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**Original Paper**

# Genetic Deletion of Galectin-3 Exacerbates Age-Related Myocardial Hypertrophy and Fibrosis in Mice

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**Key Words**
Aging • Galectin-3 • Cardiac aging • Hypertrophy • Fibrosis

**Abstract**
***Background/Aims:*** Aging is accompanied by progressive and adverse cardiac remodeling characterized by myocardial hypertrophy, fibrosis, and dysfunction. We previously reported that galectin-3 (Gal-3) is a critical regulator of inflammation and fibrosis associated with hypertensive heart disease and myocardial infarction. Nevertheless, the role and mechanism of Gal-3 in age-related cardiac remodeling have not been previously investigated. We hypothesized that Gal-3 plays a critical role in cardiac aging and that its deficiency exacerbates the underlying mechanisms of myocardial hypertrophy and fibrosis. ***Methods:*** Male C57BL/6 (control) (n=24) and Gal-3 knockout (KO) (n=29) mice were studied at 24 months of age to

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evaluate the role of Gal-3 in cardiac aging. We assessed 1) survival rate; 2) systolic blood pressure (SBP) by plethysmography; 3) myocardial hypertrophy, apoptosis, and fibrosis by quantification of histological and immunohistochemical analysis; 4) cardiac expression of angiotensin (Ang) II, Ang (1–7) by Radioimmunoassay; 5) transforming growth factor-β (TGF-β), sirtuin (SIRT) 1, SIRT 7 and metalloproteinase 9 (MMP-9) by RT-qPCR and 6) ventricular remodeling and function by echocardiography. ***Results:*** We found that aged Gal-3 KO mice had a lower survival rate and exhibited exacerbated myocardial hypertrophy and fibrosis without changes in SBP. Similarly, myocardial apoptosis and MMP-9 mRNA expression was significantly increased in the hearts of Gal-3 KO mice compared to controls. Additionally, cardiac Ang II and TGF-β expression were higher in aged Gal-3 KO mice while SIRT1 and SIRT7 expression were reduced. ***Conclusion:*** Our findings strongly suggest that Gal-3 is involved in age-related cardiac remodeling by regulating critical mechanisms associated with the development of pathological hypertrophy. The gene deletion of Gal-3 reduced the lifespan and markedly increased age-dependent mechanisms of myocardial hypertrophy, apoptosis, and fibrosis, including Ang-II, TGF-β, and MMP-9. At the same time, there was diminished cardiac-specific expression of SIRT1 and SIRT7, which are extensively implicated in delaying age-dependent cardiomyopathies.



## Introduction

Increases in lifespan and an aging population worldwide have amplified the prevalence of chronic diseases and placed heightened importance on expanding the understanding of the increasingly prevalent cardiovascular diseases [1]. Aging is a complex and time-dependent physiological process associated with progressive decline and loss of a variety of physiological cell functions, leading to an increased risk of health complications [2, 3]. Aging notably affects the cardiovascular system, significantly increasing the risk of heart disease and the development of heart failure. Studying the underlying mechanisms associated with cardiac aging thus has relevant clinical implications [1, 4].

Constitutive aging of the heart increases its susceptibility to stress and contributes to elevated cardiovascular morbidity and mortality in the elderly [1]. The hallmark of cardiac aging is a progressive and adverse remodeling characterized by myocardial hypertrophy, inflammation, fibrosis, apoptosis, and dysfunction due to a wide variety of interconnected factors promoted by aging. Angiotensin II (Ang II), the main effector peptide of the Renin Angiotensin System (RAS), has been highly implicated in cardiac remodeling and it is recognized to play an important contribution to aged-related myocardial hypertrophy and fibrosis [5]. Ang-(1-7), converted from Ang II by angiotensin converting enzyme 2 (ACE-2) and considered an antagonist of Ang II, attenuated the myocardial hypertrophy and fibrosis in Ang II induced hypertension [6] and diminished the sarcopenia and osteoporosis in old mice [7] but its role in cardiac aging is less known. Likewise, chronic pharmacologic inhibition of RAS markedly prevented the adverse cardiac remodeling associated to aging and significantly enhanced the longevity [8]. In addition, other factors such as transforming growth factor-β (TGF-β), metalloproteinase (MMP), and sirtuins (SIRT) [9–13] and closely linked to the RAS are important contributors to morphological and functional changes of cardiac aging.

Over the last few years, clinical and experimental studies have strongly suggested that galectin-3 (Gal-3), a β-galactosidase–binding lectin, is highly increased in a variety of pathological conditions and promotes cardiac remodeling by regulating the underlying mechanisms of myocardial hypertrophy, inflammation, and fibrosis [14–19]. Additionally, Gal-3 has been found to be an independent prognostic factor for heart failure [14, 15, 20, 21]. Although the role of Gal-3 in cardiovascular pathology appears to be clearly defined, the involvement of Gal-3 in age-related cardiac remodeling is still unknown. Gal-3 regulates cell growth and proliferation as well as apoptosis by orchestrating cell–cell and cell–extracellular matrix interactions [14, 22]. Gal-3 was found to be increased in elderly patients and was

proposed as a biomarker associated with frailty in subjects with systolic heart failure [23, 24]. In addition, Gal-3 was associated with elevated left ventricular (LV) mass in age- and sex-matched analysis, suggesting a role in age-related cardiac remodeling [25]. A lack of Gal-3 in old mice with exacerbated renal fibrosis, oxidative stress, and renal dysfunction suggests that an absence of Gal-3 during aging amplifies age-related organ damage [26]. However, the role of Gal-3 in age-induced cardiac remodeling is unknown. Here we hypothesized that genetic deletion of Gal-3 exacerbates the age-related myocardial hypertrophy, fibrosis and apoptosis through the mechanisms involving ANGII, TGF-β, MMP-9 and SIRT and, independently of blood pressure.

