Universidade de Aveiro Departamento de Química
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Efeito do exercício físico na remodelação do ventrículo direito induzida pela hipertensão arterial pulmonar

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica do Doutor Daniel Moreira-Gonçalves, Professor Auxiliar da Faculdade de Medicina da Universidade do Porto, e da Doutora Rita Ferreira, Professora Auxiliar do Departamento de Química da Universidade de Aveiro.

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palavras-chave Exercício físico, hipertensão arterial pulmonar, remodelação cardíaca, terapia, metabolismo

resumo A hipertensão arterial pulmonar (HAP) é uma doença grave, caracterizada pela proliferação excessiva de células endoteliais e musculares lisas das artérias pulmonares, com aumento da resistência vascular pulmonar e sobrecarga do ventrículo direito, sendo este o principal determinante de prognóstico associado à patologia. O exercício físico confere vários benefícios ao nível da função cardiovascular, tanto em condições fisiológicas como patológicas, pelo que se questionou a sua potencial utilização como medida terapêutica na HAP. Assim, neste trabalho, numa primeira parte, reuniu-se o conhecimento atual sobre a adaptação do ventrículo direito face às alterações impostas pela HAP (Capítulo II), discutindo-se os mecanismos modelados pelo exercício físico. De um modo geral, o exercício físico aparenta prevenir a remodelação da matriz extracelular (diminuição da fibrose e modulação das MMPs/TIMPs), estimular a angiogénese, reduzir a inflamação (diminuição da infiltração celular e dos níveis de TNF-α e IL-6) e o stress oxidativo. Na segunda parte deste trabalho (Capítulo III), estudou-se os efeitos cardioprotetores do exercício físico na HAP, utilizando o modelo animal de monocrotalina submetido a duas semanas de exercício físico (5 dias/semana, 60 min/dia, 25 m/min). Os resultados evidenciaram que o exercício físico retarda a progressão da doença, atendendo a que os ratos treinados apresentaram melhoria da função diastólica (pressão diastólica final inferior e Tau), apesar da presença de sobrecarga cardíaca (aumento da pressão sistólica, pressão diastólica final e elastância arterial). Concomitantemente, observou-se um aumento da captação de glucose para os cardiomiócitos do ventrículo direito através do GLUT4 seguido da sua oxidação a lactato, não se observando alteração da oxidação de ácidos gordos. Este efeito do exercício está associado a um aumento da expressão de proteína PGC1α e PPARγ. De um modo geral, o presente trabalho suporta a recomendação de exercício físico para o tratamento da HAP.

keywords Exercise training, pulmonary arterial hypertension, cardiac remodelling, therapy, metabolism

abstract Pulmonary arterial hypertension (PAH) is a deadly disease characterized by progressive remodelling of the pulmonary arteries, causing a rise in pulmonary vascular resistance and overloading the right ventricle (RV). The RV response to the overload is the main prognostic determinant. Since exercise training provides several cardiovascular benefits in both physiological and pathological conditions, its use as a therapeutic tool for PAH has been hypothesised. In this work, we first performed a narrative review summarizing the current evidence about the mechanisms underlying the cardioprotective effects of exercise training in PAH (Chapter II). Our review suggests that exercise training prevents the remodelling of ECM (decrease fibrosis and modulation of MMPs/TIMPs), stimulate angiogenesis, reduce inflammation (decreased cell infiltration and levels of TNF-α and IL-6) and oxidative stress. Secondly, we extended the comprehension of the cardioprotective effects of exercise training in PAH by exploring the metabolic changes promoted by exercise. In order to do so, we used the monocrotaline animal model of PAH submitted to two weeks of treadmill exercise training (5 days/week, 60 min/day, 25 m/min). Our data shows that exercise training delays the progression of the disease, as trained rats had improved diastolic function (lower end-diastolic pressure and tau) despite the presence of cardiac overload (increased peak systolic pressure, end-diastolic pressure and arterial elastance). This improved hemodynamic response was paralleled by an increased uptake of glucose to cardiomyocytes through GLUT4 followed by its oxidation to lactate. Exercise did not revert the decrease of fatty acid oxidation related to PAH. This metabolic remodelling was associated to an increase of PGC1α and PPARγ protein expression. Overall, our work supports the recommendation of exercise training for the management of PAH.

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CHAPTER I

General Introduction

Pulmonary arterial hypertension (PAH) is a rare, chronic and progressive form of pulmonary hypertension which is characterized by elevated pulmonary arterial pressure and pulmonary vascular resistance in blood vessels carrying the blood from right side of heart through lungs.[1] It occurs due to the tightening and stiffening of the small pulmonary arteries leading to the right ventricular dysfunction and vessel obstruction. Without effective therapeutic interventions, patients with PAH eventually die from right heart failure.[2] According to USA and Europe register, idiopathic subtype of PAH is the most common (50-60% of all cases).[3,4] Prevalence of PAH varies occurring to the geographical distribution, environmental factors, sex, age and socioeconomic factors. The reported incidence of pulmonary arterial hypertension in the developed world is 1.1-7.6 *per* million adult *per* year.[4] PAH generally affects predominantly females and female sex is considered a risk factor for PAH; however, females have better survival than males. This disease is characterized by a hemodynamic worsening, and the treatment aims to reduce pulmonary arterial pressure as well as normalize the cardiac debit.[2,9]

