# Role of epinephrine in cardiovascular function after chronic exercise


Raquel da Silva Martinho
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**Orientador**
Professora Doutora Mónica Moreira-Rodrigues, Professora Auxiliar Convidada no Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Laboratório de Fisiologia Geral, da Universidade do Porto

**Coorientador**
Mestre Priscila Mendes, Assistente Convidada na Escola Superior de Tecnologia da Saúde do Porto e Técnica de Diagnóstico e Terapêutica nos Serviços Farmacêuticos, Centro Hospitalar do Porto

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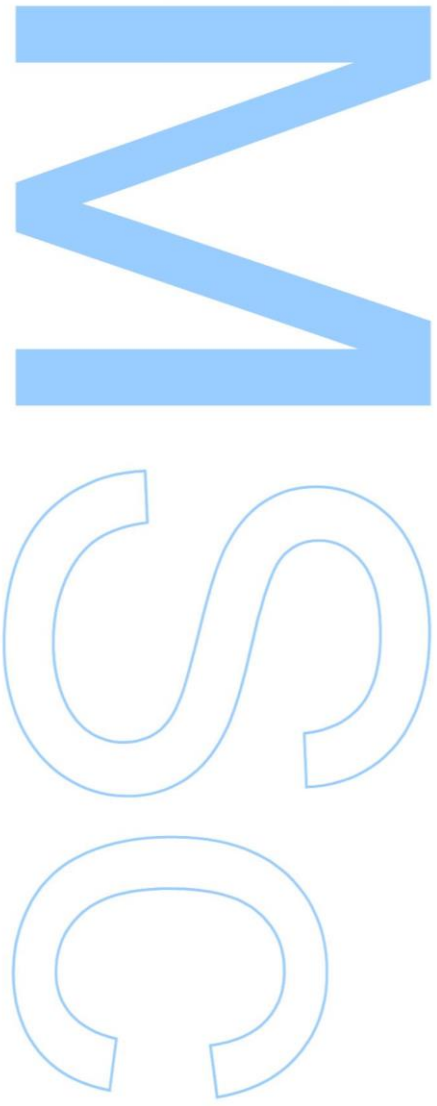

*It does not matter how slowly you go as long as you do not stop.*

Confucius

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# Publications

Mendes, P., Martinho, R., Leite, S., Leite-Moreira, A., Lourenco, A., & Moreira-Rodrigues, M. (2016). Exercise-induced hypertension and cardiac hypertrophy in epinephrine-deficient mice. *Journal of Hypertension, 34 Suppl 2*, e98. doi:10.1097/01.hjh.0000491586.92904.fb

# Oral presentation

Martinho, R., Mendes, P., Leite, S., Leite-Moreira, A., Lourenco, A., & Moreira-Rodrigues, M. (2016). Partial conversion of endogenous norepinephrine into epinephrine prevents left ventricle hypertrophy in chronic exercise. in XLVI Reunião da Sociedade Portuguesa de Farmacologia, Porto, Fevereiro de 2016.

# Resumo

A Feniletanolamina-*N*-metiltransferase é a enzima necessária para a conversão de norepinefrina (noradrenalina) em epinefrina (adrenalina). Bao et al. (2007) descreveu que ratinhos que não possuem esta enzima (Pnmt-KO) têm um aumento na proporção entre a espessura da parede posterior do ventrículo esquerdo e a dimensão interna do ventrículo esquerdo, mas não apresentam hipertrofia cardíaca. Os ratinhos Pnmt-KO apresentam, em repouso, uma pressão arterial normal mas tornam-se hipertensos durante o exercício físico agudo quando comparando com a estirpe selvagem (WT). O papel da epinefrina na resposta cardiovascular ao exercício crónico permanece pouco esclarecida. O objetivo deste estudo foi avaliar a morfologia cardíaca, bem como alterações funcionais e moleculares após o exercício crónico em animais Pnmt-KO.

Genotipagem por PCR foi realizada no locus da Pnmt em ratinhos Pnmt-KO ($Pnmt^{-/-}$) e WT ($Pnmt^{+/+}$). Os níveis de norepinefrina e epinefrina nas glândulas suprarrenais foram quantificados por RP-HPLC-ED. Os animais foram submetidos a um programa de exercício físico crónico durante 6 semanas. Em repouso, a pressão arterial foi medida após o exercício crónico. A morfologia e a função cardíaca foram avaliadas por ecocardiografia e hemodinâmica, seguidas de análise morfométrica. Marcadores moleculares de hipertrofia e fibrose cardíaca foram avaliados por PCR em tempo real.

Nos animais sedentários não houve diferenças na pressão arterial mas, pelo contrário, com o exercício crónico, um aumento da pressão arterial sistólica foi observado nos ratinhos Pnmt-KO em comparação com os WT. Após exercício crónico, foi observado um aumento do peso do coração normalizado pelo peso corporal nos animais Pnmt-KO comparando com os WT. A espessura da parede posterior e a massa do ventrículo esquerdo bem como a expressão de mRNA do IGF-1 e ANP apresentaram-se aumentadas e a expressão de mRNA do DDR2 não se alterou em animais Pnmt-KO treinados quando comparado com animais WT treinados. Contrariamente aos animais WT treinados, não se observaram diferenças nos parâmetros EF, $LVEDV_i$ e CI nos animais Pnmt-KO treinados, após perfusão com dobutamina.

Em conclusão, o aumento da pressão arterial sistólica em animais Pnmt-KO em resposta ao exercício crónico parece estar associada com o aumento do peso do coração, da espessura da parede posterior e da massa do ventrículo esquerdo bem com o aumento da expressão de mRNA do IGF-1 e ANP e sem diferenças na expressão de mRNA do DDR2, sugerindo hipertrofia concêntrica e ausência de fibrose no ventrículo esquerdo. A perfusão de dobutamina não levou a alterações dos parâmetros EF, $LVEDV_i$ e CI em animas Pnmt-KO contrariamente aos animais WT, sugerindo um

possível estado inicial de hipertrofia patológica concêntrica no exercício crónico em animais Pnmt-KO. Assim, a epinefrina parece ser essencial para a manutenção de uma pressão arterial normal, provavelmente através da vasodilatação induzida pelos recetores adrenérgicos $\beta_2$, prevenindo a hipertrofia do ventrículo esquerdo durante o exercício crónico.

Palavras-chave: ratinho Pnmt-KO, epinefrina, exercício crónico, pressão arterial, hipertrofia cardíaca.

# Abstract

Phenylethanolamine-*N*-methyltransferase (Pnmt) is required for the conversion of norepinephrine (NE) to epinephrine (EPI). Bao et al. (2007) described that Pnmt-knockout (Pnmt-KO) mice have an increased ratio of left ventricular (LV) posterior wall thickness to internal dimensions (LVPW/LVID), but not overall cardiac hypertrophy. Pnmt-KO mice showed normal resting blood pressure (BP), but became hypertensive during acute exercise when compared to wild-type (WT) mice. The role of EPI in cardiovascular response to chronic exercise remains unclear. The aim of this study was to evaluate cardiac morphology, and functional and molecular alterations after chronic exercise in Pnmt-KO mice.

PCR-based genotyping was performed at the Pnmt locus of Pnmt-KO ($Pnmt^{-/-}$) and WT mice ($Pnmt^{+/+}$). EPI and NE were quantified by RP-HPLC-ED, in adrenal glands. Animals were submitted to a 6-week exercise training program. BP was measured after chronic exercise, at rest. Cardiac morphology and function were evaluated by echocardiography and hemodynamics, followed by morphometric analysis. Molecular markers of cardiac hypertrophy and fibrosis were evaluated by real-time PCR.

There were no differences in BP between sedentary groups, however, after chronic exercise, Pnmt-KO mice showed a significant increase in systolic BP when compared with WT mice. After chronic exercise, heart weight (HW/BW) was significantly increased in Pnmt-KO mice when compared to WT mice. LV posterior wall thickness (LVPWd and LVPWs), LV mass ($LVM_i$) and mRNA expression of IGF-1 and ANP were significantly increased and there were no changes in DDR2 expression in trained Pnmt-KO mice when compared to trained WT mice. After dobutamine perfusion, trained Pnmt-KO mice did not had any changes in EF, $LVEDV_i$, and CI, contrary to trained WT mice.

In conclusion, the increased systolic BP in Pnmt-KO mice in response to chronic exercise appears to be associated with an increase in heart weight, LV wall thickness and mass and an increased IGF-1 and ANP mRNA expression and no changes in DDR2 expression, suggesting a concentric hypertrophy and no fibrosis of the LV. Futhermore, dobutamine infusion did not led to any changes in EF, $LVEDV_i$ and CI in trained Pnmt-KO, contrary to trained WT, altogether suggesting a possible initial pathological concentric hypertrophy stage in Pnmt-KO mice, with chronic exercise. Therefore, EPI appears to be essential for maintaining a normal BP, probably through $\beta_2$-adrenoceptors induced vessel relaxation, thus preventing LV hypertrophy during chronic exercise.

Keywords: Pnmt-KO mouse, epinephrine, chronic exercise, blood pressure, cardiac hypertrophy.

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# Abbreviations

A- Peak Doppler velocity of late filling wave (A-wave) in transmitral flow

α-ARs - α-adrenoceptors

AG(s) - Adrenal gland (s)

αMHC - α myosin heavy chain

ANP - Atrial natriuretic peptide

AR(s) - Adrenoceptor(s)

AT1R - Angiotensin II type 1 receptor

AT2R - Angiotensin II type 2 receptor

AU - Arbitrary unit

β-AR - β-adrenoceptors

$\beta_i$ - Left ventricular chamber stiffness constant derived from end-diastolic pressure-volume relationship

βMHC - β myosin heavy chain

BNP - Brain natriuretic peptide

BP - Blood pressure

BSA - Body surface area

BW - Body weight

CI - Cardiac index

CNS - Central nervous system

CO - Cardiac output

DA - Dopamine

DBH - Dopamine-β-hydroxylase

DBP - Diastolic blood pressure

DDC - DOPA decarboxylase

DDR2 - Discoidin domain receptor 2

E - Peak Doppler velocity of early filling wave in transmitral flow

E/A - Ratio between peak velocity of early filling to peak velocity of late filling in transmitral flow pulsed-wave Doppler

$Ea_i$ - Arterial elastance index

ECM - Extra-cellular matrix

EDP - End-diastolic pressure

EDPVR - End-diastolic pressure volume relationship

E/E' - Ratio of peak early transmitral pulsed-wave Doppler flow velocity (E) to peak early diastolic

E' - Peak mitral annulus tissue Doppler velocity in early diastole

$E_{ES}i$, End-systolic elastance derived from end-systolic pressure-volume relationship

EF - Ejection fraction

EIA - Enzyme Immunoassay

EPI - Epinephrine

ESPVR - End-systolic pressure volume relationship

FS - Fractional shortening

GAPDH - Glyceraldehyde-3-phosphate dehydrogenase

GW - Gastrocnemius muscle weight

GW/BW - Ratio of gastrocnemius muscle weight to body weight

HR - Heart rate

HW/BW - Ratio of heart weight to body weight

IGF-1 - Insulin-like growth factor-1

IGF-1R - Insulin-like growth factor 1 receptor

IVA- Isovolumetric acceleration

IVC - Inferior vena cava

IVS - Interventricular septum

IVSd - Interventricular end-diastolic septal thickness

IVSs - Interventricular end-systolic septal thickness

IVSW/BW - Ratio of interventricular septum weight to body weight

LV - Left ventricle or left ventricular

L-DOPA - L-3,4-dihydroxyphenylalanine

LVEDV - Left ventricular end-diastolic volume

$LVEDV_i$ - Indexed left ventricular end-diastolic volume

LVEndoAd - Left ventricular end-diastolic endocardial area

LVEndoAs - Left ventricular end-systolic endocardial area

LVEpiAd - Left ventricular end-diastolic epicardial area

LVEpiAs - Left ventricular end-systolic epicardial area

$LVESV_i$ - Indexed left ventricular end-systolic volume

LVH - Left ventricular hypertrophy

LVIDd - Left ventricular end-diastolic internal dimension

LVIDs - Left ventricular end-systolic internal dimension

LVM - Left ventricular mass

$LVM_i$ - Left ventricular mass indexed for body surface area

LVPW - Left ventricular posterior wall thickness

LVPWd - Left ventricular end-diastolic posterior wall thickness

LVPWd/LVIDd – Ratio of left ventricular end-diastolic posterior wall thickness to left ventricular end-diastolic internal dimension

LVPWs - Left ventricular end-systolic posterior wall thickness

LVW/BW - Ratio of left ventricular weight to body weight

MAP - Mean arterial blood pressure

Minus RT mix - Reverse transcriptase control master mix

NE - Norepinephrine

Pmax - Maximum pressure

Pnmt - Phenylethanolamine-*N*-methyltransferase

Pnmt-KO - Phenylethanolamine-*N*-methyltransferase-knockout

Pnmt-KO ex - Chronic exercise trained Phenylethanolamine-*N*-methyltransferase-knockout

PNS - Peripheral nervous system

PRSW - Preload recruitable stroke work

PV - Pressure volume

PW - Pulsed-wave

qPCR - Real-time PCR

RAAS - Renin-angiotensin-aldosterone system

RR - Respiratory rate

RP-HPLC-ED - Reversed-phase high-performance liquid chromatography with electrochemical detection

RT mix - Reverse transcription master mix

RV - Right Ventricle

RVW/BW - Ratio of right ventricular weight to body weight

S' - Peak systolic mitral annulus tissue Doppler velocity

SBP - Systolic blood pressure

SV - Stroke volume

$SV_i$ - Indexed stroke volume

TD - Tissue Doppler

TH - Tyrosine hydroxylase

$VVC_i$ - Ventricular-vascular coupling index

w/v - Weight/volume

WT - Wild-type

WT acute ex - Acute exercise trained wild-type

WT ex - Chronic exercise trained wild-type

# 1. Introduction

## 1.1. Catecholamines: Historical perspective

Vulpian, in 1856, applied a solution of ferric chloride to slices of adrenal glands (AGs) and observed that the medulla stained green while the cortex did not. Also, he noticed that the same reaction happened in samples of venous blood leaving the adrenal, but not in the arterial blood entering the gland. Therefore, Vulpian assumed that the medulla synthesized a substance that was released into circulation (Vulpian, 1856).