## Materials and Methods

*Mice and experimental design*

Male C57BL/6J (C57, n=24) and Gal-3 Knockout (Gal-3 KO; n=29) mice were followed up for 24 months from weaning. Animals maintained the light/dark cycle of 12 h with access to water and food ad libitum grouped with 4 animals per cage. Mice were obtained and followed up in our Bioresources facilities at the Biomedical Research Institute (BIOMED UCA-CONICET) of the Pontificia Universidad Católica Argentina. The experimental protocol was approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL) of BIOMED in line with the NIH's Guide for the Care and Use of Laboratory Animals [27].

*Water and food intake and body weight*

Body weight, food and water intake were monitored weekly throughout the experimental period. Body weight (gr) evolution per animal was recorded and averaged for each group.

*Systolic Blood Pressure*

At 24 months of follow-up the animals were trained for one week for the measurement of blood pressure by plethysmography according to the tail cuff method [19]. After training, systolic blood pressure (SBP) values were recorded for three consecutive days. Measurements were made at the same time for each group in a quiet environment and avoiding stressful conditions for the animals. To facilitate statistical analysis and data interpretation, the results of the three measurements were averaged and a single SBP value at 24 months was considered for each animal.

*Echocardiography*

Age-related cardiac remodeling and function were studied at 24 months by two-dimensional echocardiography. All Two-dimensional echocardiograms were performed using a Vivid 7 machine (General Electric Medical Systems, Horten, Norway) with a phased array 10 MHz transducer. Mice were slightly anesthetized with tribromoethanol (Avertin); 1.15 ml/kg. Briefly, the heart was visualized in the long axis parasternal view for M-mode. Left ventricle (LV) dimension measurement and posterior wall and left ventricular (LV) end-diastolic and end-systolic diameters (LVEDD and LVESD) and LV-posterior wall thicknesses (PWT) and anterior wall thickness (AWT) were measured in systole and diastole. LV mass and fractional shortening (FS) were calculated as described elsewhere [18] as follows:

1) LV mass = $1.055\ [(AWT + LVEDD + PWT)^3 - (LVEDD)^3]$

2) FS (%) = [(LVEDD - LVESD)/LVEDD] x 100

*Necropsy*

At 24 months, the animals were euthanized with an overdose of sodium pentobarbital (150 mg/kg). After euthanasia, hearts, lungs, kidneys and spleens were removed and weighed and tibia lengths were measured. Organ weights were normalized to tibia length or body weight. In order to corroborate the age-cardiac remodeling at 24 months, heart weight, cardiac mass index and blood pressure were matched between young and old animals (Supplementary Fig. 1 – for all supplementary material see www.cellphysiolbiochem.com).

*Histology*

Hearts were harvested and fixed in formaldehyde. After that, hearts were cut from apex to base, and paraffin embedded. Serial cuts were performed and stained with hematoxilin-eosin (H&E) for measuring myocyte cross-sectional area (MCSA) and Picrosirius red for quantifying myocardial interstitial collagen by using the image-analysis software (ImageJ). In images obtained with light microscope outlines of myocytes were traced, and cell areas were measured by using an image analyzer-software (Image J) [18].

The percentage of collagen for each region was measured with picrosirius red staining, and calculated as: collagen area/tissue area of the high-power field (400 X) as described previously [19].

*Cardiac expression of ANG II and ANG-(1-7)*

Cardiac Ang II, and Ang-(1-7) expression was measured by radioimmunoassay. Briefly, cardiac samples were homogenized in acid ethanol (0.1 mol/L HCl/80 % ethanol) containing 0.44 mmol/L o-phenanthroline, 1 mmol/L Na+ para-chloromercuribenzoate, 0.12 mmol/L pepstatin A and 25 mmol/L EDTA. Homogenates were centrifuged at 20000 g for 30 min at 4 °C. Proteins in the supernatant were quantified. The supernatant was subsequently lyophilized and Ang extraction and recovery was performed as described elsewhere [28]. Each sample was corrected for each recovery. Ang II level was quantified by radioimmunoassay using Ang-labelled as described previously. Results were expressed as pg/mg tissue [28].

*Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR)*

mRNA expression was determined using 5× HOT FIREPOL EVAGREEN qPCR (Solis BioDyne, Estonia) in a StepOnePlus Real-Time PCR System. Parameters were as follows: 52 °C for 2 min, 95 °C for 15 min, and 40 cycles at 95 °C for 15 sec, 63 °C (for TGF-β) 62 °C (for β-actin and MMP-9), 57 °C (for SIRT7) or 56 °C (for SIRT1), for 30 sec and 72 °C for 1 min. Normalization was carried out using actin RNA. Quantification was performed using the comparative threshold cycle (Ct) method, as all the primer pairs (target gene/reference gene) were amplified using comparable efficiencies (relative quantity, $2^{-\Delta\Delta Ct}$) [29, 30]. Primer sequences:

β-Actin: forward: 5′-GGCTGTATTCCCCTCCATCG-3′, reverse: 5′-CCAGTTGGTAACAATGCCATGT-3′
SIRT1: forward: 5′-GCGGCTGACGACTTC-3′, reverse: 5′-GCTGGCGTGTGACGTTC-3′
SIRT7: forward: 5′-GCCGAGAGCGAGCT-3′, reverse: 5′-GCCCGTGTAGACAACCA-3′
MMP-9: forward: 5′-CAGACCAAGGGTACAGCCTGTT-3′, reverse: 5′-AGTGCATGGCCGAACTC-3′
TGF-β: forward: 5′-CACCGGAGAGCCCTGGATA-3′, reverse: 5′-TGTACAGCTGCCGCACACA-3′

*Apoptosis determinations*

The fragmented DNA in cells undergoing apoptosis was detected in slices from middle sections of the hearts using ApoptagTM plus peroxidase *in situ* apoptosis Detection Kit (Millipore, MA, USA), according to the manufacturer's instructions. Tunel-positive cells were quantified in at least 10 high power field (HPF) and the results expressed as Tunel + cells/HPF at 400x magnification.

*Statistical analysis*

Continuous variables are presented as means ± SEM. Parametric data were analyzed using Student's unpaired t-test to compare the two groups using Prism 6.0 (GraphPad Software, San Diego, CA). Survival analysis was performed by the Kaplan–Meier method with the log-rank test. Values of $p< 0.05$ were considered statistically significant.

## Results

*Decreased survival rate in aged Gal-3 KO mice*

During the follow-up survival rate was significantly lower in Gal-3 KO mice compared with C57 mice ($p < 0.05$ Gal-3 KO vs C57) (Fig. 1A). At 24 months 67 % of C57 (n= 16/24) and 55 % (n= 16/29) of Gal3-KO mice achieved 24 months of age. There were no differences in body weight over the study period (Fig. 1B).

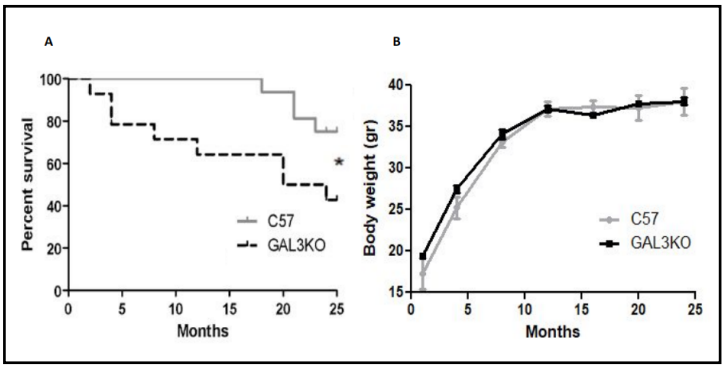


**Fig. 1.** A: Survival rate after 24-month follow-up for both genotypes. *p<0.05 Gal-3 KO (n = 29 with total number of death events n = 13) vs. C57 (n = 24 with total number of death events n = 8). B: Temporal evolution of body weight in C57 (n = 16) and Gal-3 KO mice (n = 13) (p = NS between groups).

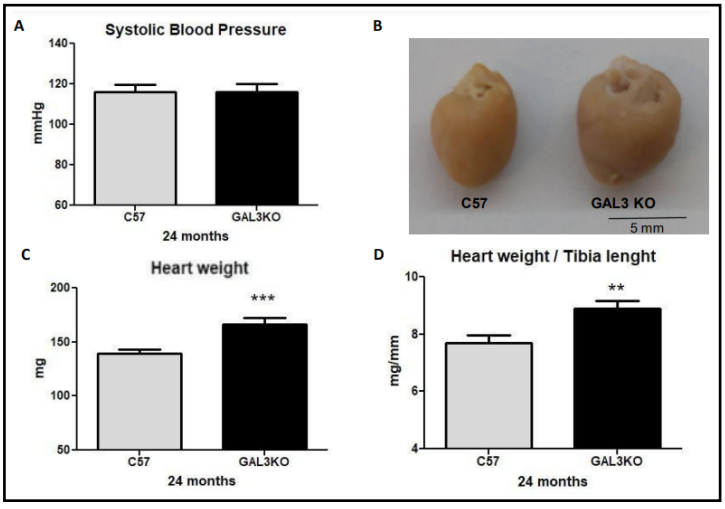


**Fig. 2.** A: Systolic blood pressure after 24-month follow-up in C57 (n = 10) and Gal-3 KO (n = 10) mice (p = NS). B: Representative macroscopic images of hearts from C57 (left) and Gal-3 KO mice (right). C–D: Cardiac mass index for both genotypes. **p<0.01, ***p<0.001 for GAL-3 KO (n = 13) vs. C57 (n = 18) mice.

