


RESEARCH ARTICLE

Exercise and Cardiac Remodeling in Normal and Athletic States

## Exercise-mitigated sex-based differences in aging: from genetic alterations to heart performance

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### Abstract

The prevalence of cardiovascular diseases dramatically increases with age; therefore, striving to maintain a physiological heart function is particularly important. Our aim was to study the voluntary exercise-evoked cardioprotective effects in aged male and female rats, from genetic alterations to changes in heart performance. We divided 20-month-old female and male Wistar rats to control and running groups. After the 12-wk-long experimental period, echocardiographic measurements were performed. Afterwards, hearts were either removed for biochemical measurements or mounted into a Langendorff-perfusion system to detect infarct size. The following genes and their proteins were analyzed from heart: catechol-O-methyltransferase (Comt), endothelin-1 (Esm1), Purkinje cell protein-4 (Pcp4), and osteoglycin (Ogn). Recreational exercise caused functional improvements; however, changes were more prominent in males. Cardiac expression of Comt and Ogn was reduced as a result of exercise in aged males, whereas Pcp4 and Esm1 showed a marked overexpression, along with a markedly improved diastolic function. The key result of this study is that exercise enhanced the expression of the Pcp4 gene and protein, a recently described regulator of calcium balance in cardiomyocytes, and suppressed Comt and Ogn gene expression, which has been associated with impaired cardiac function. In addition, as a result of exercise, a significant improvement was observed in the size of infarct elicited by left anterior descending coronary artery occlusion. Our results clearly show that age and sex-dependent changes were both apparent in key proteins linked to cardiovascular physiology. Exercise-moderated fundamental genetic alterations may have contributed to the functional adaptation of the heart.

**NEW & NOTEWORTHY** Voluntary exercise has proved to be an effective therapeutic tool to improve cardiac function in aged rats with clearly visible sex differences. Long-term exercise is associated with decreased Ogn and Comt expression and enhanced presence of Pcp4 and Esm1 genes. Sex-dependent changes were also observed in the expression of the cardiovascular key proteins. Fundamental alterations in gene and protein expression may contribute to the improvement of cardiac performance.

exercise; aging; echocardiography; gene expression; Pcp4

### INTRODUCTION

Cardiovascular diseases (CVDs) are the leading cause of morbidity and mortality worldwide and have a pronounced prevalence with age (1, 2). Longevity and aging are the two sides of the coin as they depend on environmental and lifestyle factors, sex differences, and genetic markers as well.

Aging implies numerous pathological alterations in the cardiovascular system, among others. Cardiac aging is first characterized by molecular and cellular changes, which then lead to structural, morphological and functional abnormalities. Besides, older individuals tend to suffer from chronic inflammation called “inflamm-aging” that manifests itself in the appearance of inflammatory cytokines and oxidative

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stress. Although developing inflammation is a powerful risk factor in itself, cardiac remodeling further increases the chances of CVDs and the vulnerability of the heart (3, 4). Cardiac remodeling process is a compensatory mechanism to the loss of cardiomyocytes which affects extracellular matrix (ECM) structure (5). Cardiovascular homeostasis greatly depends on the structure of the ECM; thus, imbalanced collagen accumulation caused ECM disruption contributes to the appearance of pathological cardiovascular lesions (6). These adverse changes play an important part in the development of CVDs by deteriorating elastic properties in left ventricle (LV) and eventually causing diastolic dysfunction (7). Petretto et al. (8) demonstrated that LV mass (LVM) is partly influenced by genetic factors as well; they have found that osteoglycin (Ogn) is an effective regulator of LVM in human and animal studies. Namely, its increased expression was found to be associated with elevated LVM. In line with these observations, elevated Ogn expression was found in the case of myocardial infarction and coronary heart diseases in human studies as well (9). Besides Ogn, other principal genes might also be informative in the pathogenesis of CVD. Xie et al. (10) proved that the upregulation of the endogenous calmodulin regulator, Purkinje cell protein-4 (Pcp4), contributes to a compensatory mechanism against cardiac hypertrophy and arrhythmia. As Pcp4 is able to reduce the release of  $Ca^{2+}$  from the sarcoplasmic reticulum of cardiac cells, it is presumable that it has a prominent role in cardioprotection. Intracellular  $Ca^{2+}$  level in cardiomyocytes is affiliated with catechol-*O*-methyltransferase (Comt) enzyme function as well. Increased amount of catecholamines causes intracellular  $Ca^{2+}$  overload in myocytes and leads to cardiac dysfunction (11). Consequently, Comt, the enzyme that catalyzes catecholamine metabolism, has been also linked to CVD. It is related to a higher risk of adverse cardiovascular events, such as elevated systolic blood pressure and atherosclerotic disease (12); however, long-term physical exercise has been shown to be effective in reducing Comt activity in human studies (13).

The above-mentioned lifestyle factors have always been exceedingly important in maintaining cardiovascular health. Sedentary lifestyle has long been linked to unfavorable cardiovascular events and shortened life expectancy (14). According to today's perception, it is the leading contributor to the development and progression of CVDs (15). It is well documented that people with active lifestyles are less threatened by coronary artery diseases than sedentary individuals (16). Dozens of studies discuss that regular exercise contributes to the improvement of cardiovascular function by normalizing blood pressure, resting heart rate, and myocardial perfusion (17, 18). Regular exercise is able to lower the degree of lipid peroxidation and improve antioxidant properties in adult and in aged individuals as well (19). Moreover, endothelium and vascular network also indicate complex adaptive changes in response to exercise. Physical exercise is associated with increased vascularization and angiogenesis in intact and injured heart as well (20). Although the underlying mechanisms are still unclear, a recent study by Rocha et al. (21) proved that endothelin (Esm1) plays an important role in angiogenesis through the modulation of vascular endothelial growth factor (VEGF) in endothelial cells.

CVDs are responsible for approximately 17.9 million deaths per year (22); therefore, it should be considered as a priority health issue. The characterization of molecular and genetic markers in connection with these pathophysiological abnormalities is pivotal for a deeper understanding of cardiac aging. Extensive data highlight the prominent part of exercise in the therapeutic options focused on cardiovascular health (18, 22, 23). Taken together, we hypothesized that recreational exercise may improve not only the functional properties of the heart and moderate the non-functional part of the infarcted myocardium but might also cause modifications in the expression of Comt, Ogn, Pcp4, and Esm1 genes in aged rats in a sex-dependent way.

## METHODS

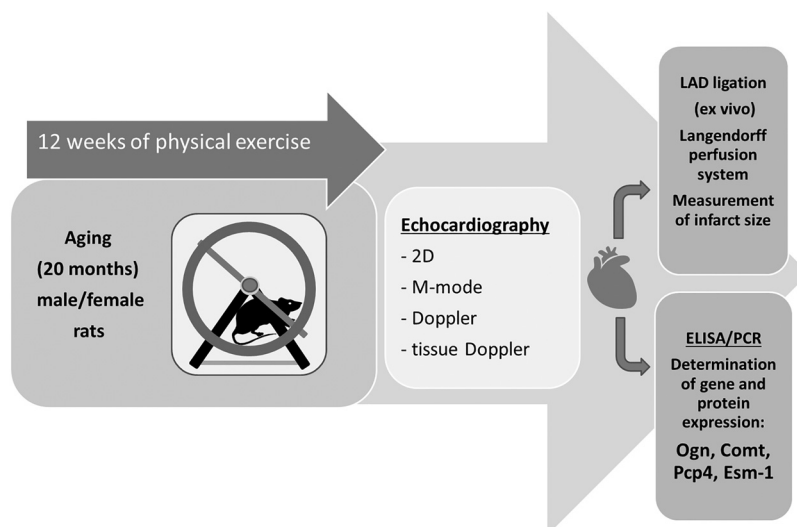
### Experimental Protocol

In this current study, 20-month-old male and female Wistar rats were used. Animals were kept at a standard 20–23°C temperature, and appropriate light/dark cycle was provided; all circumstances were accomplished according to Directive 2010/63/EU. At the beginning of the study, female and male rats were divided into two main groups: control (aging male, aging female) and running animals (aging male R, aging female R). Control rats were placed into standard cages, whereas running animals were provided with a running wheel-equipped cage. This protocol was defined as a voluntary exercise model; animals were not forced to exercise but had free access to the wheel 24 h a day. At the end of the 12-wk experimental period, echocardiographic measurements were performed under anesthesia. Following this procedure, all rats were euthanized and their hearts were excised. Hearts were randomly divided into two subgroups: 1) stored at –80°C until biochemical measurements or 2) subjected immediately to ex vivo isolated heart experiments. To detect the effects of voluntary exercise in the case of an acute injury, left anterior descending coronary artery (LAD) occlusion was applied ex vivo, via Langendorff system. The detailed experimental protocol is shown in Fig. 1. All procedures were approved by the National Scientific Ethical Committee on Animal Experimentation (XX/4801/2015, 25/2013DEMÁB) and complied with the ARRIVE guidelines.

