Universidade de Aveiro Departamento de Química
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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, especialização em Bioquímica Clínica, realizada sob orientação científica da Doutora Rita Marisa Nogueira Ferreira, Investigadora Auxiliar do Departamento de Cirurgia e Fisiologia da Faculdade de Medicina da Universidade do Porto, e da Doutora Iola Melissa Fernandes Duarte, Investigadora Principal do Laboratório Associado CICECO – Instituto de Materiais de Aveiro, Departamento de Química da Universidade de Aveiro.

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Dedico este trabalho à minha família.

o júri

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palavras-chave

insuficiência cardíaca com fração de ejeção preservada, exercício fisíco, músculo esquelético, metabolismo

resumo

A insuficiência cardíaca com fração de ejeção preservada (ICFEP) é uma síndrome clínica complexa caracterizada por congestão pulmonar, dispneia, pressões de enchimento do ventrículo esquerdo elevadas, fração de ejeção ventricular esquerda normal (FEVE ≥ 50%) e intolerância ao exercício físico. Embora seja uma síndrome cada vez mais comum, o seu tratamento continua bastante limitado, sendo que as terapias disponíveis se focam maioritariamente no alívio dos sintomas. A intolerância ao exercício fisíco é um dos principais sintomas apresentados pelos pacientes com ICFEP. Devido aos benefícios já conhecidos da prática de exercício físico para o sistema cardiovascular, este tem vindo a ser recomendado como terapia não farmacológica adjuvante no tratamento da ICFEP. Embora os benefícios do exercício fisíco tenham já sido documentados na ICFEP, os mecanismos moleculares subjacantes a esses benefícios permanecem pouco compreendidos. Este trabalho teve como objetivo global estudar as alterações moleculares subjacentes à prática de exercício fisíco na ICFEP. Foi utilizado um modelo animal de ICFEP, nomeadamente ratos ZSF1 obesos. Os animais foram submetidos a um protocolo de exercício em tapete rolante durante 4 semanas, ao fim das quais se procedeu ao teste de intolerância ao esforço e à avaliação hemodinâmica. Após o sacrifício dos animais, o músculo *gastrocnemius* foi colectado para realização de uma análise metabólomica por espetroscopia de Ressonância Magnética Nuclear e avaliação da expressão proteica por Western Blot. A comparação entre os perfis metabólicos do *gastrocnemius* de ratos ZSF1 obesos com o de ratos ZSF1 magros (controlo) permitiu caracterizar as alterações inerentes à doença. Os resultados mostram que a ICFEP promoveu a degradação do glicogénio em pequenos oligossacarídeos, de onde resultou a libertação de glucose-1-fosfato, havendo também acumulação de glucose-6-fosfato e glucose. Para além disso, destacam-se a diminuição da expressão da AMPK, o comprometimento do metabolismo de corpos cetónicos e um aumento da metabolização de aminoácidos como a glutamina e o glutamato. O impacto do exercício físico no metabolismo do *gastrocnemius* deste modelo animal foi avaliado através da comparação entre animais obesos sedentários e exercitados. Os resultados obtidos mostram que, tal como nos animais sedentários, também os animais exercitados apresentaram uma elevada taxa de degradação de glicogénio, não se notando grande impacto do exercício no metabolismo da glucose. Também o metabolismo dos aminoácidos permaneceu inalterado. No entanto, o grupo exercitado mostrou alterações que sugerem um aumento da beta-oxidação de ácidos gordos e da fosforilação oxidativa, bem como o restabelecimento do metabolismo de corpos cetónicos. Globalmente, este trabalho demonstrou que o exercício físico tem um impacto positivo no metabolismo do *gastrocnemius* suportando assim a sua prescrição supervisionada a pacientes com ICFEP.

keywords heart failure with preserved ejection fraction, exercise training, skeletal muscle, metabolism

abstract

Heart failure with preserved ejection fraction (HFpEF) is a complex clinical syndrome characterized by pulmonary congestion, dyspnea, elevated left ventricular filling pressures, normal left ventricular ejection fraction (LVEF ≥ 50%) and exercise intolerance. Although it is an increasingly common syndrome, its treatment remains quite limited, with available therapies focusing mostly on symptom relief. Intolerance to exercise training is one of the main symptoms presented by patients with HFpEF. Due to the known benefits of exercise training at cardiovascular levels, it has been recommended as adjuvant non-pharmacological therapy in the treatment of HFpEF. Indeed, the benefits of exercise training have already been documented in HFpEF, however the molecular mechanisms underlying those benefits remain poorly understood. Thus, this study aimed to study the molecular changes underlying exercise training effects in HFpEF. An animal model of HFpEF was used, namely obese ZSF1 rats. The animals were submitted to a treadmill exercise protocol for 4 weeks, after which exercise intolerance test and hemodynamic evaluation were performed. After animal sacrifice, the *gastrocnemius* muscle was collected for metabolic analysis by Nuclear Magnetic Resonance Spectroscopy and Western Blot protein expression evaluation. Comparison between the metabolic profiles of *gastrocnemius* of ZSF1 obese rats and ZSF1 lean (control) rats allowed to characterize the changes inherent to the disease. The results show that HFpEF promoted glycogen degradation in small oligosaccharides, resulting in the release of glucose-1-phosphate, as well as glucose-6-phosphate and glucose accumulation. In addition, decreased AMPK expression, impaired metabolism of ketone bodies and increased metabolism of amino acids such as glutamine and glutamate were also observed in the sedentary HFpEF group. The impact of exercise on *gastrocnemius* metabolism of this animal model was evaluated by comparing sedentary and exercised obese animals. The results obtained show that, as in sedentary animals, the exercised animals also showed a high rate of glycogen degradation, with no significant impact of exercise on glucose metabolism. Also, amino acid metabolism remained unchanged. However, the exercised group showed changes that suggest an increment in fatty acid beta-oxidation and oxidative phosphorylation, as well as the reestablishment of ketone body metabolism. Overall, this work has shown that exercise has a positive impact on *gastrocnemius* metabolism thus supporting its supervised prescription to patients with HFpEF.

Contents

List of abbreviations and acronyms

ADP	adenosine diphosphate
AGE	advanced glycation end product
Ala	alanine
AMP	adenosine monophosphate
AMPK	5'-AMP-activated protein kinase
ATP	adenosine triphosphate
BCAA	branched-chain amino acids
BNP	B-type natriuretic peptide
BSA	bovine serum albumin
Ca^{2+}	calcium
cAMP	cyclic AMP
cGMP	cyclic guanosine monophosphate
cidea	cell death-inducing DFF-like effector A
CO	cardiac output
CR	caloric restriction
DD	diastolic dysfunction
DM	diabetes mellitus
DTNB	5,5'-dithiobis-82-nitrobenzoic acid
ECM	extracellular matrix
EDL	*extensor digitorum longus*
EF	ejection fraction
eNOS	endothelial nitric oxide synthase
ES	effect size
ET	exercise training
ETFDH	electron transfer flavoprotein dehydrogenase
eva	epithelial v-like antigen
FAO	fatty acid β-oxidation
G1P	glucose-1-phosphate
G6P	glucose-6-phosphate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
Gln	glutamine

Glu	glutamate
GLUT 1	glucose transported type 1
GLUT 4	glucose transporter type 4
HF	heart failure
HFmEF	heart failure with mid-range ejection fraction
HFpEF	heart failure with preserved ejection fraction
HFrEF	heart failure with reduced ejection fraction
HIIT	high-intensity interval training
HMDB	human metabolome database
IL-6	interleukin-6
JNK	c-Jun N-terminal protein kinase
LC3	microtubule-associated protein A1/1B-light chain
LDH	lactate dehydrogenase
LV	left ventricle
LVEF	left ventricle ejection fraction
LVH	left ventricle hypertrophy
MCT	moderate-continuous training
Mfn2	mitofusin 2
MMP1	matrix metalloproteinase-1
MMP2	matrix metalloproteinase-2
MMP9	matrix metalloproteinase-9
MMPs	matrix metalloproteinases
mRNA	messenger ribonucleic acid
MuRF1	muscle Ring Finger protein-1
MuRF2	muscle Ring Finger protein-2
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
NMR	nuclear magnetic resonance
NPs	natriuretic peptides
NT-proBNP	N-terminal pro-BNP
$ONOO^-$	peroxynitrite
OXPHOS	oxidative phosphorylation

pAMPK	phosphorylated AMP
Par	pareto scaling
PCA	principal component analysis
PDE	phosphodiesterase
PGC1-α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
ph-eNOS	phosphorylated eNOS
PHR	peak heart rate
PKG	protein kinase G
PLS-DA	partial least squares-discriminant analysis
ROS	reactive oxygen species
SD	standard deviation
sGC	soluble guanylate cyclase
SOD	superoxide dismutase
TCA	tricarboxylic acid cycle
TGF-β	transforming growth factor-β
TIMP-1	tissue inhibitor of matrix metalloproteinase-1
TMA	trimethylamine
TNF-α	tumor necrosis factor-α
TOCSY	total correlation spectroscopy
TSP-d_4	2,2,3,3-d_4-3-(Trimethylsilyl)propionic acid sodium salt
UC	usual care
UCP 3	uncoupling protein-3
UDP-glucose	uridine diphosphate-glucose
UV	unite-variance scaling
VCAM	vascular adhesion molecule
VIP	variable importance to projection
VO_2	oxygen consumption
XO	xanthine oxidase
ZSF1	Zucker fatty spontaneously hypertensive heart failure F1 hybrid rat

List of tables

List of figures

1. Introduction

Heart failure with preserved ejection fraction (HFpEF) is a complex clinical syndrome associated with diastolic dysfunction (DD), whilst systolic function is normal or near normal [1]. This syndrome is characterized by lung congestion, dyspnea, elevated left ventricle (LV) filling pressures, exercise intolerance [2,3] and normal left ventricular ejection fraction (LVEF ≥ 50%) [4]. The prevalence of HFpEF is higher in patients who are elderly, female, and with a high prevalence of comorbidities [4–6]. The pathophysiology of HFpEF is still poorly understood and current pharmacological treatments show modest beneficial effects in patients' mortality and morbidity [7,8]. In order to develop effective therapies, further research is needed on the molecular mechanisms underlying this syndrome. Recently, a new paradigm was proposed, where comorbidities are an important contributor to HFpEF development and progression. In particular, it has been suggested that comorbidities induce functional and structural remodeling in the heart of HFpEF patients through systemic inflammation [9]. Skeletal muscle abnormalities have been suggested to be an important contributor to the reduced exercise capacity displayed by HFpEF patients [10]. However, studies addressing the molecular changes induced by HFpEF in this tissue are lacking, and further investigation is needed.

Exercise training (ET) was already shown to have beneficial effects in cardiovascular diseases, and in comorbidities frequently displayed by HFpEF patients such as obesity and diabetes mellitus (DM) [11–13], suggesting that ET may also have beneficial effects in HFpEF individuals. Indeed, ET has been shown to improve the quality of life of HFpEF patients and to reduce exercise intolerance [14,15], however the molecular changes underlying these benefits remain poorly comprehended. Thus, the present work focuses on the study of the effects of exercise training in the remodeling of *gastrocnemius* muscle in HFpEF, using an animal model of this syndrome.

1.1. Heart Failure with Preserved Ejection Fraction

Heart failure (HF) is a clinical condition that results from functional and/or structural cardiac abnormalities, which causes a reduction in the cardiac output (CO) and/or an elevation of the intracardiac pressure both at rest and in stress conditions [8]. Heart failure may be related with a wide spectrum of LV abnormalities which can range from patients with reduced LV ejection fraction (LVEF) (LVEF < 40%, considered HF with reduced

ejection fraction (EF) (HFrEF)), mid-range ejection fraction (LVEF 40-49%, considered HF with mid-range EF (HFmEF)), and preserved LVEF (LVEF ≥ 50%, considered HF with preserved EF (HFpEF)) [4]. Heart failure with preserved ejection fraction is the most common condition within the three HF groups. Indeed, studies have reported that the prevalence of HFpEF in patients with clinical HF is approximately 50% [5,6]. The mortality among patients with HFpEF ranges from 30 to 60%, hospitalization rates are high and the quality of life of these patients is often severely affected [16,17].

This clinical syndrome is associated with DD whilst systolic function is normal or near normal [1]. Furthermore, HFpEF is characterized by lung congestion, dyspnea, high LV filling pressures (both at rest and during exercise) and exercise intolerance [2,3]. Usually, patients presenting symptoms (such as dyspnea and fatigue) and signs (elevated jugular venous pressure, alterations in the heart rate, among others) of HF, while presenting normal LVEF are suspected to suffer from HFpEF [8,18]. However, these parameters are usually non-specific, and often do not allow the differentiation between HF and other non-cardiac conditions [8]. Thus, in recent years, more specific diagnose criteria have been elaborated. These new criteria comprise clear evidence of DD, elevated LV filling pressures, symptoms and/or signs of HF, elevated plasma concentrations of natriuretic peptides (NPs) (B-type natriuretic peptide (BNP) > 35 pg/mL and/or N-terminal pro-BNP (NT-proBNP) > 125 pg/mL) and structural heart disease (left atrial enlargement, and/or left ventricle hypertrophy (LVH)) [8,18].

Several epidemiological studies show that HFpEF patients are generally old, female, and have a high prevalence of comorbidities [4–6], such as obesity, renal or pulmonary disease, atrial fibrillation, DM, hypertension and anemia [19–23]. Among these, obesity, hypertension and DM are the most common in HFpEF scenario [16]. Epidemiological studies also suggest that, with the aging of population and the increment in comorbidities burden, HFpEF may reach epidemic proportions [6,22,24,25]. The heterogeneity among HFpEF patients, due to the different comorbidities displayed by these patients, makes it harder to unveil the pathophysiological mechanisms behind this syndrome [3,24] and contributes to the lack of specific treatments [3,18]. Thus, considering the proportions that HFpEF is reaching and the incomplete knowledge of its pathophysiology, it is mandatory to study this syndrome, in order to better understand the underlying mechanisms and therefore develop more efficient therapies.

1.2. Pathophysiology of HFpEF

Heart failure with preserved ejection fraction was previously referred to as "diastolic heart failure" [26,27], but over the past years it has been shown that HFpEF is not caused only by DD, but by a complex interplay of several impairments [28,29]. Some of the frequent impairments presented by HFpEF patients are pulmonary hypertension, endothelial dysfunction, impaired ventricular systolic and diastolic reserve function, heart rate reserve and rhythm, vasodilatation, atrial dysfunction, stiffening of the vasculature and peripheral abnormalities, such as skeletal muscle alterations [3,29–31]. Also, investigators have been recently focusing their attention on systemic abnormalities, such as oxidative stress, mitochondrial dysfunction and inflammation [9,32,33].

1.2.1. Cardiac remodeling in HFpEF

The main changes in cardiac function of HFpEF patients occur in the diastolic properties and manifest as incomplete myocardial relaxation and elevated LV stiffness, which results in increased filling pressures of the LV, hence dyspnea symptoms and decreased exercise tolerance [31,34–37]. Still, the pathophysiology of HFpEF is not completely understood, a novel paradigm has been recently proposed [9]. According to this paradigm, comorbidities such as obesity, DM and hypertension promote a systemic inflammatory state that leads to endothelium dysfunction, together with functional and structural remodeling of the heart (Figure 1), thus playing an important role in HFpEF pathophysiology [9]. In fact, a systemic inflammatory state was evident in HF patients, given the high circulating levels of proinflammatory molecules, such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) [38]. The correlation between the systemic inflammatory state induced by comorbidities and the inflammation of the coronary microvascular endothelium was established through the evident expression of endothelial adhesion molecules in myocardial samples of HFpEF individuals. Both E-selectin and vascular cell adhesion molecule (VCAM) were shown to be highly expressed in the myocardium of HFpEF patients [39,40]. The expression of these molecules triggers the activation of subendothelial migration of circulating monocytes [39]. Monocytes infiltration through the inflamed endothelium is proposed to have a role in myocardial collagen deposition observed in HFpEF. Indeed, a histological study of HFpEF patients' myocardium biopsies revealed an increased collagen volume fraction, high levels of collagen type I expression and more collagen cross-linking [41]. In another study, high

levels of collagen type III were reported in myocardial biopsies of HFpEF individuals. Furthermore, increased collagen expression was coupled with reduced levels of matrix metalloproteinase-1 (MMP1), a major human collagenase, and increased tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) expression [39]. Additionally, increased serum levels of pro-collagen type I, a marker of collagen production, were also reported. It has been proposed that collagen deposition results from the differentiation of fibroblasts into myofibroblasts due to release of transforming growth factor-β (TGF-β) by monocytes [39,42]. Endothelial dysfunction was also reported in the aorta of two HFpEF experimental models, namely male Zucker fatty spontaneously hypertensive heart failure F1 hybrid rats (ZSF1) and female Dahl salt-sensitive rats [43,44]. In the ZSF1 obese rat model, the expression levels of endothelial nitric oxide synthase (eNOS) did not differ between Control and HFpEF animals [43]. However, the ratio of phosphorylated eNOS (ph-eNOS) (the activated form of eNOS) over total eNOS was found to be reduced in the HFpEF group compared to the Control group. Moreover, increased levels of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and nitrotyrosine modified proteins expression were reported in the HFpEF animals, in comparison with the non-HFpEF animals [43]. The activation of the c-Jun N-terminal protein kinase (JNK) pathway, in the aorta of ZSF1 obese rats, was also evaluated. Two different isoforms of JNK were evaluated in this study, namely p54 and p46 splice isoforms. The phosphorylation of the p46 splice isoform did not differ between HFpEF and non-HFpEF control animals, whereas the phosphorylation of the p54 splice isoform was found to be increased in the HFpEF group, compared with the Control group. It has been speculated that the increment in inflammation mediated by the activation of JNK, results in an increment of NADPH expression, which culminates in an increment of reactive oxygen species (ROS) production [43]. In the female Dahl salt-sensitive rats, eNOS protein expression was reduced in the HFpEF group, when compared with the Control group, while no difference was observed in the expression of NADPH oxidase between HFpEF and non-HFpEF animals [44]. Furthermore, both studies revealed molecular alterations that promote vascular stiffness, such as increased matrix metalloproteinases (MMPs) activity, namely matrix metalloproteinase-2 (MMP2) and matrix metalloproteinase-9 (MMP9) [44], increased advanced glycation end product (AGE)-modified proteins [44], and reduced collagen I/III ratio [43].