*Exacerbated myocardial hypertrophy in aged Gal-3 KO mice independent of blood pressure*

SBP (mmHg) at 24 months was similar between groups and within physiological values (Fig. 2A) while comparing with previous information of young animals, no significant changes were observed between young and aged animals in both groups (Supplementary Fig. 1). Cardiac weight, heart weight/body weight ratio and heart weight/tibia length ratio were significantly increased with aging in both groups (Fig. 2C-D) (Supplementary Fig. 1). However, the increases in cardiac weight and cardiac mass index were higher in aged Gal-3 KO mice than in C57 mice (Fig. 2B-D; $p < 0.05$). Gal-3 KO mice demonstrated increased renal hypertrophy expressed as kidney weight/tibia length (mg/mm) ratio (27 ± 6 in C57 vs 37 ± 4 in Gal-3 KO; $p < 0.05$). Additionally, the lung weight/ tibia length (mg/mm) ratio was similar between groups (14.21 ± 5.93 in C57 and 13.69 ± 4.58 in Gal-3 KO mice; p=ns). Spleen weight/ tibia length (mg/mm) were similar in both genotypes (7.13 ± 3.12 in C57 and 6.35 ± 2.21 in Gal-3 KO mice; p=ns).

*Myocardial hypertrophy in aged Gal-3 KO mice with preserved systolic function*

At 24 months, cardiac mass index as calculated by echocardiography was significantly increased in the Gal-3 KO group compared with the C57 group (Fig. 3A; $p < 0.01$). Additionally, posterior wall thickness and anterior wall thickness in both systole and diastole were significantly increased in the Gal-3 KO group compared to the C57 group (Fig. 3B and C; $p < 0.0001$). LV end-systolic dimensions (LVESD) and LV end-diastolic dimensions (LVEDD), as well as shortening fraction, were similar between genotypes (Fig. 3D–G; p = NS).

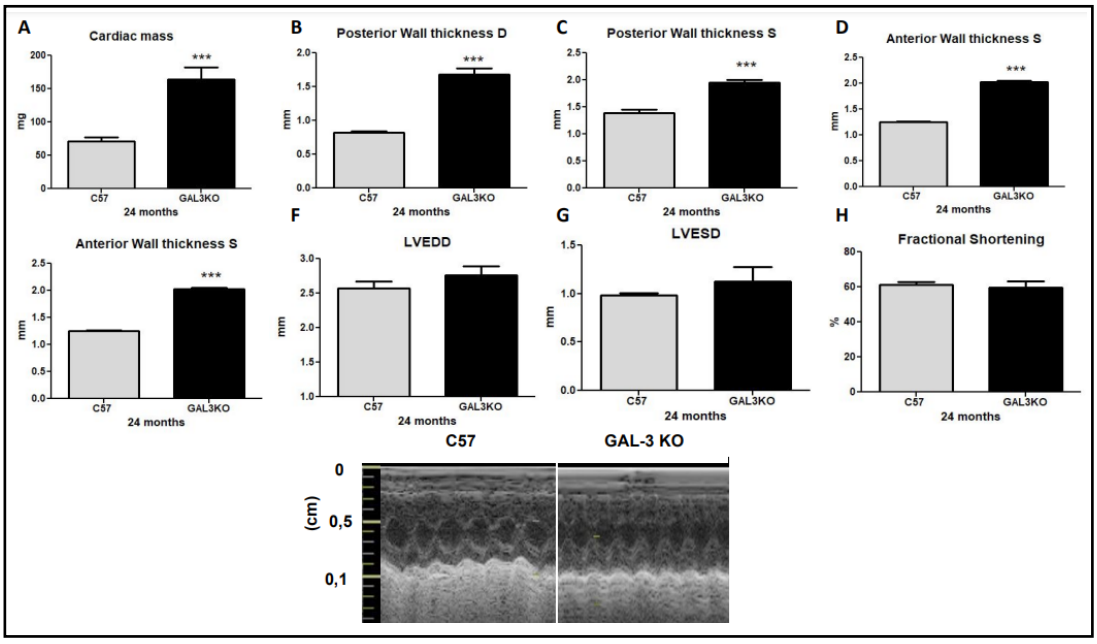


**Fig. 3.** A: Cardiac mass index as calculated by echocardiography in aged C57 and Gal-3 KO mice. B–C: Posterior wall thickness in systole (S) and diastole (D) in both genotypes. D–E: Anterior wall thickness in systole (S) and diastole (D) in both genotypes. F–G: Left ventricular end-diastolic and end-systolic dimension (LVEDD and LVESD) in both genotypes. H: Fractional shortening (%) in both genotypes. I: Representative images of echocardiograms of C57 mice (left) and Gal-3 KO mice (right) obtained after 24-month follow-up. ***p<0.0001 for C57 (n = 7) vs. Gal-3 KO (n = 5) mice.

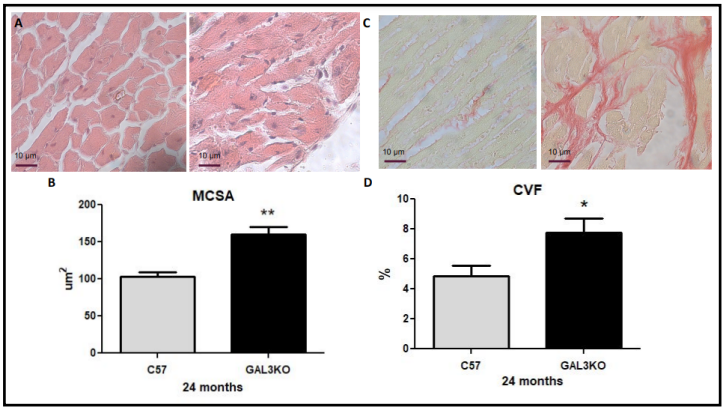


**Fig. 4.** A: Representative images of myocyte cross-sectional area (MCSA) from HE stained section (magnification 400x; scale bars: 10 µm) in aged C57 (left) and Gal-3 KO mice (right). B: quantification of MCSA in C57 (n = 5) and GAL-3 KO (n = 5) at 24 months. C: Representative images of picrosirius red-stained images (magnification 400x, scale bars: 10 µm) in aged C57 (left) and Gal-3 KO mice (right). D: quantification of collagen volume fraction in C57 (n = 5) and Gal-3 KO (n = 5) at 24 months. **p<0.01, *p<0.05 for C57 vs. Gal-3 KO mice.