### Transthoracic Echocardiography

Echocardiographic measurements of rats were carried out under intramuscular anesthesia (ketamine 50 mg/kg, xylazine 5 mg/kg) at the end point of the study. The chest of each animal was shaved, and the rats were positioned in a dorsal decubitus position. Data acquisition was performed using a Vivid E9 sonograph (GE Healthcare, Chicago, IL), with an i13L linear array probe at 14 MHz. Two-dimensional and M-mode measurements at parasternal long axis and short axis views were carried out as recommended by the American Society of Echocardiography (24). Electrocardiograms were recorded during all measurements. M-mode tracings were performed at the midpapillary muscle level, in either parasternal long or short axis views. Echocardiographic measurements included interventricular and left ventricular free-wall thickness in diastole and systole (IVSs, IVSd), left ventricular internal diameter at end-diastole (LVIDd) and

**Figure 1.** Detailed experimental protocol. LAD, left anterior descending artery; Ogn, osteoglycin; Comt, catechol-O-methyltransferase; Pcp4, Purkinje cell protein-4; Esm1, endothelin 1. This figure was created using modified Servier Medical Art templates, which are licensed under a Creative Commons Attribution 3.0 License; <https://smart.servier.com>



end-systole (LVIDs), aortic root diameter (Ao) and left atrial diameter (LA). Fractional shortening was computed by using the equation  $[(LVIDd - LVIDs)/LVIDd] \times 100\%$ , and the ejection fraction (EF) was calculated by the software of the sonograph. Relative wall thickness was calculated as  $2 \times LVPWd/LVIDd$ . Mitral annular peak systolic excursion (MAPSE) and tricuspid annular peak systolic excursion (TAPSE) were assessed with M-mode, measuring the distance of mitral or tricuspid annular movement between end-diastole to end-systole. All measurements were averaged over three to five consecutive cardiac cycles. Doppler (PW) imaging of the mitral, tricuspid, and aortic valves was obtained from the apical three-, four-, and five-chamber views. From the mitral inflow velocity image, the following measurements were obtained: left ventricular peak E and peak A waves (mitral early and late filling velocities) and the E- to A- ratio (E/A). Mitral valve closure to opening time (MCOT), ejection time (ET), isovolumic contraction and relaxation time (IVCT and IVRT, respectively) were measured, and myocardial performance index (MPI or Tei-index) was calculated. Left ventricular outflow tract (LVOT) parameters (velocity and pressure gradients) were also obtained: LVOT  $V_{max}$ ,  $V_{mean}$ ; LVOT maxPG, meanPG; and VTI. Pulsed wave Doppler measurements were also carried out on the right side of hearts, and RV E, RV A waves, as well as the E/A ratio of the right ventricle, were also determined. Tissue velocity imaging (TVI) measurements were analyzed from the apical four-chamber view and from the parasternal long axis and short axis views as well. A 2-mm tissue sampling volume was obtained at the mitral annulus from both septal and lateral walls. From the acquired images, the following functional parameters were measured:  $e'$  and  $a'$  wave velocities, septal  $e'/a'$  ratio,  $E/e'$  (early diastolic mitral inflow velocity divided by average value of septal tissue Doppler early diastolic velocity) (25).

EchoPAC PC software (GE Healthcare, Chicago, IL) was used to analyze the recorded measurements by a blinded reader. Shapiro-Wilk normality test was used to estimate Gaussian distribution, and Student's *t* test was applied to estimate differences between groups.

## Determination of Gene Expression

### RNA extraction and quantitative real-time PCR.

Total RNA was isolated from rat right and left ventricle tissues with Qiagen miRNeasy Mini Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). On-column DNase digestion was carried out with the RNase-Free DNase Set (Qiagen GmbH). The quality and quantity of the isolated RNA were measured with NanoDrop1000 Version 3.8.1. (Thermo Fisher Scientific, Waltham, MA). RNA samples were stored at  $-80^{\circ}\text{C}$  until use. Reverse transcription from  $2\mu\text{g}$  of total RNA was performed with the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) in a total volume of  $20\mu\text{L}$  according to the manufacturer's protocol.

After dilution with  $80\mu\text{L}$  of PCR-grade water (Roche Applied Science, Mannheim, Germany),  $1\mu\text{L}$  of the diluted reaction mix was used as a template in the quantitative real-time PCR (qRT-PCR) with FastStart Essential DNA Green Master (Roche Applied Science, Mannheim, Germany) using the following protocol: 10 min at  $95^{\circ}\text{C}$  followed by 45 cycles of  $95^{\circ}\text{C}$  for 15 sec.,  $60^{\circ}\text{C}$  for 25 s and  $72^{\circ}\text{C}$  for 25 s. The fluorescence intensity of SYBR Green dye was detected after each amplification step. Melting temperature analysis was done after each reaction to check the quality of the products. Primers were designed using the online Roche Universal Probe Library Assay Design Center. The quality of the primers was verified by MS analysis provided by Bioneer (Daejeon, Korea). Primer sequences and accession numbers are listed in Table 1. Relative expression ratios were calculated as normalized ratios to rat Hprt1 and Gusb genes. After amplification, the melting curve was checked to verify the specificity of the PCRs. The presented relative gene expression ratios were  $\Delta\Delta\text{CT}$  values ( $\log_2$ ).

### LightCycler 1536 system.

Identical reaction volumes were prepared by the Agilent Bravo Liquid Handling Platform (Agilent Technologies) in a  $24 \times 64$ -well design two times according to Agilent and Roche's recommendations. Each  $2\mu\text{L}$  reaction mixture

**Table 1.** Primers used in qRT-PCR analysis

Gene Symbol	Gene Product	Accession Number	Forward Primer	Reverse Primer
Comt	Catechol-O-methyltransferase	NM_012531.2	gcactgcatcaccagtagg	tgttattggcgtctggaca
Esm-1	Endothelial cell-specific molecule-1	NM_022604.2	gaccacgctcctgattcct	gcaatccaccgcatacttg
Ogn	Osteoglycin	NM_001106103.1	gagccacatgtaggcaatca	ttgctaagttggctctgtacca
Pcp4	Purkinje cell protein-4	NM_013002.4	ggagtcaggccaacatgag	gaccttctctgccatcatt

qRT-PCR, Quantitative real time polymerase chain reaction, Comt, catechol-O-methyltransferase; Esm1, endothelin-1; Ogn, osteoglycin; Pcp4, Purkinje cell protein-4.

contained 8 ng cDNA, 10 pmole gene-specific primers, and 1  $\mu$ L 2 $\times$  qPCRBIO SyGreen Mix (PCR Biosystems, London, UK). The following amplification protocol was used: 95°C for 2 min (activation), 60 cycles of 95°C for 10 s, and 60°C for 10 s, followed by 40°C for 10 s final cooling. Amplification was performed on the LightCycler 1536 System (Roche Applied Science) using 42 gene-specific primers and 2 reference genes. Data were collected and processed using the LightCycler 1536 SW 1.0 software. Curves were analyzed by using dynamic tube and slope correction methods. Relative expression of the analyzed genes was normalized to the mean.