In recent years, exercise training has been recognized as a non-pharmacological therapeutic tool in several chronical diseases.[10] In PAH, the presence of physiological/hemodynamic and cellular alterations reduce the functional capacity and tolerance to exercise training. Thus, until 2015 the exercise was considered forbidden in patients with this pathology.[1] However, there is still a broad path ahead in knowing for certain whether physical exercise is responsible for attenuating or reversing structural and molecular alterations present in PAH. Despite these imposed PAH limitations, there is a growing number of studies in humans[11–14] and animal models,[15–19] which indicate that the practice of supervised physical exercise can promote beneficial effects on patients and animal models with PAH. However, the types of exercises to be carried out, the intensity and the duration of the exercise are not yet established. The results obtained in human studies show an improvement in the quality of life and functional capacity, an increase in the capacity of resistance to physical exercise and ventilatory efficiency, a general improvement of the cardiac function and muscle metabolism.[12,16,20–22] To add new insights on this topic, the present work begins with a narrative review followed by an experimental work. In the first, we discuss what is known so far about the role of exercise training in the modulation of the molecular pathways underlying right ventricle dysfunction in PAH. On the second,

we evaluated the therapeutic effect of exercise training in the right ventricle metabolic remodelling in an animal model of PAH.

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CHAPTER II

Review. **The role of exercise training in the modulation of the molecular pathways underlying right ventricle dysfunction in pulmonary arterial hypertension**

1. Pulmonary arterial hypertension

Pulmonary hypertension (PH) is a pathophysiological disorder encompassing multiple clinical conditions.[1] It is defined by an increase in mean pulmonary artery pressure at rest ≥ 25 mmHg, as assessed by right heart catheterisation, and can complicate the majority of cardiovascular and respiratory diseases.[1] Pulmonary hypertension is currently classified into five categories (**Table 1**).

Table 1 – Clinic classification of pulmonary hypertension according to the 5th World Symposium on Pulmonary Hypertension (2013)

1. Pulmonary Arterial Hypertension (PAH) **1.1.** Idiopathic PAH **1.2.** Heritable PAH **1.2.1.** *BMPR2* mutation **1.2.2.** *ALK-1*, *endoglin*, *Smad9*, *caveolin-1*, *KCNK3* mutation **1.2.3.** Unknown **1.3.** Drug and toxin induced **1.4.** Associated with: **1.4.1.** Connective tissue disease **1.4.2.** Human immunodeficiency virus infection **1.4.3.** Portal hypertension **1.4.4.** Congenital heart disease **1.4.5.** Schistosomiasis **1'** Pulmonary veno-oclusive disease and/or pulmonary capillary haemagiomatosis **1''** Persistent pulmonary hypertension of the new-born
2. Pulmonary hypertension due to left heart disease **2.1.** Left ventricular systolic dysfunction **2.2.** Left ventricular diastolic dysfunction **2.3.** Valvular disease **2.4.** Congenital/acquired left heart inflow/outflow tract obstruction and congenital cardiomyopathies
3. Pulmonary hypertension due to lung diseases and/or hypoxia **3.1.** Chronic obstructive pulmonary disease **3.2.** Interstitial lung disease **3.3.** Other pulmonary diseases with mixed restrictive and obstructive pattern **3.4.** Sleep-disordered breathing **3.5.** Alveolar hypoventilation disorders **3.6.** Chronic exposure to high altitude **3.7.** Development lung diseases
4. Chronic thromboembolic pulmonary hypertension (CTEPH)
5. Pulmonary hypertension with unclear multifactorial mechanisms **5.1.** Haematologic disorders: chronic haemolytic anaemia, myeloproliferative disorders, splenectomy **5.2.** Systemic disorders: sarcoidosis, pulmonary histiocytosis, lymphangioleiomyomatosis **5.3.** Metabolic disorders: glycogen storage disease, Gaucher disease, thyroid disorders **5.4.** Others: tumoral obstruction, fibrosing mediastinitis, chronic renal failure, segmental pulmonary hypertension

This classification categorises the multiple clinical conditions culminating in PH according to their similar clinical presentation, pathological findings, hemodynamic characteristics and treatment strategy.[1] This review will focus on category 1, Pulmonary Arterial Hypertension (PAH) (**Table 1**).

1.1. Definition, epidemiology, clinical presentation and diagnostic

Pulmonary arterial hypertension is a vastly underdiagnosed condition, with an estimated incidence of 2.4-7.6 million cases[5] per year and prevalence situated between 15-26 million cases[6] worldwide. It is defined by the presence of a pre-capillary pattern in the invasive hemodynamic evaluation and characterised by a resting mean pulmonary artery pressure (PAP) superior to 25 mmHg, pulmonary vascular resistance (PVR) higher than 3 Wood units, and pulmonary capillary wedge pressure inferior to 15mmHg (in the absence of other causes of PAH).[1] In the presence of a suspected PAH patient, multiple exams are required to confirm the diagnosis, clarify the specific aetiology within the PH group and evaluate the functional and haemodynamic impairment. Importantly, the diagnosis of PAH (particularly of idiopathic PAH) is a diagnosis of exclusion.[1] Regarding the clinical presentation, the symptoms of PAH are non-specific and include fatigue, breathlessness, weakness, angina, syncope and abdominal distension. Importantly, symptoms at rest are only reported in advanced stages of disease. The signs of PAH include right ventricle (RV) third sound, tricuspid insufficiency murmur, prominent right ventricular impulse and accentuated pulmonic valve component.[1,4] The electrocardiogram (ECG) could provide evidence of PAH by showing RV hypertrophy, and right atrial dilatation. RV hypertrophy on ECG are present in 87% and 79% of patients with idiopathic PAH. The absence of these findings does not exclude the presence of PAH nor does it exclude severe hemodynamic abnormalities.[5] Pulmonary function and arterial blood gases tests are also performed. Arterial blood gas analysis may help to exclude hypoxia as supplier to PH. Pulmonary function tests are essentials to establish airflow obstruction or restrictive pulmonary pathology.[1] Cardiac catheterization should be performed in patients with unexplained pulmonary hypertension and remains the gold standard for PH diagnosis and quantification. The following parameters must be recorded during right heart catheterization: pulmonary arterial pressure (systolic,