*Increased MCSA, fibrosis, and apoptosis in aged Gal-3 KO mice*

In order to further confirm that Gal-3 deletion induced cardiac hypertrophy, MCSA was measured at 24 months in Gal-3 KO and control mice. As shown in Fig. 4A, the MCSA in aged Gal-3 KO mice was significantly elevated compared to control mice ($p < 0.05$). Similarly, interstitial myocardial fibrosis was significantly increased in aged Gal-3 KO mice compared to control mice (Fig. 4B; $p < 0.02$).

Finally, we quantified myocardial apoptosis as the quantity of TUNEL+ cells and observed that aged Gal-3 KO mice presented with significantly higher numbers of TUNEL+ cells than aged C57 mice (Fig. 5; $p < 0.05$), indicating increased apoptosis.

*Increased myocardial expression of Ang II in aged Gal-3 KO mice*

Cardiac expression of Ang II was significantly increased in old Gal-3 KO mice compared with control mice (Fig. 6A; $p < 0.02$). Cardiac Ang (1–7) and Ang 1-7/Ang II expression were similar between genotypes (Fig. 6B and C; p=NS).

*Increased cardiac expression of TGF-β and MMP-9 and reduced SIRT1 and 7 in aged Gal-3 KO mice*

Cardiac expression of SIRT1 and 7 were significantly lower in aged Gal-3 KO mice than in control mice (Fig. 7C–D); $p < 0.05$). On the contrary, cardiac mRNA expression for the fibrotic factor TGF-β and the hypertrophy promotor MMP-9 were significantly increased in aged GAL-3 KO mice compared with C57 mice (Fig. 7A–B); $p < 0.05$).

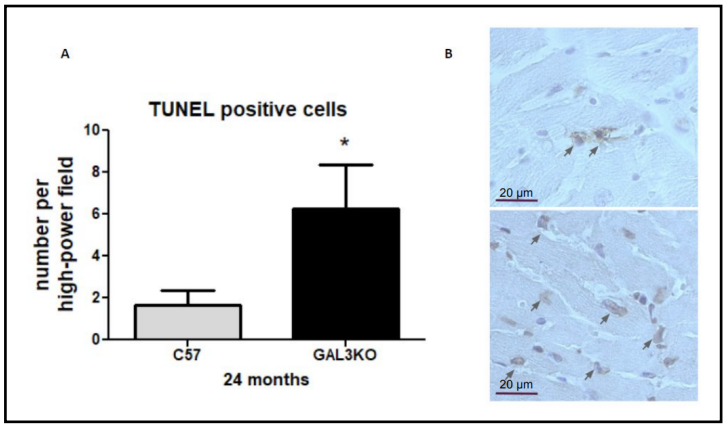


**Fig. 5.** A: Average of numbers of apoptotic cells per each high-power field at 24 months with positive TUNEL staining in both genotypes. *p<0.05 for C57 (n = 6) vs. Gal-3 KO (n = 4) mice. B: Representative images of TUNEL+ staining slices (magnification 400x, scale bars 20µm). Arrows indicate TUNEL-positive cells.

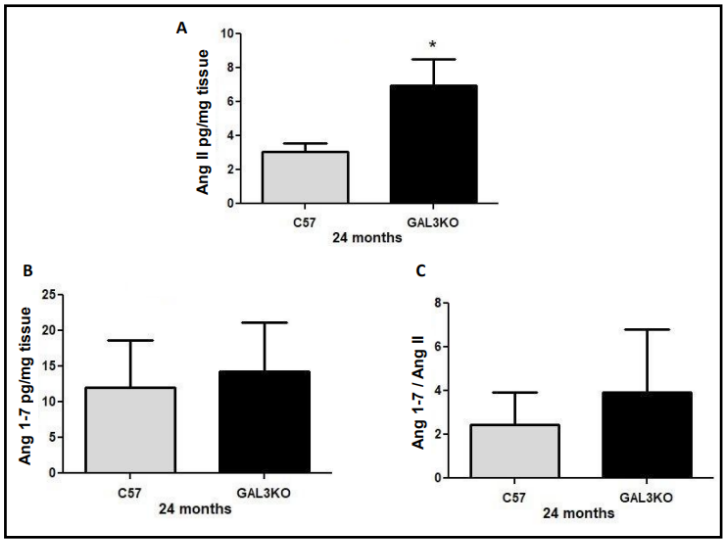


**Fig. 6.** A: Cardiac expression of angiotensin (Ang) II in old C57 (n = 11) and Gal-3 KO (n = 9) mice. B: Cardiac Ang (1-7) expression in C57 (n = 12) and Gal-3 KO (n = 8). C: Ang 1-7/Ang II ratio in cardiac tissue in C57 (n = 13) and Gal-3 KO (n = 9). *p< 0.05 for C57 vs. Gal-3 KO mice.