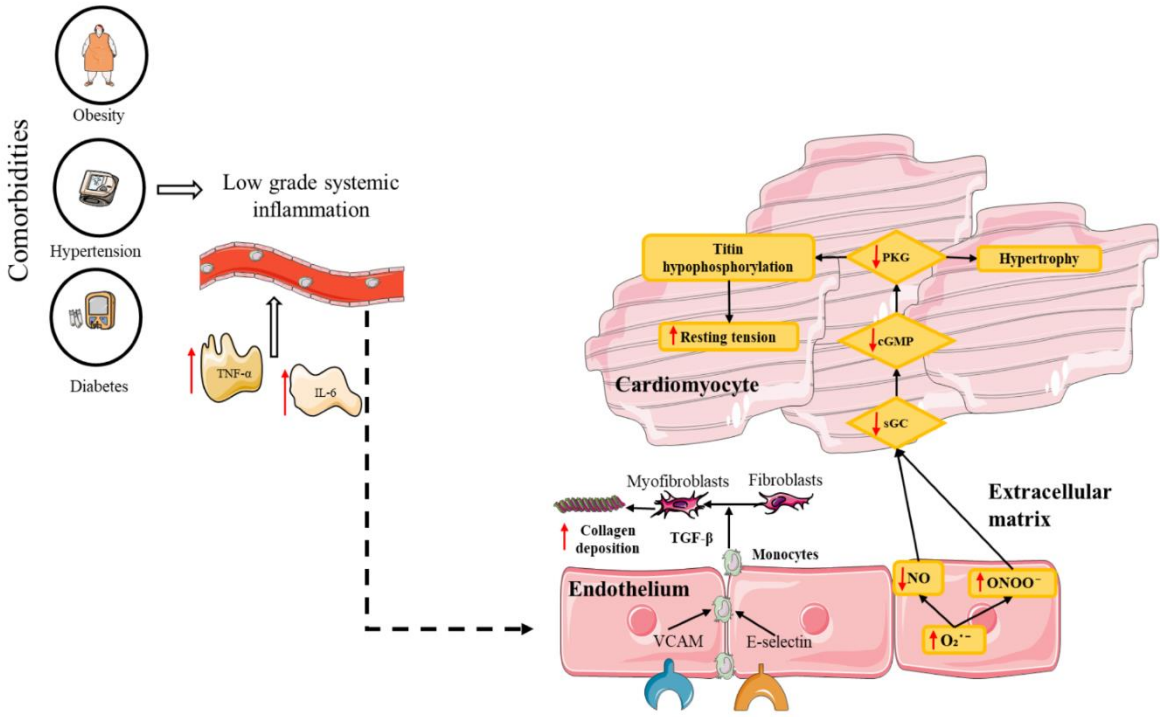

Figure 1: The role of systemic inflammation in HFpEF. The presence of comorbidities induces a low-grade systemic pro-inflammatory state, with increased circulating levels of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) that, when prolonged, will promote coronary microvascular endothelium dysfunction, through increased production of reactive oxygen species (ROS) and increased expression of cell adhesion molecules, such as E-selectin and vascular cell adhesion molecule (VCAM). The dysfunctional endothelium will affect the signaling from the endothelium to adjacent cardiomyocytes. The production of ROS induces the formation of peroxynitrite ($ONOO^-$), hence a decrease in nitric oxide (NO) bioavailability, that leads to a reduction of soluble guanylate cyclase (sGC) activity in adjacent cardiomyocytes. Lower sGC activity decreases cyclic guanosine monophosphate concentration (cGMP) and protein kinase G (PKG), which promotes cardiomyocytes hypertrophy and increases resting tension due to titin hypophosphorylation. Moreover, cell adhesion molecules promote the infiltration of monocytes which release transforming growth factor-β (TGF-β). TGF-β triggers the differentiation of fibroblasts into myofibroblasts, thus increasing collagen deposition in interstitial space. Figure made using Servier Medical Art (https://smart.servier.com/).

Cytokines also induce endothelial production of ROS through activation of NADPH oxidase [45]. ROS production leads to the formation of peroxynitrite ($ONOO^-$), hence limiting nitric oxide (NO) bioavailability due to the deviation of NO to $ONOO^-$ production [9]. Low NO bioavailability and high $ONOO^-$ levels in cardiomyocytes adjacent to dysfunctional endothelium, result in lower activity of soluble guanylate cyclase (sGC) in the cardiomyocytes, hence, decreased cyclic guanosine monophosphate (cGMP) concentration and protein kinase G (PKG) activity [36,46]. Myocardial sGC-cGMP-PKG signaling pathway is fundamental for normal cardiac physiology, by inhibiting maladaptive hypertrophy, and enhancing cardiomyocyte compliance through PKG-mediated phosphorylation of the sarcomeric protein titin [47–49]. The second messenger cGMP is

generated through activation of sGC by NO [48]. cGMP activates PKG allowing PKG-mediated phosphorylation of several target proteins, which have a vast range of downstream effects, such as inhibition of calcium (Ca^{2+}) influx, enhanced reuptake of Ca^{2+} into the sarcoplasmic reticulum, suppression of hypertrophic signaling through inhibition of G-protein coupled receptors and the transient receptor potential canonical channel and stimulation of LV relaxation and distensibility by phosphorylation of titin [48]. Titin is a sarcomeric protein which functions as a bidirectional spring and is responsible for early diastolic recoil and late diastolic distensibility [47]. Titin phosphorylation by PKG increases its compliance, promoting cardiomyocyte stiffness reduction [36,40,50–52]. Titin-based cardiomyocyte stiffness results from changes in the expression of compliant (N2BA) and stiff (N2B) titin isoforms, as well as in isoform phosphorylation status [47]. Dynamic changes in the expression of both isoforms, favoring the expression of N2B and hypophosphorylation of this isoform results in increased cardiomyocyte stiffness [47,49,51]. Several studies with endomyocardial tissue samples from HFrEF, HFpEF and aortic stenosis patients, demonstrated significantly stiffer cardiomyocytes [36,40,50–52]. The described molecular alterations are associated with structural and functional remodeling of the heart. Structural changes comprise cardiomyocyte hypertrophy [36,40,50–52], varying degrees of myocardial interstitial fibrosis [40,50,51,53] and reduced density of capillaries [53].

In addition to the molecular, structural and functional alterations mentioned in the heart, energy metabolism impairments also seem to contribute to HFpEF pathophysiology [54]. Indeed, new evidence is restoring interest in metabolic impairment as an important contributor to HFpEF development and progression [9,32,55–58]. In HFpEF, the myocardium undergoes major metabolic changes, although not all of those changes are well characterized [59]. Myocardial energy metabolism is compromised in HF due to impairments in metabolic flexibility, adenosine triphosphate (ATP) transfer and utilization, mitochondrial tricarboxylic acid cycle (TCA) activity, and overall oxidative metabolism [60,61]. Myocardial ATP content can decrease by 40% in ischemic HF in comparison with normal heart, due to impaired mitochondrial function and oxidative capacity. This energy deficit can contribute to exercise intolerance in HFpEF patients [59,60,62,63]. Some studies have shown decreased phosphocreatine and ATP levels in HF myocardium of both animals and humans [64–67]. This energy deprivation in myocardium could result from an

uncoupling between glucose oxidation and glycolysis [59]. In fact, it has been reported in experimental models of HFpEF that this syndrome is usually accompanied by an increment in glucose uptake, and an overall increment in glycolytic efflux, while glucose oxidation is found to be reduced. Even though, there is an higher glycolytic efflux, this is not sufficient to fulfill the energetic needs of the heart, since the energy obtained through glycolysis is lower than the energy obtained when glucose is completely oxidized [59].

Furthermore, inefficient β-oxidation in HFpEF was been described in recent studies where the metabolic profiles of patients' blood serum or plasma were characterized by metabolomics [57,58]. In the first study, 181 serum metabolites were quantified by nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography mass spectrometry [57]. Higher serum concentrations of carnitine, short-, -medium and long-chain acylcarnitines were reported in HFpEF patients in comparison with the age-matched non-HF Controls [57]. Changes in carnitine and acylcarnitines had already been associated with HF progression [68]. Indeed, both carnitine and its acyl derivates play an important role in fatty acids uptake and oxidation [69,70]. Consequently, an increment in the serum levels of these molecules could reflect inefficient β-oxidation in HFpEF patients [71]. Furthermore, the serum concentrations of creatinine and several amino acids (e.g., arginine, asparagine, histidine, phenylalanine, threonine and alanine) were higher in HFpEF individuals than in non-HF Controls. High serum levels of amino acids could be due to a hypercatabolic state that has been documented in HF patients [72], while high serum levels of creatinine could reflect renal impairment, a condition frequently observed in HF patients [73]. Furthermore, HFpEF patients presented lower levels of lysophosphatidylcholines, phosphatidylcholines and sphingomyelins compared to the Control group [57]. This decrease in serum phosphatidylcholines was accompanied by an increase in serum betaine levels. These findings could indicate a shift in choline metabolism towards more betaine and less phosphatidylcholines production [57]. Indeed, choline deficiency has been reported to induce cardiac dysfunction in male Wistar Albino rats, and it is thought to be involved in HF pathophysiology [74]. In another study, 63 metabolites were quantified in fasting plasma by tandem flow-injection targeted mass spectrometry and enzymatic assays [58]. Long-chain acylcarnitine levels were also found elevated in the HFpEF group compared to the non-HF Control group. A drawback of these studies is the fact that both evaluated circulating molecules, which are difficult to trace back to where they were synthesized or

to where they took action. Still, while these studies do not allow the determination of the exact tissues were metabolic dysregulations occur, they demonstrate that such abnormalities are present in HFpEF and are reflected at the systemic level.

The molecular changes in HFpEF go beyond the heart and vascular system. As the pathophysiology of HFpEF is unveiled, more attention has been paid to alterations in other tissues, namely in skeletal muscle. Its study could be especially relevant, since little is known about the alterations induced by HFpEF in this tissue. Considering the importance of exercise intolerance in HFpEF it is reasonable to suggest that skeletal muscle may represent a potential therapeutic target in HFpEF [10,75].

1.2.2. Skeletal muscle remodeling in HFpEF

Exercise intolerance is one of the main symptoms reported by HFpEF patients [3], and it can be objectively measured by oxygen consumption (VO_2) [76]. This symptom limits patients' daily activities and is a major contributor to the reduced quality of life displayed by HFpEF patients [3]. New evidence shows that peripheral "non-cardiopulmonary" factors are important contributors to reduced VO_2 in HFpEF patients [76,77], hence reduced exercise capacity. Skeletal muscle alterations have been suggested as contributors to the development of exercise intolerance by patients. In particular, skeletal muscle dysfunctions reported in HFpEF comprise muscle atrophy, fiber-type shifting, impaired contractile function and mitochondrial dysfunction [10,78]. All these impairments contribute to impaired oxygen utilization [79].

Lean muscle mass and VO_2 were measured in HFpEF patients older than 60 years and in 40 age-matched healthy Controls, using dual-energy X-ray absorptiometry [80]. HFpEF individuals had reduced percentage of total and leg lean mass, and severely reduced VO_2 in comparison with healthy Controls [80]. Furthermore, the skeletal muscle of HFpEF patients has been found to display increased intermuscular fat content in comparison to both HFrEF and age-matched healthy Control individuals [81,82]. Intermuscular fat content was inversely correlated with exercise capacity [81].

Multiple molecular changes induced by HFpEF in skeletal muscle have already been documented both in humans and animal models (Figure 2) [10,78,83–85].

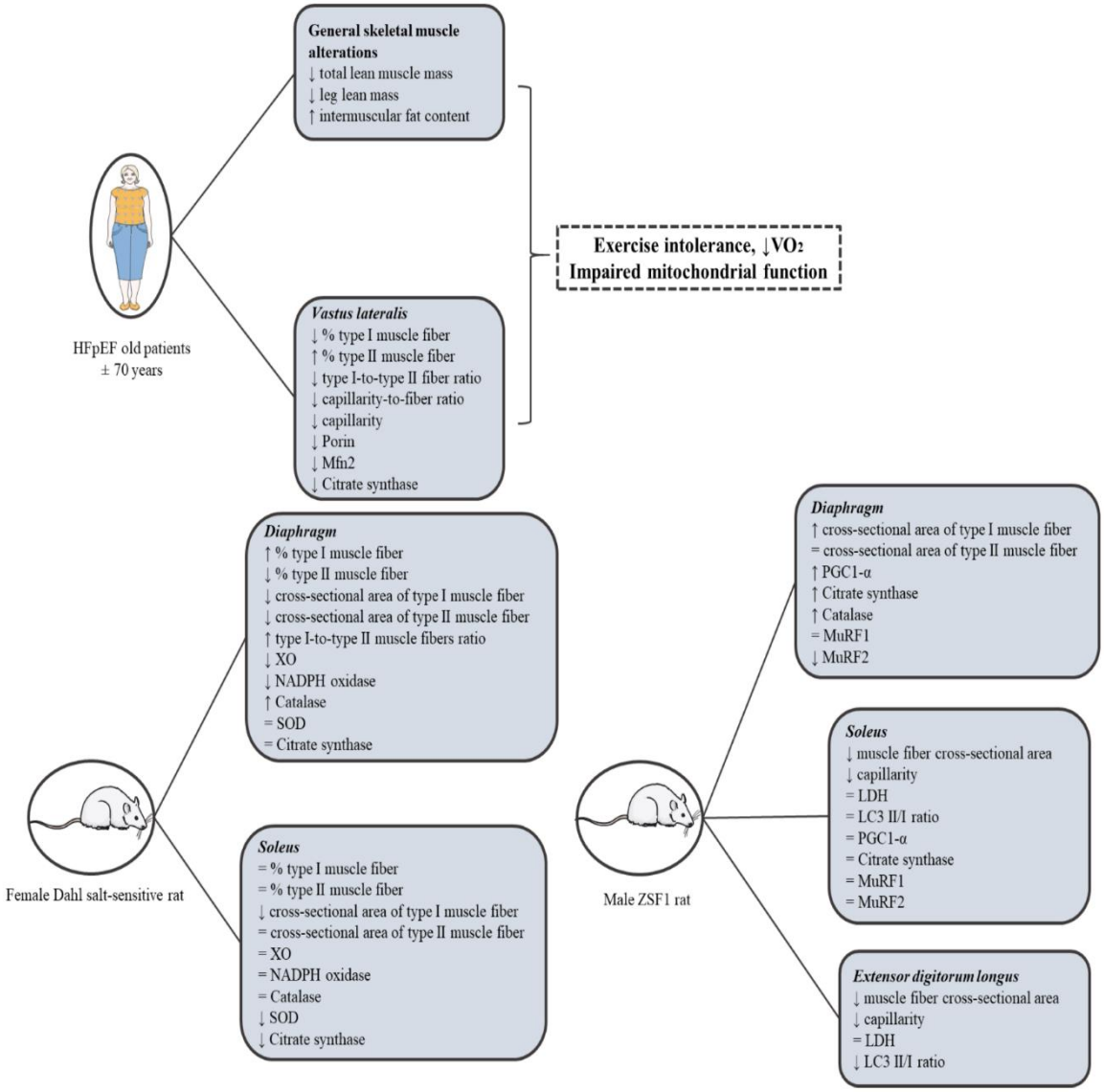

Figure 2: Skeletal muscle changes induced by heart failure with preserved ejection fraction in patients and in experimental models of heart failure with preserved ejection fraction. Figure made using Servier Medical Art (https://smart.servier.com/). Legend: ↑, increase; ↓, decrease; =, no change; IL-6, interleukin-6; LC3, microtubule-associated protein A1/1B-light chain; LDH, lactate dehydrogenase; Mfn2, mitofusin 2; MuRF1, muscle Ring Finger protein-1; MuRF2, muscle Ring Finger protein-2; NADPH, nicotinamide adenine dinucleotide phosphate; PGC1-α, peroxisome proliferator-activated receptor-γ coactivator-1α; SOD, superoxide dismutase; VO_2, oxygen consumption; XO, xanthine oxidase; ZSF1, Zucker fatty spontaneously hypertensive heart failure F1 hybrid rat.

An histological analysis of *vastus lateralis* muscle biopsies from HFpEF patients revealed a reduced percentage of oxidative (type I) and a greater percentage of glycolytic (type II) muscle fibers [10]. Furthermore, lower ratio of type I-to-type II muscle fibers and lower capillary-to-fiber ratio have been linked to the reduced exercise tolerance displayed by HFpEF old patients [10]. Skeletal muscle mitochondrial content and oxidative capacity were also evaluated in *vastus lateralis* muscle biopsies of HFpEF patients [83]. The *vastus*

lateralis muscle of HFpEF patients showed lower levels of porin expression, and citrate synthase activity, suggesting reduced mitochondrial content, hence oxidative capacity [83]. Deficits in these mitochondrial parameters may have a role in reduced exercise capacity displayed by HFpEF patients [83]. In the same study, the expression of mitofusin 2 (Mfn2), an important mediator in the elimination of dysfunctional mitochondria through autophagy, was also reduced in HFpEF patients compared to healthy Controls. Low expression of Mfn2 may lead to the accumulation of dysfunctional mitochondria, compromising the overall oxidative capacity of the skeletal muscle [83]. Skeletal muscle alterations induced by HFpEF were also evaluated in two different animal models of HFpEF, namely Dhal salt-sensitive and ZSF1 obese rat models [78,84,85]. The analysis of *extensor digitorum longus* (EDL) muscle of ZSF1 obese male rats revealed a reduction in muscle fiber cross-sectional area and in capillarity, in the HFpEF group compared to non-HFpEF group [84]. These findings were accompanied by the measurement of lactate dehydrogenase (LDH) activity, a glycolytic enzyme, while using microtubule-associated protein A1/ 1B-light chain (LC3) II/I ratio as an autophagy marker. In the EDL muscle of HFpEF animals, LC3 II/I ratio was reduced, while LDH activity levels did not vary in comparison to non-HFpEF animals. An histological analysis of the *soleus* muscle in female Dahl salt-sensitive rats showed no differences in muscle fiber type percentage, while only type I muscle fibers cross-sectional area was reduced, and no differences were found in type II muscle fibers cross-sectional area [78]. In another study, the *soleus* muscle of ZSF1 obese male rats showed a reduction in both muscle fibers cross-sectional area and capillarity [84]. Mitochondrial volume density was evaluated, through the measurement of citrate synthase activity, in the *soleus* muscle of Dahl salt-sensitive rat model, where its activity was reduced [78], suggesting reduced mitochondrial content. In addition, NADPH oxidase and xanthine oxidase (XO) expression, which represent possible ROS sources, did not vary between HFpEF and Control groups, neither did catalase activity. However, superoxide dismutase (SOD) activity was reduced in the *soleus* muscle of this animal model [78]. The molecular analysis of the *soleus* muscle of the ZSF1 obese rat model reveal that, LDH activity and LC3 II/I ratio did not differ between HFpEF animals and non-HFpEF animals [84]. Furthermore, markers of mitochondrial content and antioxidant capacity, such as peroxisome proliferator-activated receptor-γ coactivator-1 α (PGC1-α) and citrate synthase activity, as well as catalase activity did not differ between the *soleus*

muscle of HFpEF and non-HFpEF animals. The expression of atrophy-related proteins, such as muscle Ring Finger protein-1 (MuRF1) and muscle Ring Finger protein-2 (MuRF2), was also evaluated in the *soleus* muscle of the ZSF1 obese rat model. The expression levels of both MuRF1 and MuRF2 did not vary between HFpEF and non-HFpEF animals [85]. In the *diaphragm* muscle of female Dahl salt-sensitive rats, a reduction in the percentage of type II muscle fibers and an increment in the percentage of type I muscle fibers was reported [78]. These alterations resulted in an increment in the type I-to-type II muscle fiber ratio in HFpEF animals, in comparison to the non-HFpEF Control group. Furthermore, a reduction on cross-sectional area of both type I and type II muscle fibers was observed in *diaphragm* muscle of Dhal salt-sensitive rats [78]. In contrast, ZSF1 obese rats *diaphragm* muscle showed an increment in fiber cross-sectional area of type I muscle fibers, while fiber cross-sectional area of type II muscle fibers did not vary between HFpEF and Control animals [85]. A molecular analysis of the *diaphragm* muscle of female Dahl salt-sensitive rats showed no difference in citrate synthase activity, between HFpEF and Control animals. In addition, NADPH oxidase and XO expression were reduced in the *diaphragm* muscle of HFpEF animals, while catalase activity was higher and SOD activity showed no changes between HFpEF and Control animals [78]. The expression levels of atrophy-related proteins were also measured in the *diaphragm* muscle of ZSF1 obese rats, and it was reported that MuRF1 expression levels were not different between HFpEF and non-HFpEF animals, while MuRF2 expression levels were reduced in HFpEF group [85]. Furthermore, citrate synthase and catalase activity, as well as PGC1-α expression levels were increased in *diaphragm* muscle of ZSF1 obese animals, compared to the Control group [85].