### Determination of Cardiac Comt, Esm1, Ogn, Pcp4 Concentrations

Right and left ventricle tissues were homogenized in phosphate buffer (PBS) (pH 7.4) for 20 s and placed into the centrifuge for 20 min at 3,000 rpm, at 4°C. Centrifuged supernatants were collected and used for enzyme linked immune sorbent assay (ELISA; GenAsia, Shanghai). Standard solutions were prepared and added into the wells together with 50  $\mu$ l streptavidin-HRP. Into the sample wells, 40  $\mu$ l of supernatants, 10  $\mu$ l antibodies, and 50  $\mu$ l streptavidin-HRP were added. The plate was covered with a seal plate membrane and incubated for

**Table 2.** Echocardiographic outcomes

	Aging Male		Aging Female		Aging Male Running			Aging Female Running				
	mean	mean $\pm$ SE	mean	mean $\pm$ SE	vs. aging		vs. aging		vs. aging		vs. aging	
					male	female	male	female	male	female		
LA/Ao	0.8583	0.0514	0.8859	0.0697	ns	0.9776	0.0446	ns	0.8667	0.0602	ns	ns
RWT%	0.4331	0.0355	0.3467	0.0217	ns	0.4217	0.0228	ns	0.3792	0.0412	ns	ns
LV mass, g	0.9652	0.0546	0.6155	0.0763	##	0.9854	0.0621	ns	0.5744	0.0248	ns	###
EF (%)	76.3333	1.2019	75.8333	1.4926	ns	84.3333	0.4944	***	80.5000	0.2236	*	####
%FS	40.0000	1.0000	40.1667	1.3520	ns	48.6667	0.6667	****	44.0000	0.4472	*	###
MV E, m/s	0.5917	0.0259	0.5917	0.0138	ns	0.5983	0.0199	ns	0.6100	0.0289	ns	ns
MV A, m/s	0.4883	0.0190	0.4333	0.0217	ns	0.3400	0.0173	***	0.3400	0.0350	*	ns
MV E/A	1.1800	0.0410	1.4050	0.0992	ns	1.8050	0.1129	***	1.8583	0.2474	ns	ns
DecT, ms	64.8333	1.4240	44.5000	2.0777	####	50.8333	1.4701	****	41.6667	1.4063	ns	#
MAPSE, mm	1.8840	0.1060	1.9232	0.1272	ns	2.3668	0.1411	*	2.4280	0.0762	**	ns
LVOT V <sub>max</sub> , m/s	0.8183	0.0192	0.6367	0.0311	###	0.7933	0.0332	ns	0.7583	0.0521	ns	ns
LVOT maxPG, mmHg	2.6983	0.1171	1.6367	0.1746	###	2.5300	0.2010	ns	2.3483	0.3018	ns	ns
HR, beats/min	229.8333	8.2033	246.6667	10.7072	ns	275.5000	10.8374	**	225.6667	7.8387	ns	#
Sept E', mm/s	30.5000	2.5658	30.5000	1.6882	ns	43.3333	2.7528	**	42.3333	3.9805	*	ns
Sept A', mm/s	49.6667	6.6767	44.3333	1.9607	ns	55.5000	6.7614	ns	40.3333	2.9515	ns	ns
Sep E'/A'	0.6343	0.0403	0.6894	0.0286	ns	0.8175	0.0779	ns	1.0629	0.1006	**	ns
E/e'	19.8905	1.4415	19.7083	1.1878	ns	14.1169	1.0534	**	14.7956	0.9315	**	ns
ET, ms	73.0000	2.0656	64.0000	3.1728	#	69.5000	2.7049	ns	71.3333	3.3133	ns	ns
Tei-Index	0.7977	0.0501	0.9070	0.0545	ns	0.6146	0.0460	*	0.6937	0.0509	*	ns
IVCT, ms	18.6667	1.9437	18.5000	1.3844	ns	15.3333	1.3824	ns	18.0000	2.5430	ns	ns
IVRT, ms	39.3333	1.6262	32.8333	0.9458	##	26.6667	2.0440	***	30.3333	1.8012	ns	ns
RV E/A	0.7900	0.1272	0.6167	0.0562	ns	0.7517	0.1024	ns	0.8283	0.0511	*	ns
TAPSE, mm	2.7415	0.2194	2.3223	0.1003	ns	2.8892	0.1276	ns	2.6580	0.2530	ns	ns
SV, ml	0.5592	0.0790	0.3356	0.0419	#	0.5428	0.0504	ns	0.4159	0.0339	ns	ns
CO, ml/min	127.7507	16.7011	82.4105	10.2439	#	147.9433	10.6617	ns	93.9640	8.4432	ns	#

Shapiro-Wilk method was used to estimate data distribution, and two-tailed Student's *t* test was performed to estimate differences between groups. Result shown as means  $\pm$  S.E.; *n* = 6/group. LA/Ao, ratio of left atrial and aortic diameters; RWT%, relative wall thickness; LVmass, left ventricle mass; FS, fractional shortening of the left ventricle; EF, ejection fraction of the left ventricle; SV, stroke volume of the left ventricle; CO, cardiac output of the left ventricle; MAPSE, mitral annular plane systolic excursion; E/A, peak mitral early diastolic inflow velocity/peak atrial diastolic inflow velocity of the left ventricle; DecT, E wave deceleration time; HR, heart rate; E/e', peak mitral inflow velocity/average of spectral tissue Doppler peak early diastolic velocities at the lateral corner of mitral annulus; LVOT V<sub>max</sub>, maximal velocity of left ventricle outflow; LVOT maxPG, maximum pressure gradient of left ventricle outflow tract; e'/a', peak early diastolic velocity of the septal wall/peak atrial diastolic velocity of the septal wall; MPI or Tei-index, myocardial performance index; IVCT, isovolumic contraction time; IVRT, isovolumic relaxation time; ET, ejection time; RV E/A, peak early diastolic inflow velocity/peak atrial diastolic inflow velocity of right ventricle; TAPSE, tricuspid annular plane systolic excursion.\*Statistical significance between running and non-running counterparts; #statistical significance between matching female and male groups. Symbols denote the level of significance: \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001; \*\*\*\**P* < 0.0001; and #*P* < 0.05; ##*P* < 0.01; ###*P* < 0.001; ####*P* < 0.0001; ns, not significant.

60 min at 37°C. After incubation, the plate was washed and chromogen solutions A and B were added to the wells, which was followed by a 10-min incubation at 37°C. To stop the reaction, stop solution was added to each well, absorbance (OD) was measured under 450 nm wavelength. Protein determination was performed with Bradford method and measured spectrophotometrically at 595 nm. Comt, Ogn, and Pcp4 levels were expressed as pg/ $\mu$ g protein; Esm1 values were defined as pg/mg protein.

### Implementation of Ischemia-Reperfusion Injury

Rats were anesthetized, and their hearts were removed appropriately. Using an ice-cold Krebs-Henseleit buffer (1.24 mM  $\text{KH}_2\text{PO}_4$ , 20.1 mM  $\text{NaHCO}_2$ , 11.2 mM glucose, 119 mM NaCl, 1.25 mM  $\text{CaCl}_2$ , 4.7 mM KCL, 1.24 mM  $\text{MgSO}_4$ ), hearts were tied up to a Langendorff perfusion system and a retrograde perfusion was started through the aorta. The perfusion was performed under the following constant conditions: 75 mmHg pressure, 5%  $\text{CO}_2$ -95%  $\text{O}_2$ , 37°C. By occluding the LAD for 30 min, the ischemic injury was evolved, which was followed by a 120-min reperfusion process. At the end of the reperfusion, LAD occlusion and perfusion were stopped and Evans blue solution (1%) was injected to the aorta, which led to the exposure of the area at risk. Hearts were placed into  $-20^\circ\text{C}$  for overnight.

### Determination of Infarct Size

Hearts frozen overnight were sliced into 2-mm-thick pieces and dipped into 1% 2,3,5-triphenyltetrazolium chloride (TTC) staining solution for 10 min. Slices were immersed in a 10% formalin solution, which was followed by a phosphate buffer (pH 7.4) rinse. Both sides of the heart slices

were photographed with a camera, and infarct size was computed as the percentage of the area at risk.