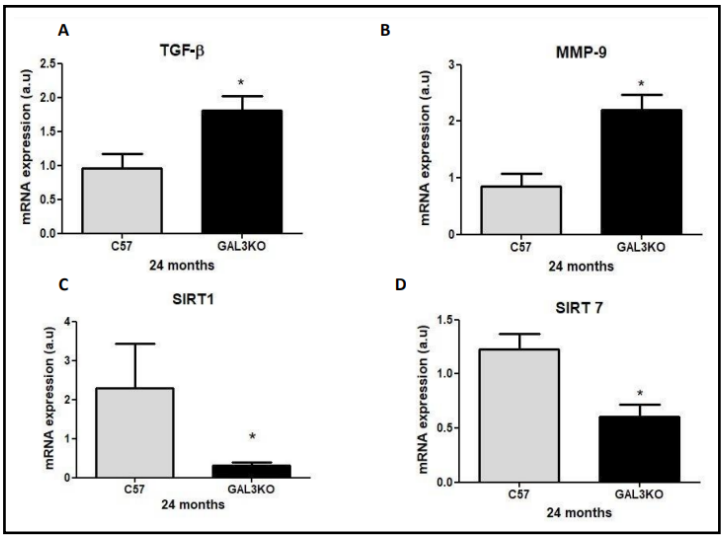


**Fig. 7.** A–B: Quantification of mRNA of profibrotic (TGF-β) and antifibrotic factors (MMP-9) by quantitative real-time PCR (RT-qPCR) in the hearts of 2-year-old C57 and Gal-3 KO mice. C–D: Quantification of myocardial mRNA expression of SIRT 1 and 7 in both genotypes. *p<0.05 for C57 (n = 5) vs. Gal-3 KO (n = 5) mice.

## Discussion

In the present study, we investigated the role of Gal-3 on age-related ventricular remodeling and function in mice. To our knowledge, this is the first study to investigate Gal-3 as a potential factor involved in the temporally evolving pathophysiology of cardiac aging. Based on our findings, aged Gal-3 KO mice demonstrated 1) a significantly reduced survival rate; 2) a marked increase in myocardial hypertrophy and fibrosis independent of blood pressure; 3) elevated expression of cardiac Ang II, a hormone largely involved in myocyte growth, fibrosis, and apoptosis, which was not followed by changes in Ang 1-7 or the ratio between Ang 1-7/Ang II; 4) Increased myocardial expression of proinflammatory and profibrotic factors such as TGF-β and MMP-9; 5) significantly increased myocardial apoptosis; and 6) reduced myocardial expression of two antihypertrophic factors, SIRT1 and SIRT7. Our results describe a central role of this galectin in the process of cardiac aging and suggest that total inhibition may lead to adverse aging outcomes.

During aging, the heart undergoes multiple complex morphological and functional changes distinguished by cardiomyocyte growth and fibrosis. Age-related cardiac remodeling is also marked by a decrease in the number of myocardial cells either by apoptosis or necrosis, which progressively leads to cardiac dysfunction [9, 31, 32]. In our work for studying the role of Gal-3 in age-related cardiac remodeling, we employed a 24-month follow-up and compared the histological, functional, and biochemical features of cardiac remodeling between C57 and Gal-3 KO mice. Complementary, cardiac mass index and blood pressure between young and old animals were matched to corroborate the myocardial response to aging (Supplementary Fig. 1). Thus, we found that aged control mice recapitulated most age-related cardiac changes described previously for rodents and humans [8, 33, 34]. Since in previous studies performed in young animals, we did not find differences in the cardiac mass index, myocardial fibrosis and cardiac function, other measurements such as transcriptomics and proteomics were not performed. Interestingly, after 24 months of follow-up, we found that the genetic deletion of Gal-3 significantly reduced survival rate and exacerbated myocardial hypertrophy, apoptosis, fibrosis, and the mechanism of age-related cardiomyopathy independent of blood pressure and without affecting LV systolic function.

Gal-3 has been proposed as a marker of cardiac fibrosis and associated with an elevated risk of both heart failure and all causes of cardiovascular-related mortality. Ho *et al*. reported that Gal-3 is associated with elevated LV mass in age-adjusted and sex-adjusted analyses and observed a strong association between Gal-3 and kidney dysfunction [25, 26, 35]. Kasacka *et al*. reported increased Gal-3 expression in the hearts of patients over 45 years of age

without a previous history of cardiac disease [35]. However, observational studies cannot determine a cause–effect relationship between Gal-3 and cardiac aging. Here, we report for the first-time experimental evidence that Gal-3 plays a key role in the pathophysiology of the temporal evolution of cardiac aging and that genetic deletion of Gal-3 is associated with cardiac hypertrophy and fibrosis. Consistent with our results, Iacobini *et al*. reported that aged Gal-3 KO mice showed higher levels of renal fibrosis and dysfunction than aged control mice [26]. However, under pathological conditions such as myocardial infarction, hypertrophy, hypertension and heart failure, Gal-3 KO mice demonstrated reduced inflammation and consequently myocardial fibrosis. The reason of this discrepancy between those studies and the results presented here are not entirely clear. One possibility is that those pathologies are strongly associated with inflammation while in aging inflammation is clearly lower when compared with myocardial infarction or hypertensive hearts. This apparent discrepancy underlines the importance of identifying and deeply studies the underline mechanism of action of Gal-3 in cardiac remodeling aging as an independent condition. Our findings and others [26] strongly suggest that during aging, Gal-3 could have effects opposite to those usually known in a profibrotic and proinflammatory lectin. In our study, the genetic deletion of Gal-3 in aged mice significantly reduced survival and exacerbated the mechanism associated with the development of myocardial hypertrophy, apoptosis, and interstitial fibrosis, similar to the findings stated above observed in aged kidneys [26].