diastolic and mean), right atrial pressure, pulmonary wedge pressure and RV pressure.[1,6] All patients should perform routine biochemistry and haematology tests. A range of biomarkers has been described in PAH, which may be of diagnostic and prognostic significance. They include markers of heart failure and cardiac myocyte damage.[5] Examples are atrial natriuretic peptide (ANP) and B-typenatriuretic peptide (BNP).[7] More recently, interest has turned to the N-terminal fragment of BNP (NT-proBNP) as an alternative biomarker to BNP, because it seems to provide the same information, but has advantages in terms of stability. Endothelin 1 (ET-1), troponin T, nitric oxide (NO), acid uric, cyclic guanosine monophosphate (cGMP), 5-hydroxytryptamine (5-HT) and von Willebrand factor are among other biomarkers that might be of interest.[5,7] Assessment of functional capacity is also informative to evaluate the disease severity. Two commonly used tests are the 6 minute walk test (6MWT) and the cardiopulmonary exercise testing.[8]

Thus, the data of the clinical history of the patient and a careful physical examination are crucial to the correct diagnosis of pulmonary hypertension. Attention should be given to previous medical conditions, drug use and family history.

1.2. Pathophysiology of pulmonary arterial hypertension

As illustrated in **Table 1**, PAH may be idiopathic or secondary to various conditions, but regardless of the underlying aetiology, patients exhibit similar pathological changes in the pulmonary vasculature. A wide array of pulmonary vascular changes can occur in PAH, predominantly affecting the small pulmonary arteries (diameter <500 mm), which include enhanced pulmonary arteriole contractility, endothelial dysfunction, remodelling and proliferation of booth endothelial and smooth muscle cells.[9,10] The physiological outcome of these disturbances is the partial and/or total occlusion of small pulmonary arteries, causing an increase in PVR and imposing a hemodynamic overload to the RV. Initially, the RV will adapt by developing compensatory hypertrophy but ultimately cardiac dysfunction and failure, and premature death will ensue.[11]

1.2.1. Vasoconstriction impairment

Vasoconstriction of pulmonary arteries is a fundamental factor in the pathogenesis of PAH. When present without microthrombosis and vascular remodelling (**Figure 1**) it represents a highly reversible state in the development of the disease.[12] Vasoconstriction is mostly mediated by an imbalance between vasoactive and vasodilating factors (i.e. an excess of vasoconstrictors and/or a deficiency in vasodilators). Over the past years, three major pathways have been identified to modulate vasoconstriction and vasodilatation: prostacyclins, endotelin-1 or nitric oxide pathways.[13,14]

Prostacyclin and thromboxane A_2 belong to the prostanoid family and are produced by cyclooxygenase during the metabolism of arachidonic acid. The former is an effective vasodilator and inhibitor of platelet activation whereas the latter has the reverse effects.[14] In PAH patients, the expression of prostacyclin synthase in the pulmonary arteries is reduced, which leads to a diminished production of prostacyclin in the pulmonary endothelial cells (**Figure 1**).[15,16]

Endothelin (ET-1) is a peptide mostly produced by the vascular endothelium with potent vasoconstrictive and proliferative actions on the vascular smooth muscle cells. ET-1 exerts its effects through the interaction with two types of receptors: ET receptor type A (ET_A) and ET receptor type B (ET_B), which belong to the G-protein-coupled receptor family.[17] ET_A receptors are located predominately on vascular smooth muscle cells and mediate vasoconstriction, as well as proliferation, hypertrophy and cell migration. ET_B are found on both endothelial and smooth muscles. ET_B activation on smooth cells produces vasoconstriction, however ET_B activation on endothelial cells leads to vasoconstriction and antiproliferation by increasing NO and prostacyclin production. [14] As PAH progresses, the cellular distribution of ET-1 receptors changes (**Figure 1**), with increased expression of both constrictive ET_A and ET_B on smooth cells and decreased expression of vasodilatory endothelial ET_B. In animals and patients with PAH, it was reported that ET-1 levels were increased in lungs and in circulation.[17–19]

NO is a lipophilic gaseous molecule that can be synthetized in mammalian tissues *via* activation of one of the three NO synthase isoforms. This enzyme catalyses the formation of NO from L-arginine in a two-step reaction. NO is a vasodilator that modulates several physiologic processes, and is also capable of inhibiting leucocyte adhesion, platelet

aggregation, thrombus formation and vascular proliferation.[20] Some studies have shown the relevance of NO in pulmonary physiology, particularly, in the maintenance of low blood pressure, which is characteristic of the circulatory system in this organ.[21,22] Reduced availability of this gas is a main feature described in both clinical and preclinical settings of PAH (**Figure 1**).[23]