The first pharmacological action of catecholamines was demonstrated by Oliver and Schäfer when they revealed that the administration of extracts of AGs led to the increase of blood pressure (BP) (Oliver & Schäfer, 1895). Takamine, in 1901, reported the first successful crystallization of a hormone named “adrenalin” (Takamine, 1901). Catecholamines soon became the center of various scientific discussions, with extraordinary breakthroughs that crossed the twentieth century (Costa, 2012).

## 1.2. An overview on the role of catecholamines

Epinephrine (EPI), norepinephrine (NE) and dopamine (DA) are known as catecholamines, as they have an amine side-chain, a catechol moiety, and they are all derived from tyrosine (Purves, 2001) (Figure 1). With 5-hydroxytryptamine, catecholamines share the amino side chain and altogether are known as biogenic amines (Purves, 2001; Costa, 2012). They form a class of hormones and chemical neurotransmitters that have an important role in the regulation of physiological processes and the development of cardiovascular, endocrine and neurological diseases. As such, catecholamines and the catecholamine endocrine and neuronal systems in which they are produced continue to have enormous research attention (Eisenhofer, Kopin, & Goldstein, 2004).

### 1.2.1. Epinephrine

EPI (also called adrenaline), is preserved throughout vertebrate evolution and is an important determinant of responses to metabolic or global challenges to homeostasis (Bao et al., 2007; Goldstein, 2010). The chromaffin tissue in the adrenal medulla is the main source of this catecholamine and is present in specific parts of the brain, however at lower levels than the other catecholamines (Purves, 2001; Stanford, 2001). The synthesis of EPI is also present in several tissues outside of the adrenal medulla, such as heart (Axelrod, 1962) and blood vessels (Spatz et al., 1982). EPI found in other peripheral tissues possibly derives from uptake from the circulation (Stanford, 2001).

EPI has positive inotropic and chronotropic actions on the heart (Furnival, Linden, & Snow, 1971). This catecholamine also increases oxygen consumption (Berne, 1958) and can cause angina pectoris (Levine, Ernstene, & Jacobson, 1930).

The systemic infusion of EPI has been reported to amplify increases in both BP and plasma NE during a cold pressor test and isometric exercise, suggesting that the augmented pressor responses to these stimuli are due to facilitated NE release (Vincent, Boomsma, Man in 't Veld, & Schalekamp, 1986). Also, the short-term administration of EPI seems to increase glucose and fatty acids, while high urinary EPI levels are associated with a favorable lipid pattern. In contrast, chronic EPI infusion enhance rat muscle insulin sensitivity (Jensen, Ruzzin, Jebens, Brennesvik, & Knardahl, 2005).

### 1.2.2. Norepinephrine

Von Euler was the first to show that NE (also called noradrenaline) was the main neurotransmitter in the sympathetic nervous system (von Euler, 1946). NE is found in most, but not all, postganglionic sympathetic neurons. Stimulation of sympathetic neurons is inseparable from the “freeze, fight or flight” response (Bracha, 2004), which results in the release of EPI and NE by the adrenal medulla and sympathetic neurons and the secretion of corticosteroids by the adrenal cortex (Wang, Xu, Gainetdinov, & Caron, 1999). EPI, corticosteroids and NE help to coordinate different body part responses for adaptation to a stressful situation (Costa, 2012) resulting in an enhanced energy mobilization and neural reflexes, increased heart rate (HR) and BP (Wang et al., 1999).

### 1.2.3. Dopamine

DA is a neurotransmitter in cell groups in the brain and in a few peripheral groups (Rios et al., 1999) and is the metabolic precursor of NE and EPI (Costa, 2012) (Figure 1). The major DA-containing area of the brain is the corpus striatum, which receives major input from the substantia nigra and has a significant role in the coordination of body movements (Purves, 2001). Also, two important central dopaminergic systems are located in the chemoreceptor trigger zone and in the anterior pituitary, outside the blood-brain barrier (Costa, 2012).

When released, DA binds to DA receptors, present in the brain (Purves, 2001) and in coronary, renal, mesenteric, and intracerebral arteries (Bloom, 2006). Clinically, DA is used to treat shock because it dilates renal arteries by activating DA receptors and increases cardiac output (CO) by activating β-adrenoceptors (β-ARs) in the heart (Purves, 2001).

## 1.3. Biosynthesis of catecholamines

The various mechanisms of catecholamine synthesis, storage, release, and binding have been regularly studied in sympathetically innervated organs and in the adrenal medulla (Westfall & Westfall, 2006). The different steps that occur in the synthesis of DA, NE, and EPI are shown in Figure 1.

The biosynthetic pathway of catecholamines begins with uptake of the amino acid tyrosine into the cytoplasm of sympathetic neurons, adrenomedullary cells, possibly para-aortic enterochromaffin cells and specific centers in the brain. Circulating tyrosine derives from hepatic hydroxylation of phenylalanine and from the diet (Goldstein, 2010). The hydroxylation of tyrosine by tyrosine hydroxylase (TH) is the rate limiting step in the biosynthesis of catecholamines (Zigmond, Schwarzschild, & Rittenhouse, 1989). TH catalyzes the conversion of tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) (Rios et al., 1999) and is activated after stimulation of sympathetic nerves or adrenal medulla (Costa, 2012). Low cerebrospinal fluid levels of NE and DA metabolites, like HVA and 3-methoxy-4-hydroxy-phenylethylene glycol are detected in humans with TH deficiency (Wevers et al., 1999, Carson and Robertson, 2002). The TH knockout is unviable in mice as they die in the embryonic stage, maybe because catecholamine loss results in altered cardiac function (Zhou et al., 1995).

In the neuronal cytoplasm, DOPA decarboxylase (DDC), catalyzes the conversion of L-DOPA to DA (Goldstein, 2010). In noradrenergic neurons in the

sympathetic nervous system, in the brainstem, and in chromaffin cells, dopamine-β-hydroxylase (DBH) catalysis the conversion of DA to NE (Rios et al., 1999; Goldstein, 2010). NE deficiency (or DBH deficiency) is a rare congenital disorder (Robertson et al., 1986; Man in't Veld, Moleman, Boomsma, & Schalekamp, 1987). NE-deficient patients display deficits in autonomic regulation of cardiovascular function, prompting them to orthostatic hypotension. Moreover, these patients show undetectable levels of NE and its metabolites and highly elevated DA and its metabolites (Kim et al., 2011).

The last step in this biosynthetic pathway is the conversion of NE to EPI by a cytoplasmic enzyme that requires the methyl donor S-adenosylmethionine (SAMe) as a cofactor, phenylethanolamine-*N*-methyltransferase (Pnmt) (Stanford, 2001). Pnmt is present in the chromaffin tissue of the adrenal medulla and in a few neuronal groups in the lower brainstem (Rios et al., 1999), but it is also found in the retina, brain, and heart (Stanford, 2001).

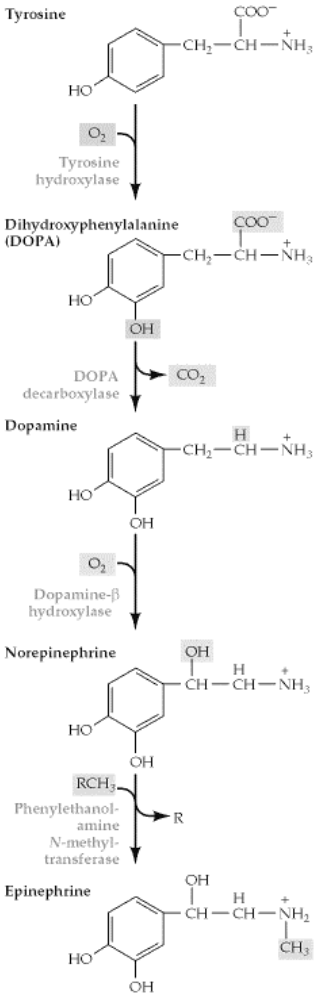


Figure 1. The biosynthetic pathway of catecholamines. From Purves, Augustine et al. (2001).

## 1.4. Adrenoceptors

In the membranes of effector cells, adrenoceptors (ARs) determine the metabolic and physiological effects of catecholamines (Goldstein, 2010). The adrenoceptor family was first divided into two subtypes, the α- and β-AR as determined by pharmacological studies in isolated tissues (Ahlquist, 1948). The ARs are targets for many therapeutically drugs, including those for some cardiovascular diseases, asthma and obesity (Guimarães & Moura, 2001).

### 1.4.1. α-Adrenoceptors

The α-adrenoceptors (α-ARs) subtypes have been classified as $\alpha_1$-ARs ($\alpha_{1A}$- (Schwinn et al., 1990), $\alpha_{1B}$- (Cotecchia et al., 1988; Schwinn et al., 1990) and $\alpha_{1D}$- (Lomasney et al., 1991; Perez, Piascik, & Graham, 1991; Bylund et al., 1994; Ford, Williams, Blue, & Clarke, 1994)) and $\alpha_2$-ARs ($\alpha_{2A}$- ($\alpha_{2D}$- species variation of the human $\alpha_{2A}$-) (Bylund et al., 1992; Trendelenburg, Limberger, & Starke, 1996; Paiva, Mota, Moura, & Guimaraes, 1997; Guimaraes, Figueiredo, Caramona, Moura, & Paiva, 1998), $\alpha_{2B}$- (Lomasney et al., 1990) and $\alpha_{2C}$- (Weinshank et al., 1990)).

Via G-protein coupled receptors, the $\alpha_1$-ARs mediate their response and are located in the central nervous system (CNS) and peripheral nervous system (PNS). In the CNS they are predominantly located post-synaptically where they mediate an excitatory role (Robinson & Hudson, 1998). Peripheral $\alpha_1$-ARs are located on both non-vascular and vascular smooth muscle where activation of the receptor results in contraction (Aboud, Shafii, & Docherty, 1993). They are also located on the heart where they mediate negative or positive inotropic effects (Wang et al., 2006).

The $\alpha_2$-ARs mediate their functions through a variety of G-proteins (Robinson & Hudson, 1998). $\alpha_2$-ARs are found in both the CNS and PNS and located both pre and post synaptically (French, 1995). Effects of $\alpha_2$-ARs include the regulation of BP, hypothermia and cognitive function. Peripheral functions include inhibition of lipolysis through $\alpha_2$-ARs located on fat cells, hyperpolarization of sympathetic ganglia and contraction of vascular smooth muscle (Robinson & Hudson, 1998).

### 1.4.2. β-Adrenoceptors

β-adrenoceptors (β-ARs) (Dixon et al., 1986) are members of the superfamily of G-protein-coupled receptors and have important roles in respiratory, cardiovascular,

reproductive, and metabolic functions (Chruscinski et al., 1999). At present, three distinct β-ARs subtypes ($\beta_1$-, $\beta_2$-, and $\beta_3$-AR), have been identified (Dixon et al., 1986; Frielle et al., 1987; Emorine et al., 1989).

The activation of $\beta_1$-ARs leads to increased HR and force of contraction (Brodde, 1991). Although they represent a smaller population in the heart than $\beta_1$-ARs, $\beta_2$-ARs play a role in regulating cardiac function in several of species (Kaumann, 1986; Brodde, 1991; Takei et al., 1992).

$\beta_2$-AR stimulation leads to activation of adenylyl cyclase and contributes to both chronotropic and inotropic responses (Brodde, 1991). β-ARs also regulate peripheral vascular tone where the stimulation of these ARs, especially $\beta_2$-ARs, in skeletal muscle, produces vasodilation (Goldstein, 2010).

## 1.5. Cardiovascular effects of Epinephrine

The cardiovascular effects of adrenal medullectomy in experimental animals are controversial, with both change and no change in HR, CO, total peripheral resistance and BP reported in resting dogs (Harakal, Reidenberg, Sevy, & Ohler, 1966; Ashkar, 1971). The role of EPI has been hard to decipher because the adrenal medullectomy procedure can damage the adrenal cortex, altering the release of corticosteroids and it also removes the release of other adrenal amines and peptides, such as NE, chromogranin A, catestatin, and neuropeptide Y (Harrison and Seaton, 1966).

EPI is the prototypical stress hormone and its actions are both mimicked and blocked by numerous commonly used medications (Ziegler, Elayan, Milic, Sun, & Gharaibeh, 2012).

The administration of isoproterenol, a β-AR agonist, depletes the energy reserves of cardiac muscle cells and causes biochemical and structural changes leading to cell damage and necrosis (Padmanabhan & Prince, 2006). The infusion of isoproterenol for 7 days has been demonstrated to induce cardiac dysfunction in mice (Oudit et al., 2003).

Dobutamine, a $\beta_1$-AR agonist, is a synthetic catecholamine that increases myocardial contractility, while the reflex reduction in sympathetic tone, in response to augmentation of stroke volume (SV), leads to a decreased total peripheral resistance (Vallet, Dupuis, & Chopin, 1991). Dobutamine chronotropic (HR increase) and positive inotropic (dP/dtmax increase) effects have been evidenced in C57BL/6 mice (Calligaris, Ricca, & Conget, 2013).

Cardiac catheterization used to measure in vivo responsiveness to dobutamine showed an enhancement of LV dP/dt max in mice lacking NE and EPI ($dbh^{-/-}$) mice, suggesting increased $\beta_1$-AR coupling and sensitivity to $\beta_1$-agonist stimulation (Cho et al., 1999).