The direct association that we observed between cardiac hypertrophy and survival in aged Gal-3 KO mice has been previously shown in both rodents [4] and humans [36]. However, survival is dependent on several mechanisms and may also be attributable to non-cardiac causes, such as tumors, kidney failure, or multiple organ dysfunctions. Unfortunately, we did not perform necropsies of the mice to determine the precise causes of death in both genotypes.

Aging is associated with hypertension, and increased SBP can lead to cardiac hypertrophy [37]. In Gal-3 KO mice, SBP was similar to control mice without signs of hypertension. A lack of hypertension in aged mice has also been shown in other studies [8], although early stages of altered vascular tone were not assessed in the current study. Thus, the increase in myocardial hypertrophy and fibrosis observed in aged Gal-3 KO mice in this study was independent of blood pressure changes.

Direct evidence indicates that the renin–angiotensin system (RAS) is a key neurohumoral system linked to cardiac aging independent of hemodynamic overload. Cardiac Ang II increases with age and promotes cardiomyocyte growth, fibroblast proliferation, collagen synthesis and fibrosis, and inflammation and cell death by apoptosis [8, 37, 38]. All components of RAS have been identified in cardiac tissue [39, 40]. Different studies support that cardiac Ang I and II may be produced locally [41]. Sadoshima *et al*. showed that cardiac Ang II released from isolated cardiomyocytes, in an autocrine manner induced cardiomyocyte hypertrophy [42]. In addition, cardiac Ang II was increased in aged mice and linked to hypertrophy, fibrosis and diastolic dysfunction [5]. Furthermore, pharmacologic inhibition of RAS prevented the cardiac hypertrophy associated with aging. Because the Ang II is the main effector of the RAS and it is critically involved in the pathophysiology of myocardial hypertrophy and apoptosis, we studied the Ang II as one of the factors involved in the exacerbation of myocardial hypertrophy in Gal-3 KO mice. Although, we found that Ang II is an important factor involved in the development of myocardial hypertrophy in aged Gal-3KO mice, we cannot rule out that other components of RAS may be increased. In rodents, chronic treatment with AT1-receptor blockers has been found to prevent cardiac remodeling and mortality associated with aging [38, 43]. We found that cardiac expression of Ang II was two-fold higher in aged Gal-3 KO than in control mice. Pharmacological treatment with ACE inhibitors or AT1-receptor blockers delayed the progression of age-related organ damage, particularly in the heart, kidney, and vessels [8, 38, 43]. Thus, the exacerbation of pathological hypertrophy observed in Gal-3 KO mice could be explained by the increase in the expression of Ang II. Although cardiac Ang II expression was increased in Gal-3KO mice, the mechanism of this association remains elusive and further studies are warranted to better assess the role of

Gal-3 in the regulation of the whole renin-angiotensin-system (RAS). In reference to these results, we decided to study the expression of Ang 1-7/Ang II for determining if the lack of Gal-3 shifted the RAS balance towards an increase of Ang II and reduction of ACE-2/Ang (1-7) axis in aged hearts. Previous studies in experimental models of hypertension, obesity and metabolic syndrome showed that ACE-2 and Ang 1-7 elicits protective cardiovascular effects [44, 45]. Moreover, in an animal model of Ang II induced hypertension it has been demonstrated that Ang 1-7 significantly attenuated the myocardial hypertrophy and fibrosis by mechanisms that involved SIRT independently of hypertension [6]. In this study, we did not find differences in cardiac expression of Ang 1-7 nor in the ratio between Ang 1-7/ Ang II in both groups, suggesting that the regulation of RAS by Gal-3 would be restricted to the classic RAS and independently of ACE-2/Ang (1-7) axis.

According to our results, the absent Gal-3 in aged KO mice suggests that elevated levels of Gal-3 reported in elderly patients and aged animals may antagonize the profibrotic and hypertrophic action of Ang II [23]. However, this requires further investigation.

During aging, the myocardium undergoes various intracellular changes that cause a decrease in the absolute number of cardiomyocytes, either by apoptosis, autophagy or necrosis [9]. In this study, we found that deletion of Gal-3 in aged mice led to increased cardiac apoptosis. An antiapoptotic function of Gal-3 has been previously demonstrated in experimental myocardial infarction [46] and damage induced by metabolic disorders [47], in agreement with our findings. After a proapoptotic stimulus, Gal-3 is translocated from the cytosol to the nucleus and prevents apoptosis by inhibiting mitochondrial membrane potentials [36]. However, contrasting results have also been reported by Li *et al*. and Zhang *et al*. who found that Gal-3 overexpression may promote cardiomyocytes apoptosis [48, 49]. Meanwhile, Ang II is proposed as a proapoptotic molecule that selectively induces cardiomyocyte apoptosis [50]. It has been also shown that Gal-3 is necessary for proper autophagy and cell repair, therefore we are not able to discard that adverse remodeling observed in aged Gal-3KO hearts may be a consequence of autophagy dysregulation. However, if Gal-3 is involved in the pathways of autophagy in cardiac aging should be determined. Thus, in our study, the lack of Gal-3 or the increase in Ang II individually or in combination may have led to increased apoptosis.

