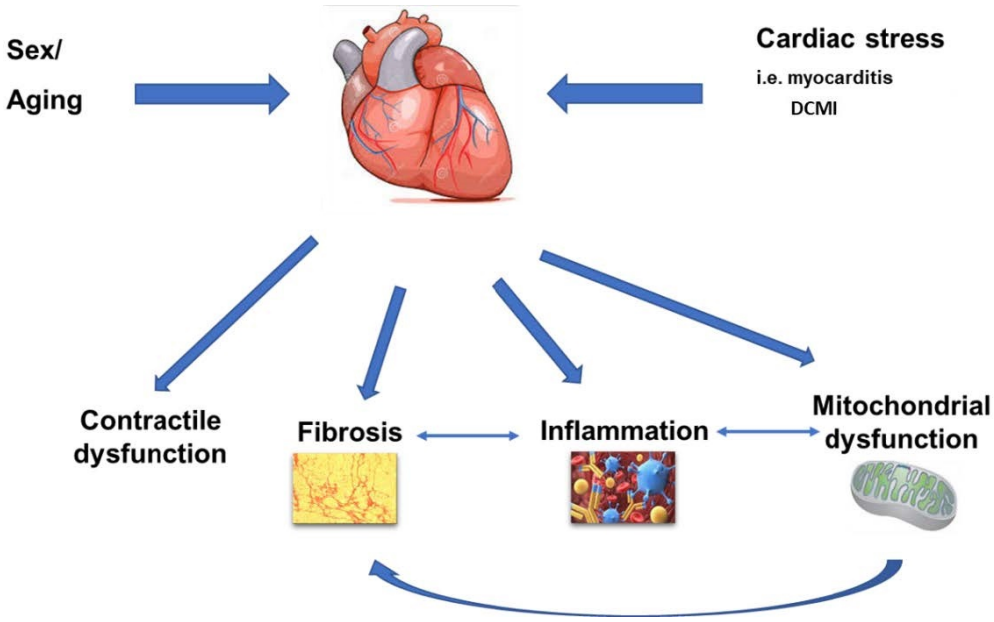


**Figure 4: Schematic representation of the effects of age and sex on cardiovascular conditions.** Age and sex profoundly modulate mitochondrial function, inflammation, and fibrosis formation, influencing cardiac function in myocarditis, DCMI and DCM.

# 5 Summary and outlook

Aging processes and biological sex influence physiological and pathophysiological mechanisms in the heart and may, therefore, affect the treatment effects in patients with CVD.

The research projects included in this habilitation thesis contribute to better understanding of age and sex differences in inflammatory processes and mitochondrial homeostasis in cardiomyopathies (i.e., dilated cardiomyopathy and inflammatory dilated cardiomyopathy).

We demonstrated age and sex-specific mechanisms in the healthy aging heart, leading to a female-specific impaired metabolic sensing and a pro-inflammatory shift due to downregulation of two key $NAD^+$-dependent deacetylases (Sirt1 and Sirt3) in older women.

In addition, we identified an impaired mitochondrial homeostasis and reduced anti-oxidative defense in older hearts of patients with end-stage DCM, which was accompanied by an enhanced pro-inflammatory response in both sex-dependent and -independent manners.

To further demonstrate the role of age and biological sex in cardiomyopathies, we examined their effects on inflammation, mitochondrial homeostasis, autophagy, and cellular senescence in patients with end-stage inflammatory dilated cardiomyopathy. Our study demonstrated a compromised expression of mitochondrial proteins, which was accompanied by disturbed autophagy, pro-inflammatory shift and cellular senescence in older women with DCMI. In contrast, older male DCMI hearts showed a preserved mitochondrial homeostasis and pro-inflammatory/anti-inflammatory balance, but accelerated autophagy.

The study of sex differences in autoimmune myocarditis, using the animal model of experimental autoimmune myocarditis, revealed a pro-inflammatory and pro-fibrotic phenotype in male EAM rats, which was accompanied by a severe cardiac dysfunction in males with autoimmune myocarditis. In this animal model, female rats showed less inflammation and cardiac remodeling, leading to a preserved cardiac function.

Part of this thesis deals with the study of sex differences in the polarization of murine macrophages, which might influence the development and progression of myocarditis and dilated cardiomyopathy. The study suggests a stronger pro-inflammatory response to a pro-inflammatory environment in male murine bone marrow macrophages, which was accompanied by a sex-dependent pro-inflammatory and pro-fibrotic responses in

male activated cardiac fibroblasts. The sex-dependent macrophage and fibroblast activation might have important effects on cardiac inflammatory diseases, e.g., myocarditis.

Taken together, our studies demonstrated the influence of age and sex in cardiac inflammation, which may impair mitochondrial dysfunction and autophagy, and enhance cellular senescence in healthy and diseased hearts. The sex and age differences described in this thesis may help to optimize the personalized therapy of cardiovascular diseases and prolong the life expectancy of cardiovascular patients.

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## 7 Acknowledgments

I want to thank Prof. Dr. Dr. hc. Vera Regitz-Zagrosek for her support and the opportunity to perform this work at the Institute of Gender in Medicine.

I also would like to thank Prof. Dr. Ursula Müller-Werdan very much for the opportunity to habilitate at the Clinic for Geriatrics and Medical Gerontology.

I thank PD Dr. Yury Ladilov for the great cooperation and the endless discussions.

I thank PD Dr. Shokoufeh Mahmoodzadeh for the revision of this habilitation thesis.

I also want to thank all the members of the Institute of Gender in the Medicine and the AG Immuno Aging. My special thanks go to Natalie Haritonow and all my students who made this work possible.

*Was nichts kostet, ist nichts wert*

Albert Einstein

# 8 Statement

Erklärung

**§ 4 Abs 3(k) der HabOMed der Charité**

Hiermit erkläre ich, dass

- weder früher noch gleichzeitig ein Habilitationsverfahren angemeldet noch durchgeführt wurde.
- die vorgelegte Habilitationsschrift ohne fremde Hilfe verfasst, die beschriebenen Ergebnisse selbst gewonnen sowie die verwendeten Hilfsmittel, die Zusammenarbeit mit anderen Wissenschaftlern/Wissenschaftlerinnen und mit technischen Hilfskräften sowie die verwendete Literatur vollständig in der Habilitationsschrift angegeben wurden.
- mir die geltende Habilitationsordnung bekannt ist.

Ich erkläre ferner, dass mir die Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis bekannt ist und ich mich zur Einhaltung dieser Satzung verpflichte.

Datum Unterschrift