In order to determine whether EPI plays an important role during development and to identify adrenergic cell descendants in the developing mouse, Ebert et al. (2004) inserted a Cre-recombinase gene into the mouse Pnmt locus (Pnmt-Cre). Specifically, a Cre-recombinase gene was inserted into exon 1, creating a gene fusion of the 5' leader sequences from Pnmt directly to Cre-recombinase open reading frame. Additionally, germ-line transmission of the Pnmt-Cre allele was verified firstly by Southern blotting and then by polymerase chain reaction (PCR) (Figure 2). The nonexistence of Pnmt expression was predicted to result in mice that cannot produce EPI. To test this hypothesis, in adrenal extracts from $Pnmt^{+/+}$, $Pnmt^{+/Cre}$, and $Pnmt^{Cre/Cre}$ mice, the authors measured EPI and NE concentrations by radioimmunoassay. EPI was not detectable in extracts from $Pnmt^{Cre/Cre}$ mice, and the concentration of NE was found to be significantly greater in $Pnmt^{Cre/Cre}$ relative to $Pnmt^{+/+}$ and $Pnmt^{+/Cre}$ extracts. In contrast, NE and EPI concentrations were similar in extracts from $Pnmt^{+/+}$ and $Pnmt^{+/Cre}$ mice. All this data shows that insertion of Cre-recombinase into the Pnmt locus created a functional knockout of Pnmt expression, with loss of EPI in homozygous $Pnmt^{Cre/Cre}$ mice. Also, despite the reduction in Pnmt expression and EPI production in $Pnmt^{Cre/Cre}$ mice, these mice were viable and fertile, with no developmental defects (Ebert et al., 2004).

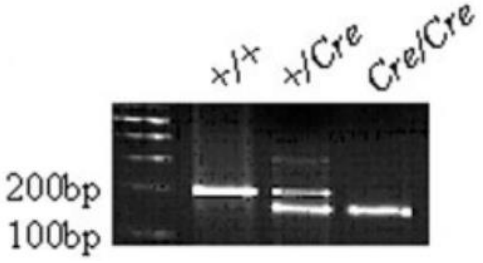


Figure 2. Polymerase chain reaction analysis distinguishes homozygous wild-type (+/+), heterozygous mutant (+/Cre), and homozygous mutant (Cre/Cre) animals. From Ebert et al. (2004).

Bao et al. (2007) also produced an EPI-deficient Pnmt-knockout (Pnmt-KO) mouse to further define the role of EPI in cardiovascular function. In 129/SvJ mice, a Cre-Neo cassette replaced the PNMT gene from the translational start codon ATG to the exon 3 *Cla*I site. Furthermore, Cre-recombinase was inserted into the PNMT 5' untranslated region sequence so that its transcription was controlled by Pnmt regulatory elements. Germ-line transmission of the Pnmt allele was verified by Southern blot analysis (Figure 3) (Bao et al., 2007).

EPI-deficient Pnmt-KO mice presented normal levels of NE in plasma and increased adrenal NE content. However, NE stimulates $\beta_2$-ARs poorly compared with EPI, so Pnmt-KO mice probably lacked the $\beta_2$-AR induced vasodilation caused by EPI (Lands, Arnold, McAuliff, Luduena, & Brown, 1967; Bao et al., 2007; Sun et al., 2008; Moreira-Rodrigues et al., 2014)

During exercise, plasma EPI increases very rapidly (Tidgren, Hjemdahl, Theodorsson, & Nussberger, 1991) in order to decrease peripheral vascular resistance, lower exercise BP and enhance insulin sensitivity (Ziegler, Elayan, Milic, Sun, & Gharaibeh, 2012). EPI deficiency changed the cardiovascular response to acute stress caused by exercise. Mice with and without EPI had similar HR increases during acute exercise. Also, during acute exercise, Pnmt-KO mice presented a higher mean arterial BP (MAP) than control animals. Echocardiography under light anesthesia and during the stress of restraint showed that Pnmt-KO mice had an increase in the ratio of left ventricular posterior wall thickness to the internal dimension in diastole. During restraint, the left ventricular end-diastolic volume (LVEDV) was lower, causing a significantly smaller stroke volume (SV). As a result, CO decreased even though there was no change in cardiac contractility or in HR. Thus, the EPI-deficient mice had concentric remodeling of the left ventricle (LV) and decreased LVEDV during stress. This data suggests that EPI is necessary for normal BP and cardiac filling responses to acute stress, but is not necessary for tachycardia during stress or normal cardiovascular function at rest (Bao et al., 2007).

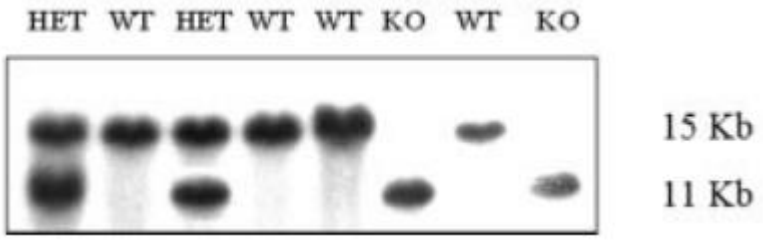


Figure 3. The Southern blot with XbaI revealed a 15-kb band with the WT allele and an 11-kb band with the KO allele. WT, wild- type; HET, heterozygous Pnmt-KO; KO, homozygous Pnmt-KO mice. From Bao et al. (2007).

## 1.6. Cardiac hypertrophy

Left ventricular hypertrophy (LVH) is defined as an increase in myocardial muscle mass due to the increased number or size enlargement of the myocytes (Weber et al., 1988; Gosse & Dallocchio, 1993; Messerli & Ketelhut, 1993). A compensatory mechanism for an increased pressure or volume load imposed on the heart is the

hypertrophy of myocardial tissue, as it provides the mechanical adjustment necessary to overcome the extra burden (Panidis et al., 1984).

Physiological and pathological LVH are caused by different stimuli and are related with different molecular and structural phenotypes (McMullen & Jennings, 2007). It has also been shown that both physiologic and pathologic LVH may display distinct biochemical and molecular signaling pathways (Iemitsu et al., 2001; Heineke & Molkentin, 2006; Bernardo, Weeks, Pretorius, & McMullen, 2010).

### 1.6.1. Physiological cardiac hypertrophy

Exercise training is thought to produce a physiological LVH which is characterized by myocardial adaptations sufficient to meet increased demands while maintaining normal function (Colan, Sanders, & Borow, 1987; Weber, Clark, Janicki, & Shroff, 1987; Weber et al., 1988).

Physiological LVH includes different hypertrophy patterns, but these may be influenced by different forms of exercise training (Richey & Brown, 1998). Exercise increases peripheral resistance (afterload), thus stimulating concentric hypertrophy (Fleck, 1988; Alpert, Pape, Ward, & Rippe, 1989).

Structurally, exercise-induced physiological LVH can be marked by an adaptive growth of myocytes without fibrosis or alterations in intramural arteries frequently seen with hypertensive LVH (Weber et al., 1988; Vogt, Motz, Schwartzkopf, & Strauer, 1993).

The functional consequences of physiological LVH can be related with a normal diastolic function and maintenance or enhancement of contractility, capillary density and the mitochondrial to myocyte volume ratio (Keul, Dickhuth, Simon, & Lehmann, 1981; Keul, Dickhuth, Lehmann, & Staiger, 1982; Douglas, 1989).

After 4 weeks of treadmill exercise it has been reported that C57BL/6J mice developed physiological cardiac hypertrophy, confirmed by an increase in ventricular weights and cardiomyocyte dimensions and normal cardiac function and structure (Kemi, Loennechen, Wisloff, & Ellingsen, 2002). Also, an increase in heart weight to body weight ratio (HW/BW) has been showed after 4 weeks of voluntary wheel training (Allen et al., 2001) and swimming exercise (Kaplan et al., 1994) in C57BL/6J mice.

IGF-1 is a 70-amino acid basic peptide (Rinderknecht & Humbel, 1978) which has growth-promoting actions on a variety of tissues including the heart (Mathews, Norstedt, & Palmiter, 1986; Murphy, Bell, Duckworth, & Friesen, 1987; Isgaard et al., 1988). It has been previously reported that a significant increase in heart weight after IGF-1 treatment was present in rats (Lisa, Haleagrahara, & Chakravarthi, 2011).

Moreover, rats undergoing LVH by several mechanisms have an increase in IGF-1 mRNA and protein levels within the myocardium coincident with the development of hypertrophy (Wahlander, Isgaard, Jennische, & Friberg, 1992; Donohue et al., 1994). Other reports showed that mice with constitutive overexpression of IGF-1 may develop an increase in heart size, characterized by both hyperplasia and cardiomyocyte hypertrophy, and are protected against ischemic injury and heart failure (Reiss et al., 1996; Li et al., 1999; Kajstura et al., 2001; Yamashita et al., 2001; Welch et al., 2002). Activation of the IGF-1 receptor (IGF-1R) recapitulates the hypertrophic phenotype, with preserved contractile function and increased cardiomyocyte size (McMullen et al., 2004).

The reversibility of physiological LVH is a factor used to differentiate it from pathological LVH (Richey & Brown, 1998). The importance of exercise intensity is indicated by studies demonstrating that high aerobic intensity of the exercise training yields a larger magnitude of effects than low-to-moderate exercise intensity, including the cardiac hypertrophy (Kemi et al., 2002; Haram et al., 2009). No threshold intensity of exercise for inducing observable cardiac hypertrophy has yet been identified, but cardiac hypertrophy is only sustained if exercise training is maintained, and lost within weeks if exercise is not continued (Kemi, Haram, Wisloff, & Ellingsen, 2004).

### 1.6.2. Pathological cardiac hypertrophy

A pathological pressure overload of the LV might result from systemic hypertension, increased peripheral resistance, aortic stenosis or aortic coarctation. This pathological concentric LVH can be accompanied by a parallel addition of new myofibrils (wall thickening), normally with myocyte necrosis and increased fibrosis possibly leading to diastolic dysfunction with stiffness and decreased contractility (Richey & Brown, 1998).

A pathological volume overload of the LV may be stimulated by such a condition as valvular regurgitation. Pathological eccentric LVH also suffers from increases in interstitial fibrosis caused by structural remodeling (Richey & Brown, 1998). In the ischemic or infarcted myocardium, decompensated eccentric LVH, or ventricular dilation, often occurs. This condition is characterized by the side-to-side slippage of myocytes in the affected region which may result in mechanical dysfunction (Anversa, Olivetti, & Capasso, 1991; Beltrami et al., 1994).

Pathological cardiac hypertrophy may be manifested by alterations in cardiac contractile proteins (α-skeletal actin and the $\alpha$- to β- myosin heavy chain (MHC) switch) and increased expression of genes such as atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) (Iemitsu et al., 2001). ANP and BNP are hormones that affect

BP and plasma volume status through natriuretic, diuretic, and vasodilator activities (Woodard & Rosado, 2008). Also, they signal locally in the cardiac myocyte where they can antagonize hypertrophy (Kehat & Molkentin, 2010).

It has been demonstrated that transgenic mice overexpressing ANP have a lower heart weight (HW) and BP than WT mice (John et al., 1995). Moreover, low-salt fed ANP null mice exhibited concentric LV hypertrophy (Feng et al., 2003). Mice lacking the ANP receptor specifically within cardiac myocytes also showed enhanced cardiac hypertrophy (Holtwick et al., 2003). Taken together, these observations suggest that ANP plays an important role in protecting against the development of cardiac hypertrophy (Kehat & Molkentin, 2010). Also, in a report were physiological hypertrophy was induced by swimming in C57/BL6 mice, in order to exclude the occurrence of pathological hypertrophy, mRNA levels of ANP and BNP were determined and both genes were not changed in swimming mice, indicating that possibly physiological hypertrophy other than pathological was induced in these mice (Xiao et al., 2014).

The change from the $\alpha$ to $\beta$MHC phenotype is considered as a hallmark of pathologic hypertrophy, which is more intense in pressure-overload than in volume-overload hypertrophy (Gupta, 2007). In animal models of pressure overload, such as aortic constriction in rats, rabbits and mice, the relative proportion of $\alpha$MHC has been shown to decrease during pathologic hypertrophy, but it was never found to be completely eliminated (Lompre et al., 1979; Mercadier et al., 1981; Litten, Martin, Low, & Alpert, 1982; Pillai, Russell, Raman, Jeevanandam, & Gupta, 2005).

The growth in fibrous tissue content is based on the maintained proliferative potential of fibroblasts, and the synthesis of extra-cellular matrix (ECM) proteins, predominantly by fibroblasts (MacKenna, Summerour, & Villarreal, 2000). Discoidin domain receptor 2 (DDR2) belongs to a family of collagen specific receptor tyrosine kinases (Shrivastava et al., 1997; Vogel, Gish, Alves, & Pawson, 1997). This receptor mediate a variety of cell functions, including growth, migration, morphology and differentiation. The tissue distribution of DDR2, a marker for cardiac fibroblasts (Goldsmith et al., 2004) varies, and its expression has been detected in both rat and mouse heart (Lai & Lemke, 1991).

### 1.6.3. Concentric vs Eccentric hypertrophy

LV remodeling and hypertrophy is determined by the type of overload. The commonly accepted scheme of classification of patterns of LV remodeling follows as

normal, concentric remodeling, concentric hypertrophy, and eccentric hypertrophy (Lang et al., 2006) (Figure 4).

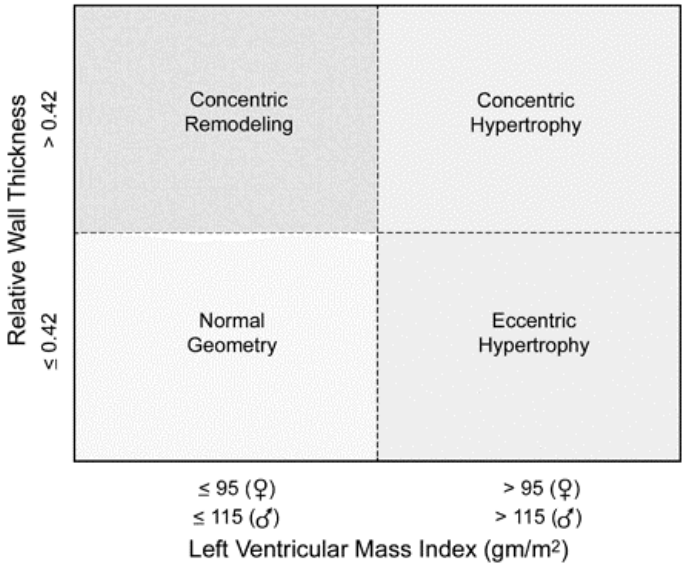


Figure 4. Classification of patterns of left ventricular remodeling. Patients with normal LV mass can have either concentric remodeling or normal geometry and normal LV mass. Patients with increased LV mass can have either concentric or eccentric hypertrophy. From Lang et al. (2006).