1.3. Experimental models of HFpEF

Animal models are a fundamental tool to better understand the pathophysiology of cardiovascular diseases like HFpEF, even though the experimental results obtained may not be readily transferable to the human setting. Ideally, an animal model should mimic all the clinical features of the disease, although this is rarely possible, particularly in HFpEF [2,24]. The heterogeneity and complexity of HFpEF makes it difficult to establish an adequate animal model [21,24]. In fact, only a few animal models of HFpEF have been proposed. However, none of the models present all the characteristics displayed by patients, significantly reducing their utility for testing new therapies [24]. Until now only a

few animal models have been validated for the preclinical evaluation of novel therapies for HFpEF [2].

In spite of the difficulties mentioned, animal models of HFpEF have become more widely available and more representative of the fundamental pathophysiological mechanisms of the disease. These models try to replicate the main factors reported to cause DD, particularly, hypertension, aging and DM, and can be obtained through surgical procedure, exposure to specific drugs or through genetic modification [2]. Heart failure models have been developed in both small and large animals. Large animal models are highly desirable since they better mimic human physiology, but the research using these models is limited mainly by ethical concerns [2]. On the other hand, small animal models have lower maintenance and housing costs [2,86], which favors their utilization in research.

The HFpEF rodent models can be divided into groups according to risk factor, such as hypertension (such as Dahl salt-sensitive rat, aldosterone-infused uninephrectomized mouse, angiotensin II-infused mouse and transverse aortic constriction-induced pressure overload mouse), DM and obesity (such as leptin-deficient *ob/ob* mice, leptin receptor-deficient *db/db* mice, obese Zucker rat and obese diabetic Zucker rat), cardiometabolic syndrome (such as Dahl salt-sensitive obese rat and ZSF1) and age (spontaneous senescence-prone mouse and Fischer 344 rat) [2,87]. Within the available models, only aldosterone-infused uninephrectomized mouse, angiotensin II-infused mouse, *db/db* mouse and ZSF1 obese rat model have shown HFpEF phenotype, presenting concentric LVH, preserved systolic function, DD, pulmonary congestion and exercise intolerance [87].

1.3.1. Murine models of HFpEF

The aldosterone-infused uninephrectomized mouse model is achieved by subjecting the animals to uninephrectomy and aldosterone infusion for four weeks, in combination with 1% sodium chloride intake. The animals develop HFpEF with moderate hypertension, pulmonary congestion, concentric LVH, echocardiographic evidence of DD, while LVEF remains normal/preserved [88,89], and exercise intolerance [90]. The angiotensin II-infused mouse model is obtained through the administration in mice of angiotensin II for a variable period of time (1 to 8 weeks). The administration of angiotensin II results in elevated blood pressure accompanied by cardiac remodeling and hypertrophy [91–94]. The *db/db* leptin receptor-deficient mouse has a point mutation in the diabetes (*db*) gene

encoding the leptin receptor. This mutation results in spontaneous morbid obesity accompanied by type II DM [95]. Even though both hyperleptinemia and hyperinsulinemia are present, mice do not show cardiac hypertrophy at an early age [95–97], but it eventually develops at older ages [98,99]. Furthermore, at 12 weeks of age this model displays severely reduced exercise tolerance [100] and shows evidence of pulmonary congestion [101]. Recently, ZSF1 obese rat has been described as a new model of cardiometabolic syndrome [102]. The ZSF1 obese rat model, used in this work, is presented in more detail in the next section.

1.3.2. The ZSF1 obese rat model

The ZSF1 obese rat model results from the crossing of a lean female Zucker diabetic fatty rat (ZDF, *+/fa*) with a lean spontaneously hypertensive heart failure prone male rat (SHHF/Mcc, *+/facp*) [103]. This model was previously described as a model of diabetic cardiomyopathy [104]; however, a recent study proposed it as a promising experimental model for preclinical research in HFpEF [49]. Both obese and lean ZSF1 rats present high blood pressure, which suggest that the ZSF1 rats inherits a hypertensive gene from the spontaneously hypertensive parent [49,103].

At 20 weeks of age, ZSF1 obese rats have already developed HFpEF and represent a robust model of cardiometabolic syndrome, displaying obesity, hypertension, insulin resistance, type II DM, hyperinsulinemia, hypercholesterolemia and heart failure [49,105]. Besides, this animal model also presents concentric LV remodeling, DD, evidenced by echocardiographic evaluation, preserved LVEF, prolonged Tau (τ - time constant of relaxation), elevated arterial elastance and pulmonary congestion [49,106]. Additionally, one of the most important characteristics displayed by ZSF1 obese rats is reduced VO_2 hence, effort intolerance [49,107]. In terms of systolic function, ZSF1 obese rats have a similar systolic function to HFpEF patients, since the indexes of LV systolic function, such as LVEF, are preserved [49].

The similarities between this model and the human phenotype of HFpEF go beyond functional parameters. At the cellular level, ZSF1 rats present cardiomyocyte hypertrophy [49]. Cardiac fibrosis was also reported, but it develops later, and it seems to be induced by a hyperproliferative vascular reaction, which results from the replacement of functional vascular structures by fibrotic tissue [106]. This model also shows increased myocardial stiffness, largely due to increased cardiomyocyte stiffness resulting from

hypophosphorylation of N2B titin isoform [49]. These findings are very similar to what is observed in HFpEF patients, where both increased cardiac expression and hypophosphorylation of the N2B titin isoform were described [52]. All these features make the ZSF1 obese rats an adequate preclinical model of HFpEF. Additionally, the ZSF1 obese rat model has other advantages compared to the other models mentioned. One of the major limitations of mice models is their size, which complicates surgical procedures and limits the amount of tissue available for post-mortem analysis [2,86]. Being a rat model, ZSF1 shares many attractive features of mice models (such as, easy handle and reduced maintenance cost), while having enough size to facilitate surgical procedures and provide a higher amount of tissue for subsequent analysis [108]. Another drawback of the three mice models mentioned above is the fact that they represent only one or two risk factors associated with HFpEF [87–89,91–94], when the most common scenario in the human clinical setting is a combination of different risk factors [109]. The ZSF1 obese rat model, on the other hand, is representative of a substantial portion of HFpEF patients, since it displays a combination of the most prevalent comorbidities presented by patients [109]. Thus, the ZSF1 obese rat model seems to be an appropriated model to provide insights into the mechanisms underlying HFpEF development and potential therapeutic strategies.

1.4. Treatment of HFpEF

Even though a large number of clinical trials have already been performed in HFpEF to evaluate the effects of distinct pharmacological therapies, only a few showed modest beneficial effects in patients' mortality and morbidity [7,8]. An efficient treatment for HFpEF has not been identified yet, given the incomplete understanding of its pathophysiology and the difficulty in reproducing the human clinical setting in animal models [24]. Currently, the treatment is largely empirical and based on the comorbidities displayed by patients [3,7,8,110]. Most of the clinical trials performed in HFpEF evaluated the effects of drugs used in the treatment of HFrEF. However, treatments that showed benefits in HFrEF mortality and morbidity did not show the same beneficial effects in HFpEF [7,32,111].

The general approach to HFpEF consists on treating the comorbidities, intending to improve the functional capacity and the patients' quality of life. Nowadays, the treatment of HFpEF is based on pharmacological and non-pharmacological strategies [2]. The pharmacological strategies are summarized in Table 1.

Table 1: Pharmacological therapies for HFpEF.

Drug	Route	Recommendations and Observations	References
Renin-angiotensin system antagonists			
Candesartan	O	Mostly used to control blood pressure in hypertensive HFpEF patients	[110,112,113]
Perindopril	O		
β-Blockers			
Nebivolol	O	Used to control blood pressure	[110,114,115]
Carvedilol	O		
Diuretics			
Indapamide	O	Used in the relief of symptoms related with volume overload	[110,116,117]
Chlorthalidone	O		
Statins			
Simvastatin	O	Induces beneficial effects on arterial stiffness, inflammation and endothelial dysfunction; used in combination therapy	[110,118–120]
Aldosterone antagonist			
Spironolactone	O	Used in the relief of symptoms related with volume overload	[110,121]
Phosphodiesterase-5 inhibitors			
Sildenafil	O	Improves RV function, LV ventricular relaxation and distensibility and PAP	[122]
Nitrates			
Organic nitrates	O	Does not improve significantly the quality of life, exercise capacity and NT-proBNP levels	[123]
Inorganic nitrates	In	Acute administration reduces biventricular filling pressures and PAP at rest and during exercise	[124]

Legend: BNP, B-type natriuretic peptide; In, inhaled; LV, left ventricle; NT-proBNP, N-terminal pro-B-type natriuretic peptide; O, oral; PAP, pulmonary artery pressure; RV, right ventricle.

The non-pharmacological strategies consist in the modification of patients lifestyle, through dietary calorie restriction (CR) and exercise training (ET) [7]. Long-term CR effects were evaluated in male Sprague-Dawley rats, subjected to surgical myocardium infarction [125]. This study demonstrated that, when initiated after the development of HF, long-term CR can improve cardiac function. At the molecular level, chronic CR showed

the ability to significantly improve sympathetic cardiac innervation and β-adrenergic receptor levels in the failing heart [125]. Recently, some investigators assessed the role of CR in old, obese HFpEF patients [126]. Participants with comorbidities other than obesity were excluded. This study had a duration of 20 weeks in which the effects of CR or aerobic ET alone or in combination were evaluated. Both CR and aerobic ET alone, reduced body weight and adipose tissue mass, and showed similar improvements in exercise capacity and overall quality of life [126]. It was also reported that CR and aerobic ET combined had a complementary effect, with greater improvements in exercise capacity, but showed no differences in body composition [126]. In spite of the beneficial effects of CR in old obese HFpEF patients, caution must be taken in its prescription. Caloric restriction also has associated risks, since people who are more prone to develop HF usually are also more prone to muscle waste. Thus, removal of vital nutrients from the patients diet [79] may contribute to the worsening of physical and health conditions.

In the past years, ET has been recommended as an adjuvant therapy in multiple diseases, especially in diseases where increased adiposity is present [11–13]. Indeed, ET has effects on different tissues such as adipose tissue and cardiac and skeletal muscle [12,127,128]. Furthermore, the anti-inflammatory effects of ET have already been addressed and these effects can be important in HFpEF where a low-grade systemic inflammation state is observed [9,128]. Indeed, ET has already been reported to have beneficial effects in HFpEF [14,15].

1.5. Exercise training as a non-pharmacological approach for HFpEF treatment

Exercise training has already shown to have beneficial effects in cardiovascular diseases, and in the comorbidities frequently displayed by HFpEF patients such as obesity and DM [11–13]. The beneficial effects of ET in type II DM patients are well documented. Indeed, ET has been considered one of the cornerstones in DM treatment and prevention [11]. Several studies have shown that exercise can increase insulin sensitivity, improve glycemic control and is associated with the amelioration of cardiovascular risk factors related with DM [129–132]. Exercise has also proved to be a desirable adjuvant therapy in obesity treatment [12]. Obesity is characterized by high percentage of adipose tissue, especially abdominal. Regular ET results in a considerable reduction of abdominal fat, even when there is no apparent weight loss [133]. Further, regular ET is associated with lower circulating levels of pro-inflammatory molecules such as leptin, IL-6, TNF-α and

retinol-binding protein 4, and higher circulating levels of adiponectin, which participates in glucose and lipid metabolism [134,135]. Exercise has the potential to augment peripheral insulin sensitivity, induce white adipose tissue browning and increase muscular mass and strength [12,136].

It has been documented that ET has several physiological benefits in the cardiovascular system such as reduction of resting heart rate, improvement of endothelial function, increased flow-mediated vasodilation during exercise and several metabolic changes in myocardium [137–139]. In a small trial, 101 male patients (>70 years) with stable coronary artery disease were randomized to a 12 month bicycle ergometer training (20 minutes/day), or to percutaneous coronary intervention [140]. By the end of the trial, the training group showed improvements in event-free survival and exercise capacity compared with the group that had interventional therapy [140]. Exercise training is also relevant in HF therapy. Multiple prospective randomized ET studies in HFrEF patients have documented that aerobic endurance training intervention is both effective and safe [141,142]. Indeed, endurance training reverses cardiac remodeling with reduction of end-diastolic volume-pressure, and improves systolic and diastolic function in HFrEF [141,143,144]. These ameliorations result in improved exercise capacity, quality of life and reduced HF-related hospitalizations [141,143].

Exercise training has already shown to have cardioprotective effects in HFpEF individuals. An improvement in exercise capacity, quality of life scores and LV diastolic function in HFpEF patients have been reported after 12 weeks of aerobic (bicycle or ergometer cycling) and resistance training [14,15]. In one of the studies, the patients participated in a 30 minutes training session 3 times *per* week and exercised for five intervals (3 minutes each) at 80% VO_2 (each interval was separated by a 3-minutes active rest at 40% VO_2) [14]. Improvements in exercise capacity, such as increment of VO_2, and general quality of life scores were reported. In the other study, the exercise protocol was adjusted over time [15]. In the first 4 weeks of training the participants performed aerobic endurance training 2 times *per* week. The duration of the exercise session increased from 20 minutes to 40 minutes during the first 4 weeks of the exercise program. From week 5 onward, weekly training frequency increased from 2 to 3 times *per* week. In week 5 the participants also started the resistance training program (leg press, bench press, *triceps* dip, *latissimus* pull down, rowing machine). The participants would perform the resistance

training 2 times *per* week, and the training session consisted of 15 repetitions of each exercise [15]. After 12 weeks of ET, VO_2 increased, NT-pro BNP serum levels were not altered in trained HFpEF patients in comparison with usual care (UC) HFpEF patients; exercise capacity and cardiac function, such as LV diastolic function were also improved. Furthermore, pro-collagen type I serum levels were also reduced in trained HFpEF patients [15].

The peripheral effects of ET in HFpEF have been assessed by multiple randomized controlled trials [145–147]. Old HFpEF patients were assigned to a 16 weeks endurance (walking and ergometer cycling) ET program (Figure 3). The participants would exercise 3 times *per* week, for approximately 60 min [145–147]. The participants exercised at 40% to 50% peak heart rate (PHR) initially and the intensity of training session increased over the weeks until a 60% to 70% PHR. Following 16 weeks of endurance training, there was no difference in resting or exercise cardiac function. Also, no differences were found in LV end-diastolic volume, end-systolic volume, stroke volume, nor diastolic, systolic or pulse pressures, between the trained and sedentary HFpEF patients [145–147]. Moreover, following ET no differences were observed in echocardiography measurements, such as LVEF, LV mass, LVH and remodeling [145]. Endothelial function and atrial stiffness also did not improve after 16 weeks of endurance exercise training [146]. However, following the 16 weeks of ET, VO_2, exercise capacity, quality of life scores and 6-minute walk distance were improved. These findings suggest that ET benefits in HFpEF patients may be associated with improvements in the peripheral vascular and microvascular function as well as in skeletal muscle function [146,147]. The metabolic pathways that underlie these ameliorations are still poorly comprehended. Nevertheless, it is thought that ET might attenuate some of the metabolic dysfunctions in HFpEF patients [148].

A recent study has unveiled some of the metabolic alterations induced by ET in HFpEF [148]. This study consisted in a post-hoc analysis of the Exercise Training in Diastolic Heart Failure Pilot (Ex-DHF-P) trial [15]. Herein, the plasma concentration of 188 endogenous metabolites (from 5 classes) were evaluated in 44 ET and 20 UC, HFpEF patients, at baseline and after 12 weeks of exercise. The exercise program followed was the same as previously described [15]. Targeted metabolomic profiling of the plasma samples was performed through combined flow injection analysis and liquid chromatography mass spectrometry selective detection using multiple reaction monitoring pairs [148]. Significant

differences were found in ten metabolites regarding the progression from baseline to follow-up, both in the exercised and UC groups. The exercised group showed a slight increment in glutamine and a slight decrease in three sphingolipids (SM C18:0, SM C24:0 and SM (OH) C16:1), while in UC patients these metabolites remained constant. Further, carnitine and acetylornithine were found to be increased in UC patients, while three glycerophospholipids (PC aa C28:1, PC aa C34:2 and PC aa C36:2) were modestly reduced. Regarding these metabolites, ET did not induce any alteration in their plasma concentrations [148]. The authors suggested that one possible mechanism underlying improvements in cardiac function of exercised HFpEF patients could result from improved energy homeostasis through the regulation of carnitine availability. Carnitine plays an important role in fatty acid β-oxidation (FAO), and high plasma concentrations of this metabolite are associated with disturbed energy metabolism [69–71]. Carnitine plasma concentration was elevated in UC group, but remained constant in the exercised group, suggesting the potential of ET to modulate carnitine availability [148]. Two other metabolites, namely spermidine and spermine, which are related with autophagy induction, were associated with improvements in VO_2 and cardiac remodeling. However, ET did not have the same effects, regarding spermidine and spermine, in all participants. The reasons behind the different effects of ET in these metabolites within the trained group remain unclear and require further investigation. In this paper, an outcome-specific metabolic signature was also reported in the ET group with very little intersection between echocardiographic, cardiorespiratory and ventilatory parameters, suggesting that the previously described ameliorations induced by ET in HFpEF patients [15] could involve multiple metabolic pathways [148]. Another very important finding in this study was the identification of two distinct metabolic signatures amongst the trained HFpEF patients. Strong differences were found in 39 metabolites between the two identified subgroups in trained HFpEF patients. This finding suggests that even though all patients who performed the ET program did show the same improvements in exercise capacity and LV dysfunction, the metabolic pathways underlying these ameliorations could be dependent on patient characteristics. Overall, this study demonstrated that, even though the metabolic response to ET was heterogenous among the participants, its effects were beneficial for both identified groups [148].