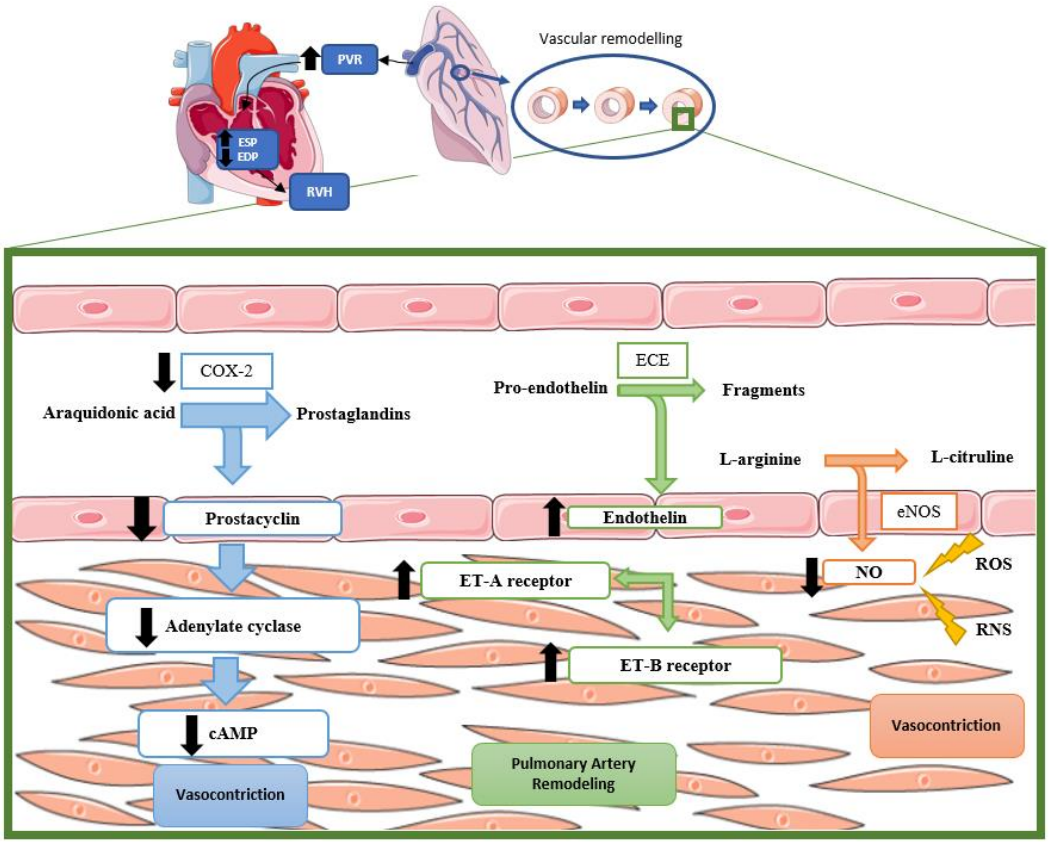

Figure 1 - **Pathophysiology of pulmonary arterial hypertension in heart and lungs.** Vascular remodelling and endothelial dysfunction are markers of the pathology, leading to a progressive increase in PVR and ultimately to RVH with increased RV systolic and diastolic pressures. Due to progressive increase in PVR, RV function deteriorates, eventually leading to RV failure. Figure made using Servier Medical Art.by Servier (https://smart.servier.com/) and modified by the author under the Creative Commons Attribution 3.0 Unported License (CCBY 3.0). Abbreviations: cAMP, cyclic adenosine monophosphate; COX-2, cyclooxygensase 2; ECE, endothelin converting enzyme; EDP, end-diastolic pressure; ESP, end-systolic pressure; ET, endothelin; NO, nitric oxide; PVR: pulmonary vascular resistance; RONS, reactive oxygen and nitrogen species; RVH: right ventricle hypertrophy

The reduction of NO may be related with decreased expression and/or activity of the endothelial nitric oxide synthase (eNOS) enzyme, the uncoupling of eNOS, the activation of iNOS or by further degradation of NO by reactive oxygen and nitrogen species (RONS).[24]

1.2.2. Pulmonary vascular remodelling

The vascular obstruction seen in PAH results from a combination of increased vasoconstriction, abnormal vascular remodelling and *in situ* thrombosis of pulmonary vessels.[25,26] Pulmonary vascular remodelling in PAH is characterised by the thickening of all three layers of the blood vessel wall. This thickening is the result not only of hypertrophy and/or hyperplasia of the predominant cell type within each of the layers (fibroblasts, PASMCs and endothelial cells (EC)) but also of increased deposition of extracellular matrix components (collagen, elastin and fibronectin).[27] Additionally, it is also characterised by increased cellular proliferation and resistance to apoptosis.[26] These changes arise from the loss of antimitogenic endothelial substances, like prostacyclins and NO, and increased levels of mitogenic substances, such as ET-1.[28] Other stimuli appear from locally activated platelets, which release thromboxane A_2 and serotonin, promoting hypertrophy and hyperplasia of vascular smooth muscle cells.[24] The aforementioned increased deposition of extracellular matrix components leads to intimal narrowing and increased resistance to blood flow.[20]