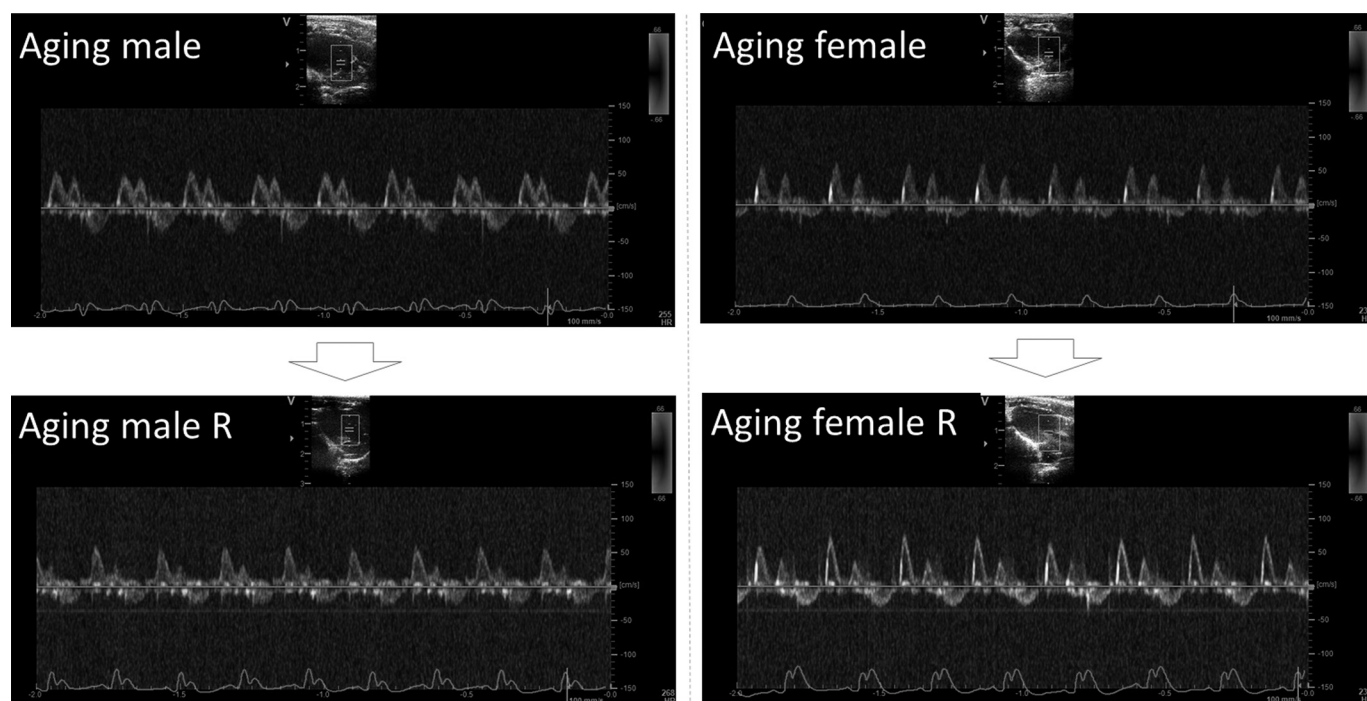
### Data Representation and Statistical Analysis

Data are presented as the average outcome in a group (mean  $\pm$  SE) or as minimum to maximum and mean, with individual data points (the way of data representation is indicated in figure and table legends). Shapiro-Wilk normality test was used to estimate Gaussian distribution. Differences between groups were calculated using two-tailed Student's *t* test, and probability values ( $P \leq 0.05$ ) were considered significant. Figures were edited with GraphPad Prism 8.4.2. (San Diego, CA).

## RESULTS

### Echocardiographic Analyses

All echocardiographic examinations were completed within 15 minutes with stable heart rates and respiratory frequencies during the procedure. FS and EF of running male and running female animals were significantly increased in comparison with this outcome evaluated in aged animals of matched control groups (Table 2). The increase in EF and FS was more prominent in running male rats than in running females (EF,  $P < 0.0001$  and  $P = 0.0114$ ; FS,  $P < 0.0001$  and  $P = 0.0226$ , respectively). No significant differences were found in relative wall thickness among groups. Left ventricle systolic function was also evaluated measuring mitral annular plane systolic excursions: MAPSE values of both running male and running female groups increased in comparison to nonrunning counterparts ( $P = 0.0210$  and  $P = 0.0067$ , respectively).



**Figure 2.** Representative Doppler echocardiographic images of transmitral inflow from listed groups. R, running.

Diastolic function of the left ventricle was slightly improved in both running groups, but the changes were again more prominent in running male rats (Fig. 2). Transmitral A wave velocity decreased significantly in running male rats in comparison to aging males ( $P = 0.0002$ ), thus E/A ratio increased ( $P = 0.0004$ ). A wave velocity tended to decrease in running females ( $P = 0.0470$ ) and E/A ratio failed to improve in comparison to aging female controls. E wave deceleration time (DecT) was relatively lengthened in aging males, but significantly shortened in running males ( $P < 0.0001$ , compared to aging males). Value of DecT remained in the normal range in aging females and was unchanged in running females. Left ventricle outflow tract velocities and pressure gradients also remained unchanged. Tissue Doppler echocardiography revealed that TDI  $e'$  wave increased in both running male and running female rats in comparison to nonrunning counterparts ( $P = 0.0067$  and  $P = 0.0209$ , respectively). The ratio of  $e'/a'$  increased in running females compared to aging females ( $P = 0.0051$ ) and showed trend toward increase in running males versus nonrunning animals ( $P = 0.0632$ ). The ratio of E/ $e'$  (indicative for left ventricle filling pressure) improved significantly in both running male and

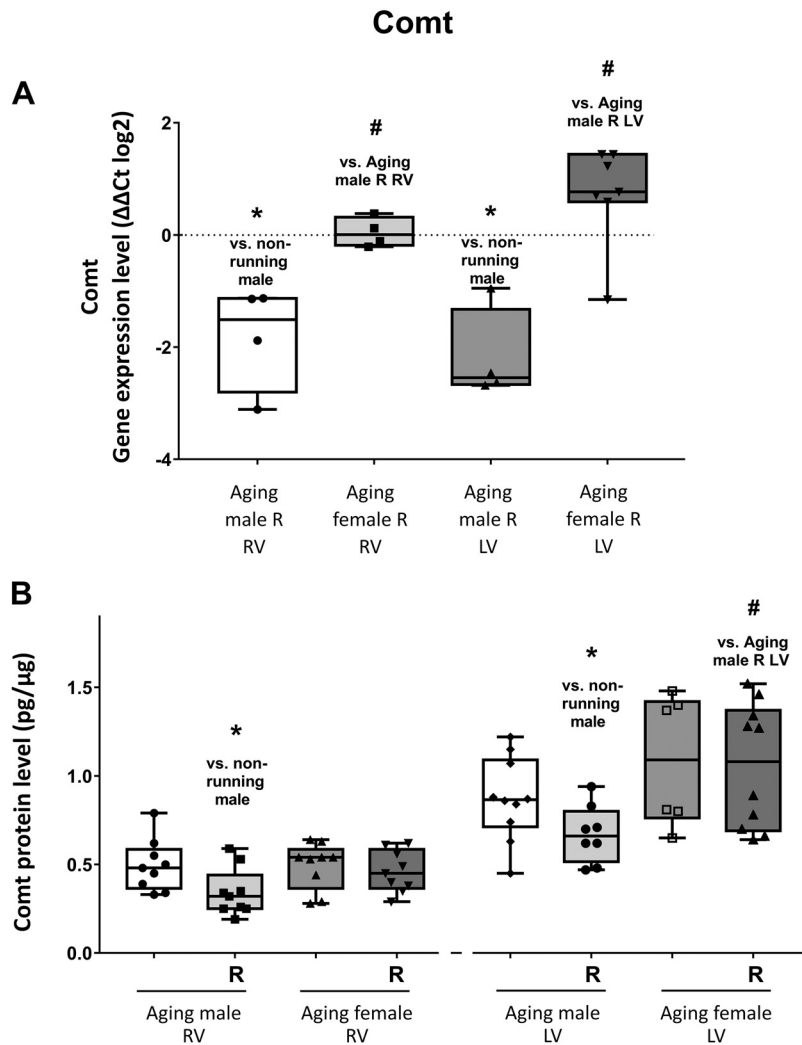
running female groups in comparison to matched aging controls ( $P = 0.0090$  and  $P = 0.0087$ , respectively). Isovolumic relaxation time (IVRT) was relatively long in aging males, but significantly shortened in running males ( $P = 0.0007$ ). This parameter failed to improve in female rats. Tei-index decreased in both running females ( $P = 0.0170$ ) and running males ( $P = 0.0226$ ) compared to aging controls. In relation to right ventricle function, TAPSE parameters remained unchanged among groups; however, a slight improvement could be detected in right ventricle diastolic function expressed as RV E/A ratio in running female animals compared to aging female controls ( $P = 0.0192$ ).

### Determination of Gene Expression and Protein Concentrations

Gene expression alterations are represented relative to aging control groups; 12-wk voluntary exercise-caused changes are depicted.