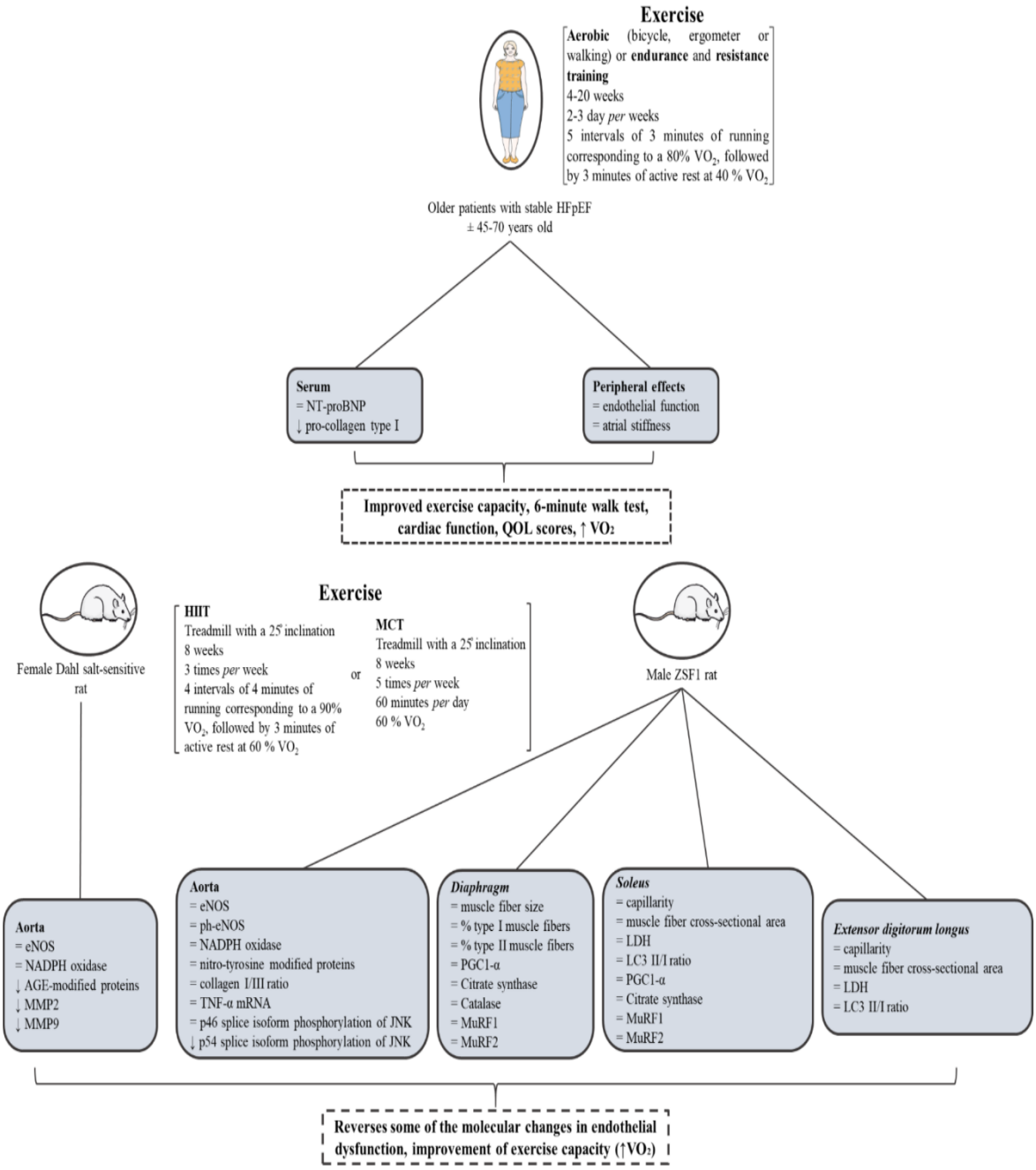

Figure 3: Molecular changes associated with exercise training effects in patients and in experimental models of heart failure with preserved ejection fraction. Figure made using Servier Medical Art (https://smart.servier.com/). Legend: ↑, increase; ↓, decrease; =, no change; AGE, advanced glycation end product; eNOS, endothelial nitric oxide synthase; HIIT, high-intensity interval training; JNK, c-Jun N-terminal protein kinase; LC3, microtubule-associated protein A1/1B-light chain; LDH, lactate dehydrogenase; MCT, moderate-continuous training; MMP2, matrix metalloproteinase-2; MMP-9, matrix metalloproteinase-9; MuRF1, muscle Ring Finger protein-1; MuRF2, muscle Ring Finger protein-2; NADPH, nicotinamide adenine dinucleotide phosphate; NT-proBNP, N-terminal pro-B-type natriuretic peptide; PGC1-α, peroxisome proliferator-activated receptor-γ coactivator-1α; ph-eNOS, phosphorylated eNOS; QOL, quality of life; TNF-α, tumor necrosis factor-α; VO2, oxygen consumption; ZSF1, Zucker fatty spontaneously hypertensive heart failure F1 hybrid rat.

To date, only four studies have evaluated the molecular alterations induced by ET in preclinical models of HFpEF [43,44,84,85]. In particular, ET effects on endothelial dysfunction [43,44] and skeletal muscle alterations [84,85] were assessed. The effects of high-intensity interval training (HIIT) and moderate-continuous training (MCT) on endothelial dysfunction were evaluated in the aorta of male ZSF1 obese rats [43]. The exercise protocol performed in this study had a duration of 8 weeks, for both training modalities (HIIT and MCT). The bioavailability of NO was evaluated through the analysis of eNOS, ph-eNOS, NADPH oxidase and nitrotyrosine modified proteins expression. Protein expression levels of eNOS and ph-eNOS did not vary between trained (HIIT and MCT) and sedentary animals. The expression levels of NADPH oxidase were also not different in either of the trained groups, when compared with the sedentary group. Furthermore, the levels of nitrotyrosine modified proteins, which function as an indirect marker of $ONOO^-$ formation, and therefore an indirect marker of ROS production, did not vary in trained animal groups [43]. The same lack of variation was found for the collagen I/III ratio [43]. Additionally, the role of ET in endothelial inflammation was evaluated through the measurement of TNF-α mRNA expression and the phosphorylation of both p46 and p54 splice isoforms of JNK. No differences were found in TNF-α mRNA expression between trained groups (HIIT and MCT) and the sedentary group. The phosphorylation of the p46 splice isoform of JNK did not differ in trained animals compared to the sedentary group, while the phosphorylation of the p54 splice isoform of JNK was reduced in animals of both training modalities in comparison with the sedentary group [43]. Overall, these findings suggest that ET, independently of the training modality (HIIT or MCT), has the potential to partially reverse some of the known molecular alterations that underlie endothelial dysfunction in HFpEF. In another study, the effects of HIIT on endothelial dysfunction were evaluated in the female Dahl salt-sensitive rat model [44]. The exercise protocol had a duration of 8 weeks and the animals trained 3 times *per* week on a treadmill with a 25º inclination. The training session consisted of four intervals (4 minutes each) of running, at 90% VO_2, separated by 3 minutes of active rest at 60% VO_2. The exercise session was preceded and followed by a 10 min run at 40% to 50% VO_2. High-intensity interval training did not increase eNOS expression, while a reduction in AGE-modified proteins and MMPs (MMP2 and MMP9) activity in the aorta of HFpEF animals was reported. Furthermore, NADPH oxidase expression did not vary between

trained and sedentary groups [44]. These findings suggest that ET has the potential to reduce vascular stiffness, contributing to endothelial dysfunction amelioration in HFpEF.

Exercise training effects on skeletal muscle abnormalities in a HFpEF animal model have also been evaluated [84,85]. The animals were randomized to perform either HIIT or MCT for 8 weeks, on a treadmill with 25º inclination. The HIIT group trained 3 times *per* week and the training session consisted on four intervals (4 minutes each) of running, at 90% VO_2, followed by 3 minutes intervals of active rest at 60% VO_2. The MCT group trained 5 times *per* week, 1 hour a day at 60% VO_2. The exercise session was preceded and followed by 10 min run at 40% to 50% VO_2, independent of the exercising regime. After 8 weeks of ET (HIIT or MCT) most of skeletal muscle abnormalities induced by HFpEF were not reversed, nor attenuated in ZSF1 obese rat model [84]. Exercise training was unable to increase capillarity or fiber cross-sectional area in EDL muscle of trained ZSF1 obese rats, and to reverse the shift from oxidative to glycolytic metabolism, since LDH activity did not vary between both trained and sedentary groups. Autophagy was assessed by measuring LC3 II/I ratio, which also did not differ in both HIIT and MCT groups in comparison to the sedentary group [84]. The results obtained for *soleus* muscle were similar to those obtained for EDL muscle, since ET was unable to increase capillarity or fiber muscle cross-sectional area. Autophagy activation was also not reduced, independently of the ET regime, since LC3 II/I ratio did not differ between trained and sedentary animals. The activity of LDH was also not different in both HIIT and MCT groups compared with non-trained animals [84]. These findings are in agreement with the results from another study, which evaluated the effects of ET in the *diaphragm* and *soleus* muscles of male ZSF1 obese rats [85]. The exercise protocol in this study was the same performed in the previous study [84]. In the *diaphragm* muscle, muscle fiber size did not change in any of the 3 groups and the same was observed in the expression of atrophy-related proteins MuRF1 and MuRF2. Furthermore, no shift from glycolytic to oxidative metabolism was evident after ET sessions, since no muscle fiber type shift was observed in both trained groups comparing to sedentary group. Moreover, mitochondrial density markers, such as PGC1-α and citrate synthase activity, as well as catalase activity did not differ between ZSF1 trained (HIIT or MCT) and sedentary animals [85]. In the *soleus* muscle, PGC1-α, as well as citrate synthase activity were not different between the trained (HIIT and MCT) animals, and the sedentary animals. Additionally, the expression of

atrophy related proteins in the *soleus* muscle did not vary in either of the trained groups, in comparison with the sedentary group [85]. Nevertheless, both studies reported an increment of exercise capacity in both trained groups with VO_2 increased by approximately 15% [84,85], supporting the beneficial effects of ET in exercise capacity in HFpEF.

2. Aims of this work

Heart failure with preserved ejection fraction is the most commonly observed syndrome in patients suffering from HF and it is estimated that its prevalence will continue to increase over the next years. Exercise intolerance is one of the major symptoms that characterize HFpEF, and one of the major contributors to the impairment in quality of life reported by HFpEF patients. Moreover, efficient therapies for HFpEF are lacking due to the complexity of this syndrome. It has already been documented that HFpEF patients can benefit from ET, since it is associated with an improvement in quality of life, exercise capacity and HF symptoms. However, the molecular mechanisms underlying the beneficial effects of exercise in these patients are still poorly understood, calling for further in-depth studies.

This study aims at contributing to improved understanding of the molecular events underlying exercise training effects in HFpEF. For that purpose, a morphometric, protein profiling and metabolomics analysis of *gastrocnemius* muscle from ZSF1 obese rats submitted to an exercise training protocol was performed.

3. Material and Methods

3.1. Experimental scheme

The experimental scheme followed in this work is summarized in Figure 4. As detailed in section 3.2, animals were divided into three groups - Control, sedentary HFpEF and exercised HFpEF. The assays performed on all groups included: peak effort testing and hemodynamic analysis (sections 3.3 and 3.4), morphometric analysis (section 3.5), metabolic profiling, protein expression analysis (sections 3.6 and 3.7) and citrate synthase activity of *gastrocnemius* muscle (section 3.8).

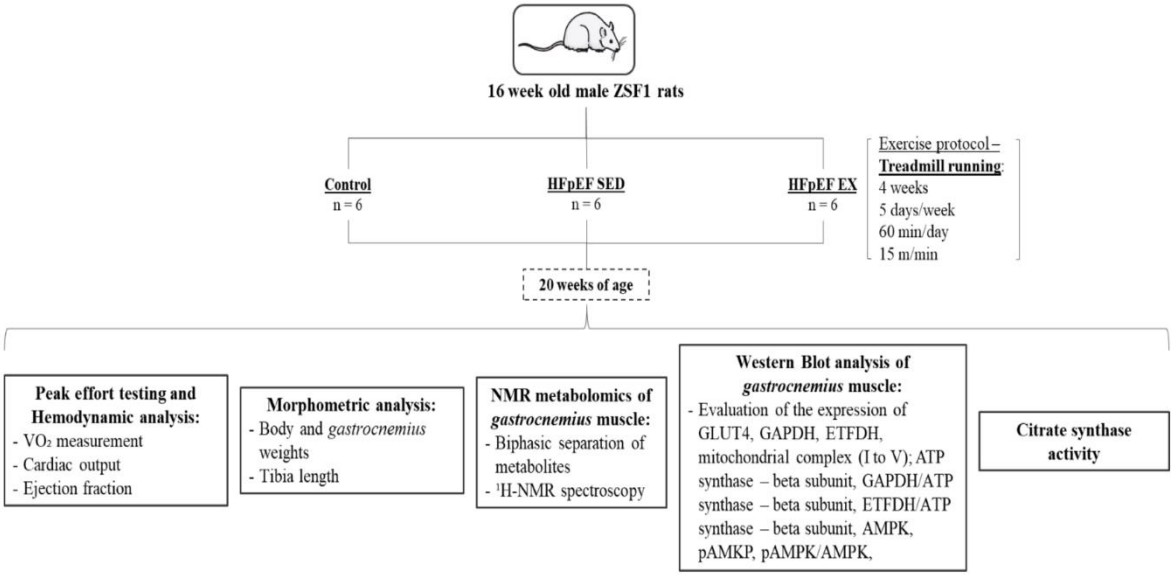

Figure 4: Experimental scheme of animal groups considered, and assays performed. Legend: AMPK, 5'-AMPK-activated protein kinase; ATP synthase, adenosine triphosphate synthase; ETFDH, electron transfer flavoprotein dehydrogenase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GLUT4, glucose transporter type 4; ^{1}H-NMR, nuclear magnetic resonance spectroscopy; pAMPK, phosphorylated 5'-AMPK-activated protein kinase; VO_2, oxygen consumption; ZSF1, Zucker fatty spontaneously hypertensive heart failure F1 hybrid rat.

3.2. Animal protocol

Animal experiments were conducted according to the Portuguese law on animal welfare and conform to the Guide for the Care and Use of Laboratory Animals published by US National Institute of Health (NIH Publication No. 85-23, Revised 2011). Nine-week-old male ZSF1 lean (n=6) and obese (n=12) rats (Charles River Laboratories (Barcelona, Spain)), were housed in groups of 2 animals *per* cage, maintained on a 12-hour light-dark cycle, at room temperature (22ºC), fed with Purina Diet (#5008) and water *ad libitum*. Before experiments, the rats had a 1-week acclimatization period to the laboratory conditions. At 16 weeks of age, the ZSF1 obese rats were randomly divided into two

groups, obese sedentary (HFpEF SED, n=6) and obese exercised (HFpEF EX, n=6). The number of animals *per* group was determined based on the potential biological variability to exercise training, mortality rate and 3Rs policy of animal experimentation [149]. Exercise training sessions consisted on treadmill running (76-0896, PanLab Harvard Apparatus) 5 days *per* week, 1 hour *per* day, at a speed of 15 m/min, for 4 weeks. HFpEF SED group was submitted to a stationary treadmill to minimize environmental confounders among the animals not subjected to the exercise protocol. The protocol was interrupted 48 hours before terminal hemodynamic evaluation and sample collection in order to avoid confounding with acute effects of the last training session.

3.3. Peak effort testing with oxygen consumption determination

With 20 weeks of age, animals were submitted to an exercise tolerance test with VO_2 measurement (LE8700C, OxyletPro System, PanLab Harvard Apparatus). Animals underwent a 3 min adaptation period to the treadmill, at 9 m/min, followed by a rapid effort escalation to evaluate peak oxygen consumption. Velocity was changed to 18 m/min and then stepped up 3 m/min at 1 min intervals. Whenever the animals were unable/unwilling to maintain pace, the treadmill was stopped, as defined by incapability to come of the back of the treadmill lane for >3s. Gas concentrations, namely O_2 and CO_2, were continuously recorded. Data was analyzed with the support of software Metabolism V2.2.01 (PanLab Harvard Appartus ®).

3.4. Hemodynamic evaluation

Terminal hemodynamic evaluation was performed 48 hours after the last training session. The animals were sedated (100 µg.Kg^{-1} and 5 mg.Kg^{-1} intraperitoneal fentanyl and midazolam, respectively), and then anesthetized by inhalation of a mixture of sevoflurane (2.5 – 3% sevoflurane; Penlon Sigma Delta) and oxygen, intubated for mechanical ventilation (TOPO, Kent Scientific) and placed over a heating pad (body temperature was maintained at 38ºC). A peripheral venous catheter was introduced in the right femoral vein for intravenous warm Ringer's solution infusion (8 mL. $Kg^{-1}.h^{-1}$; NE-1000, New Era Pump Systems). A left thoracotomy was performed to allow the access to the heart for insertion of a pressure-volume catheter in the LV through the apex (SPR-838 Millar Instruments). A probe (Transonics) was placed around the ascending aorta that allowed CO measurement (Active Redirection Transit Time Flowmeter, Triton Technology). The signals were continuously acquired (MPVS 300, Millar Instruments), recorded at 1000 Hz (ML880

PowerLab 16/30, ADinstruments), and analyzed (PVAN 3.5, Millar Instruments). Recording were obtained at suspended end-expiration.

3.5. Morphometric analysis

After hemodynamic evaluation, animals were sacrificed (100 $mg.Kg^{-1}$ intravenous pentobarbital). Samples from *gastrocnemius* muscle were collected, weighed and immediately snap frozen on liquid nitrogen, and then stored at -80ºC for biochemical analysis. Tibia length was measured and then used to normalizations.