The uncontrolled proliferation of ECs occurs in the irreversible plexogenic lesions, which are complex plexiform lesions (glomeruloid-like structures) forming channels in branches of the pulmonary artery, They originate from remodelled pulmonary arteries and constitute a hallmark of severe PAH.[26] The prevailing consensus is that plexiform lesions are formed by a proliferating network of endothelial-lined vascular channels supported by a core of specialized, apoptosis-resistant myofibroblasts, smooth muscle cells, or even undifferentiated mesenchymal cells[29], ultimately obliterating the vascular lumen.[18,26] Regarding the ECs present in these complex lesions, Masri *et al*. found evidence that idiopathic PAH pulmonary artery EC have a hyperproliferative, apoptosis-resistance phenotype when compared with cells from healthy lung.[9] Therefore, in this pathology, the proliferation of ECs is enhanced, while apoptosis is repressed.[30]

Vascular endothelial growth factor (VEGF) and VEGF receptor 2 (VEGFR-2) are overexpressed in the plexiform lesions of patients with PAH, suggesting a role for this angiogenic factor in the pathogenesis of PAH.[29] However, pre-clinical data have been inconsistent in supporting this role. For instance, while VEGF was shown to be upregulated in rats with chronic hypoxia-induced PH[31], it was downregulated in rats with monocrotaline-induced PH.[26] Moreover, when rats with established PH were treated with VEGF, the disease

was mitigated. This could be the result of VEGF helping in the maintenance of endothelial cell function, such as in the production of antiproliferative factors.[31] At the same time, while VEGF overexpression was capable of slowing down the development of pulmonary hypertension in both the monocrotaline and hypoxia-induced PH, inhibition of VEGF signalling by sorafenib (a small multikinase inhibitor) was also able to attenuate experimental PH.[32] Altogether, these findings delineate an intriguing and complex role for VEGF in PAH pathophysiology.

1.3. Current therapeutic strategies

The knowledge of the aetiology of the disease provides the basis for the choice of a pharmacological approach. However, since PAH appears to have a multifactorial nature, the treatment has remained primarily palliative. However, even if palliative, the improvement in the understanding of PAH pathogenesis and underlying molecular pathways over the last years has allowed for a significant upgrade in the treatment of this syndrome.[8] Unfortunately, the available therapies are only capable to slowdown the progression of the disease and alleviate symptoms but cannot afford a definite cure. The current therapeutic options are summarized in **Table 2**, and include supportive therapies, calcium channel blockers, synthetic prostacyclin and prostacyclin analogues, ET-1 receptor antagonists, type 5 phosphodiesterase inhibitors and interventional procedures.[14] Importantly, if left untreated, survival after diagnosis is estimated to be as low as 2.8 years[33], with death occurring in most cases from right ventricular failure.[1] It should be noted that despite the major importance of the RV to the patient prognosis, there are no specific therapies targeting the RV function and remodelling.

Table 2 – Current pharmacological therapies

Drug Class	Generic name	Route; Dosage	Side effects	Comments and Recommendations	References
Synthetic prostacyclin and prostacyclin analogues	Epoprostenol	cIV; up to 30 ng/Kg/min	Jaw pain Headache Flushing Nausea Diarrhoea	Catheter related infections	34
	Treprostinil	cSC or O; up to 50-60 ng/kg/min		Infusion site pain can limit dose escalation when given SC	35–37
	Iloprost	Inhaled; 5 µg, 6-12 times daily			38
	Beraprost	O; 120 µg four times per day	Headache Flushing	Only modest benefit in the short term	39,40
Prostacyclin receptor agonist	Selexipag	O; up to 1600 mg bid	Jaw pain Headache Flushing Nausea Diarrhoea		41,42
Endothelin receptor antagonists	Bosentan	O; 125 mg twice per day	Anaemia Nasopharyngitis Hepatoxicity	Teratogenic	43,44
	Ambrisentan	O; 10 mg daily	Anaemia Nasopharyngitis		45,46
	Macitentan		Fluid retention		47
Type 5 phosphodiesterase inhibitors	Sildenafil	O; 20 mg three times per day	Headache Flushing, Nasal congestion Dizziness Visual disturbance	Must not be combined with nitrates (severe hypotension)	48
	Tadalafil	O; 40 mg daily	Headache Flushing, Dizziness visual disturbance		49

cIV, continuous intravenous; cSC, continuous subcutaneous; O, oral

2. The right ventricle in pulmonary arterial hypertension

Under physiological conditions, the amount of blood ejected by the RV is identical to that ejected by the left ventricle (LV). However, the structural and functional characteristics of the RV are quite different from those of the LV.[50] Contrary to the LV, the RV has a thin wall, which allows it to have high diastolic compliance, thus adapting to fast fluctuations in volume load.[51] On the other hand, RV pumping function is impaired to a much greater extent in comparison with that of the LV when exposed to equal amounts of pressure overload.[50] Chronic pressure overload imposed by increased PVR leads to RV hypertrophy, followed by maladaptation and subsequent RV failure, which is the main cause of premature death in PAH patients.[52] In fact, RV dysfunction and failure are reportedly important predictors of mortality in PAH.[53] However, contrasting to the well-characterized pulmonary vascular pathology, the RV adaptation to PAH remains poorly understood. In order to further advance this knowledge, we will next review the main pathophysiological features of RV remodelling, providing an overview of the main structural, functional, cellular and molecular modifications already described in PAH.