Extracellular matrix remodeling and fibrosis contribute to cardiac-specific senescence. In line with increased hypertrophy and higher expression of Ang II, we found that myocardial fibrosis and the expression of TGF-β were also exacerbated in aged Gal-3 KO mice and accompanied by increased MMP-9 mRNA expression. Gal-3 is upregulated in several fibrotic pathologies and regulates fibrosis through factors such as TGF-β and MMP [14, 18, 19]. TGF-β is a powerful cytokine that contributes to cardiac fibrosis; its expression is increased when fibroblasts and cardiomyocytes are activated, causing collagen synthesis and deposition in areas where necrosis or apoptosis has occurred [51]. We previously showed that Gal-3 regulates the expression of TGF-β during the healing process after infarction [18]. However, all previous studies demonstrating the role of Gal-3 as a mediator of repair were performed on acute pathological conditions and in young mice, which highlights the importance of applying a variety of scenarios, such as aging, to the study of these physiological processes. Our study found that the elevated myocardial hypertrophy and Ang II expression observed in aged Gal-3 KO mice were accompanied by increased fibrosis and TGF-β expression compared with the control group. Our results are in line with Iacobini *et al*.'s aging model that showed increased kidney fibrosis and TGF-β expression in the same model of aging and with the same mice [26]. Both studies together confirm that Gal-3 is a key regulator of aging.

Finally, to further investigate the mechanisms of cardiac aging, we quantified the cardiac expression of SIRT1 and SIRT7. SIRTs are a family of evolutionarily conserved enzymes that act as nicotinamide adenine dinucleotide ($NAD^+$)-dependent deacetylases and ribosyl transferases that modulate aging, from yeast to mammalian [12]. In our study, we found that the pathological myocardial hypertrophy developed by Gal-3 KO mice significantly reduced the expression of SIRT1 and SIRT7. Our results are in agreement with previous findings in which SIRT7 KO mice developed cardiac hypertrophy and inflammatory cardiomyopathy

and were also characterized by an increase in fibrosis, where mild/moderate expression (2.5- to 7.5-fold overexpression) of SIRT1 in the heart attenuated age-dependent induction of hypertrophy, apoptosis/fibrosis, and consequent LV dysfunction, which were accompanied by decreases in the expression of senescence markers [11, 12]. SIRT1 has been proposed as a key regulator of MMP-9 expression [52, 53]. We found that cardiac expression of MMP-9 was increased in old Gal-3 KO mice, which supports previous findings that postulate that the overexpression of MMP-9 promotes cardiac hypertrophy [13]. These results suggest an association between Gal-3, MMP-9, and SIRT1 in age-related cardiac hypertrophy.

## Conclusion

Here we present novel experimental evidence showing that Gal-3 is a critical regulator of age-related cardiac remodeling in mice. Genetic deletion of Gal-3 increased mortality and exacerbated myocardial hypertrophy, fibrosis, and apoptosis in association with an increase in cardiac Ang II, MMP-9, and TGF-β expression and reduction in the longevity genes SIRT1 and SIRT7. Thus, our results strongly support the hypothesis that Gal-3 may be an upstream factor closely associated with the regulation of age-related cardiac remodeling and may be responsible for the cardio-protective regulation of multiple factors involved in mice aging.

*Study limitations*

Aged-related cardiac remodeling is the final result of the temporal evolution of several mechanisms that exponentially increase the rate of cardiovascular disease and mortality. In our study, the role of Gal-3 in age-related cardiac remodeling was analyzed at a single time point of 24 months. Although the animals found dead did not present visual signs of cardiac decompensation, the fact that we did not perform the necropsy on those animals, we are unable to determine the real cause of death. Consequently, we cannot determine when the Gal-3 KO mice began to exhibit signs of exacerbated cardiac hypertrophy and fibrosis or if the absence of Gal-3 was related to the higher mortality observed in the old Gal-3 KO mice. Additional studies are warranted to further understand the temporal evolution of these findings. In addition, cardiac remodeling associated with aging is accompanied by diastolic dysfunction. In this study, we only evaluated systolic function based on echocardiography; thus, additional studies investigating diastolic function are required to fully understand the role of Gal-3 in age-related cardiac remodeling.

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*Author Contributions*

FSFS, MCB, VM, IMS, VT, CS, AV, and GEG: Conceived and designed the protocol, performed the experiments, collected the data, performed the analysis, and wrote the paper. MS and MMG: processed the hearts, and performed the RIA and data analysis. FP and NG: processed the hearts, and performed and analyzed the RTq-PCR data as well as wrote the paper. TFC contributed to perform and analyzed the echocardiogram data; VM and CM performed the analysis of histopathological samples.

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*Statement of Ethics*

The experimental protocol conforms to internationally accepted standards and it was approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL) of BIOMED in line with the NIH's Guide for the Care and Use of Laboratory Animals.

## Disclosure Statement

The authors declare that no conflicts of interest exist.

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