3.6. NMR metabolomics assays

3.6.1. Tissue homogenization and metabolite extraction

Representative slices of frozen *gastrocnemius* were cut in a Petri dish, placed on top of ice, with a disposable scalpel and weighed. The cut tissue was then homogenized (in the proportion of 50 mg of tissue to 1 mL of homogenization buffer) with a Potter – Elvehjem glass homogenizer and a Teflon pestle at 0-4°C, in cold homogenization buffer (phosphate buffer 100mM, pH 7.4). After tissue homogenization, the metabolites were extracted using a biphasic extraction protocol with methanol:chloroform:water (1:1:0.7). First, 2 mL of cold methanol (-80°C) was added to the tube, followed by the addition of 2 mL of cold chloroform (-20°C) and 500 µL of cold ultra-pure water; the samples were vortexed (60s) between each solvent addition. Next, the samples were kept on ice for 20 min and then centrifuged at 2000g for 15 min. The resulting aqueous phase was transferred to a microcentrifuge tube and the organic phase was transferred to an amber glass vial. Polar extracts were vacuum dried (SpeedVac, Eppendorf), while organic extracts were dried under a gaseous nitrogen flow. All samples were stored at -80°C.

At the time of NMR analysis, the polar extracts were resuspended in 600 µL of deuterated phosphate buffer (PBS 100 mM, pH 7.0) containing 0.1 mM TSP-d_4 (2,2,3,3-d_4-3-(Trimethylsilyl)propionic acid sodium salt), and 550 µL of each sample were transferred to a 5 mm NMR tube.

3.6.2. ^{1}H-NMR data acquisition and treatment

Samples were analyzed in a Bruker Advance III HD 500 NMR spectrometer (University of Aveiro, PT NMR Network) operating at 500.13 MHz for ^{1}H observation, at 298 K. Standard one-dimensional (1D) ^{1}H-NMR spectra with water presaturation (pulse program ‘noesypr1d’, Bruker library) were recorded for all samples, with 32K points,

7002.8 Hz spectra width, a 2s relaxation delay and 512 scans. Spectral processing was then carried out using TopSpin 4.0.6 (Bruker Biospin, Rheinstetten, Germany). Each FID was multiplied by a cosine function, with a *ssb* value of 2, zero filled to 64k data points and Fourier-transformed. The obtained spectra were manually phased, baseline corrected and calibrated to the TSP signal at δ 0 ppm.

In order to identify the metabolite signals detected in the 1D spectra, two-dimension (2D) NMR spectra, namely ^{1}H-^{1}H TOCSY, *J*-resolved and ^{1}H-^{13}C HSQC, were also recorded for selected samples. The assignment of metabolites was carried out by matching the spectral information of 1D and 2D spectra to reference spectra available in Chenomx database, BBIOREFCODE-2-0-0 (Bruker Biospin, Rheinstetten, Germany) and human metabolome database (HMDB) [150,151].

1D spectra were visualized and prepared for multivariate analysis in Amix-Viewer 4.0.1 (Bruker Biospin, Rheinstetten, Germany). All spectra were normalized by total area, excluding water-suppression regions and contaminant signals (ethanol, chloroform, methanol). Additionally, the citrate signals were also excluded in spectra corresponding to the exercise group, since this signal was only present in 3 spectra of this particular group. After normalization, the data were organized into matrices ('bucket tables'), containing the information of signals intensity (variables) at each chemical shift in the different spectra (observations).

Data matrices were then uploaded into SIMCA-p 11.5 (Umetrics, Umeå, Sweden), where PCA (Principal Component Analysis) and PLS-DA (Partial Least Squares-Discriminant Analysis) were performed. Data were scaled using either unit-variance (UV) or pareto (Par) scaling, whereby each column (containing the intensities at a particular chemical shift) was divided by its respective standard deviation (UV) or the square root of its standard deviation (Par). This allows for variations in less abundant metabolites to have similar weight to that of more intense signals in multivariate models. The results were then visualized through factorial coordinates ('scores') and factorial contributions ('loadings') colored according to variable importance to projection (VIP). In respect to PLS-DA models, R^2 and Q^2 values, respectively reflecting explained variance and predictive capability, obtained through sevenfold internal cross validation, were utilized to assess the strength of class discrimination.

Integration of selected signals was carried out in in Amix-Viewer 4.0.1 (Bruker Biospin, Rheinstetten, Germany), to provide a quantitative measurement of metabolic variations. Representative signals of each metabolite, that were found to be relatively free of overlap, were integrated and normalized by total spectral area. For each metabolite, differences between exercised or sedentary HFpEF animals in relation to Control animals were assessed through the percentage of variation, the effect size [152] and the p-value (one-way analysis of variance (ANOVA)). Variations with medium-large magnitude (|ES|>0.5) were represented in heatmaps colored as a function of % of variation, using the R-statistical software 3.6.0 (R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/).

3.7. Western blotting analysis

Gastrocnemius sections were weighed and homogenized in 100mM phosphate buffer, pH 7.4 (50mg tissue/mL of buffer), supplemented with protease (Sigma P8340) and phosphatase (Sigma P0044, Sigma P5726) inhibitors (1:100), using a Teflon pestle on a motor-driven Potter-Elvehjem glass homogenizer at 0-4°C. Total protein content of the skeletal muscle homogenate was spectrophotometrically assayed with the Bio-Rad DC method, following the instructions of the manufacturer, using bovine serum albumin (BSA) as standard.

Equivalent amounts of protein of each experimental group (20-40µg, depending on the target protein) were electrophoresed in a 12.5% SDS-PAGE as described by Laemmli [153]. Gels were then blotted onto a nitrocellulose membrane (Amersham™, Protan™) in transfer buffer (25 mM Tris, 192 mM glycine, pH 8.5 and methanol) for 2 h at 200 mA. After, nonspecific binding was blocked with 5% (w/v) nonfat dry milk in TBS-T (100 mM Tris, 1.5 mM NaCl, pH 8.0, and 0.5% Tween 20). Later, the membranes were incubated with primary antibody solution diluted 1:1000 (mouse anti-ATPB, ab14730, Abcam; rabbit anti-GAPDH, ab 9485, Abcam; mouse anti-Total OXPHOS Rodent WB Antibody Cocktail, ab110413, Abcam; mouse anti-GLUT4, ab48547, Abcam); 1:500 (mouse anti-AMPK α 1 + AMPK α 2, ab80039, Abcam; rabbit anti-AMPK α 1 (phospho T183) + AMPK α 2 (phospho T172), ab23875, Abcam; rabbit anti- ETFDH, ab91508, Abcam) overnight at 0-4 °C with agitation. Membranes were then washed with TBS-T and incubated with IRDye 800CW goat anti-mouse IgG or IRDye 800CW goat anti-rabbit IgG

secondary antibodies (LI-COR) diluted 1:10000 in 5% (w/v) nonfat dry milk in TBS-T, for 1 h at room temperature with agitation. Membranes were imaged with Odyssey Infrared Imaging System (LI-COR) and densitometric analysis of immunoreactive bands was determined using the Image Studio Lite software (v5.2, LI-COR). Protein loading was controlled by Ponceau S staining.

3.8. Evaluation of citrate synthase activity

The activity of citrate synthase was measured in *gastrocnemius* muscle homogenates using the method previously described by Coore and colleges [154]. Briefly, CoASH released from the reaction of acetyl-CoA with oxaloacetate was measured by its reaction with 5,5' – dithiobis-82-nitrobenzoic acid (DTNB) at 412 nm (molar extinction coefficient of 13.6 $mM^{-1}cm^{-1}$).

3.9. Statistical analysis regarding oxygen consumption, hemodynamic, morphometric, western blotting and citrate synthase activity data

Statistical analysis was performed with GraphPad Prism software (v6.0), and values are given as mean ± standard deviation (SD) for all variables. Kolmogorov-Smirnov test was performed to check the normality of the data. Significant differences between the groups were evaluated using one-way ANOVA followed by the Tukey multiple comparisons post hoc test. Results were considered significantly different when $p < 0.05$.

4. Results

4.1. Effects of exercise training on oxygen consumption in ZSF1 obese animals

In order to assess exercise capacity in ZSF1 obese rats and evaluate the effects of ET in this parameter, an exercise tolerance test was performed. The results are presented in Figure 5.

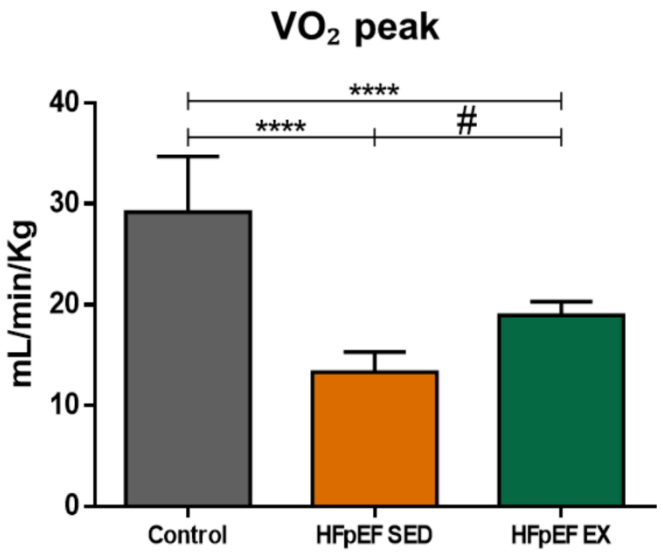

Figure 5: VO_2 peak evaluation of Control, HFpEF sedentary and HFpEF animals submitted to exercise training. Legend: VO_2, oxygen consumption. Values are expressed as mean ± standard deviation. **** $p < 0.0001$ *vs.* Control; # $p < 0.05$ *vs.* HFpEF SED.

The HFpEF SED group showed a reduction in exercise capacity in comparison to the Control group ($p < 0.0001$). Exercise training showed potential to increase exercise capacity as shown by an increment of VO_2 in HFpEF EX group, compared to HFpEF SED group ($p < 0.05$).

4.2. Effect of exercise training on cardiac hemodynamics in ZSF1 obese animals

In order to assess the effects of ET in the cardiac function of ZSF1 obese animals, LV hemodynamic evaluation was performed. The results are presented in Table 2.

Table 2: Hemodynamic evaluation of Control, HFpEF sedentary and HFpEF animals submitted to exercise training.

	Control	HFpEF SED	HFpEF EX
Cardiac output (µL/min)	65193.52 ± 16746.77	80884.71 ± 19682.88	88214.43 ± 8500.30*
EF (%)	53.12 ± 8.75	57.94 ± 6.75	54.38 ± 2.27

Legend: EF, ejection fraction. Values are expressed as mean ± standard deviation. * $p < 0.05$ *vs.* Control.

As shown in Table 2, HFpEF EX group showed a significant increase in the cardiac output ($p < 0.05$). Regarding EF, no significant differences were found between the experimental groups.

4.3. Effects of exercise training on morphometric parameters in ZSF1 obese animals

Absolute and normalized (to tibia length) morphometric parameters are presented in Table 3.

Table 3: Morphometric analysis of Control, HFpEF sedentary and HFpEF animals submitted to exercise training.

	Control	HFpEF SED	HFpEF EX
BW (g)	416.08 ± 26.13	604.29 ± 49.01****	615.44 ± 29.26****
TL (cm)	4.26 ± 0.05	3.82 ± 0.05****	3.88 ± 0.07****
Gas (g)	2.66 ± 0.33	1.99 ± 0.10****	2.24 ± 0.12**
Gas/TL (g/cm)	0.63 ± 0.08	0.51 ± 0.02**	0.58 ± 0.03

Legend: BW, body weight; Gas, *gastrocnemius* weight; TL, tibia length. Values are expressed as mean ± standard deviation. ** $p < 0.01$ *vs.* Control; **** $p < 0.0001$ *vs.* Control.

Both HFpEF animal groups showed an increment in body weight ($p < 0.0001$ *vs.* Control). Decreases in tibia length ($p < 0.0001$ *vs.* Control), *gastrocnemius* weight (HFpEF SED: $p < 0.0001$ *vs.* Control; HFpEF EX: $p < 0.01$ *vs.* Control) and *gastrocnemius* weight-to-tibia length ratio (HFpEF SED: $p < 0.01$ *vs.* Control) were observed. Four weeks of ET did not promote a significant alteration of body weight, tibia length and *gastrocnemius* weight. Despite not being statistically significant, four weeks of ET prevented the reduction in *gastrocnemius* weight-to-tibia length ratio in HFpEF EX animals.

4.4. NMR metabolomics of ZSF1 *gastrocnemius* muscle

4.4.1. Metabolic profile of ZSF1 *gastrocnemius* muscle

The metabolic profile of *gastrocnemius* muscle of ZSF1 rats was assessed by ^{1}H-NMR spectroscopy of polar extracts obtained from tissue homogenates. Liquid state ^{1}H NMR allows for the detection of practically all ^{1}H-containing molecules, above a certain concentration threshold (> μM). This detection is possible because protons are NMR-active nuclei (nuclear spin number $I = \frac{1}{2}$) and will give rise to signals in different positions

of the chemical shift scale depending on their chemical environments [155]. Additionally, the ^{1}H NMR signals observed in the 1D spectrum display distinctive splitting patterns (multiplicities) as a result of scalar coupling to neighboring protons. Hence, each ^{1}H-containing molecule has a characteristic set of NMR signals. Complex mixtures, such as tissue extracts, typically show a high degree of overlap between signals arising from the various molecules present. To annotate and resolve overlapping resonances, bidimensional (2D) NMR experiments are required, such as total correlation spectroscopy (^{1}H-^{1}H TOCSY). This is a homonuclear shift correlation experiment, in which correlations between protons distanced by up to 5-6 bonds are shown as cross peaks, symmetrically positioned around the plot diagonal.

In this work, 1D and 2D ^{1}H-^{1}H TOCSY spectra were recorded to aid metabolite assignment, as exemplified in Figures 6 and 7. By matching this spectral information to reference databases, a total of 35 metabolites were identified (Supplementary Table S1). These comprised organic acids (acetate, lactate), several amino acids, a few peptides (carnosine, anserine), glucose and related molecules, adenosine and AMP, among others.

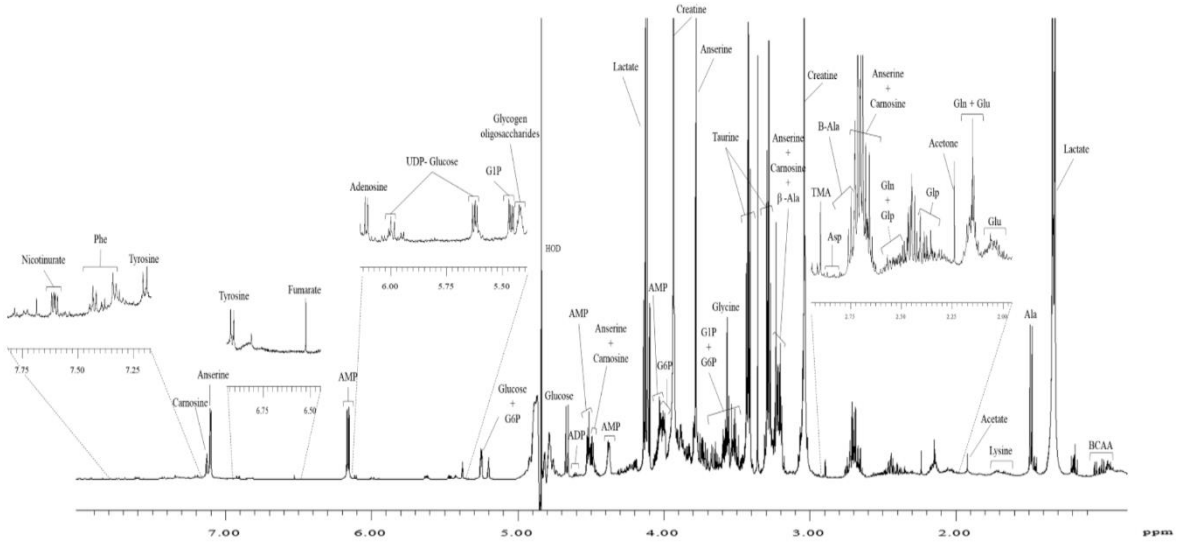

Figure 6: 500 MHz ^{1}H-NMR spectra of polar extracts of *gastrocnemius* muscle homogenate from HFpEF sedentary animals. Legend: Ala, alanine; AMP, adenosine monophosphate; BCAA, branch chain amino acids, G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; Gln, glutamine; Glu, glutamate, UDP-glucose, uridine diphosphate-glucose.

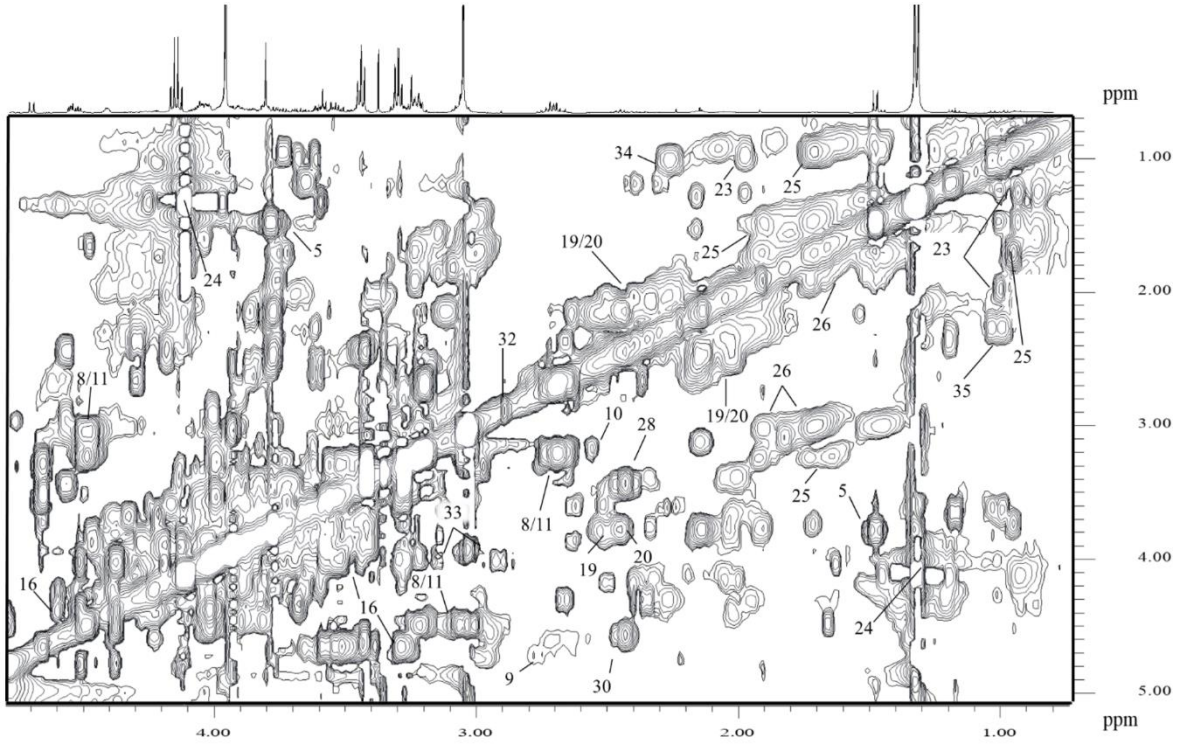

Figure 7: Expansion of ^{1}H-^{1}H TOCSY spectra of polar extracts of *gastrocnemius* muscle homogenate with some metabolite's assignment. Signals are numbered in accordance to Table S1 (Supplementary information).