2.1. Functional and structural modifications

Increased RV afterload is the primary cause of right ventricular adaptation in PAH. The normal right ventricle has a thin crescent shaped wall. These features allow the RV to adapt rapidly to changes in volume load, but not so much to pressure load.[54] The normal heart has a pressure–volume relationship in which the RV responds to an increase in load with an increase in contractility. In patients with PAH, the RV initially copes with the progressive increase in afterload (imposed by PVR and reduced pulmonary vascular compliance) by increasing contractility and by developing compensatory hypertrophy. These changes allow right atrial pressure to remain normal and to maintain cardiac output (CO) despite the elevated overload.[50,51] However, RV hypertrophy is only a short-term solution, with chronically sustained overload leading to failure of the compensatory mechanisms (ventricular pulmonary arterial uncoupling) and, eventually, to a transition to cardiac dilatation, characterised by elevated RV filling pressure and increased end-diastolic volume. The limits of the compensatory adaptation are poorly understood, although there is increasing

evidence that a mismatch between cardiac oxygen supply and demand has an important role.[51] When the afterload rises because of PAP elevations, RV hypertrophy sustains a normal RV ejection fraction (RVEF) and diminishes wall stress.[53] The RV also assumes a more rounded shape, compressing the LV. When the RV dilates, LV fillings is altered which may help explain the inadequate CO response to metabolic demand.[51,55] In this setting, systolic right coronary artery flow is reduced which can lead to RV ischemia. Therefore, it has been suggested that the RV coronary circulation becomes more like the one seen in the LV, with a greater oxygen extraction at rest and a higher dependence on an increase coronary flow to meet an increase in myocardial oxygen demand. The increasing pressures during the RV filling and decreased capacity in blood ejection may be a cause of RV ischemia and decrease contractility.[51] However, even if this mechanism first preserves cardiac function, ultimately it leads to diastolic dysfunction, ischemia, dilatation of the RV and even to heart failure.[52,53] In addition, RV diastolic function is also affected in PAH, as shown by their altered filling pattern, prolonged relaxation times, and intrinsic diastolic stiffness.[56]

Several studies have evaluated the haemodynamic function in preclinical models of PAH by echocardiography.[24,57–61] In monocrotaline studies, this compound resulted not only in increased peripheral vascular resistance,[60,62] but also in increased systolic and diastolic blood pressure in the RV,[24,57–60,63] which worsened RV contractility and relaxation. At the same time, monocrotaline lead to an overall impairment of diastolic function,[62] decreased artery flow velocity, and reduce the heart rate,[58,62] leading to premature development of heart failure.[57,64] (**supplementary Table 1**) It was also found that the rate of acceleration/ejection time of pulmonary artery was lower in the animals injected with monocrotaline.[24,60,65] In sum, the overall analysis of these results evidences PAH-induced heart failure, characterized by RV dysfunction.

Structurally, the foetal/neonatal RV is a thick-walled chamber that ejects blood at a relatively high pressure into a high-resistance vascular bed. The wall thickness of both ventricles concomitantly increases, and while the RV presents with a concentric geometry, LV's geometry is in a quite distinct cone-shaped form. The RV can be subdivided into 3 segments: **1)** the inlet, comprising the tricuspid valve, chordae tendineae, and papillary muscles; **2)** the trabeculated apical myocardium; and **3)** the outflow region which has a smooth myocardial surface.[66] A superficial, circumferential layer of RV myocardial fibres

are in continuity with the LV fibres, accounting for the systolic motion of the RV free wall toward the outflow tract.[54] When systolic and diastolic ventricular pressures rise, an increase in diastolic and systolic stretch on the RV wall is verified, which initially leads to an increase in muscle mass (adaptative hypertrophy) due to increased protein synthesis and an increase in cardiomyocyte size through the addition of new sarcomeres.[52] Archer and associates were able to distinguish two different phenotypes, depending on the cardiac adaptation to pressure elevation: the adaptive phenotype, where the RV is less dilated and fibrotic, the RVEF is preserved and remodelling is concentric; and the maladaptive phenotype, where the RV is dilated and fibrotic, and the remodelling is eccentric.[67] However, the RV cannot maintain adaptative hypertrophy in the face of sustained pressure overload, and eventually there is a transition to dilatation. At this point there is no further increase, and even a decrease in RV contractility can occur, despite a further increase in load.[51] One consequence of RV dilatation is an increase in wall tension, which increases myocardial oxygen demand and simultaneously decreases right ventricular perfusion, leading to further compromised contractility and dilatation.[68] An increase in ventricular volume may also lead to functional tricuspid regurgitation, imposed by annular valve dilatation and chordal traction, which in turn results in RV volume overload, and thus further progressive annular dilatation and RV remodelling.[69] As a consequence of the decline in RV function, the increase in RV contraction time and subsequent ventricular asynchrony, and decrease in RV stroke volume, underfilling of the LV starts to occur.[70] Therefore, the LV is also impaired due to the development of leftwards ventricular septal bowing, resulting from prolonged RV contraction time and reduction of LV volume during early diastole.[55] All these markers, together with systolic/diastolic RV dysfunction, contribute to the marked decline in cardiac output seen in severe PAH. When RV function has declined, a vicious cycle of events is underway, which, if not interrupted, leads to right heart failure.[51]