The limitation of the classical categories is the suboptimal categorization of dilated ventricles (Khouri, Peshock, Ayers, de Lemos, & Drazner, 2010). Khouri et al. (2010) found that concentric or eccentric LVH may each be subclassified into 2 subgroups, yielding 4 different geometric patterns. Also, this work suggests the hypothesis that the adaptation of the LV is the result of the independent increase in wall thickness and/or chamber dimension rather than the effect of their interaction (relative wall thickness (RWT)) to compensate for increased wall stress (Khouri, Peshock, Ayers, de Lemos, & Drazner, 2010) (Figure 5).

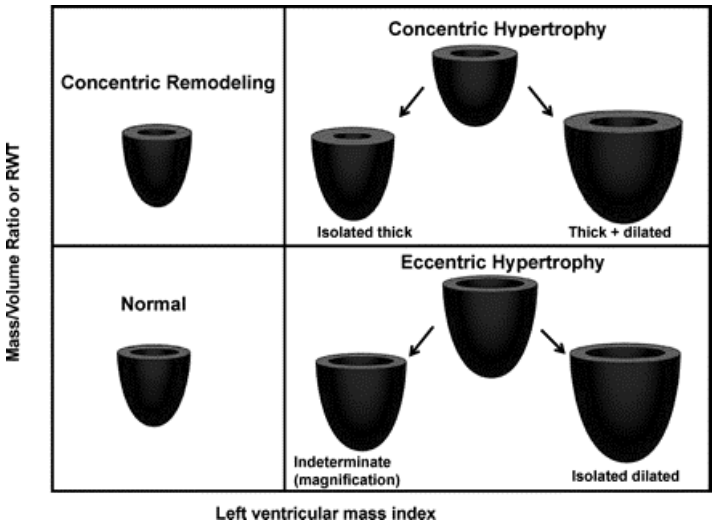


Figure 5. Representation of the 4-tiered classification of left ventricular hypertrophy (LVH).Classification may be made by presence or absence of LVH (horizontal axis) and by geometry (vertical axis), depending on mass-to-volume ratio. From Chinali & Aurigemma (2010).

Both concentric processes are normally in response to LV pressure overload, with the earliest response exhibited as concentric remodeling. This response shows normal or small LV cavity size, increased LV wall thickness and normal LVM (Marwick et al., 2015). Once the LVM is increased it is referred to as concentric hypertrophy (Lang et al., 2006). A concentric hypertrophy pattern may show the parallel addition of new myofibrils that create a thickening of the myocardial wall (Richey & Brown, 1998). This hypertrophic response is an attempt to limit wall stress to allow for the maintenance of normal LV systolic function and performance (Gaasch & Zile, 2011).

The increase in preload may produce an eccentric hypertrophy pattern characterized by the addition of new sarcomeres which results in dilated ventricular cavities, increased LVM and normal LV wall thickness (Marwick et al., 2015). The increase in chamber size produces a wall thinning effect that elevates wall stress (Richey & Brown, 1998). Consequently, this condition might produce a positive feedback loop stimulating a concentric hypertrophic response in an attempt to normalize the elevated wall stress (Hood, Rackley, & Rolett, 1968; Grossman et al., 1975; Alpert et al., 1989).

## 1.7. Objectives

The role of EPI in normal cardiovascular function associated with chronic exercise training has not been fully elucidated. Nowadays, loss-of-function studies are widely used to clarify the role of a specific gene or protein. In that basis, the aim of this study is to evaluate cardiac morphological, functional and molecular alterations after chronic exercise training in Pnmt-KO and wild-type (WT) mice. For this purpose, it was established and organized a framework including i) identification of genotypes at the Pnmt locus by PCR analysis, ii) quantification of EPI and NE in AGs, iii) evaluation of BP and HR by tail-cuff method after a 6-week exercise training program, iv) evaluation of cardiac morphology and function by echocardiography and hemodynamics, followed by v) morphometric analysis and vi) evaluation of molecular markers of cardiac hypertrophy and fibrosis.

# 2. Materials and Methods

## 2.1. Animals

All animal care and experimental procedures were performed in accordance with the European Directive 63/2010/EU, transposed to the Portuguese legislation by the Directive Law 113/2013. Ten-week-old WT (129x1/SvJ, *n*=38) and Pnmt-KO (129x1/SvJ, *n*=15) male mice were used for this study. The animals were kept in cages under controlled environmental conditions (12 hour light/dark cycle, room temperature 23 ± 1°C, humidity 50%, autoclaved drinking water, and mice breeding diet 4RF25/I; Ultragene, Porto, Portugal) and housed with the respective litter.

## 2.2. Genotyping

The Pnmt-KO mice were produced by disruption of Pnmt locus by insertion of Cre-recombinase in exon 1 (Ebert et al., 2004). A couple of Pnmt-KO mice were kindly provided by Steven N. Ebert and animals were bred in our conventional vivarium. Genotyping was done by PCR amplification analysis of the PNMT gene in ear samples by using the following primers: Primer 1, 5'-CAGGCGCCTCATCCCTCAGCAGCC-3'; Primer 2, 5'-CTGGCCAGCGTCGGAGTCAGGGTC-3'; and Primer 3, 5' GGTGTACGGTCAGTAAATTGGACACCGTCCTC-3'.

## 2.3. Acute exercise training protocol

Sedentary WT and Pnmt-KO mice were left without exercise training. The acute exercise training program was always performed at the same time of day. WT mice were adapted to the procedure 3 days before the beginning of the acute exercise training protocol. Before the training session, the animals were allowed 15 minutes to adapt to the treadmill without exercising. Exercise training was done on a motor treadmill (Panlab Harvard Apparatus, Barcelona, Spain), starting at 6 cm/s for 10 minutes with an increase in speed of 3 cm/s every 2 minutes until 20 m/min, for 55 minutes (until exhaustion if 55 minutes duration was not reached). It was considered exhaustion the animal's permanence in the shock grid (0.2 mA) for more than 5 consecutive seconds or the third time that the animal stays 2 or more seconds in the shock grid (without any attempt to

return to the treadmill). Once the animal reached one of the two exhaustion criteria, it was removed from the motor treadmill.

## 2.4. Assay of catecholamines

After acute exercise training, the animals were anesthetized (ketamine, 100 mg/kg and xylazine, 10 mg/kg, i.p.) and AGs were removed. The assay of catecholamines in AGs was performed by reversed-phase high-performance liquid chromatography with electrochemical detection (RP-HPLC-ED). In brief, catecholamines in 0.3 mL of perchloric acid, in which AGs had been kept, were eluted on Corning Costar Spin-X (Sigma-Aldrich, MO, EUA) centrifuge tube filters. After dilution and centrifugation, 50 µL of the eluate were injected into an HPLC for separation of NE and EPI (Gilson Medical Electronics, Villiers le Bel, France) (Moreira-Rodrigues et al., 2014). The lower limit of detection ranged from 350 to 1000 fmol.

## 2.5. Chronic exercise training protocol

Sedentary WT and Pnmt-KO mice were left without exercise training. The chronic exercise training program was always performed at the same time of day. Pnmt-KO and WT mice were adapted to the procedure 3 days before the beginning of the exercise training protocol. Before the training session, the animals were allowed 15 minutes to adapt to the treadmill without exercising. Exercise training was performed on a motor treadmill (Panlab Harvard Apparatus, Barcelona, Spain), starting at 6 cm/s for 10 minutes with an increase in speed of 3 cm/s every 2 minutes until 20 m/min, for 55 minutes (until exhaustion if 55 minutes duration was not reached), 5 days/week for 6 weeks (Paulino et al., 2010). It was considered exhaustion the animal's permanence in the shock grid (0.2 mA) for more than 5 consecutive seconds or the third time that the animal stays 2 or more seconds in the shock grid (without any attempt to return to the treadmill). As soon as the animal reached one of the two exhaustion criteria, it was removed from the motor treadmill.

## 2.6. Blood pressure and heart rate measurements

Systolic (SBP) and diastolic (DBP) BP and HR were measured after 6 weeks of chronic exercise training in conscious restrained animals, in a temperature control box at 37-38$^{o}$C, using a photoelectric tail-cuff pulse detector (Kent Scientific, CT, USA)

(Moreira-Rodrigues et al., 2010). Four determinations were made each time and the means were used for further analysis.

## 2.7. Echocardiographic and hemodynamic evaluation

BW was measured in WT and Pnmt-KO mice. Upon sedation and analgesia with 50 µg/kg and 5 mg/kg intraperitoneal fentanyl and midazolam, respectively, mice were anesthetized by inhalation of 8% sevoflurane in vented containers, orotracheally intubated (20G) and mechanically ventilated using a MouseVent™ Automatic Ventilator (Physiosuite, Kent Scientific, CT, USA). Anesthesia was maintained with sevoflurane (1-2.5%) titrated to avoid the toe pinch reflex. Mice were placed in left-lateral decubitus on a heating pad, the electrocardiogram was monitored (Animal Bio Amp, FE136, ADInstruments, Dunedin, New Zealand) and their temperature was automatically kept at 38ºC using a RightTemp™ Temperature Monitor & Homeothermic Controller (Physiosuite, Kent Scientific, CT, USA). The peripheral oximetry (MouseSTAT™ - Pulse Oximeter & Heart Rate Monitor, Physiosuite, Kent Scientific, CT, USA), capnography, respiratory rate (RR) and minute ventilation (CapnoScan™ - End-Tidal $CO_2$ Monitor, Physiosuite, Kent Scientific, CT, USA) were continuously motorized.

Cardiac ultrasounds were performed using an ACUSON Sequoia™ C512 ultrasound system (Siemens Medical Solutions, CA, USA). Before applying pre-warmed echocardiography gel, chest was shaved and depilated and then a linear 15MHz ACUSON™ Sequoia 15L8W (Siemens Medical Solutions, CA, USA) probe was gently positioned on the thorax. The 2-D parasternal short-axis imaging plane was used as a guide for obtaining LV end-systolic and end-diastolic endocardial and epicardial areas and also wall thickness and cavity dimensions using M-mode tracings, just above the papillary muscle. Based on this values, LV volumes, ejection fraction (EF) and fractional shortening (FS) were calculated according to Teichholdz formula, and LV mass was determined in diastole through an uncorrected cube method (Kiatchoosakun, Restivo, Kirkpatrick, & Hoit, 2002). The parasternal long axis view was used to measure LV long axis, from the mitral annulus to LV endocardial surface at the apex, and to record aortic root dimensions in M-mode. Pulsed-wave (PW) Doppler velocity tracings in the 4-chamber view allowed to assess LV filling with the peak early (E) and late (A) wave velocities of the mitral inflow. Peak early diastolic (E') and systolic (S') mitral annular velocities were measured with tissue Doppler (TDI) at lateral mitral annulus, and E/E' ratio was calculated. All the recordings were averaged from three consecutive heartbeats

For the hemodynamic evaluation, the right jugular vein was catheterized (24G) under surgical microscopy for fluid replacement with warm Ringer's lactate solution at 64

ml/Kg/h (NE-1000, New Era Pump Systems, NY, USA). After left thoracotomy, a pressure volume (PV) catheter (PV-1035, Millar Instruments, TX, USA), was inserted through the apex and signals were continuously acquired (MPVS 3000, Millar Instruments) and digitized at 1000 Hz (ML880 PowerLab 16/30, ADInstruments, Dunedin, New Zealand). Parallel conductance was assessed by 10 µL of 10% hypertonic saline injection. Baseline recordings were obtained after a stabilization period of 30 minutes and inferior vena cava (IVC) occlusion with a 5-0 silk lace were also obtained to derive load-independent indexes of contractility and compliance by linear and exponential fitting of the end-systolic and end-diastolic PV relationships (ESPVR and EDPVR). After an intravenous infusion of dobutamine ($\beta_1$-AR agonist) at 5 µg/Kg/min, IVC occlusion recordings were repeated upon obtaining a stable effect of at least 10 minutes. All acquisitions were performed with ventilation suspended at end-expiration. To account for large differences in body weight (BW) between groups, all volumes were indexed for body surface area (BSA) as estimated by 9.82*body weight $(BW)^{2/3}$ in grams (Cheung et al., 2009). Upon completion of experiments, animals were euthanized by exsanguination under anesthesia and samples were collected.

## 2.8. Morphometric analysis

Hearts were removed, weighed and dissected. Afterwards, the LV, right ventricle (RV) and interventricular septum (IVS) were weighted. Gastrocnemius muscle weight (GW) and tibia length were also measured. The ratios between heart weight (HW/BW), LV weight (LVW/BW), IVS weight (IVSW/BW), RV weight (RVW/BW), GW (GW/BW) and BW were calculated.

## 2.9. Determination of plasma Brain Natriuretic Peptide

The levels of BNP in mice plasma were measured using the Brain Natriuretic Peptide Enzyme Immunoassay (EIA) Kit (Sigma-Aldrich, MO, EUA) with detectable levels of BNP ranging from 0.1 to 1,000 pg/mL according to the manufacturer's instructions.

## 2.10. Total RNA isolation from left ventricular tissue

LV tissue samples were collected and frozen (-80°C). For tissue homogenization, 1 mL of Trizol Reagent (Sigma-Aldrich, MO, EUA) was added to the tubes with the samples and the contents were transferred to MagNA Lyser Green Beads tubes, prefilled

with 1.4 mm-diameter ceramic beads (Roche Diagnostics, Basel, Switzerland). Then, tissues were homogenized using the MagNA Lyser Instrument homogenizer (Roche Diagnostics, Basel, Switzerland). Homogenized samples were incubated for 5 minutes at room temperature to allow complete dissociation of the nucleoprotein complex. Afterwards, 200 µL of chloroform were added, the tubes were shaken by hand for 15 seconds, incubated for 2-3 minutes at room temperature and later centrifuged at 12,000 ×g for 15 minutes at 4°C. Samples separated into a red phenol-chloroform phase and an aqueous phase where the RNA remains exclusively. The aqueous phase was collected into a new tube, and 200 µL of 95% ethanol were added to the cleared lysate.