4.5.2. Metabolic impact of HFpEF and/or ET on ZSF1 *gastrocnemius* muscle

As a first approach to assess the variability in the metabolic profiles of *gastrocnemius* muscle samples, collected from the three animal groups compared, multivariate analysis was applied to their ^{1}H-NMR spectra. The resulting PCA scores scatter plot (Figure 8) showed a reasonable separation between all three experimental groups. The Control group (lean animals) was separated from the two HFpEF groups (obese animals) along PC1, while sedentary and exercised HFpEF groups were separated along PC3.

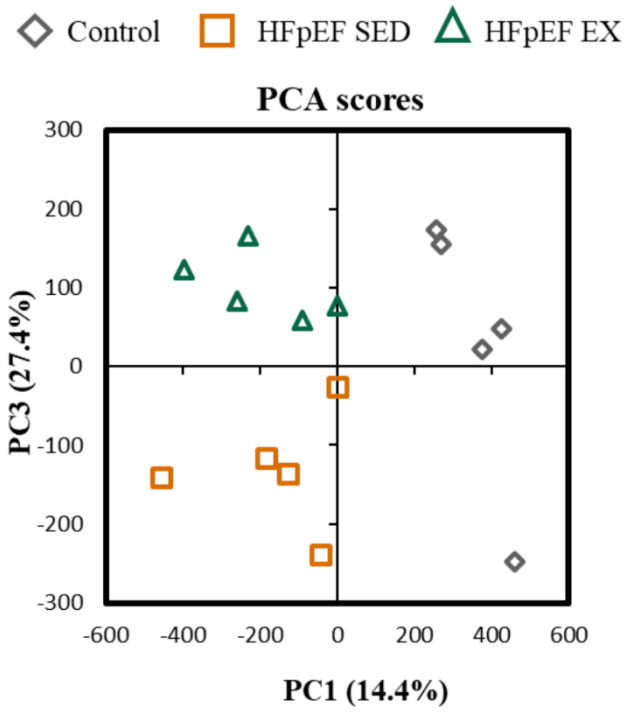

Figure 8: Principal Component Analysis (PCA) of ^{1}H-NMR spectra from polar extracts of *gastrocnemius* muscle homogenates. PCA scores scatter plot, where grey, orange and green symbols correspond, respectively, to Control, HFpEF SED and HFpEF EX.

In order to better assess the differences between groups, pairwise comparison was also made, using PCA and PLS-DA. The resulting PCA scores scatter plots, PLS-DA scores and loadings are shown in Figure 9 (left, middle and right, respectively), for HFpEF SED *vs.* Control (A), HFpEF EX *vs.* Control (B), and HFpEF SED *vs.* HFpEF EX (C).

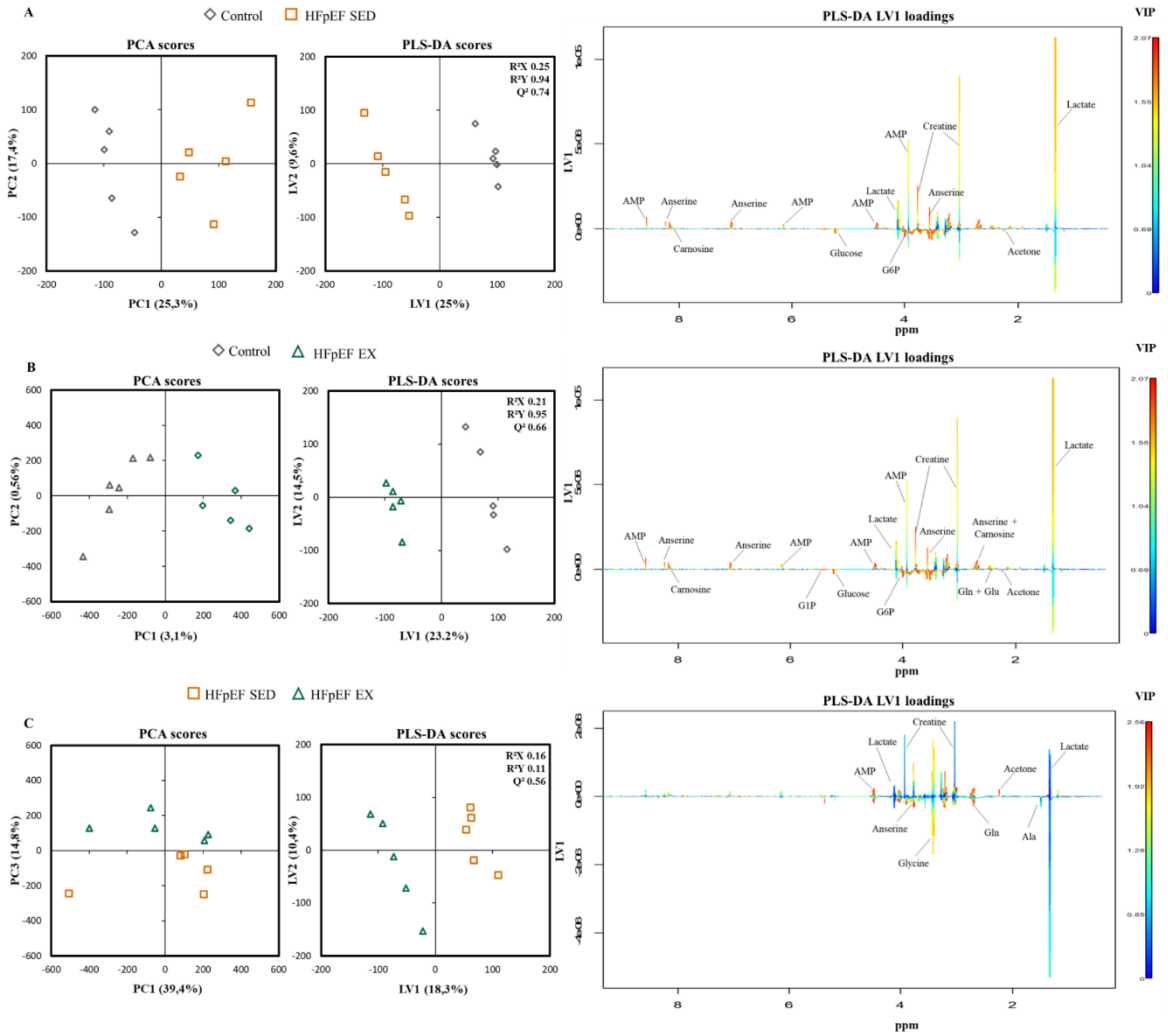

Figure 9: Pairwise multivariate analysis of ^{1}H-NMR spectra from polar extracts of *gastrocnemius* muscle homogenates. Grey, orange and green symbols correspond, respectively, to Control, HFpEF SED and HFpEF EX. PCA and PLS-DA scores scatter plots (left and center, respectively) and LV1 loadings W (right), colored according to variable importance to projection (VIP). Legend: A: Control *vs.* HFpEF SED; B: Control *vs.* HFpEF EX; C: HFpEF SED *vs.* HFpEF EX. Legend: Ala, alanine; AMP, adenosine monophosphate; BCAA, branch chain amino acids, G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; Gln, glutamine; Glu, glutamate.

All PCA scores scatter plots showed a reasonable separation between the groups compared. Such discrimination was further confirmed by PLS-DA, as robust models, where Q^2 values above 0.5, were obtained in all cases. The main metabolite differences accounting for group discrimination are displayed in LV1 loadings plots. Positive loadings correspond to metabolites with higher relative abundance in samples with positive PLS-DA scores, while negative loadings arise from metabolites increased in samples with negative PLS-DA scores. Also, the importance of metabolite variations to the scores

distribution is color-coded from blue (least important) to red (most important). The loadings corresponding to the discrimination between Control and HFpEF SED samples (Figure 9A) suggest the latter to be characterized, among others, by higher levels of glucose and acetone, together with lower levels of lactate, creatine and AMP. This metabolic signature is very similar to the one highlighted for HFpEF EX samples in relation to Controls (Figure 9B). Still, a few differences between the HFpEF groups were also apparent, for example, in the levels of acetone, glycine and anserine (Figure 9C).

Based on spectral integration, the magnitude and statistical significance in individual metabolite alterations highlighted in the PLS-DA loadings were further analyzed. The results are presented in Figures 10 and 11.

The heatmap in Figure 10 is color-coded according to the percentage of variation of each metabolite in either sedentary or exercised HFpEF animals relatively to Control lean animals (see also Supplementary Table S2). Only variations with a medium-large magnitude (|ES| > 0.5 [152]) were considered.

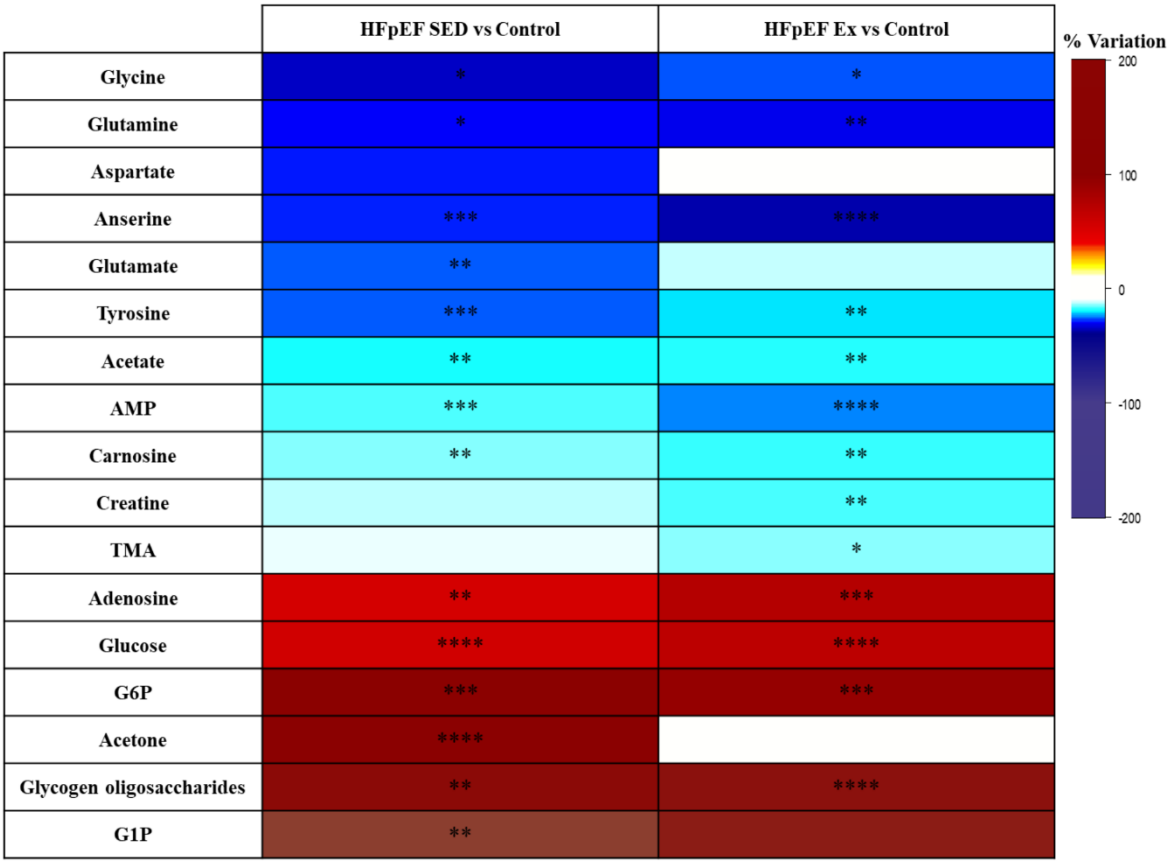

Figure 10: Heatmap of the main variations in the polar extracts of *gastrocnemius* muscle homogenates of ZSF1 animals. The color scale represents the percentage of variation relative to Control. Legend: AMP, adenosine monophosphate; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; TMA, trimethylamine. * $p < 0.05$ *vs.* Control; ** $p < 0.01$ *vs.* Control; *** $p < 0.001$ *vs.* Control; **** $p < 0.0001$ *vs.* Control.

This heatmap immediately shows that while some metabolic effects were similar for sedentary and exercised HFpEF animals (in relation to Controls), others appeared to depend on the HFpEF group considered. For instance, increased acetone and decreased aspartate levels were seen in the muscle of HFpEF SED animals, but not in samples of HFpEF rats submitted to the ET protocol. Moreover, the variation magnitude of some metabolites in relation to Controls, such as glucose-1-phosphate, acetone, glutamate and AMP differed considerably between HFpEF groups.

The graphs presented in Figure 11 allow a more detailed analysis of the above-mentioned differences. Glycogen oligosaccharides, glucose, glucose-6-phosphate (G6P) and adenosine increased significantly ($p < 0.01$) in both HFpEF groups in relation to Controls. These increases were of similar magnitude, with no difference being found between sedentary and exercised HFpEF groups. On the other hand, glucose-1-phosphate (G1P) and acetone were significantly elevated in the HFpEF SED group (compared to Controls) but not in the HFpEF EX group (no statistically significant difference relatively to Controls). As for the metabolites showing lower levels in the muscle of HFpEF animals compared to Control samples, those which decreased to similar extents in both HFpEF groups were acetate, glutamine, glycine, tyrosine anserine and carnosine. Contrastingly, glutamate and aspartate decreased only in the HFpEF SED group, while the levels in HFpEF EX samples were similar to Controls. Also, AMP, creatine and trimethylamine showed stronger decreases in exercised than in sedentary HFpEF muscle samples.

Overall, the results show that HFpEF has a wide impact on the metabolic profile of *gastrocnemius* muscle of ZSF1 rats and that this is partially modulated by exercise training.

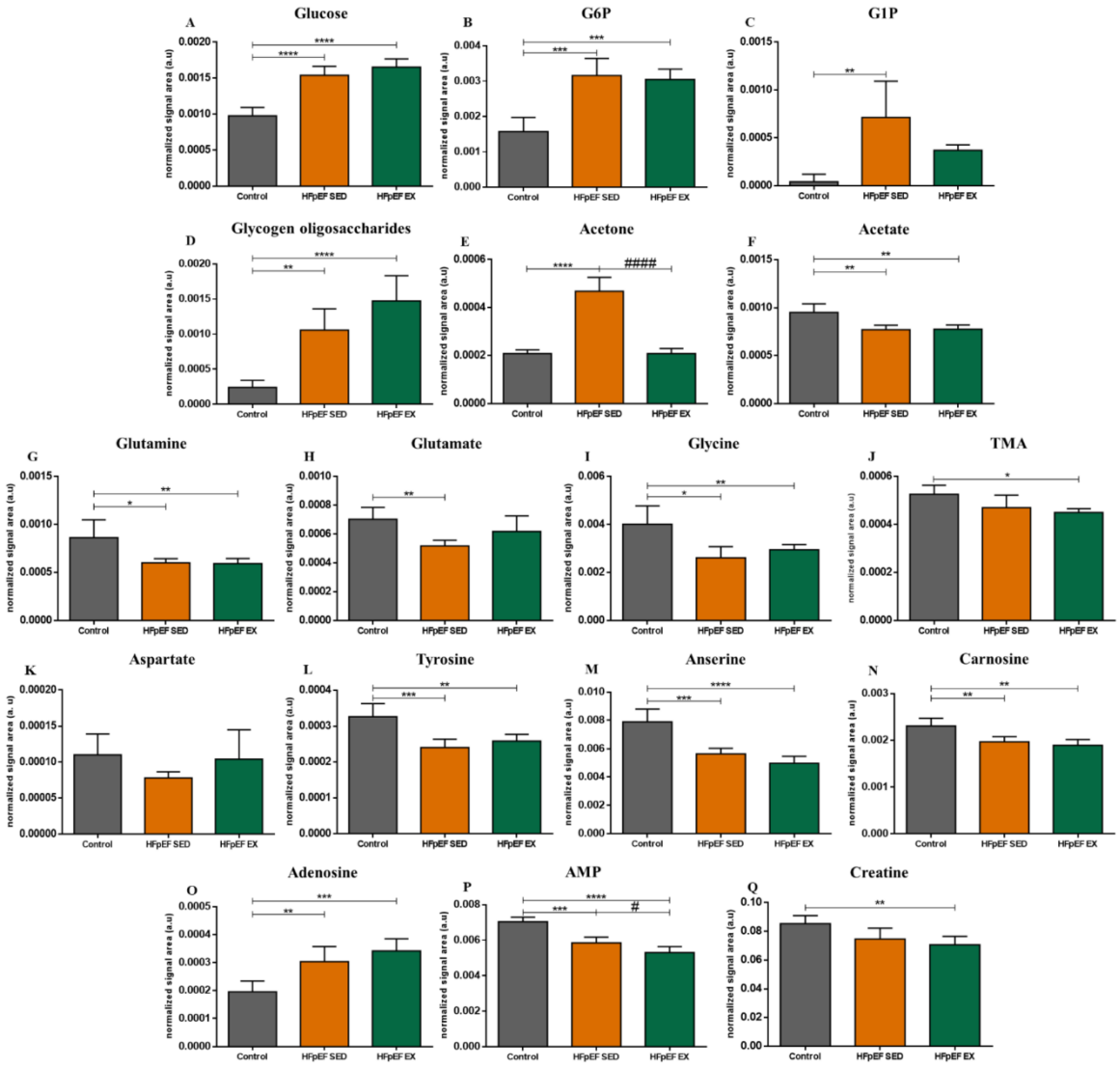

Figure 11: Relative content of metabolites was assessed by ^{1}H-NMR in *gastrocnemius* muscle of Control, HFpEF sedentary and HFpEF animals submitted to exercise training. Legend: A- glucose; B- G6P; C- G1P; D- glycogen oligosaccharides; E- acetone.; F- acetate; G- glutamine; H- glutamate; I- glycine; J- TMA; K- aspartate; L- tyrosine; M- anserine; N- carnosine; O- adenosine; P- AMP; Q- creatine. AMP, adenosine monophosphate; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; TMA, trimethylamine. Values are expressed as mean ± standard deviation. * $p < 0.05$ *vs.* Control; ** $p < 0.01$ *vs.* Control; *** $p < 0.001$ *vs.* Control; **** $p < 0.0001$ *vs.* Control; # $p < 0.5$ *vs*. HFpEF SED; #### $p < 0.0001$ *vs.* HFpEF SED.