2.2. Inflammation

Inflammation exerts a central role in the pathogenesis of PAH. The profile of circulating levels of inflammatory mediators is associated with disease severity, symptom burden and survival in PAH patients.[71] Moreover, it has been hypothesized that circulating

inflammatory mediators released from the pulmonary vasculature could generate or contribute to the inflammatory process that adversely affect the RV remodelling and function.[72] Furthermore, there is evidence of recruitment of immune cells from the circulatory system to the RV, which could also contribute to the initiation and maintenance of cardiac inflammation.[73]

Macrophages are present in all tissues, including the heart. Expansion of cardiac macrophages has been documented in models of pressure overload-induced LV remodelling and failure.[74] In opposition, data regarding the role of macrophages in RV remodelling and failure are still limited. The recruitment of macrophages into the RV appears to be triggered by increased RV afterload induced by pulmonary artery banding (PAB) in rats.[75] Increased recruitment of macrophages has also been reported in RV autopsy samples from PAH patients with RV failure.[71] However, due to the lack of studies in RV failure in PAH patients, the role of macrophages recruitment has not been well established. In fact, based on studies in LV remodelling and failure, the potential mechanism through which macrophages might contribute to RV remodelling and dysfunction include production of RONS, regulation of cardiac inflammation and mediation of ECM modifications.[74,76]

The inflammatory mediators involved in ventricular remodelling and dysfunction can be produced by infiltrating immune cells, by cells from the heart tissue, or by cells of extra-cardiac origin.[77] Interleukin-6 (IL-6) is a multi-functional cytokine with a variety of biological activities, which have been implicated in the remodelling and failure of the heart.[78] Notably, increased expression of IL-6 mRNA has been detected in RV of patients with advanced heart failure.[79] Elevated plasma levels of IL-6 and increased IL-6 expression has been described in the RV of rats with monocrotaline-induce PAH[80] and in mice subjected to PAB.[81]

The nuclear factor-κ B (NF-κB) superfamily of transcription factors has been associated with the regulation of a variety of physiological processes and plays a central role in the regulation of the inflammatory response.[71] Indeed, only a handful of studies have investigated the role of NF-κB in RV remodelling and dysfunction. In rats subjected to PAB, elevated expression of activated NF-κB was found in the RV.[75] Additionally, Nogueira-Ferreira et al. investigated the activation of the non-canonical NF-κB pathway and observed the upregulation of NF-κB's subunits p100/p52 and Rel-B in the RV of rats with

monocrotaline-induced PAH.[82] Remarkably, the NK-κB inhibitor pyrrolidine dithiocarbamate ameliorated PAB-induced RV inflammation and fibrosis, and improved RV function.[74]

Nowadays, the ongoing question is whether inflammation is the cause of pulmonary vascular disease or whether it is the consequence of a high-pressure pulmonary microenvironment. In this perspective, the increasing knowledge of the immune pathways associated with PAH supports the usefulness of tailored immunotherapy for the management of this disease.[83] Two clinical trials are addressing immuno-modulators and their potential impact on different forms of PH. Rituximab is a monoclonal antibody against the B cell antigen CD20, which is already used in rheumatology and oncology.[84] Tociizumab is a humanized monoclonal IL-6 receptor antibody, which has been approved for the treatment of rheumatoid diseases.[85] However, the exact role of these inflammatory mediators in the set of PAH is not yet well established. A better understanding of the role of inflammation in this pathology will help developing novel anti-inflammatory therapies.

2.3. Remodelling of the extracellular matrix

Cardiac fibroblasts play an important role as sensors and amplifiers of signals from immune cells and cardiomyocytes by secreting cytokines, growth factors and chemokines.[86] Both IL-1 and TNF-α can increase fibroblast migration and induce fibroblast production of other pro-inflammatory cytokines, such as IL-6 and chemokines, such as CXCL-1, -2, -5 and -8.[87,88] Additionally, IL-1 can activate the expression of tumour growth factor beta (TGF-β), which is the major profibrotic cytokine that induces differentiation of fibroblasts into myofibroblasts. Importantly, macrophages also play an important role in the differentiation of fibroblasts into myofibroblast via expression of TGF-β.[89] In this way, cardiac fibroblasts are the main effector cells in cardiac fibrosis. The presence of fibrosis in pressure-overloaded RV is associated with the activation and expansion of fibroblasts, which acquire a myofibroblast phenotype and secrete a large amount of ECM proteins, such as collagens and matricellular proteins.[90] Direct stimulation of resident cardiac fibroblasts through the activation of neurohormonal or growth factor-mediated pathways may also contribute to the fibrotic response.[91] Histological studies have revealed increased collagen deposition in the RV from PAH patients, which might play a central role in the development of RV

dysfunction and failure in this pathology.[92] In experimental models of RV pressure overload, fibrotic remodelling of the RV is often associated with decompensation, and is usually evidence of severe diastolic dysfunction.[56]