After a centrifugation at 12,000 ×g for 1 minute, total RNA isolation was carried out with the SV Total RNA Isolation System kit (Promega, WI, USA), according to the manufacturer's instructions.

Concentration and purity of the isolated RNA was measured using the NanoDrop 2000 spectrophotometer (Thermo Scientific, MA, USA). One µL of the eluted RNA was used to determine the concentration by measuring A260 absorbance for each sample. Purity was assessed by calculating A260/A280 and A260/A230 ratios. Nuclease-free water was used as blank.

A fraction of RNA from each sample was individually mixed with loading dye (Bromophenol blue 6x), loaded into a 1 %(w/v) agarose gel and ran at 90 V for 50 minutes using PowerPac™ Basic Power Supply (Bio-Rad, CA, USA) to assess RNA integrity. All samples were then stored at -70°C.

## 2.11. cDNA synthesis

Reverse transcription (20 µL) was performed in a T100™ Thermal Cycler (Bio-Rad, CA, USA) and consisted of different steps which are described below.

In the first step, after mixing 1 µg of RNA, 5x Reaction Buffer (Thermo Scientific, MA, USA), 1.7 µM oligo $(dT)_{18}$ Primer (Thermo Scientific, MA, USA), 10 U/reaction of Ribolock RNase inhibitor (Thermo Scientific, MA, USA) and Nuclease-free $H_2O$ (Thermo Scientific, MA, USA) to complete a volume of 18 µL, the tubes were incubated at 65ºC for 5 minutes and immediately placed on ice for 5 minutes.

In the second step, a reverse transcription master mix (RT mix) was prepared by adding 0.05 mM of dNTP Mix, 20 U/reaction of Ribolock RNase inhibitor and 100 U/reaction of RevertAid H Minus Reverse Transcriptase to a final volume of 2 µL. Also, a reverse transcriptase control master mix (Minus RT mix) was prepared with all the components of the RT mix, except that Nuclease-free $H_2O$ (Thermo Scientific, MA, USA)

was used as substitute for RevertAid H Minus Reverse Transcriptase (Thermo Scientific, MA, USA) to a final volume of 2 µL.

In the third step, after addition of RT and Minus RT samples to the mixtures, the tubes were incubated at 42ºC for 60 minutes. In the end of the reaction, the samples were incubated at 85ºC for 5 min in order to denature the RT enzyme, thus preventing unspecific activity or uncontrolled events.

Preparation of standard curve mix samples was made by adding 1 µL of each RT sample to a tube and diluting to a final 1/5 concentration with $H_2O$. Subsequent points of the standard curve were prepared by sequential 1:1 dilutions.

Finally, cDNA samples were diluted 1:20 and aliquots of 80 µL were made. For further use, the cDNA aliquots were stored at -20 ºC.

## 2.12. Relative quantification of mRNA expression

Real-time PCR (qPCR) was used to perform relative quantification of mRNA. For each studied mRNA molecule, standard curves were generated from the correlation between the amount of starting total mRNA and PCR threshold cycle of graded dilutions from a pool of all samples.

A StepOne™ Real-Time PCR System (Applied BioSystems, MA, USA) was used in qPCR experiments.

The qPCR reaction per well (12.5 µL) was performed by mixing 2x Maxima SYBR Green qPCR Master Mix (Thermo Scientific, MA, USA), 5 µM of gene-specific primers (Table 1) and Nuclease-free $H_2O$ (Thermo Scientific, MA, USA) to complete a final volume of 9.5 µL before adding 3 µL of cDNA (1:20). Instead of cDNA, Nuclease-free $H_2O$ (Thermo Scientific, MA, USA) was added as a negative control. The PCR reaction components were transferred to the wells in triplicate. The plate was centrifuged at 25ºC for 2 minutes at 2000 rpm. Centrifugation was performed to get all the contents at the bottom of the wells.

Table 1. Gene-specific primers sequences and biochemical features.

| Primer | Sequence (5'→3') | Size (bp) | Tm (°C) | GC% |
|---|---|---|---|---|
| GAPDH FWD | CCATCACCATCTTCCAGGAG | 20 | 54.9 | 55.0 |
| GAPDH REV | GCATGGACTGTGGTCATGAG | 20 | 55.9 | 55.0 |
| αMHC FWD | TGGTCACCAACAACCCATACGACT | 24 | 70.2 | 50.0 |
| αMHC REV | TGTCAGCTTGTAGACACCAGCCTT | 24 | 68.0 | 50.0 |
| βMHC FWD | GCCAACACCAACCTGTCCAAGTTC | 24 | 71.4 | 54.1 |
| βMHC REV | TGCAAAGGCTCCAGGTCTGAGGGC | 24 | 76.8 | 62.5 |
| ANP FWD | AGGCAGTCGATTCTGCTTGA | 20 | 65.1 | 50.0 |
| ANP REV | CGTGATAGATGAAGGCAGGAAG | 22 | 64.7 | 50.0 |
| BNP FWD | TAGCCAGTCTCCAGAGCAATTC | 22 | 64.5 | 50.0 |
| BNP REV | TTGGTCCTTCAAGAGCTGTCTC | 22 | 64.7 | 50.0 |
| DDR2 FWD | GAAATCTACAAGCGACCTGACAT | 23 | 63.2 | 43.4 |
| DDR2 REV | GATCATTTCAGAAGACGGAGTTG | 23 | 63.7 | 43.4 |
| IGF-1 FWD | GAAGTCCCCGTCCCTATCGA | 20 | 67.3 | 60.0 |
| IGF-1 REV | CCTTCTCCTTTGCAGCTTCG | 20 | 66.2 | 55.0 |
| IGF-1R FWD | AGTGACTCGGATGGCTTCGTT | 21 | 67.1 | 52.3 |
| IGF-1R REV | TTTCACAGGAAGCTCGCTCTC | 21 | 65.9 | 52.3 |
| AT1R FWD | GGCAGCATCGGACTAAATGG | 20 | 66.6 | 55.0 |
| AT1R REV | CCAGCTCCTGACTTGTCCTTG | 21 | 66.2 | 57.1 |
| AT2R FWD | AGCCAAGGCCAGATTGAAGA | 20 | 66.0 | 50.0 |
| AT2R REV | GCCACCAGCAGAAACATTACC | 21 | 65.5 | 52.3 |

The thermal cycling profile of the PCR machine was set-up as shown in Table 2. Reactions were run for 45 cycles and after that, the amplified PCR products were routinely subjected to melting curve analysis (60-95ºC) to verify specificity of the amplification. Afterwards, gel electrophoresis 1% (w/v) was performed to exclude primer-dimer formation and assess the purity of the amplification product.

Table 2. Thermal cycler profile.

| Temperature (°C) | Time (minutes:seconds) |
|---|---|
| 95 | 05:00 |
| 95 | 00:30 |
| 60 | 01:00 |
| 95 | 00:15 |
| 60 | 01:00 |

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were similar in all experimental groups, which enabled the use of this gene as an internal control. Results of mRNA quantification are expressed in an arbitrary unit (AU) after normalization for GAPDH.

Fold changes were determined using the standard curve method as described by others (Larionov, Krause, & Miller, 2005).

## 2.13. Statistics

All results are expressed as mean ± standard error of the means (SEM) for the indicated number of determinations. Student's unpaired t-test was applied to data regarding distance and total time run during chronic exercise training and catecholamine assays. Hemodynamic results were assessed by two-way ANOVA and two-way repeated measures ANOVA. Residuals normality was checked by Shapiro-Wilk's test and homogeneity of variances by Levene's or Box's M test. For the ESPVR and EDPVR analysis, volume intercept and scaling constant were included as covariates, respectively. All other statistical analysis was performed only by two-way ANOVA and log transformation of variables was applied whenever necessary to overcome violation of assumptions. When not possible to meet the assumptions at all, a Kruskal-Wallis non parametric test was used, and when appropriate, post hoc analysis was performed using Newman-Keuls' test. The comparisons regarding hemodynamic results were made using *STATISTICA* (StatSoft, Inc., OK, USA) software. All other results were assessed by two-way ANOVA (BP and HR, morphometric and echocardiographic analysis, plasma BNP, and qPCR). All other comparisons were made using GraphPad Prism statistics software package (GraphPad Software Inc., CA, USA). A statistical significant difference was set at $P<0.05$.

# 3. Results

## 3.1. Genotyping

The presence of the Pnmt-KO (mutant) allele was verified by PCR-based genotyping. Amplification reactions yielded products of 200 and 160 bp for the WT and Pnmt-KO alleles, respectively (Figure 6).

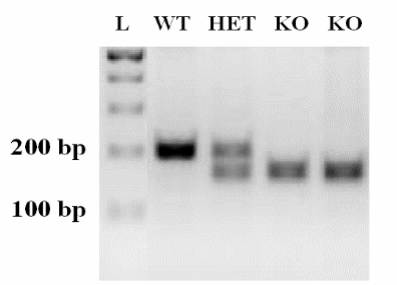


Figure 6. PCR amplification of the Pnmt gene in ear samples. The PCR products and ladder were separated in a 1.0 % agarose gel, photographed and representative examples are shown. The DNA fragments expected for the wild-type (WT, 200 bp), heterozygous (HET, 200 bp and 160 bp) and Pnmt-KO (mutant, 160 bp) mice are indicated.

## 3.2. Catecholamines

EPI levels in AGs were vestigial in Pnmt-KO when compared with WT mice (Figure 7A). On the contrary, NE levels in AGs were significantly higher in Pnmt-KO than in WT animals (Figure 7B).

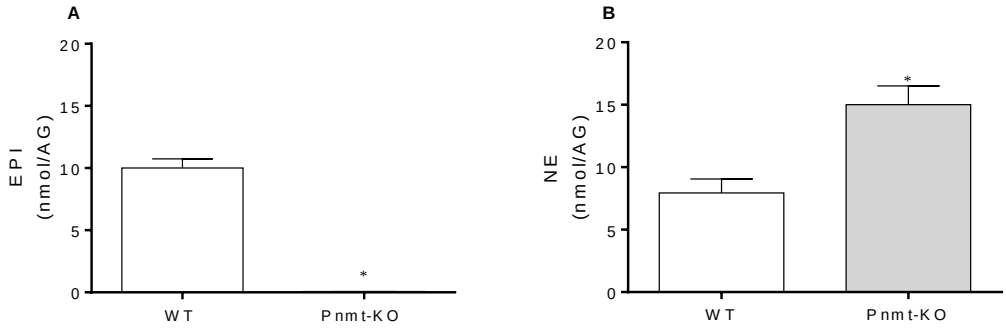


Figure 7. Quantification of A) EPI and B) NE levels in adrenal gland (AG) in WT and Pnmt-KO mice. Values are means ± SEM of 6-10 animals per group. WT, wild-type; Pnmt-KO, Phenylethanolamine-*N*-methyltransferase-knockout. *Significantly different from correspondent values in WT mice ($P < 0.05$).

Immediately after acute exercise training, NE levels in AGs were higher in trained compared to sedentary WT mice (Table 3). Also, no significant differences were found in EPI levels in AGs between acute trained and sedentary WT mice (Table 3).

Table 3. Quantification of EPI and NE in adrenal glands in sedentary and acute exercise trained WT mice.

| | | WT | WT acute ex |
|---|---|---|---|
| **EPI** | **Adrenal Gland (nmol/AG)** | 12.80 ± 1.29 | 12.91 ± 0.73 |
| **NE** | **Adrenal Gland (nmol/AG)** | 7.17 ± 0.55 | 9.73 ± 0.62* |

Values are means ± SEM of 6-7 animals per group. WT, wild-type; WT acute ex, acute exercise trained wild-type; EPI, epinephrine; NE, norepinephrine; AG, adrenal gland. * Significantly different from correspondent values in WT mice ($P < 0.05$).

## 3.3. Distance and total time run during chronic exercise training

No differences were found in distance travelled (Figure 8A) and running time duration (Figure 8B) between chronic exercise trained Pnmt-KO and WT mice.

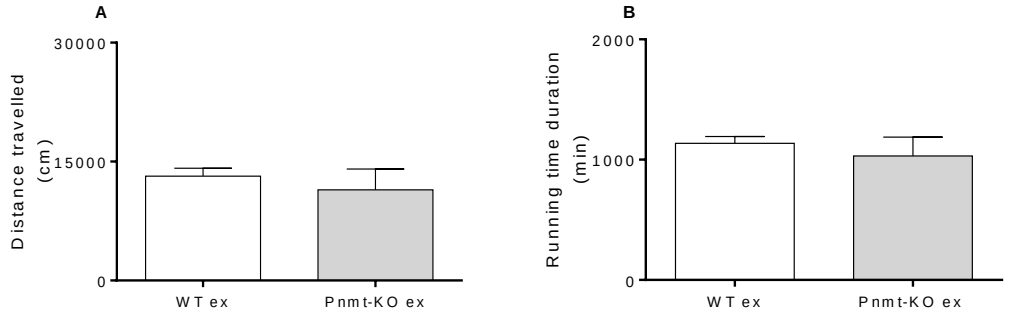


Figure 8. A) Distance travelled and B) running time duration in chronic exercise trained WT and Pnmt-KO mice. Values are means ± SEM of 5-8 animals per group. WT ex, chronic exercise trained wild-type; Pnmt-KO ex, chronic exercise trained Phenylethanolamine-*N*-methyltransferase-knockout.

## 3.4. Blood pressure and heart rate

No differences were observed in SBP (Figure 9A), DBP (Figure 9B) and HR (Figure 9C) between sedentary Pnmt-KO and WT mice. However, SBP was significantly increased in trained Pnmt-KO when compared to trained WT mice (Figure 9A).