4.5. Impact of HFpEF and/or ET on the expression of selected metabolism-related proteins

In order to help interpreting the metabolomics findings, different proteins involved in glycolytic and oxidative metabolism were evaluated through Western Blot. The results of expression levels or ratios obtained for the three groups compared (lean Control animals, sedentary HFpEF, exercised HFpEF) are shown in Figure 12. Data on citrate synthase activity is also shown.

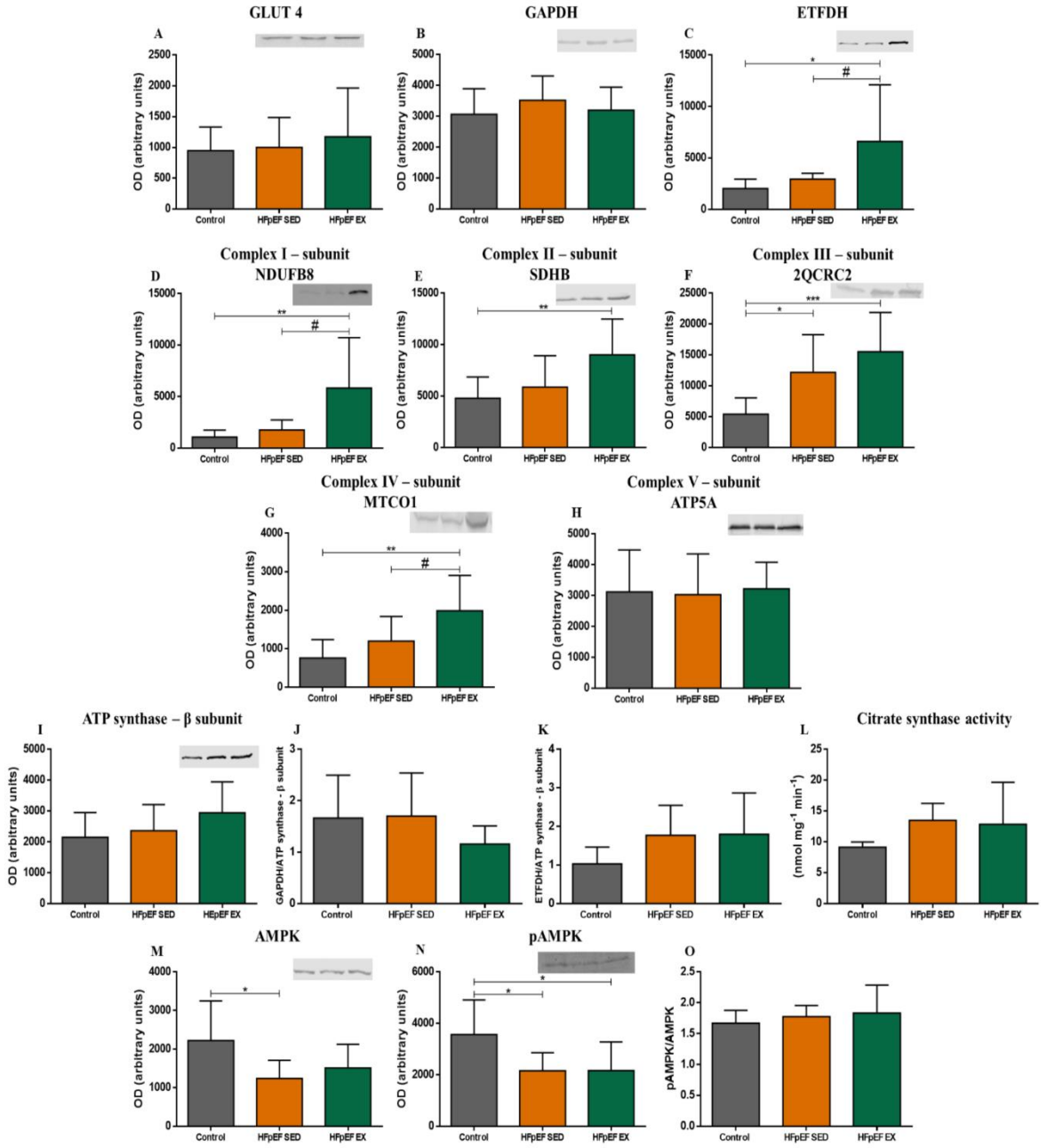

Figure 12: Expression of selected proteins and ratios: GLUT4, GAPDH, ETFDH, mitochondrial complexes (Complex I, II, III, IV and V), ATP synthase – beta subunit, AMPK, pAMPK protein levels, GAPDH/ATP synthase – beta subunit, ETFDH/ATP synthase – beta subunit and pAMPK/AMPK ratio and citrate synthase activity from *gastrocnemius* muscle of Control, HFpEF sedentary and HFpEF animals submitted to exercise training. Legend: A- GLUT4; B- GAPDH; C- ETFDH; D- complex I- subunit NDUFB8; E- complex II- subunit SDHB; F- complex III- subunit 2UQCRC2; G- complex IV- subunit MTCO1 ; H- complex V- subunit ATP5A; I- ATP synthase– β subunit; J- GAPDH/ATP synthase– β subunit; K- ETFDH/ATP synthase– β subunit; L- citrate synthase activity; M- AMPK; N- pAMPK; O- pAMPK/AMPK. Representative immunoblots are shown above the correspondent graph (sample order has correspondence to the order of the groups presented in the graph). AMPK, 5'-AMP-activated protein kinase; pAMPK, phosphorylated AMPK; ATP, adenosine triphosphate; ETFDH, electron transfer flavoprotein dehydrogenase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GLUT4, glucose transporter type 4. Values are expressed as mean ± standard deviation. * $p < 0.05$ *vs.* Control; ** $p < 0.01$ *vs.* Control; *** $p < 0.001$ *vs.* Control; # $p < 0.05$ *vs.* HFpEF SED.

The expression of electron transfer flavoprotein dehydrogenase (ETFDH) was increased in HFpEF EX group ($p < 0.05$ *vs.* Control and HFpEF SED group). Further, the exercised group showed an increment in the expression of the subunits NDUFB8, SDHB, 2UQCRC2 and MTCO1 from the mitochondrial complexes I ($p < 0.01$), II ($p < 0.01$), III ($p < 0.001$) and IV ($p < 0.01$) respectively, when compared to Control group. Exercise promoted a significant increment in the expression of the subunits NDFB8 and MTCO1 from the mitochondrial complexes I and IV, respectively ($p < 0.05$ *vs.* HFpEF SED). Additionally, 5'-AMP-ativated protein kinase (AMPK) expression levels were decreased in HFpEF SED group in comparison with the Control group ($p < 0.05$), while phosphorylated AMPK (pAMPK) expression levels were lower in both HFpEF SED ($p < 0.05$) and HFpEF EX ($p < 0.05$) when compared to Control group.

No significant differences were found among the experimental groups in glucose transporter type 4 (GLUT4), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), alpha and beta subunits from complex V expression levels, neither in citrate synthase activity. There were also no significant differences in GAPDH/ATP synthase, ETFDH/ATP synthase and pAMPK/AMPK ratios among the three experimental groups.

5. Discussion

In this work, the ZSF1 obese rat model was chosen to assess the therapeutic effect of exercise training in HFpEF, since among the animal models available to study this pathology, this is the model that better resembles the human features of HFpEF, where cardiac impairments are reinforced by several comorbidities, including obesity, hypertension, diabetes and kidney dysfunction [49,106,109]. Hemodynamic data (Table 2), namely, EF was preserved in all three experimental groups validating this model as a HFpEF model [49]. Besides, ZSF1 obese rats display one of the major symptoms of HFpEF, exercise intolerance [49,107]. Our data confirmed the reduced exercise tolerance, since a significant decrease in VO_2 was observed in both HFpEF groups, in comparison with the Control group (Figure 5). Exercise intolerance is a primary symptom in HFpEF patients and is strongly correlated with the prognosis and reduced quality of life [156]. To date, no pharmacological therapy has proven to be effective in HFpEF treatment, while ET has proven to have the ability to improve exercise capacity and quality of life [157]. Indeed, four weeks of ET resulted in a significantly increment in VO_2, in comparison to HFpEF SED group (Figure 5). Moreover, ET benefits may be related with peripheral adaptations rather than cardiac ameliorations [14,147,158]. Despite the recognized potential of ET to improve exercise capacity through peripheral adaptations, the mechanisms underlying these changes need to be clarified.

The present work provides new molecular insights about this animal model, mostly at the metabolic level. To the best of our knowledge, this is the first study characterizing metabolic alterations in *gastrocnemius* muscle in the ZSF1 obese animal model of HFpEF. The choice of the *gastrocnemius* muscle is related with the metabolic profile of this muscle, that comprises both glycolytic and oxidative features [159], potentially allowing for a better understanding of the effects of HFpEF and/or ET in both metabolic programs. For this purpose, an untargeted metabolomics approach was employed, and the expression levels of specific enzymes, involved in glucose uptake, glycolysis and mitochondrial oxidative metabolism, were assessed. An integrated view of the metabolic changes suggested to be induced by HFpEF and ET is represented in Figure 13.

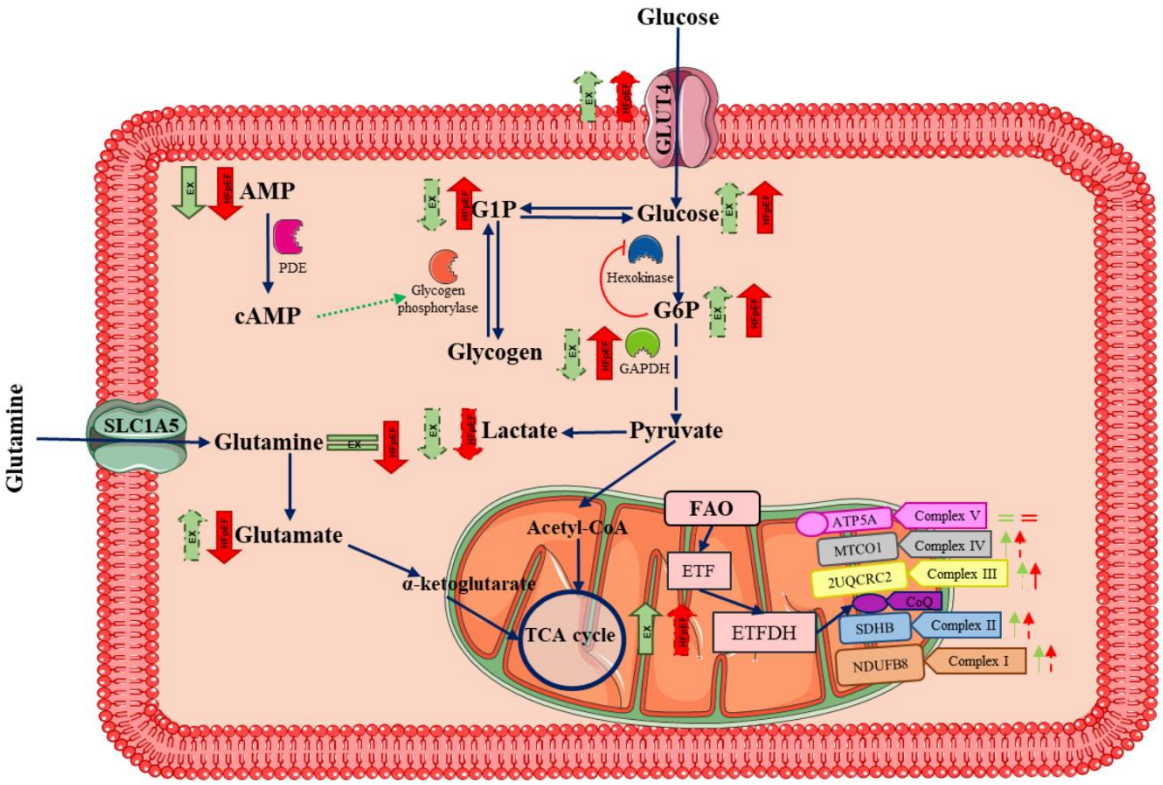

Figure 13: Schematic illustration of the main metabolic changes induced by HFpEF (red arrows and equal sign) and ET (green arrows and equal sign) in the *gastrocnemius* muscle. Dashed lines around the arrows mean no statistically significant variation. Legend: cAMP, cyclic adenosine monophosphate; CoQ, cytochrome C reductase; ETF, electron transfer flavoprotein; ETFDH, electron transfer flavoprotein dehydrogenase; FAO, fatty acid β-oxidation; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GLUT4, glucose transporter type 4; SLC1A5, glutamine transporter; TAC, tricarboxylic acid cycle.

Fatty acids and carbohydrates are the main energy sources of the skeletal muscle. Amino acids may also contribute to energy production in this tissue, but in a minor proportion [160]. Our findings revealed higher glucose levels in the HFpEF SED group when compared with the Control group. This could result from an enhancement in glucose uptake, and/or a reduction in glucose utilization by skeletal muscle cells. Since the expression levels of GLUT4 were not increased in the HFpEF SED group in comparison with Control group (Figure 12), it may be hypothesized that reduced glucose utilization is the main underlying reason for the observed increased levels. After entering the cell, glucose is converted into G6P, in an irreversible reaction catalyzed by hexokinase, and then a series of glycolytic reactions follow to produce pyruvate, which is converted into acetyl-CoA, to fuel the TCA cycle, and/or into lactate, especially under low oxygen concentrations [161]. In the present study, there were no changes in the expression levels of the glycolytic marker GAPDH (Figure 12), neither in lactate levels (Supplementary

Figure S1), suggesting no major changes in the glycolytic rate. On the other hand, the observed glucose increase was accompanied by increases in G1P and G6P (Figure 11). Together with the upsurge of signals attributed to small glycogen oligosaccharides (Figure 11), these changes suggest increased glycogen degradation in the HFpEF SED group. In turn, the G6P increase originating from upregulated glycogenolysis could explain glucose accumulation through allosteric inhibition of hexokinase. Regarding the molecular mechanisms underlying glycogenolysis upregulation, a role for cyclic AMP (cAMP), an activator of glycogen phosphorylase [162], may be hypothesized. cAMP-dependent phosphodiesterase (PDE) 3, which catalyze the breakdown of cAMP into AMP, has been found downregulated in association with heart failure [163]. In the present study, AMP levels were significantly reduced in the *gastrocnemius* muscle of the HFpEF SED in comparison to Control group (Figure 11). This decrease is consistent with the hypothesis of PDE downregulation and consequent cAMP-induced activation of glycogen phosphorylase and glycogen degradation. Low levels of AMP (Figure 11) are also consistent with reduced expression levels of AMPK and pAMPK (Figure 12) in HFpEF SED animals. Notably, decreased AMPK activity has already been linked to increased inflammation and insulin resistance, in morbidly obese patients [164]. Furthermore, AMPK activator drugs have been prescribed for treatment of type II diabetic patients [165], supporting the downregulation of AMPK activity in the diabetic setting.

Acetone relative content was found to be increased in *gastrocnemius* muscle of the HFpEF SED group (Figure 11), suggesting altered ketone body metabolism. During fasting, ketone bodies serve as an important energy substrate, replacing glucose as the primary energetic fuel to several tissues, including nervous tissue, cardiac muscle and skeletal muscle [166]. In fasting conditions, ketone bodies primarily originate from the oxidation of long chain fatty acids that result from lipolysis in adipose tissue. In the liver, fatty acids are converted into ketone bodies, and these are exported to extrahepatic tissues, such as skeletal muscle. Once in the skeletal muscle, ketone bodies are oxidized to CO_2 and water [166]. Impairment in skeletal muscle ketone body metabolism may be present in obesity, as suggested by lower ketone bodies oxidation reported in the *rectus abdominus* muscle of obese women, free of diabetes, vascular diseases or cancer, in comparison to lean women [167]. Our observation of increased acetone content in the *gastrocnemius* muscle of HFpEF SED animals suggests that ketone body oxidation in skeletal muscle may

be impaired in HFpEF. Furthermore, the production of ketone bodies in the liver could also be altered. Herein, we did not evaluate any parameter related to liver fatty acid oxidation and ketone bodies synthesis, so further investigations regarding liver metabolism in HFpEF would be beneficial to better understand the extent of ketone body metabolism dysfunction within this syndrome.

Mitochondrial functional impairments have been linked to the pathogenesis of both obesity and type II DM [168]. Despite not inducing significant differences in the expression levels of subunits NDUFB8, SDHB, MTCO1 and ATP5A from mitochondrial complexes I, II, IV and V respectively, HFpEF promoted and increment in the expression levels of subunit 2UQCRC2 of mitochondrial complex III, suggesting interference with mitochondrial oxidative phosphorylation (OXPHOS). Mitochondrial OXPHOS impairments have been reported in the *diaphragm* muscle of Dah salt sensitive rats [78]. Additionally, impairments in skeletal muscle mitochondrial metabolic pathways, such as the TCA cycle, branched-chain amino acids (BCAA) metabolism, FAO and the electron transport chain, have been described in the *gastrocnemius* muscle of a HF murine model [169]. Furthermore, the observed alterations in mitochondrial proteins seem to be independent from mitochondrial density, as no differences in citrate synthase activity (mitochondrial content marker [170]) were observed between Control and HFpEF SED groups. Our data is supported by recent studies where no changes in citrate synthase activity were observed in *soleus* muscle of the ZSF1 obese rat model, suggesting mitochondrial density in skeletal muscle to be unaltered by HFpEF in an obesity-driven model of the disease [85]. Interestingly, in contrast, biopsies collected from limb muscle of HFpEF individuals showed reduced mitochondrial content and oxidative capacity [83], so further investigation is needed in order to verify the translation of our findings to the human setting.