#### Comt.

As shown in Fig. 3A, a significant repression was found in the expression level of Comt in both ventricles of male



**Figure 3.** A: the effects of a 12-wk-long voluntary exercise on Comt gene expression in the right and left ventricles of the heart, evaluated by RT-PCR (Comt; change in relative gene expression ratios are shown as  $\Delta\Delta CT$  values ( $\log_2$ )). Data represented as minimum to maximum;  $n = 4-7$ /group (individual data points are visible on the graph). Shapiro-Wilk method was used to estimate data distribution, and two-tailed Student's  $t$  test was performed to estimate differences between corresponding running and nonrunning (\*) and between matching male or female (#) groups. B: the effects of a 12-wk-long voluntary exercise on Comt protein expression in the right and left ventricles of the heart, measured by ELISA method. Comt protein expression is shown for each group. After Shapiro-Wilk test, two-tailed Student's  $t$  test was performed to estimate differences between corresponding running and nonrunning (\*) and between matching male or female (#) groups. (Comt, expressed as pg/ $\mu$ g protein). Result shown as minimum to maximum;  $n = 6-9$ /group (individual data points are shown for each group). \* $P < 0.05$ : statistical significance between running and non-running counterparts; # $P < 0.05$ : statistical significance between matching female and male groups. Comt, catechol-O-methyltransferase; RV, right ventricle; LV, left ventricle; R, running.

animals as a result of physical exercise. However, no significant change was observed in the case of running female group. Figure 3B shows that a significant decrease was observed in the level of cardiac Comt protein in the right and left ventricles of male rats as a result of exercise. Similarly to gene expression, no significant changes were observed in heart of female animals. Moreover, sex-dependent changes were also observed in the cardiac gene and protein expression of Comt.

#### Ogn.

Figure 4A shows that cardiac Ogn expression was significantly suppressed in male running group due to the 12-wk-long exercise training. In contrast, in the heart of running female animals, no substantial change was noted at gene expression level. In line with gene expression, Ogn protein levels were also attenuated in running male rats. Moreover, a further decrease was found in the RV of females as a result of physical activity, as shown in Fig. 4B. Regarding Ogn gene and protein expression, we also observed sexual dimorphism which was considered to be significant.

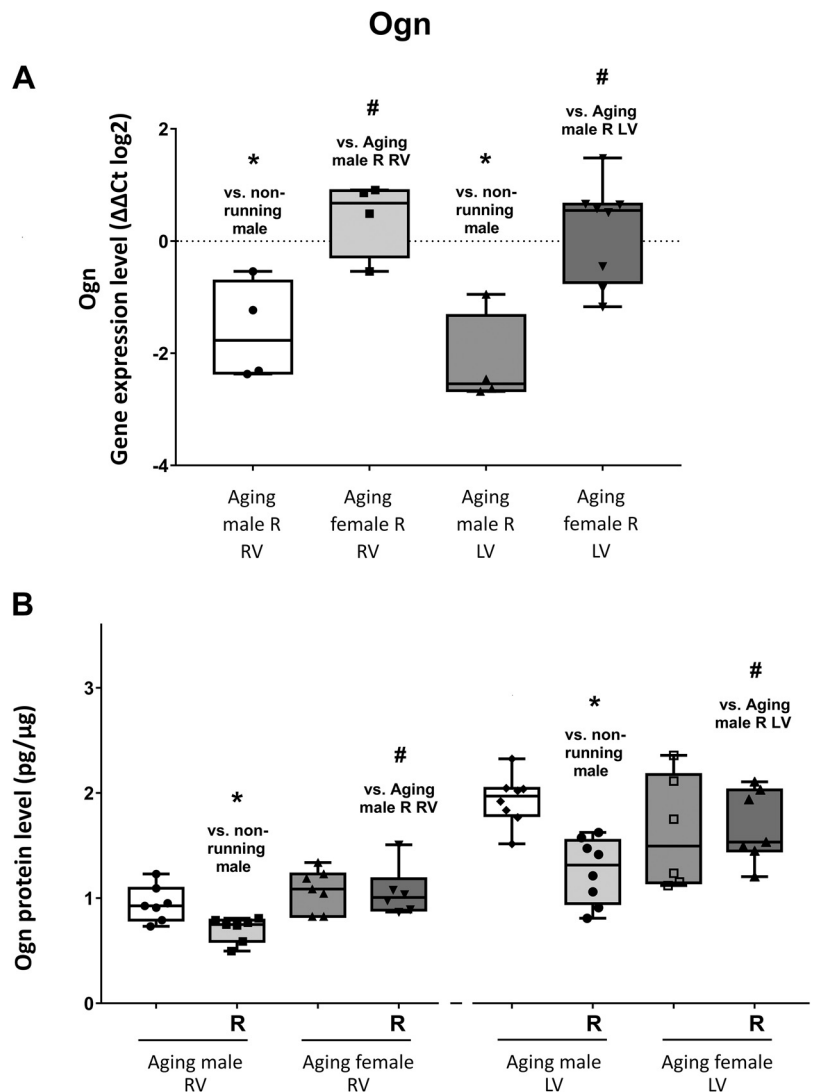
#### Pcp4.

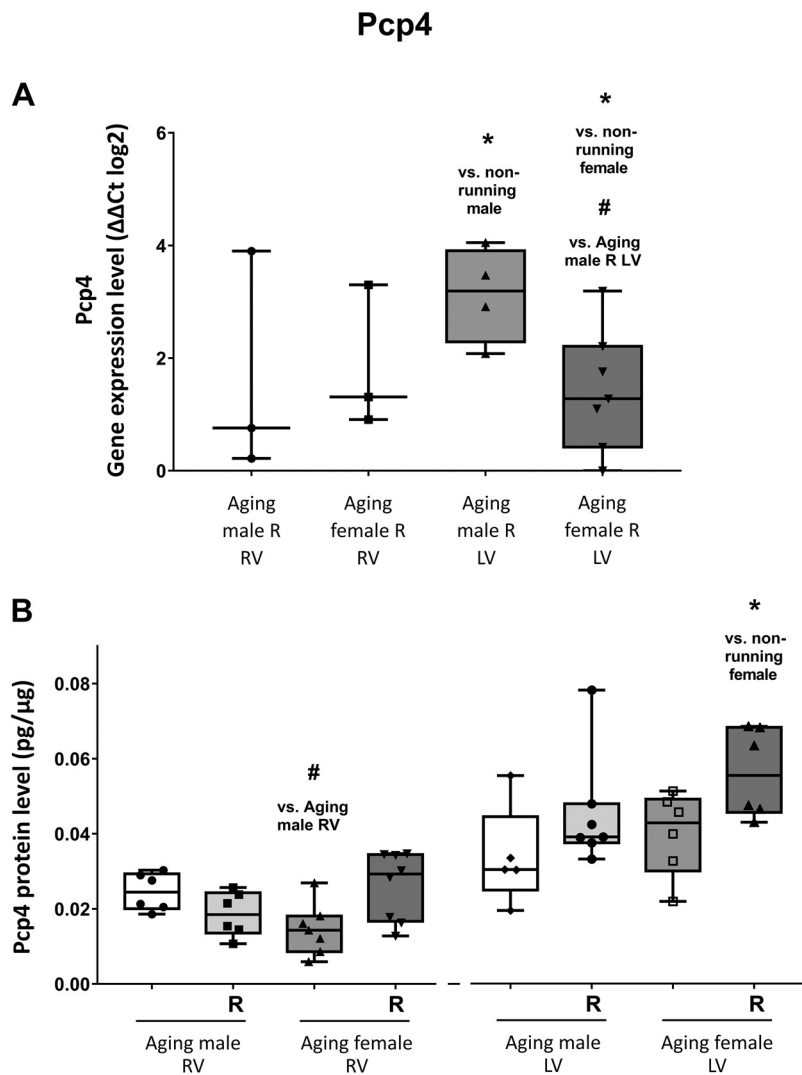
Voluntary exercise training resulted in a significant overexpression of Pcp4 gene in the LV of both running male and female rats. Moreover, Pcp4 expression displayed an elevation in the RV of both groups, but this change could not be considered significant. In the case of protein expression, an increase was noted in both ventricles of the two sexes, and changes in the female LVs were considered to be significant. In terms of cardiac Pcp4 protein and gene expression, a sex-based difference was also obtained. Data are presented in Fig. 5, A and B.