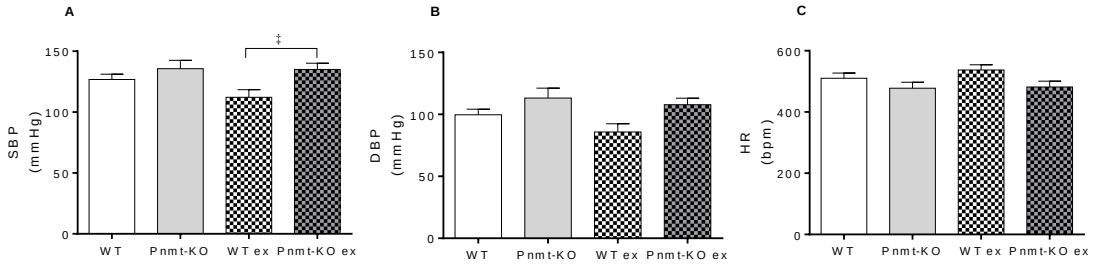


Figure 9. A) Systolic (SBP) and B) diastolic (DBP) blood pressure (BP), and C) heart rate (HR) in sedentary and chronic exercise trained WT and Pnmt-KO mice. Values are means ± SEM of 5 animals per group. WT ex, chronic exercise trained wild-type; Pnmt-KO, Phenylethanolamine-*N*-methyltransferase-knockout; Pnmt-KO ex, chronic exercise trained Phenylethanolamine-*N*-methyltransferase-knockout. ‡Significantly different from correspondent values in WT ex mice ($P < 0.05$).

## 3.5. Morphometric parameters

In sedentary Pnmt-KO mice, BW, HW/BW and LVW/BW were significantly lower than in sedentary WT mice. Moreover, in trained WT mice, only IVSW/BW was significantly higher than in sedentary WT animals. BW, HW/BW, LVW/BW and IVSW/BW were significantly increased in trained Pnmt-KO compared with sedentary Pnmt-KO mice. Trained Pnmt-KO mice also showed a significant increase in HW/BW compared to trained WT mice, and no differences in BW were observed between both groups. Additionally, no significant differences were found in RVW/BW, GW/BW and tibia length between all groups (Table 4).

Table 4. Morphometric parameters for sedentary and chronic exercise trained WT and Pnmt-KO mice.

| | WT | Pnmt-KO | WT ex | Pnmt-KO ex |
|---|---|---|---|---|
| **BW, g** | 29.0 ± 0.5 | 27.2 ± 0.3* | 29.9 ± 0.8 | 30.5 ± 1.4† |
| **HW/BW, mg/g** | 5.73 ± 0.12 | 5.01 ± 0.15* | 5.75 ± 0.23 | 6.64 ± 0.64†‡ |
| **LVW/BW, mg/g** | 2.36 ± 0.10 | 1.83 ± 0.04* | 2.18 ± 0.07 | 2.26 ± 0.12† |
| **IVSW/BW, mg/g** | 1.28 ± 0.06 | 1.31 ± 0.05 | 1.57 ± 0.08* | 1.82 ± 0.21† |
| **RVW/BW, mg/g** | 0.90 ± 0.04 | 0.90 ± 0.05 | 0.94 ± 0.06 | 1.03 ± 0.20 |
| **GW/BW, mg/g** | 4.97 ± 0.24 | 5.47 ± 0.21 | 5.07 ± 0.27 | 4.64 ± 0.31 |
| **Tibia length, cm** | 18.4 ± 0.1 | 18.2 ± 0.1 | 18.6 ± 0.3 | 18.9 ± 0.3 |

Values are means ± SEM of 5-12 animals per group. WT, wild-type; WT ex, chronic exercise trained wild-type; Pnmt-KO, Phenylethanolamine-*N*-methyltransferase-knockout; Pnmt-KO ex, chronic exercise trained Phenylethanolamine-*N*-methyltransferase-knockout. BW, body weight; HW/BW, ratio of heart weight to body weight; LVW/BW, ratio of left ventricular weight to body weight; IVSW/BW, ratio of interventricular septum weight to body weight; RVW/BW, ratio of right ventricular weight to body weight; GW/BW, ratio of gastrocnemius muscle weight to body weight. * Significantly different from correspondent values in WT mice ($P < 0.05$). †Significantly different from correspondent values in Pnmt-KO mice ($P < 0.05$). ‡Significantly different from correspondent values in WT ex mice ($P < 0.05$).

## 3.6. Echocardiographic measurements

Regarding sedentary groups, even though there was no change in LV mass ($LVM_i$) (Figure 10A), the ratio of LV end-diastolic posterior wall thickness to LV end-diastolic internal dimension (LVPWd/LVIDd) (Figure 10D) was significantly higher in Pnmt-KO compared to WT mice. In contrast, in Pnmt-KO mice, LV end-diastolic internal dimension (LVIDd) and indexed LV end-diastolic volume ($LVEDV_i$) were significantly lower than in WT mice (Table 5). Moreover, in sedentary Pnmt-KO mice, indexed stroke volume ($SV_i$) and cardiac index (CI) were decreased, with no changes in other cardiac contractility parameters (FS, EF, S' and IVA) (Table 5). Echocardiographic parameters of diastolic function (E/E' and E/A) also showed no changes between sedentary mice (Table 5).

In chronic exercise, it was observed in WT mice a significantly increased LV mass ($LVM_i$) (Figure 10A), interventricular end-diastolic septal thickness (IVSd) (Table 5), LV posterior wall thickness (LVPWd (Figure 10B) and LVPWs (Figure 10C)), and LVPWd/LVIDd (Figure 10D), without significant differences in LV volume (LVIDd, LVIDs, $LVEDV_i$ and $LVESV_i$) when compared with sedentary WT mice (Table 5). Furthermore, when compared with sedentary WT mice, there were no changes in systolic (FS, EF, S´, IVA, $SV_i$, and CI) and diastolic function (E/E' and E/A) indexes in trained WT mice (Table

5).

Moreover, in trained Pnmt-KO mice, LV mass ($LVM_i$) (Figure 10A), LV posterior wall thickness (LVPWd (Figure 10B) and LVPWs (Figure 10C), LV epicardial area (LVEpiAd and LVEpiAs) and interventricular septal thickness (IVSd and IVSs) were significantly higher compared with sedentary Pnmt-KO mice (Table 5). Also, LV volume (LVIDd and $LVEDV_i$) was significantly higher in trained Pnmt-KO mice compared with sedentary Pnmt-KO mice (Table 5). Additionally, $SV_i$ and CI increased in trained Pnmt-KO mice, with no differences in other systolic function parameters (FS, EF, S' and IVA) when compared with sedentary Pnmt-KO mice (Table 5). Parameters of diastolic function (E/E' and E/A) showed no changes between these groups (Table 5).

Furthermore, in trained Pnmt-KO mice, a significantly increase was found in LV mass ($LVM_i$) (Figure 10A), LV posterior wall thickness (LVPWd (Figure 10B) and LVPWs (Figure 10C)), and LV epicardial area (LVEpiAd and LVEpiAs), without significant differences in LV volume (LVIDd, LVIDs, $LVEDV_i$ and $LVESV_i$) when compared with trained WT mice (Table 5). Also, cardiac contractility (FS, EF, S´, IVA, $SV_i$, and CI) and relaxation (E/E' and E/A) parameters did not differ between these groups (Table 5).

Table 5. Echocardiographic parameters in sedentary and chronic exercise trained WT and Pnmt-KO mice.

| | WT | Pnmt-KO | WT ex | Pnmt-KO ex |
|---|---|---|---|---|
| **LVEndoAd, mm²** | 10.1 ± 0.8 | 8.4 ± 0.6 | 9.4 ± 0.6 | 9.3 ± 1.2 |
| **LVEndoAs, mm²** | 5.1 ± 0.4 | 4.7 ± 0.6 | 4.4 ± 0.4 | 4.7 ± 0.4 |
| **LVEpiAd, mm²** | 21.6 ± 0.6 | 19.6 ± 0.4 | 22.1 ± 0.8 | 28.1 ± 1.2†‡ |
| **LVEpiAs, mm²** | 17.9 ± 0.7 | 16.0 ± 0.7 | 18.8 ± 0.9 | 23.0 ± 1.6†‡ |
| **IVSd, mm** | 0.46 ± 0.02 | 0.51 ± 0.03 | 0.59 ± 0.03* | 0.69 ± 0.10† |
| **IVSs, mm** | 0.47 ± 0.03 | 0.49 ± 0.04 | 0.56 ± 0.05 | 0.69 ± 0.04† |
| **LVIDd, mm** | 2.8 ± 0.1 | 2.2 ± 0.1* | 3.0 ± 0.2 | 3.2 ± 0.2† |
| **LVIDs, mm** | 1.5 ± 0.1 | 1.1 ± 0.1 | 1.5 ± 0.2 | 1.8 ± 0.1 |
| **$LVEDV_i$, µL/cm²** | 0.66 ± 0.07 | 0.35 ± 0.03* | 0.77 ± 0.11 | 0.89 ± 0.14† |
| **$LVESV_i$, µL/cm²** | 0.12 ± 0.03 | 0.04 ± 0.01 | 0.12 ± 0.03 | 0.18 ± 0.03 |
| **$SV_i$, µL/cm²** | 0.54 ± 0.05 | 0.30 ± 0.02* | 0.65 ± 0.09 | 0.71 ± 0.12† |
| **CI, mL/min/cm²** | 0.22 ± 0.02 | 0.11 ± 0.03* | 0.32 ± 0.05 | 0.36 ± 0.08† |
| **FS, %** | 49 ± 3 | 52 ± 2 | 52 ± 4 | 43 ± 3 |
| **EF, %** | 81 ± 2 | 87 ± 2 | 86 ± 3 | 80 ± 3 |
| **S', cm/s** | 5.0 ± 0.3 | 4.9 ± 0.3 | 4.9 ± 0.2 | 4.2 ± 0.9 |
| **IVA, m/s²** | 4.1± 0.4 | 4.5 ± 0.5 | 4.5 ± 0.3 | 3.7 ± 0.9 |
| **E/E'** | 15.1 ± 1.43 | 16.3 ± 1.40 | 17.0 ± 1.16 | 20.1 ± 4.30 |
| **E/A** | 1.50 ± 0.05 | 1.30 ± 0.03 | 1.41 ± 0.07 | 1.29 ± 0.10 |

Values are means ± SEM of 5–12 animals per group. WT, wild-type; WT ex, chronic exercise trained wild-type; Pnmt-KO, Phenylethanolamine-*N*-methyltransferase-knockout; Pnmt-KO ex, chronic exercise trained Phenylethanolamine-*N*-methyltransferase-knockout. LVEndoAd, left ventricular end-diastolic endocardial area; LVEndoAs, left ventricular end-systolic endocardial area; LVEpiAd, left ventricular end-diastolic epicardial area; LVEpiAs, left ventricular end-systolic epicardial area; IVSd, interventricular end-diastolic septal thickness; IVSs, interventricular end-systolic septal thickness; LVIDd, left ventricular end-diastolic internal dimension; LVIDs, left ventricular end-systolic internal dimension; $LVEDV_i$, indexed left ventricular end-diastolic volume; $LVESV_i$, indexed left ventricular end-systolic volume; $SV_i$, indexed stroke volume; CI, cardiac index; FS, fractional shortening; EF, ejection fraction; S', maximum systolic tissue Doppler velocity at the lateral mitral annulus; IVA, isovolumetric acceleration; E/E', ratio between peak velocity of early filling in transmitral flow pulsed-wave Doppler and maximum velocity of early diastolic myocardial motion at the lateral mitral annulus; E/A, ratio between peak velocity of early filling to peak velocity of late filling in transmitral flow pulsed-wave Doppler. *Significantly different from correspondent values in WT mice ($P < 0.05$). †Significantly different from correspondent values in Pnmt-KO mice ($P < 0.05$). ‡Significantly different from correspondent values in WT ex mice ($P < 0.05$).

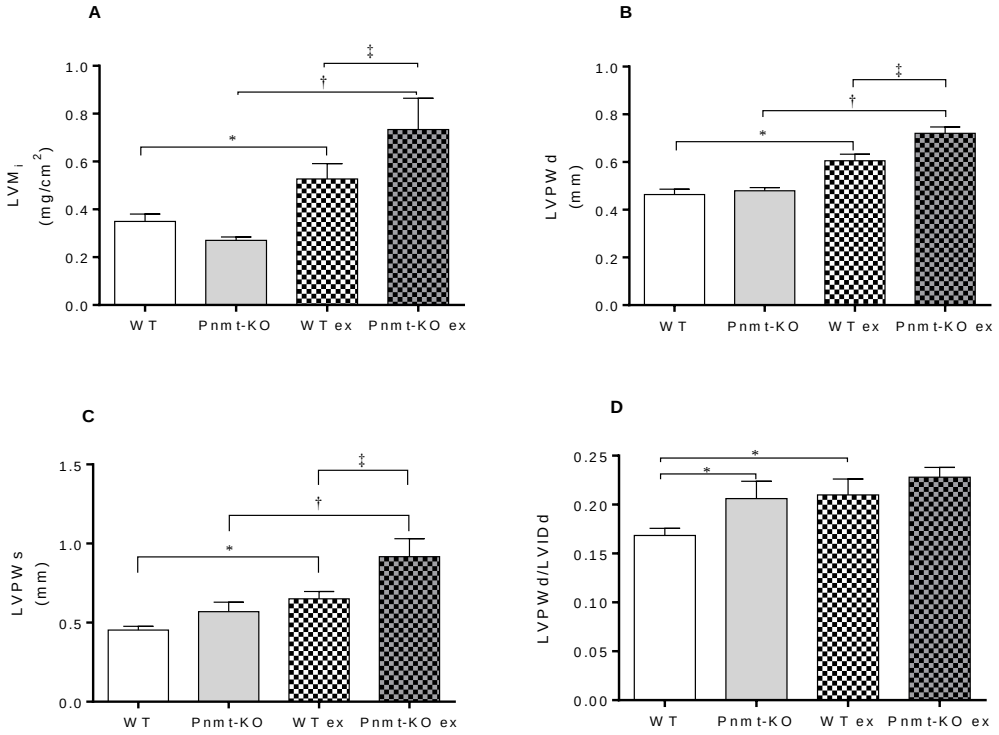


Figure 10. A) Left ventricular mass indexed for body surface area ($LVM_i$), B) left ventricular end-diastolic posterior wall thickness (LVPWd), C) left ventricular end-systolic posterior wall thickness (LVPWs) and D) ratio of left ventricular end-diastolic posterior wall thickness to left ventricular end-diastolic internal dimension (LVPWd/LVIDd) echocardiographic measurements in sedentary and chronic exercise trained WT and Pnmt-KO mice. Values are means ± SEM of 5–12 animals per group. WT, wild-type; WT ex, chronic exercise trained wild-type; Pnmt-KO, Phenylethanolamine-*N*-methyltransferase-knockout; Pnmt-KO ex, chronic exercise trained Phenylethanolamine-*N*-methyltransferase-knockout. *Significantly different from correspondent values in WT mice ($P < 0.05$). †Significantly different from correspondent values in Pnmt-KO mice ($P < 0.05$). ‡Significantly different from correspondent values in WT ex mice ($P < 0.05$).