An imbalance between protein synthesis and degradation has been described in both experimental and human HFpEF [10,78]. Also, altered amino acid metabolism has been reported in HF, both in cardiac and skeletal muscle [171]. In our study, we found that the contents of some amino acids, including glutamine, glutamate and glycine were significantly reduced in HFpEF (Figure 11). Glutamine, the most abundant circulating amino acid, is essential in situations of acute metabolic stress. It is largely produced in skeletal muscle from glutamate and ammonia via glutamine synthase [172], and it can

enter cells *via* glutamine transporter SLC1A5-mediated transportation [173]. This transport protein in largely expressed in several tissues, including skeletal muscle [173]. Moreover, glutamine is involved in signal transduction, nitrogen transport and energy metabolism [172] and has a major role in protein synthesis and breakdown within skeletal muscle [174]. In disease and/or stress conditions, skeletal muscle can display reduced glutamine synthesis, leading to a decline in skeletal muscle mass, which is a hallmark of a myriad of clinical conditions, such as sarcopenic obesity and HFpEF [10,78,175]. Glutamine also serves as an anaplerotic substrate to fuel the TCA cycle in the mitochondrial matrix, via sequential conversion into glutamate and α-ketoglutarate (TCA cycle intermediate), enabling the production of reducing equivalents that drive the mitochondrial respiratory chain [176]. Hence, a possible explanation for reduced glutamine and glutamate levels herein observed could be reduced glutamine synthesis and/or enhanced anaplerotic use. Indeed, reduced amino acid content has already been linked to increased TCA cycle anaplerosis in skeletal muscle of obese subjects, to maintain the oxidative metabolism [177]. Furthermore, the release of glutamine from skeletal muscle to blood could be increased. Indeed, increased plasma levels of glutamine were reported in obese patients who were physically inactive (< 1000 steps *per* day) for two weeks. The authors suggested that, the apparent increment in plasma glutamine levels, after two weeks of reduced physical activity, could result from greater efflux of glutamine from skeletal muscle pool into circulation, leading to reduced protein synthesis within the skeletal muscle [178]. Finally, another possible explanation for the lower glutamine and glutamate relative contents in the *gastrocnemius* muscle of ZSF1 obese rats could be a reduction in SLC1A5 expression, which would result in decreased glutamine uptake [173]. Therefore, it would be interesting to evaluate the expression of SLC1A5, in order to verify whether, in the HFpEF setting, glutamine efflux is increased and/or its uptake is reduced. We have also found reduced glycine content in the HFpEF SED group, in comparison to Control animals. Again, this may relate to increased anaplerotic replenishment of the TCA cycle. Interestingly, reduced glycine muscle content was also documented in the *vastus lateralis* muscle and plasma of obese individuals, both before and after five days of high fat diet [177]. This suggests metabolic dysregulations involving specific amino acids to be present in obesity condition [177]. Lower levels of circulating glycine were also reported in obese Zucker compared to lean Zucker rats [179], and this change has been linked to increased

plasma BCAA content. Accordingly, BCAA metabolism impairments have been reported in patients whom suffer from cardiometabolic diseases, with high contents of BCAA and related metabolites being described in obesity [180]. Moreover, recent studies have validated the strong link between increased BCAA content and the development of diabetes [181]. However, in our study, the relative content of BCAA (Supplementary Figure S1) in the *gastrocnemius* muscle of ZSF1 rats was not altered among the three experimental groups. Similarly, in obese humans, plasma glycine content was reduced, while BCAA content was not altered in comparison with lean subjects [177]. To sum up, alterations in skeletal muscle amino acids metabolism have been identified in comorbidities frequently present in HFpEF setting, namely obesity and type II diabetes. Herein, we suggest that amino acid metabolism is also altered in the *gastrocnemius* muscle of this HFpEF experimental model, however further investigation is needed to clarify the pathways involved. It should also be noted that the observed amino acid alterations in *gastrocnemius* muscle of HFpEF SED animals could relate to skeletal muscle atrophy, described in both animal models and HFpEF patients [10,79–82,84,85]. Indeed, our morphometric evaluation results are in line with published work regarding other skeletal muscles [78,84], since the weight of *gastrocnemius* muscle was significantly reduced in the HFpEF SED group (Table 3), supporting the hypothesis of HFpEF-induced muscle atrophy.

In the HFpEF EX group, the findings regarding glucose metabolism were similar to those observed in the HFpEF SED group. GLUT4 expression levels were not increased, in agreement with a previous study reporting that GLUT4 expression levels in the *vastus lateralis* muscle of obese subjects undergoing eight weeks of HIIT was not altered in comparison to Control subjects [182]. Also, GAPDH expression and lactate levels remained unaltered, while glucose, G1P, G6P and glycogen oligosaccharides increased in the muscle of HFpEF EX animals compared to Controls. Altogether, these changes suggest increased glycogenolysis and allosteric inhibition of glucose to G6P conversion. Moreover, like it was suggested for HFpEF SED animals, cAMP-induced activation of glycogen phosphorylase could be at the basis of this dysregulation, as supported by decreased AMP content and pAMPK expression.

Contrarily to what was observed in HFpEF SED animals, acetone levels did not increase in the *gastrocnemius* muscle of exercised HFpEF animals, showing no difference

to Controls (Figure 12). This indicates that ET has the potential to reestablish ketone body metabolism within skeletal muscle. Indeed, exercise has long been reported to stimulate skeletal muscle ketone body metabolism in heathy conditions [166]. Although evidence in humans is still lacking, in experimental animal models, this effect has been attributed to changes in the expression and activities of enzymes involved in both ketogenesis and ketolysis [166]. Herein, it seems that ET also promotes ketone body oxidation by skeletal muscle in the HFpEF condition. More research is needed to further elucidate the molecular pathways underlying ET beneficial effects on skeletal muscle ketone body metabolism.

Exercise training was also found to stimulate oxidative metabolism in the *gastrocnemius* muscle of HFpEF animals, as indicated by significant increases in several proteins related to the electron transport chain. Four weeks of ET resulted in higher expression of ETFDH in HFpEF EX, compared to both HFpEF SED and Control groups (Figure 12). ETFDH is an oxidoreductase located in the inner mitochondrial membrane, which plays an important role in mitochondrial FAO and in the electron transport chain. It catalyzes the transfer of electrons from electron-transferring flavoprotein into ubiquinone in the main mitochondrial respiratory chain, linking FAO to mitochondrial respiration [183]. Hence, it may be suggested that ET stimulated FAO in the muscle of HFpEF animals. Additionally, four weeks of ET promoted significant increments in the expression levels of mitochondrial complexes (I to IV) subunits, suggesting that ET has the potential to enhance mitochondrial oxidative phosphorylation (OXPHOS). This should reflect a real improvement in mitochondrial oxidative capacity, as citrate synthase activity, hence mitochondrial volume density, remained unaltered. These data are supported by recently published literature, where no variations in citrate synthase activity were found in the *soleus* and *diaphragm* muscles of ZSF1 obese male rats, after eight weeks of either HIIT or MCT [85]. Furthermore, ET was previously reported to improve mitochondrial function in female Dahl salt-sensitive rats, where eight weeks of ET prevented mitochondrial respiration impairments [78]. Interestingly, despite inducing an increment in the expression levels of the other mitochondrial complexes, ET did not promote any alterations in the expression levels of subunit ATP5A of mitochondrial complex V among the three experimental groups. These results suggest the involvement of an uncoupling protein, possibly uncoupling protein-3 (UCP3), which is highly specific for skeletal muscle and adipose tissue [184]. UCP3 creates a leakage of protons from the mitochondrial

intermembrane space to the mitochondrial matrix, blocking ATP production [185]. Additionally, it has been reported that UCP3 is involved in mitochondrial fatty acid regulation and ROS induced oxidative stress protection [186]. Due to its role in oxidative stress protection and in the amelioration of FAO, UCP3 has been suggested to have a protective role in metabolic related comorbidities such as obesity and type II DM [168]. Concordantly, enhanced UCP3 expression levels have been described in skeletal muscle of obesity-resistant mice, while in obesity-prone mice the expression levels of this uncoupling protein were lower [187]. Several experimental evidences support the role of UCP3 on energy metabolism. For instance, a slight overexpression of UCP3 in mice skeletal muscle was reported to improve FAO and to reduce ROS mitochondrial production [186]. It has also been described that nine weeks of aerobic exercise promoted an increment in UCP3 expression in the *gastrocnemius* muscle of obese Zucker rats [188]. Hence, we may hypothesize that upregulation of UCP3 could underlie ET-promoted enhanced FAO and OXPHOS in the *gastrocnemius* muscle of HFpEF animals, a hypothesis that should be verified in the future. Overall, our findings newly reveal that, while not significantly modulating glucose metabolism, ET has the potential to enhance FAO and OXPHOS, which can be linked to the observed improvements in exercise capacity of HFpEF animals.

6. Conclusion

In order to evaluate the effects of exercise training on skeletal muscle metabolism in HFpEF setting, we submitted ZSF1 obese rats (validated animal model of HFpEF) to a four-week exercise training protocol. The results obtained by NMR metabolic profiles and protein expression analysis strongly suggest that:

i. HFpEF induces an increment in glycogen degradation, accompanied by decreased expression of AMPK, an increment in amino acids metabolization and impairment of ketone body oxidation;

ii. Four weeks of ET reestablished ketone body metabolism and promoted an improvement in oxidative metabolism (fatty acid oxidation – FAO, and oxidative phosphorylation - OXPHOS).

Taken together, these data suggest that enhancement of FAO and OXPHOS in the *gastrocnemius* muscle of ZSF1 obese rats can possibly contribute to the observed improvements in exercise capacity. In the future, it would be interesting to evaluate UCP3 expression levels in the *gastrocnemius* muscle of this experimental model, in order to better understand the mechanisms underlying the observed enhancement in OXPHOS and FAO after four weeks of ET. Finally, it would also be interesting to study the effects of ET on lipid metabolism in HFpEF setting to better understand the underlying pathways in ET induced enhancement of FAO.

7. References

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8. Supplementary information

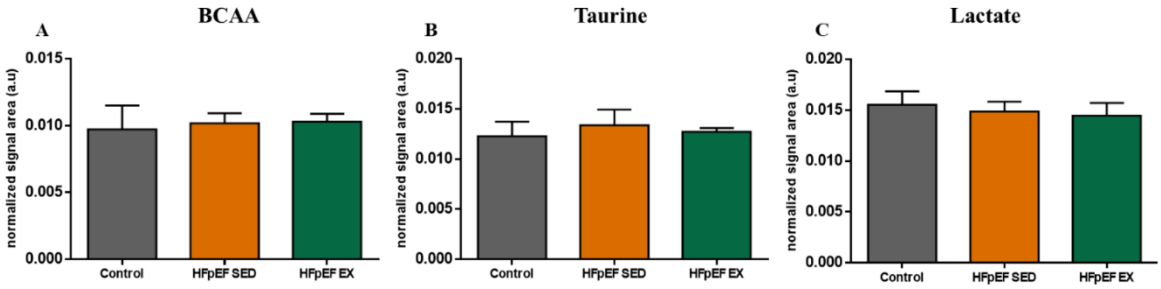

Figure S1: Relative content of metabolites was assessed by ^{1}H-NMR in *gastrocnemius* muscle of Control, HFpEF sedentary and HFpEF animals submitted to exercise training. Legend: A- BCAA; B- taurine; C- lactate. BCAA, branched-chain amino acids.

Table S1: ^{1}H-NMR chemical shifts for metabolites assigned in polar extracts of *gastrocnemius* muscle homogenates. Multiplicity: s, singlet; d, doublet; dd, doublet of doublets; dt, doublet of triplets; m, multiplet; q, quartet; t, triplet.

No.	Compound	δ ^{1}H in ppm (multiplecity)
1	Acetate	1.921 (s)
2	Acetone	2.237 (s)
3	Adenosine	3.943 (dd); 4.355 (q); 4.494 (dd); 6.110 (d); 8.121 (s); 8.360 (s)
4	ADP	4.226 (m); 4.378 (m); 4.605 (m); 6.158 (d); 8.533 (s)
5	Alanine	1.486 (d); 3.786 (q)
6	AMP	4.042 (dd); 4.379 (dd); 4.504 (dd); 6.158 (d); 8.259 (s); 8.585 (s)
7	Arginine	1.659 (m); 1.920 (m); 3.254 (t); 3.784 (t)
8	Anserine	2.691 (m); 3.035 (dd); 3.241 (m); 3.768 (s); 4.501 (q); 7.064 (s); 8.154 (s)
9	Aspartate	2.682 (dd); 2.813 (dd); 3.897 (dd)
10	β-alanine	2.657 (t); 3.270 (t)
11	Carnosine	2.701 (q,); 3.069 (m); 3.231 (q); 4.489 (q); 7.097 (s); 8.114 (s)
12	Choline	3.178 (s); 3.492 (m); 4.036 (m)
13	Creatine	3.039 (s); 3.935 (s)
14	Formate	8.465 (s)
15	Fumarate	6.525 (s)
16	Glucose	3.260 (dd); 3.422 (m); 3.473 (m); 3.549 (dd); 3.725 (m); 3.843 (m); 3.910 (dd); 4.660 (d); 5.248 (d)
17	G1P	3.404 (t); 3.489 (m); 3.770 (m); 3.880 (m); 3.935 (m); 5.461 (dd)
18	G6P	3.297 (dd); 3.495 (d); 3.575 (m); 3.743 (t); 3.898 (m); 3.948 (m); 4.000 (dd); 4.048 (m); 4.660 (d); 5.248 (d)
19	Glutamate	2.081 (m); 2.142 (m); 2.375 (m); 3.777 (dd)
20	Glutamine	2.147 (m); 2.458 (m); 3.775 (t)
21	Glycine	3.564 (s)
22	Glycogen oligosaccharides	5.437 (m)
23	Isoleucine	0.943 (t); 1.014 (d); 1.267 (m); 1.476 (m); 1.983 (m); 3.671 (d)
24	Lactate	1.331 (d); 4.115 (q)

No.	Compound	δ ^{1}H in ppm (multiplecity)
25	Leucine	0.965 (t); 1.718 (m)
26	Lysine	1.476 (m); 1.721 (m); 1.920 (m); 3.039 (t); 3.791 (t)
27	Nicotinurate	4.008 (s); 7.651 (dd); 8.266 (dt); 8.721 (dd); 8.949 (m)
28	Pantothenate	0.905 (s); 0.925 (s); 2.422 (t); 3.399 (d); 3.444 (q); 3.519 (d); 3.988 (s)
29	Phenylalanine	3.129 (m); 3.277 (dd); 3.982 (m); 7.335 (d); 7.379 (d); 7.431 (t)
30	Pyroglutamate	2.040 (m); 2.412 (m); 2.506 (m); 4.183 (dd)
31	Taurine	3.284 (t); 3.424 (t)
32	Trimethylamine	2.897 (s)
33	Tyrosine	3.066 (m); 3.205 (m); 3.946 (m); 6.914 (d); 7.203 (d)
34	UDP-glucose	3.467 (t);3.530 (dt); 3.771 (m); 3.889 (m); 4.235 (m); 4.373 (m); 5.605 (m);5.974 (m); 7.950 (d)
35	Valine	1.021 (d); 2.276 (m); 3.614 (d)

Legend: ADP, adenosine diphosphate; AMP, adenosine monophosphate; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; UDP-glucose, uridine diphosphate-glucose.

Table S2: Main metabolite variations in the polar extracts of ZSF1 *gastrocnemius* muscle homogenates. The variations are expressed as % of variation (%Var) with respective error (±), effect size (ES) and p-value (p). Variations with |ES| < 0.5 were considered to be null.

		HFpEF SED *vs.* Control	HFpEF EX *vs.* Control	HFpEF EX *vs.* HFpEF SED
BCAA	% Var	0	0	0
	±	8.75	8.44	4.17
	ES	0	0	0
	p	0.62	0.53	0.78
Alanine	% Var	0	7.85	7.24
	±	6.78	6.14	4.02
	ES	0	0.70	0.99
	p	0.94	0.27	0.12
Acetate	% Var	-19.12	-18.49	0
	±	5.21	5.14	3.69
	ES	-2.32	-2.27	0
	p	0.007	0.008	0.84
Acetone	% Var	125	0	-55.56
	±	7.93	5.85	8.15
	ES	5.54	0	-5.39
	p	0.0003	1	0.0002
Glutamate	% Var	-26.42	-2.22	19.03
	±	6.67	9.20	9.13
	ES	-2.61	0.81	1.10
	p	0.004	0.20	0.11
Glutamine	% Var	-30.16	-31.32	0
	±	11.73	12.02	5.21
	ES	-1.73	-1.76	0
	p	0.03	0.03	0.76
Aspartate	% Var	-29.02	0	33.33
	±	14.43	21.02	20.56
	ES	-1.35	0	0.79
	p	0.07	0.80	0.23

		HFpEF SED *vs.* Control	HFpEF EX *vs.* Control	HFpEF EX *vs.* HFpEF SED
TMA	% Var	-10.65	-14.45	0
	±	5.81	3.76	5.32
	ES	-1.11	-2.37	0
	p	0.09	0.008	0.45
Creatine	% Var	-12.46	-17.14	0
	±	5.31	4.70	5.89
	ES	-1.43	-2.28	0
	p	0.04	0.03	0.20
Taurine	% Var	8.80	0	0
	±	7.38	5.31	5.50
	ES	0.65	0	0
	p	0.29	0.55	0.41
Glycine	% Var	-34.88	-26.61	12.70
	±	12.03	10.21	8.25
	ES	-2.01	-1.72	0.83
	p	0.01	0.03	0.20
G6P	% Var	100.89	93.89	0
	±	11.91	9.60	8.13
	ES	3.22	3.80	0
	p	0.0006	0.0002	0.68
G1P	% Var	1600	780.95	-48.18
	±	45.71	21.23	31.58
	ES	2.22	4.28	-1.15
	p	0.02	0.00001	0.11
Glucose	% Var	57.46	69.12	7.40
	±	5.99	5.47	4.68
	ES	4.26	5.37	0.87
	p	0.00007	0.00001	0.17
Lactate	% Var	0	-6.71	0
	±	4.86	5.44	4.78
	ES	0	-0.73	0
	p	0.14	0.24	0.59
Glycogen oligosaccharides	% Var	340.83	513.33	39.13
	±	22.05	19.55	16.64
	ES	3.27	4.20	1.12
	p	0.002	0.00	0.09
AMP	% Var	-16.90	-24.65	-9.33
	±	2.89	3.06	3.70
	ES	-3.67	-5.24	-1.51
	p	0.0002	0.00002	0.03
Adenosine	% Var	55.10	74.49	12.50
	±	11.74	9.62	9.49
	ES	2.10	-5.24	-1.51
	p	0.01	0.001	0.25
Carnosine	% Var	-14.74	-17.87	0
	±	4.14	4.35	3.80
	ES	-2.19	-2.58	0
	p	0.006	0.002	0.36
Anserine	% Var	-28.71	-37.02	-11.66
	±	6.55	7.18	5.42
	ES	-2.92	3.61	-1.31
	p	0.003	0.0006	0.05

		HFpEF SED *vs.* Control	HFpEF EX *vs.* Control	HFpEF EX *vs.* HFpEF SED
Tyrosine	% Var	-26.38	-20.86	7.50
	±	6.85	6.13	5.45
	ES	-2.53	-2.11	0.76
	p	0.003	0.01	0.22

Legend: AMP, adenosine monophosphate; BCAA, branched chain amino acids; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; TMA, trimethylamine.