#### Esm1.

A stable overexpression of cardiac Esm1 gene was noted as a result of wheel-running exercise in both female and male rats compared to nonrunning counterparts, in addition changes in the LVs of both sexes were found to be significant. Voluntary exercise caused an elevation in the concentration of Esm1 protein in the RV of male rats, and in the heart of female rats, which alteration was found to be significant. Furthermore, significant sex-dependent changes were

**Figure 4.** A: the effects of a 12-wk-long voluntary exercise on Ogn gene expression in the right and left ventricles of the heart, evaluated by RT-PCR (Ogn; change in relative gene expression ratios are shown as  $\Delta\Delta\text{CT}$  values ( $\log_2$ )). Data represented as minimum to maximum;  $n = 4-8/\text{group}$  (individual data points are visible on the graph). Shapiro-Wilk method was used to estimate data distribution, and two-tailed Student's  $t$  test was performed to estimate differences between corresponding running and nonrunning (\*) and between matching male and female (#) groups. B: the effects of a 12-wk-long voluntary exercise on Ogn protein expression in the right and left ventricles of the heart, measured by ELISA method. Ogn protein expression is shown for each group. After Shapiro-Wilk test, two-tailed Student's  $t$  test was performed to estimate differences between corresponding running and nonrunning (\*) and between matching male or female (#) groups. (Ogn; expressed as  $\text{pg}/\mu\text{g}$  protein). Result shown as minimum to maximum;  $n = 6-8/\text{group}$  (individual data points are shown for each group). \* $P < 0.05$ : statistical significance between running and nonrunning counterparts; # $P < 0.05$ : statistical significance between matching female and male groups. Ogn, osteoglycin; RV, right ventricle; LV, left ventricle; R, running.





**Figure 5.** *A*: the effects of a 12-wk-long voluntary exercise on Pcp4 gene expression in the right and left ventricles of the heart, evaluated by RT-PCR (Pcp4; change in relative gene expression ratios are shown as  $\Delta\Delta C_t$  values ( $\log_2$ )). Data represented as minimum to maximum;  $n = 3-7$ /group (individual data points are visible on the graph). Shapiro-Wilk method was used to estimate data distribution, and two-tailed Student's *t* test was performed to estimate differences between corresponding running and nonrunning (\*) and between matching male or female (#) groups. *B*: the effects of a 12-wk-long voluntary exercise on Pcp4 protein expression in the right and left ventricles of the heart, measured by ELISA method. Pcp protein expression is shown for each group. After Shapiro-Wilk test, two-tailed Student's *t* test was performed to estimate differences between corresponding running and nonrunning (\*) and between matching male or female (#) groups. (Pcp; expressed as pg/ $\mu$ g protein). Result shown as minimum to maximum;  $n = 5-9$ /group (individual data points are shown for each group). \* $P < 0.05$ : statistical significance between running and non-running counterparts; # $P < 0.05$ : statistical significance between matching female and male groups. Pcp4, Purkinje cell protein-4; RV, right ventricle; LV, left ventricle; R, running.

also observed in the cardiac gene and protein expression of Esm1. Data are shown in Fig. 6, *A* and *B*.

### Measurement of Myocardial Infarct Size

According to Fig. 7, aging animals have shown a higher rate of infarct size in comparison with running counterparts and male rats represented worse outcomes. Nevertheless, as a result of the 12-wk voluntary exercise, we observed a pronounced decrease in the infarcted area of running females. In addition, a significant attenuation was ascertained in the necrotic extension of the heart in running males.

## DISCUSSION

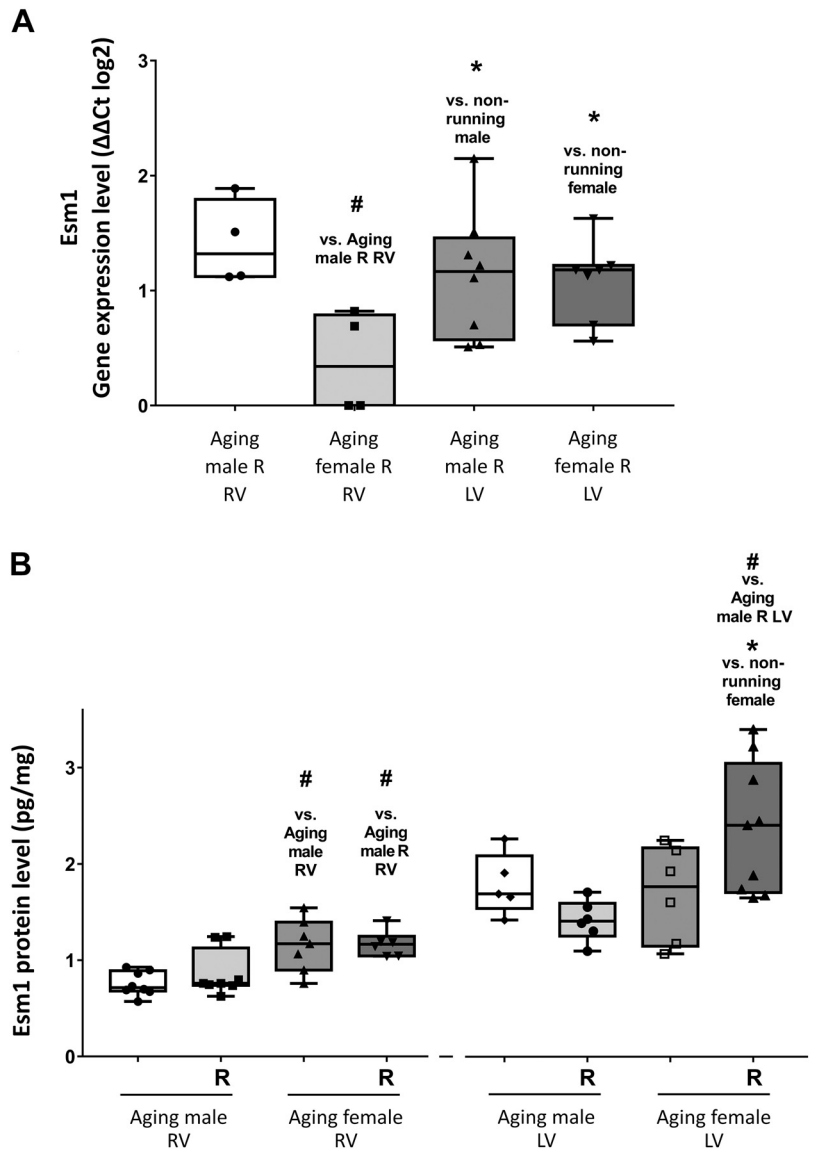
In our present study, we clearly demonstrated the prominent role of 12-wk voluntary exercise in improvement of age-related cardiovascular dysfunctionality in aged female and male rats. Our results revealed that a maintained physical activity improved cardiovascular performance particularly in aged males by affecting functional parameters and moderating the nonfunctional area in the infarcted myocardium. Moreover, it greatly affected the gene expression in both

ventricles. Obviously, it has already revealed that female sex tend to be protected against ischemia and other pathological heart diseases or even aging (26). In our recent study, cardiac function of male animals deteriorated more dramatically when compared to the females. Hence, improvement of systolic and diastolic cardiac function was more prominent in aged male animals after exercise training, along with a decreased infarcted area in hearts subjected to ischemic injury. Our main finding is that these favorable changes were accompanied by a decreased expression Comt and Ogn genes in the myocardium, whereas Pcp4 expression was found to be significantly increased.