## 3.7. Hemodynamic measurements

In sedentary mice, at baseline conditions, $LVEDV_i$ (Table 6) and CI (Figure 11B) were significantly lower in Pnmt-KO compared to WT mice. Compared to basal parameters, acute $\beta_1$-adrenergic stimulation with dobutamine decreased maximum pressure (Pmax) and $LVESV_i$ in sedentary Pnmt-KO mice and did not decreased both in sedentary WT mice (Table 6).

At baseline conditions, trained WT mice showed a significantly increase in Pmax in comparison to sedentary WT mice (Table 6). Compared to basal parameters, acute $\beta_1$-adrenergic stimulation with dobutamine increased PRSW, $LVESV_i$, $SV_i$ $LVEDV_i$, $VVC_i$, (Table 6) and CI (Figure 11B) in trained WT, and not in sedentary WT mice.

Moreover, at baseline conditions, trained Pnmt-KO mice showed a significant increase in Pmax, $LVEDV_i$ (Table 6) and CI (Figure 11B) compared with sedentary Pnmt-

KO mice. Compared to basal parameters, acute $\beta_1$-adrenergic stimulation with dobutamine decreased $LVESV_i$ (Table 6) and increased EF (Figure 11A) in sedentary Pnmt-KO, and not in trained Pnmt-KO mice (Table 6).

No differences were observed in baseline conditions between trained WT and Pnmt-KO mice (Table 6). Compared to basal parameters, acute $\beta_1$-adrenergic stimulation with dobutamine increased $SV_i$, $LVEDV_i$, $VVC_i$, (Table 6), EF (Figure 11A) and CI (Figure 11B) in trained WT mice, and did not increase in trained Pnmt-KO mice. In addition, acute $\beta_1$-adrenergic stimulation with dobutamine, compared to basal parameters, decreased $LVESV_i$ in trained WT mice, and did not decrease in trained Pnmt-KO mice (Table 6).

Compared to basal parameters, acute $\beta_1$-adrenergic stimulation with dobutamine decreased arterial elastance ($Ea_i$) in all groups (Table 6).

Moreover, after acute $\beta_1$-adrenergic stimulation with dobutamine, no significant differences were found in $E_{ESi}$, $\beta_i$ and EDP in all groups (Table 6).

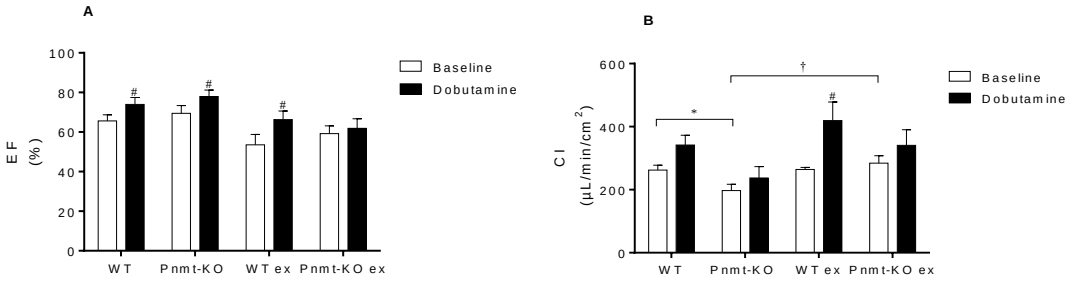


Figure 11. A) Ejection fraction (EF) and B) cardiac index (CI) hemodynamic measurements, at baseline and after dobutamine ($\beta_1$-adrenoceptor agonist) perfusion, in sedentary and chronic exercise trained WT and Pnmt-KO mice. Data are means ± SEM of 5-12 animals per group. WT, wild-type; WT ex, chronic exercise trained wild-type; Pnmt-KO, Phenylethanolamine-*N*-methyltransferase-knockout; Pnmt-KO ex, chronic exercise trained Phenylethanolamine-*N*-methyltransferase-knockout. #Significantly different from correspondent values in respective baseline ($P < 0.05$). *Significantly different from correspondent values in WT mice ($P < 0.05$). †Significantly different from correspondent values in Pnmt-KO mice ($P < 0.05$).

Table 6. Hemodynamic parameters at baseline and after dobutamine ($\beta_1$-adrenoceptor agonist) perfusion in sedentary and chronic exercise trained WT and Pnmt-KO mice.

| | | WT | Pnmt-KO | WT ex | Pnmt-KO ex |
|---|---|---|---|---|---|
| **$P_{max}$, mmHg** | **Baseline** | 89.5 ± 3.2 | 90.4 ± 2.6 | 102.9 ± 1.5* | 103.8 ± 5.2† |
| | **Dobutamine** | 88.2 ± 4.4 | 80.5 ± 2.9# | 100.1 ± 2.6 | 98.3 ± 4.6 |
| **$E_{ES}i$, mmHg/μL/cm²** | **Baseline** | 132.9 ± 11.1 | 168.1 ± 22.9 | 149.1 ± 15.4 | 160.5 ± 28.8 |
| | **Dobutamine** | 206.0 ± 40.2 | 218.5 ± 56.0 | 240.5 ± 20.1 | 183.2 ± 30.7 |
| **PRSW, mmHg** | **Baseline** | 59.4 ± 7.2 | 61.5 ± 7.4 | 36.9 ± 6.5 | 59.1 ± 6.4 |
| | **Dobutamine** | 75.9 ± 10.6 | 68.5 ± 6.9 | 85.0 ±10.3# | 102.1 ± 5.3# |
| **$LVESV_i$, μL/cm²** | **Baseline** | 0.31 ± 0.03 | 0.23 ± 0.05 | 0.48 ± 0.08 | 0.43 ± 0.08 |
| | **Dobutamine** | 0.27 ± 0.03 | 0.18 ± 0.03# | 0.39 ± 0.06# | 0.41 ± 0.09 |
| **$SV_i$, μL/cm²** | **Baseline** | 0.51 ± 0.04 | 0.38 ± 0.04 | 0.45 ± 0.02 | 0.47 ± 0.05 |
| | **Dobutamine** | 0.62 ± 0.04 | 0.45 ± 0.07 | 0.65 ± 0.07# | 0.53 ± 0.05 |
| **$\beta_I$, μL/cm²** | **Baseline** | 6.5 ± 0.8 | 7.1 ± 1.3 | 4.4 ± 0.4 | 7.3 ± 2.2 |
| | **Dobutamine** | 5.9 ± 0.70 | 7.2 ± 0.8 | 3.6 ± 0.2 | 6.7 ± 2.1 |
| **$LVEDV_i$, μL/cm²** | **Baseline** | 0.79 ± 0.04 | 0.56 ± 0.07* | 0.88 ± 0.08 | 0.84 ± 0.10† |
| | **Dobutamine** | 0.86 ± 0.05 | 0.60 ± 0.10 | 0.99 ± 0.09# | 0.86 ± 0.09 |
| **EDP, mmHg** | **Baseline** | 6.3 ± 0.8 | 7.2 ± 0.6 | 5.4 ± 0.9 | 4.2 ± 0.3 |
| | **Dobutamine** | 4.6 ± 0.4 | 5.3 ± 1.5 | 3.5 ± 0.4 | 3.9 ± 0.7 |
| **$E_a i$, mmHg/μL/cm²** | **Baseline** | 162.6 ± 13.4 | 242.9 ± 36.0 | 217.5 ± 8.0 | 208.4 ± 24.5 |
| | **Dobutamine** | 122.9 ± 10.3# | 186.9 ± 33.4# | 139.4 ± 17.8# | 163.7 ± 28.8# |
| **$VVC_i$** | **Baseline** | 0.9 ± 0.1 | 0.7 ± 0.1 | 0.7 ± 0.1 | 0.8 ± 0.1 |
| | **Dobutamine** | 1.7 ± 0.4 | 1.1 ± 0.1 | 1.8 ± 0.2# | 1.2 ± 0.3 |

Values are means ± SEM of 5–12 animals per group. WT, wild-type; WT ex, chronic exercise trained wild-type; Pnmt-KO, Phenylethanolamine-*N*-methyltransferase-knockout; Pnmt-KO ex, chronic exercise trained Phenylethanolamine-*N*-methyltransferase-knockout. $P_{max}$, maximum pressure; $E_{ES}$ i, end-systolic elastance derived from end-systolic pressure-volume relationship; PRSW, preload recruitable stroke work; $LVESV_i$, indexed left ventricular end-systolic volume; $SV_i$, indexed stroke volume; $\beta_i$, left ventricular chamber stiffness constant derived from end-diastolic pressure-volume relationship; $LVEDV_i$, indexed left ventricular end-diastolic volume; EDP, end-diastolic pressure; $E_a i$, arterial elastance index; $VVC_i$, ventricular-vascular coupling index. #Significantly different from correspondent values in respective baseline ($P < 0.05$). *Significantly different from correspondent values in WT mice ($P < 0.05$). †Significantly different from correspondent values in Pnmt-KO mice ($P < 0.05$). ‡Significantly different from correspondent values in WT ex mice ($P < 0.05$).

## 3.8. Plasma brain natriuretic peptide measurements

Plasma BNP concentration was not significant different between sedentary (7.9 ± 3.7 vs 18.5 ± 4.5 pg/mL; *n*=8-9) and chronic exercise trained (9.9 ± 4.0 vs 12.9 ± 3.4 pg/mL; *n*=5-6) Pnmt-KO and WT mice.

## 3.9. Cardiac Gene Expression

In the LV, mRNA expression of ANP (Figure 12A) and IGF-1 (Figure 12F) was significantly increased in trained Pnmt-KO mice when compared to trained WT and to sedentary Pnmt-KO mice. No differences were observed in BNP (Figure 12B), αMHC (Figure 12C), βMHC (Figure 12D), DDR2 (Figure 12E), IGF-1R (Figure 12G) and AT1R (Figure 12H) mRNA expression between all groups of animals. Also, no significant difference was found in the ratio βMHC/αMHC mRNA expression between sedentary (0.8 ± 0.4 vs 0.8 ± 0.3; *n*=6-7) and chronic exercise trained (1.5 ± 0.3 vs 3.2 ± 1.7; *n*=5-6) Pnmt-KO and WT mice. Moreover, regarding mRNA expression of AT2R, there was no amplification observed.

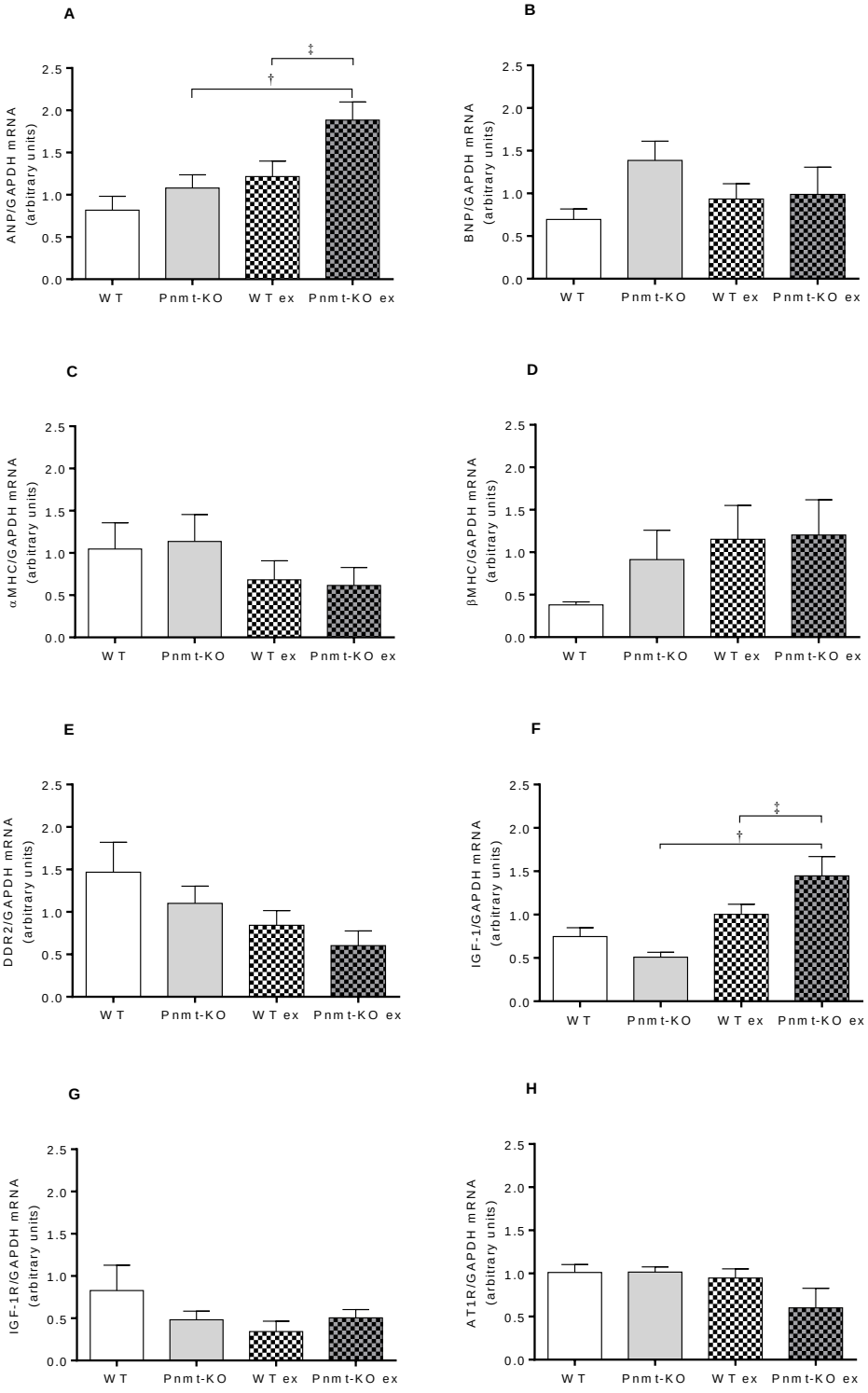


Figure 12. LV mRNA expression of A) atrial natriuretic peptide (ANP), B) brain natriuretic peptide (BNP), C) α-myosin heavy chain (αMHC), D) β-myosin heavy chain (βMHC), E) discoidin domain receptor 2 (DDR2); F) insulin-like growth factor-1 (IGF-1) ;G) insulin-like growth factor 1 receptor (IGF-1R) and H) angiotensin II type 1 receptor (AT1R) in sedentary and chronic exercise trained WT and Pnmt-KO mice. Results are expressed as arbitrary units after normalization for GAPDH. Values are means ± SEM of 6-8 animals per group. WT, wild-type; WT ex, chronic exercise trained wild-type; Pnmt-KO, Phenylethanolamine-*N*-methyltransferase-knockout; Pnmt-KO ex, chronic exercise trained Phenylethanolamine-*N*-methyltransferase-knockout. † Significantly different from correspondent values in Pnmt-KO mice ($P < 0.05$). ‡ Significantly different from correspondent values in WT ex mice ($P < 0.05$).

# 4. Discussion and conclusion

The present work has successfully assessed that chronic exercise training in Pnmt-KO mice resulted in functional cardiovascular adaptations and significant cardiac hypertrophy. Also, epinephrine appeared to be important for maintaining a normal BP, probably through $\beta_2$-AR induced vessel relaxation, and preventing LV hypertrophy in chronic exercise.

In this study, an epinephrine-deficient mouse model in which the EPI-synthesizing enzyme Pnmt is knocked out was used (Ebert et al., 2004), and the absence of the $Pnmt^{+/+}$ alele was confirmed by genotyping. Several authors showed that EPI was absent in AGs whereas the NE content of the AGs was significantly increased in Pnmt-KO mice (Ebert et al., 2004; Bao et al., 2007; Sun et al., 2008; Moreira-Rodrigues et al., 2014). The results in the present work were similar. Thus, taken together the results regarding genotyping and quantification of catecholamines, it was found that the disruption in the PNMT gene abolished its expression, therefore preventing the synthesis of EPI in Pnmt-KO mice.

Immediately after acute exercise training, NE levels in AGs were significantly higher in trained compared with sedentary WT mice, possibly in order to support the increased heart functioning, vasoconstriction and redistribution of blood to exercising muscles, and increased ventilatory needs (Garrett & Kirkendall, 2000). Therefore, it is expected that during chronic exercise, the increase of NE in both WT and Pnmt-KO mice may be favored. In order to prove this, NE levels should be quantified in AGs of sedentary and acute trained Pnmt-KO mice. Acute exercise training did not change EPI levels in AGs of WT mice. Since EPI has a short half-life (3- to 5-minutes) (Khan, 2006), this could make EPI detection and quantification by RP-HPLC-ED difficult.

In sedentary animals, systolic and diastolic BP did not differ and morphometric parameters, such as BW, HW/BW and LVW/HW were lower in Pnmt-KO mice. In addition, the observed increased ratio of diastolic LV wall thickness to LV cavity (LVPWd/LVIDd) and normal LV mass ($LVM_i$), suggests a concentric remodeling of the LV of sedentary EPI-deficient Pnmt-KO mice, and this echocardiographic results are similar to those previously reported by Bao et al. (2007) (Bao et al., 2007).

Furthermore, despite no changes in HR, echocardiographic $SV_i$ and consequently echocardiographic CI were reduced and this decreased CI may be explained by a decreased cardiac filling with a smaller $LVEDV_i$ (Bao et al., 2007) in sedentary Pnmt-KO mice. $LVEDV_i$ may be determined by venous tone and so, the reduced echocardiographic $LVEDV_i$ in sedentary Pnmt-KO mice is consistent with

findings in previous reports (Eckstein & Hamilton, 1957; Trippodo, 1981; Zhang, Weaver, Ibeawuchi, & Olson, 1998; Skals, Skovgaard, Abe, & Wang, 2005; Sandblom, Axelsson, & Farrell, 2006), that suggest that EPI has effects to constrict veins in vivo, possibly through $\alpha_1$ and $\alpha_2$-ARs (Flavahan & Vanhoutte,1987; Faber, 1988; Faber, et al., 1991). There were no changes in cardiac contractility echocardiographic parameters, as demonstrated by no changes in FS, EF, S' and IVA in sedentary groups. Also, in sedentary mice, diastolic function was similar, as showed by no changes in E/E' and E/A. Thus, in sedentary Pnmt-KO mice, EPI deficiency possibly has no impact on cardiovascular functions at rest such as BP and HR.

Numerous studies in mice (Calligaris et al., 2013; Cho et al., 1999) have shown that cardiac catheterization can be used to measure in vivo responsiveness to dobutamine, a $\beta_1$-AR agonist (Vallet et al., 1991). The increased EF observed may be due to dobutamine positive inotropic effect, and the decreased arterial elastance ($Ea_i$) observed might be due to dobutamine peripheral vasodilatation effect (Ruffolo, 1987).

Stress hormones, EPI and NE, may be responsible for many adaptations both at rest and during exercise. Previous studies have shown that exercise may induce cardiac hypertrophy in mice, namely treadmill exercise (Kemi et al., 2002), voluntary wheel training (Allen et al., 2001) and swimming exercise (Kaplan et al., 1994). In the present work, in order to access if EPI as a role in the adaptation to chronic exercise, WT and EPI-deficient Pnmt-KO mice were subjected to a chronic exercise training protocol. Exercise intensity was measured indirectly and no differences in distance and total time run were found between all groups, thus suggesting that both Pnmt-KO and WT mice adapted to the chronic exercise training protocol similarly.

In chronic exercise, a hypertrophied interventricular septum (IVSW/BW) was possibly associated with a significantly increased LV posterior wall thickness (LVPWd and LVPWs), LVPWd/LVIDd and LV mass ($LVM_i$) in WT mice when compared to sedentary WT mice. Taken all together, maybe related with no changes in mRNA expression of ANP (marker for pathological cardiac hypertrophy) (Grandi et al., 2004), and mRNA expression of DDR2 (marker for cardiac fibroblasts) (Goldsmith et al., 2004), suggests a physiological concentric hypertrophy, in an attempt to limit wall stress (Gaasch & Zile, 2011), and no cardiac fibrosis in chronic trained WT mice. Furthermore, when compared with sedentary WT mice, there were no changes in systolic (FS, EF, S´, IVA, $SV_i$ and CI) and diastolic function (E/E' and E/A) echocardiographic indexes in trained WT mice.

After acute exercise, NE content increased in AGs of WT mice, and it is conceivable that it could even be higher in acute trained Pnmt-KO mice, since exercise may lead to increased catecholamine levels. Therefore, chronic trained mice may be

exposed to high levels of NE in each exercise session. It is known that high levels of NE may stimulate renal $\beta_1$-ARs, increase renin secretion, angiotensin II production and thus adrenocortical secretion of aldosterone (Goldstein, 2010). In addition, angiotensin II via AT1 receptor is known to contribute to an increase in parameters that lead to an increased dilation in the ventricular cavity (McBride, 2006).

When compared with sedentary Pnmt-KO mice, a hypertrophied heart (in particular IVS/BW and LVW/BW) perhaps related with an increased LV mass ($LVM_i$), LV wall thickness (LVPWd and LVPWs), LV internal volume (LVIDd) and mRNA expression of IGF-1 (Wahlander, Isgaard, Jennische, & Friberg, 1992; Donohue et al., 1994) and ANP and no changes in mRNA expression of DDR2, may be associated with augmented NE levels that, by stimulating $\beta_1$-ARs, could increase angiotensin II production, possibly contributing to a thick and dilated concentric LV hypertrophy in chronic trained Pnmt-KO mice, which may be pathologic, and no cardiac fibrosis. Even though it is just a hypothesis, since there were no differences in mRNA expression of AT1R and no amplification of AT2R, angiotensin II seems not to contribute to LV hypertrophy. In order to confirm if the renin-angiotensin-aldosterone system (RAAS) plays a role in LV hypertrophy, it would be necessary to evaluate renin, angiotensin II and aldosterone levels in plasma and in cardiac tissue of WT and Pnmt-KO mice after chronic exercise.

Although there was no alteration in echocardiographic myocardial contractility (FS, EF, S' and IVA) and indexes of diastolic function (E/E' and E/A), chronic exercise training led to an increased echocardiographic and hemodynamic preload ($LVEDV_i$) in Pnmt-KO mice, possibly due to an increased venous return, which is increased maybe as a result of a combination of an increase in sympathetic activity to veins and the use of skeletal muscle and respiratory muscle pumps (Kay & Evans, 2014). This increase in venous return may lead to an increase in cardiac muscle fiber stretch, thus possibly increasing echocardiographic stroke volume ($SV_i$) and consequently cardiac index (CI) in trained Pnmt-KO when compared to sedentary Pnmt-KO mice, suggesting an improvement in systolic function and therefore functional cardiovascular adaptations with chronic exercise in EPI-deficient Pnmt-KO mice.

In this work, after chronic exercise, a significant increase in systolic BP was observed in Pnmt-KO when compared with WT mice, possibly suggesting that EPI may be required to prevent BP overshoot during exercise and to maintain a normal BP, probably through $\beta_2$-AR induced vasodilation. In fact, a previous study in our group showed that $\beta_2$-AR induced vasodilation in Pnmt-KO mice is impaired (Moreira-Rodrigues et al., 2014). Therefore, in Pnmt-KO when compared with WT mice, chronic exercise, maybe associated with an increase in systolic BP, led to an hypertrophied heart (HW/BW), an increased LV mass ($LVM_i$), LV wall thickness (LVPWd and LVPWs) and

mRNA expression of IGF-1 and ANP, thus possibly stimulating a concentric hypertrophic response, in an attempt to normalize the elevated LV wall stress (Alpert et al., 1989; Grossman et al., 1975; Hood et al., 1968). These adaptations may protect against the development of full pathological cardiac hypertrophy (Kehat & Molkentin, 2010) and prevent a further increase in BP via the vasodilatory and natriuretic effects of ANP (Woodard & Rosado, 2008) in trained Pnmt-KO mice. Moreover, hypertrophy was not associated with cardiac fibrosis because no changes in mRNA expression of DDR2 were observed in trained Pnmt-KO when compared with trained WT.

Furthermore, after acute $\beta_1$-adrenergic stimulation with dobutamine, trained Pnmt-KO mice did not had any changes in EF, CI, $LVESV_i$, $SV_i$, $LVEDV_i$ and $VVC_i$, possibly suggesting that EPI deficiency and chronic exercise may lead to these animals not to react to dobutamine stress in the same way as trained WT mice. Taken together all these observations, concentric hypertrophy might be in an initial pathologic stage in trained Pnmt-KO mice.

There are a few limitations associated with the present study that should be emphasized. First, this study assessed that Pnmt-KO mice adapted to the chronic exercise training protocol similarly to WT mice, however the duration of the protocol and lack of motivation led some animals in both groups to give up running during the training. Nevertheless, the substantial duration of the exercise stimulus appeared to be sufficient for the development of cardiovascular adaptations associated with exercise.

Second, because of the hemodynamic effects of the anesthetic agents, echocardiographic studies in conscious animals are preferred when possible. However, it is difficult to train mice to remain still in the conscious state, and movement and excitement all affect arterial pressure (Zhao et al., 2011).

Third, because in this study there was not enough plasma in order to conduct experiences regarding evaluation of renin, angiotensin II and aldosterone, it would be important to repeat the chronic exercise training protocol in order to extract more plasma of WT and Pnmt-KO mice.

In conclusion, the increased systolic BP in response to exercise appears to be associated with LV concentric hypertrophy and no fibrosis in Pnmt-KO mice. Also, acute $\beta_1$-adrenergic stimulation with dobutamine did not led to any changes in EF, $LVEDV_i$ and CI in trained Pnmt-KO contrary to trained WT, altogether suggesting a possible initial pathological concentric hypertrophy stage with chronic exercise in Pnmt-KO mice. Therefore, EPI appears to be essential for maintaining a normal BP, probably through $\beta_2$-AR induced vasodilatation, thus preventing LV hypertrophy during chronic exercise.

In the future, because the presence of mRNA transcripts alone does not prove similar regulation of a translated product, a Western blot analysis should be made in order to confirm if the increased LV IGF-1 and ANP transcripts are translated into a protein in chronic trained Pnmt-KO mice.

Moreover, in order to evaluate if acute exercise changes NE and EPI levels in Pnmt-KO mice, these catecholamines would have to be quantified in AGs and plasma by RP-HPLC-ED.

In order to confirm if the renin-angiotensin-aldosterone system (RAAS) plays a role in LV hypertrophy, it would be necessary to evaluate by RP-HPLC-ED, renin, angiotensin II and aldosterone levels in plasma and in cardiac tissue of WT and Pnmt-KO mice after chronic exercise.

Cardiac myocytes are presumed to enlarge with LV hypertrophy (Tracy & Sander, 2011). So, assessments of cardiomyocyte number (by enzymatic disaggregation and direct cell counting (Naqvi et al., 2014)), length and width (by histology (Coelho-Filho et al., 2013)) in WT and Pnmt-KO mice would be of interest in order to confirm the type of cardiac hypertrophy.

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