The outcomes of echocardiographic measurements revealed the beneficial effects of exercise training on aged male and female rats. In comparison to nonrunning counterparts, left ventricular EF and FS measured in running male and running female animals were significantly increased. Moreover, MAPSE values of running groups were significantly increased compared to aged controls. These parameters together show a marked improvement in left ventricular systolic function in both male and female rats receiving exercise training. Data obtained



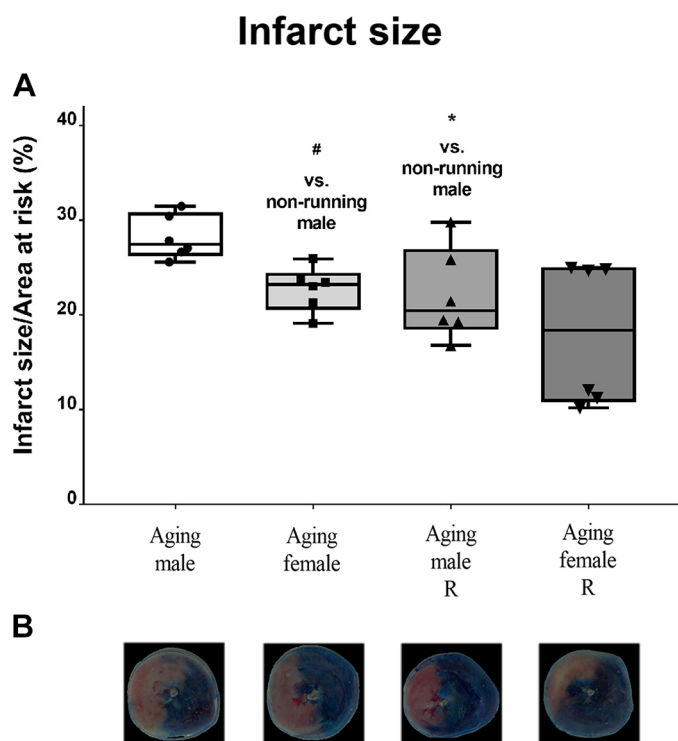
## Esm1



**Figure 6.** A: the effects of a 12-wk-long voluntary exercise on Esm1 gene expression in the right and left ventricles of the heart, evaluated by RT-PCR (Esm1; change in relative gene expression ratios are shown as  $\Delta\Delta Ct$  values ( $\log_2$ )). Data represented as minimum to maximum;  $n = 4\text{--}8/\text{group}$  (individual data points are visible on the graph). Shapiro-Wilk method was used to estimate data distribution, and two-tailed Student's  $t$  test was performed to estimate differences between corresponding running and nonrunning (\*) and between matching male or female (#) groups. B: the effects of a 12-wk-long voluntary exercise on Esm1 protein expression in the right and left ventricles of the heart, measured by ELISA method. Esm1 protein expression is shown for each group. After Shapiro-Wilk test, two-tailed Student's  $t$  test was performed to estimate differences between corresponding running and nonrunning (\*) and between matching male or female (#) groups. (Esm1; expressed as pg/mg protein). Result shown as minimum to maximum;  $n = 5\text{--}9/\text{group}$  (individual data points are shown for each group). \* $P < 0.05$ : statistical significance between running and non-running counterparts; # $P < 0.05$ : statistical significance between matching female and male groups. Esm1, endothelin 1; RV, right ventricle; LV, left ventricle; R, running.

from Doppler imaging expose that diastolic function of the left ventricle was significantly improved in running male rats, illustrated by increased E/A ratio (decreased atrial wave velocity), normalized DecT, and IVRT. Since prolonged DecT and IVRT indicate impaired myocardial relaxation, relatively shorter durations of these parameters in running male group point out an improved diastolic function associated with exercise training (27). These changes were absent in the running female group in comparison to aging female controls; however, it needs to be indicated that diastolic function of the aging female rats was relatively preserved. Tissue Doppler echocardiography also revealed that  $e'$  wave velocity increased, while E/ $e'$  ratio (indicative for LV filling pressure) decreased in both male and female groups as a result of exercise training in comparison to nonrunning counterparts, and  $e'/a'$  ratio slightly improved in running female

rats, indicating better myocardial motility. In conclusion, aging male animals showed mild diastolic dysfunction (impaired relaxation and elevated LV filling pressures) which was absent in male counterparts received exercise training. Differences in diastolic parameters were less marked in female rats, as echocardiographic parameters of aging female group only showed mild impairment. Myocardial performance index is a valuable parameter expressing both systolic and diastolic ventricle function (28). Elevated values of MPI were found in aging female group, which was significantly counteracted by exercise, showing beneficial effects of training on global heart functions. Male animals only showed trends toward decrease in Tei-index. Right ventricle E/A ratios were significantly increased in running female animals in comparison to aged females, and no differences were found in TAPSE values among groups. These observations are in



**Figure 7.** A: the effects of a 12-wk-long voluntary exercise on the extension of the infarct size, evaluated by TTC staining after I/R injury. Infarct size was lower in the aged females, and voluntary exercise decreased infarcted area in the aged male rats compared to the nonrunning counterparts. Shapiro-Wilk method was used to estimate data distribution, and two-tailed Student's *t* test was performed to estimate differences between corresponding running and nonrunning (\*) and between matching male or female (#) groups. Infarct size was calculated as the percentage of the area at risk. Results are shown as minimum to maximum and mean; *n* = 6/group. \**P* < 0.05: statistical significance between running and nonrunning counterparts; #*P* < 0.05: statistical significance between matching female and male groups. R, running. B: representative images of TTC-stained rat heart sections from listed groups, 24 hours after I/R injury. Images are positioned below the corresponding groups.

line with several human clinical studies, which corroborate the cardiac-improving effects of exercise (29–31). Here we showed that aging affects both systolic and diastolic parameters of the rat heart, but diastolic impairment is more significant in males. Voluntary exercise training successfully improved systolic function in both groups (EF, FS, MAPSE, Tei-index), but its effects were more prominent in male rats, since all signs of the early diastolic dysfunction (decreased E/A ratio, lengthened DecT and IVRT, and increased E/e') were absent in the running male group, in contrast to sedentary aging controls.

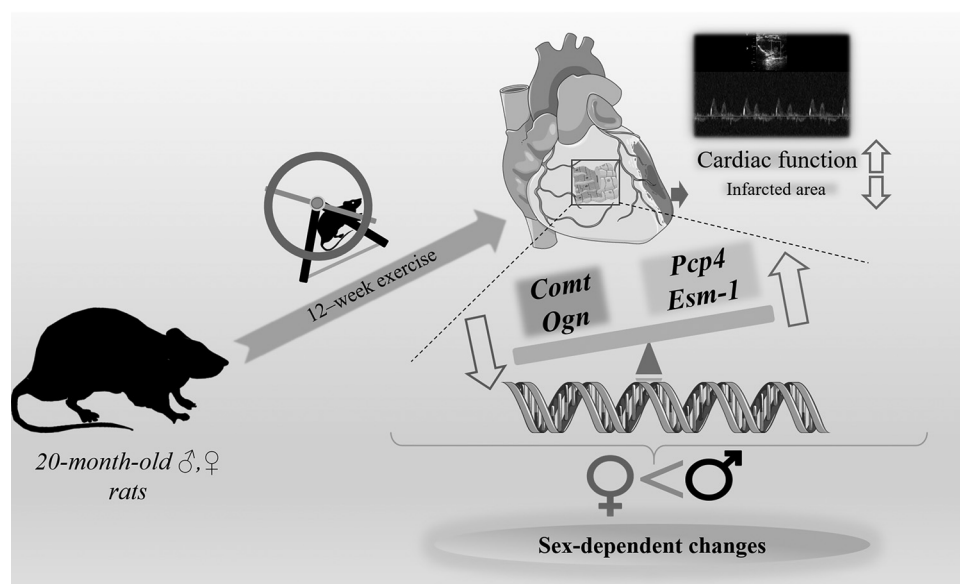
Sex-dependent changes were also observed in the expression of genes (Comt, Ogn, Pcp4, Esm1) in the myocardial tissue in response to aging and exercise training. Comt is a key enzyme in the degradation of catecholamines and catechol estrogen, hence its role in the cardiovascular regulation is substantial. A number of studies support that elevated catecholamine levels implicate cardiac dysfunction and induced intracellular calcium overload in cardiomyocytes (11). Long-term exercise training improves heart function by lowering blood pressure and heart rate, which is considered to be an evidence of decreased catecholamine levels. Moreover, low

activity of the Comt gene is associated with a decreased risk of myocardial infarction in human subjects (32). It is noteworthy that Graham et al. (33) discussed that MAO-B inhibitors and COMT inhibitors do not increase mortality compared to placebo; however, the link between the COMT inhibitors and acute myocardial infarction remains to be proven.

We verified that 12-wk exercise resulted in a significant suppression of Comt gene expression in the heart of male rats; however, the aforementioned change was not observed in female animals. Similar to gene expression, protein levels of cardiac Comt were decreased as a result of voluntary exercise, which was seen in both sexes. Carneiro et al. (13) first reported the plausible connection between exercise therapy and decreased Comt expression which is consistent with our results. These findings suggest that the attenuated presence of Comt can be proportional to the reduced level of catecholamines, thus improved cardiac function in response to exercise training.

Ogn (osteoglycin or mimecan) is reportedly expressed in fibroblasts, vascular smooth muscle cells, and cardiomyocytes, however, its precise role in subcellular signaling is yet to be determined. Ogn expression was found to be increased in the infarcted myocardium, where it may play a role in collagen maturation (34). Also, Ogn expression was increased in patients with coronary heart disease (CHD) and correlated to the severity of coronary lesions (9). Here, we found decreased expression of the Ogn gene and protein in the ventricles of the running male group, in line with the improved echocardiographic markers and reduced infarcted area. Although our knowledge of Ogn function in the trained heart is limited, our results are further corroborated by Feng and colleagues (35), who reported a similar decrease in Ogn expression after a 6-wk exercise training in the aortic wall of hypertensive rats. Additionally, injection of irisin, a recently described pro-myogenic factor (exercise-induced myokine) also promotes the down-regulation of the Ogn gene in the skeletal muscle (36). Regarding translational aspects, our knowledge is limited about Ogn function; furthermore, the expression of Ogn may be not only tissue-dependent, but also species-dependent. In contrast to data obtained in rodent models, proteoglycan genes (biglycan, osteoglycin, serglycin) in human striated muscles are rather increased in response to exercise, indicating extracellular matrix remodeling (37). The picture is further complicated considering that Ogn is posttranscriptionally regulated by micro-RNAs (miR-22) for the control of proliferation and differentiation of muscle cells during myogenesis (38). Additionally, Ogn was recently identified as a cardiac hypertrophy marker, and its increased expression was associated with elevated left ventricle mass (8). As regards our data, we did not find significant differences in left ventricle mass of running and sedentary groups. Notwithstanding, this result was expected, as according to previous studies, a 12-wk-long voluntary exercise (unlike forced training or swimming) is not sufficient to cause significant cardiac hypertrophy and elevated LV mass (39). Physiological stimuli (voluntary exercise) may rather decrease cardiac hypertrophy and remodeling along with elevated Ogn levels (8). Although demonstration of the Ogn mechanism causing a hypertrophic response requires further investigation, it is proved to be a decisive factor in this

**Figure 8.** Summary of the study. Exercise was associated with decreased Ogn and Comt expression; the presence of Pcp4 and Esm1 was enhanced in aged rats. Sex-dependent changes were also observed in the expression of the cardiovascular key proteins. Comt, catechol-O-methyltransferase; Esm1, endothelin-1; Pcp4, Purkinje cell protein-4; Ogn, osteoglycin. This figure was created using Servier Medical Art templates, licensed under a Creative Commons Attribution 3.0 License; <https://smart.servier.com>



process. As Ogn expression was found to be decreased in the myocardium of the running male animals, we conclude that in terms of exercise, Ogn is rather a marker of susceptibility to MI than cardiac hypertrophy in the aged rat. These results suggest that exercise training–caused Ogn suppression may ameliorate aging-associated adverse changes of the heart.

Pcp4 (or PEP-19) encodes a small IQ-motif protein that is a  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (CaMKII) regulator, thus it plays a major role in the intracellular calcium homeostasis (40). This system has recently been proposed to counteract cardiac hypertrophy and apoptosis in disease models (10). Kim et al. previously observed ventricular arrhythmias in a Pcp4-null model in vivo and proved the cardiac excitability modulator properties of Pcp4 (41). In addition, they showed that Pcp4 is down-regulated in disease models (aortic banding, phenylephrine infusion), along with marked echocardiographic changes showing impaired cardiac function and hypertrophy. Consistently, Xie et al. (10) showed that Pcp4 has antihypertrophic effects through inhibition of CaMKII and calcineurin (CaN) activation during development of cardiomyocyte hypertrophy. Pcp4 transfection inhibited apoptosis and protected HEK293T cells against death due to  $\text{Ca}^{2+}$  overload. In addition, Pcp4 reduced  $\text{Ca}^{2+}$  release from the sarcoplasmic reticulum of cardiomyocytes and also inhibited angiotensin-induced overload of calcium ions. As  $\text{Ca}^{2+}$  overload is a major cause of arrhythmias, hypertrophy, and increased susceptibility to ischemia-reperfusion (I/R) injury, it is proposed that Pcp4 plays an important role in cardioprotection via normalizing Ca handling of myocytes through the CaMKII system (42). Our current results show for the first time that a 12-wk-long exercise period was able to enhance the expression of Pcp4 gene in the heart of both male and female rats. Aligned with gene expression, Pcp4 protein levels were also elevated in the right ventricles of both sexes. These results suggest that the exercise-induced Pcp4 elevation may contribute to cardioprotection in the aged heart. Aging-associated calcium mishandling may directly affect echocardiographic diastolic function (IVRT,  $E/e'$ ) through the SERCA/PLB pathway, a major

regulator of cardiac relaxation (43, 44). Consistent to gene expression data, IVRT and  $E/e'$  parameters were significantly improved in running animals, further suggesting the normalized  $\text{Ca}^{2+}$  handling in cardiomyocytes, as a result of exercise. Pcp4 overexpression may contribute to intracellular calcium balance, normalized cardiac cell excitability; and counteract  $\text{Ca}^{2+}$  overload, a significant contributor of hypertrophy and increased susceptibility to I/R injury. Notwithstanding, as limitation of this study, these factors were not investigated and may be addressed in future investigations.

Esm1 has recently been associated with vascularization processes. By connecting to the VEGF signaling, it is suspected to play an important role in neoangiogenesis (45). It is well known that exercise is able to enhance VEGF production, which is one of the main inducers of Esm1 (21). In this study, we observed a strong overexpression of cardiac Esm1 in all groups as a result of the 12-wk voluntary exercise compared to nonrunning counterparts. In line with genetic modifications, Esm1 protein levels were also elevated in the heart of female rats and in the right ventricle of male animals. Our findings demonstrate that a long-term exercise training may promote angiogenesis through the induction of Esm1; however, the exact underlying molecular mechanisms need to be investigated in the future. As a limitation of this study, we did not measure VEGF levels or other angiogenesis markers, thus evaluating the role of Esm1 in cardiac aging and response to exercise training would be speculative at the moment.

In addition to the beneficial effects of sport on general cardiac function, it also has a protective effect in response to I/R-induced injury. The ligation of LAD via Langendorff system imitates the occurrence of myocardial infarction-related pathophysiological aspects. Therefore, the ratio of necrotic area followed by I/R injury has been clearly evaluated in both running and nonrunning groups. In a previous work of ours, we have already demonstrated that voluntary exercise reduced myocardial infarct size, and thus provided protection against I/R injury (46). In addition, several studies revealed that exercise was able to modify favorably the

adverse changes after myocardial infarction (30, 47). Similarly, in this study we observed a marked improvement in the extension of infarct size as a result of 12-wk voluntary exercise. In support of echocardiographic results, it can be stated that long-term exercise training has a protective effect even in the case of acute injury.

These results are consistent with exercise-induced gene expression changes observed in the heart. Although the central role of maintained physical activity is already cleared, the underlying molecular and genetic mechanisms affecting cardiac functions are still being extensively analyzed. Our results clearly highlight that long-term exercise can profoundly induce beneficial changes in the cardiovascular performance by affecting gene expression (Fig. 8).

## CONCLUSIONS

Over the years, average life expectancy has more than doubled; therefore, understanding and reversing the aging-associated processes have never been more important. Gene-targeted investigations may contribute to the understanding of overall physiological changes influenced by exercise and exercise-related therapies in the future. In addition, a better insight into cardiac aging may unravel molecular pathways related to cardiac pathophysiology and contribute to improved prevention of cardiovascular diseases.

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## DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

## AUTHOR CONTRIBUTIONS

A.P. conceived and designed research; D.B., D. P., R.S., M.B., Z.K., Z.R., K.K., A.M.B., and A.P. performed experiments; D.B., D.P., R.S., L.Z.F., B.J., and A.P. analyzed data; R.S., M.B., L.G.P., A.M.B., and B.J. interpreted results of experiments; D.B., D.P., and Z.R. prepared figures; D.B., D.P., Z.K., and L.Z.F. drafted manuscript; K.K., C.V., B.J., and A.P. edited and revised manuscript; C.V. and A.P. approved final version of manuscript.

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