Extracellular matrix (ECM) is regulated by proteolytic enzymes, such as metalloproteinases (MMPs), and their endogenous inhibitors, the tissue inhibitors of MMPs (TIMPs).[93] As aforementioned, during cardiac stress induced by PAH, the cardiac fibroblast undergoes a transition to activated myofibroblast, that is responsible for fibrosis and the secretion of MMPs and other ECM enzymes, which contribute to adverse ventricular remodelling.[94,95] An imbalance of MMPs and TIMPs, leading to the accumulation of ECM in the heart has been observed in several diseases, such as PAH, lung fibrosis and myocardial infarction. Elevated levels of serum MMP-2, MMP-9; TIMP-1 and TIMP-4 [95,96] and urinary MMP-2 and MMP-9 have been reported in patients with PH.[97] In the rodent monocrotaline-induced PAH model, elevated MMP-2, MMP-9 and TIMP-1 levels were observed in lung tissue[98,99] as well as in the RV.[92] Myocardial expression of specific MMPs, like gelatinase MMP-9 and stromelysin MMP-3, was shown to be increased in both human and experimental dilated cardiomyopathy.[100] MMPs can be induced by several molecules/pathways, such as the renin-angiotensin system (RAAS), bioactive peptides, and cytokines, such as TNF-α.[101,102] In the RAAS, angiotensin II plays a central role in LV remodelling, which is supported by evidences of a great reduction in myocardial collagen deposition in patients treated with the angiotensin receptor blocker losartan.[103] Whether the same response would be seen in PAH patients with RV hypertrophy is unknown.

2.4. Cardiac vascularity

Myocardial perfusion is a key requisite for myocardial homeostasis. Pathophysiological stresses like pressure overload enhance oxygen demand. As such, the inability to induce enough neovascularization results in deficient oxygen supply and, subsequently, in atrophy and loss and/or degeneration of cardiomyocytes, which could represent the main cause of the myocardial dysfunction present in PAH.[104,105] There is ample evidence that some forms of pathological cardiac hypertrophy, in particular those produced by pressure overload are associated with a decrease in capillary density, which is more notorious in the subendocardial layers of the hypertrophied ventricle.[31,51,106,107]

2.5. Cellular and molecular modifications in the cardiomyocyte

There are several possible contributing factors explaining RV diastolic stiffness in PAH. The above referred hypertrophy and fibrosis (**Figure 2**) are known to increase ventricular stiffness; however, this could also be caused by modifications in the contractile apparatus of cardiomyocytes.[56] The contractile function of cardiomyocytes is directly associated with Ca^{2+} ion homeostasis, which is involved both in the excitability of cardiomyocytes and in the activation of contractile filaments, promoting cellular contraction. As for the expression of Ca^{2+} regulatory proteins in the RV, PAH is known to decrease the expression of ryanodine receptors (RyRs) and sarco(endo)plasmic reticulum calcium ATPase 2a (SERCA2a).[62,64] Increased afterload of the RV induces a switch in myosin heavy chain (MHC) isoform composition from the fast α-MHC to the slower, but energetically favourable β-MHC.[108]

PAH imposes an adverse remodelling to the RV (**Figure 2**), which includes hypertrophy, associated with extended interstitial fibrosis, in response to pulmonary artery thickening and increased afterload. Myocyte hypertrophy results from increased protein synthesis without cell replication. It can be stimulated by stretch, noradrenaline, ET, angiotensin and inflammatory cytokines, such as IL-1β, all of which are thought to be increased in patients with PAH.[103] Angiotensin, ET-1 and catecholamines, such as noradrenaline, bind to specific seven-transmembrane-spanning receptors that are coupled to heterotrimeric G_q proteins. These G_q proteins may be coupled to phospholipase Cβ (PCβ). This coupling is responsible for the generation of diacylglycerol (DAG), which acts as an intracellular ligand for protein kinase C (PKC), and for the formation of inositol-1,4,5-triphosphate (IP3). The accumulation of this second messenger leads to the mobilization of internal Ca^{2+} by directly binding to the IP3 receptor located in the endoplasmic reticulum. Moreover, the generation of IP3 and subsequent mobilization of internal Ca^{2+} stores has been shown to mediate hypertrophic signalling.[109,110] The MAPK signalling cascade is initiated in cardiac myocytes by receptors tyrosine kinase (fibroblast growth factor receptors) and stress stimuli, like stretch. The activation of this pathway results in the phosphorylation and consequent activation of p38, c-Jun N-terminal kinases (JNKs) and ERKs.[111] In addition to the signalling events that are initiated by ligand-receptor interactions, cardiac myocytes directly detect mechanical deformation or stretch through an internal sensory apparatus. This

apparatus involves integrins, which are heterodimeric transmembrane receptors, consisting of α- and β-subunits, that link the extracellular matrix to the intracellular cytoskeleton.[112] Thus, hypertrophy of the RV is an initial and adaptative response to increased overload.

The adult healthy heart is an organ with high energy utilization rates, which is derived from various oxidizable energy sources, such as fatty acids, glucose, lactate, ketones and amino acids. In the adult heart, fatty acid oxidation (FAO) is the predominant energy source, but glucose metabolism contributes to adenosine triphosphate (ATP) production, albeit in a lower percentage.[54,113] Glucose metabolism initiates with cytosolic glycolysis, which ultimately converts glucose to pyruvate. Pyruvate is transferred to the mitochondria where it serves as subtract for pyruvate dehydrogenase (PDH). If this enzyme is activated, pyruvate is converted to acetyl-CoA, powering the Krebs cycle and providing reducing equivalents, like NADH and $FADH_2$, to the oxidative phosphorylation (OXPHOS) system for ATP generation.[114] In the hypertrophied and failing heart (**Figure 2**), as previously mentioned, the increased systolic wall stress in the ventricular wall will lead to an increase in the energy demand, whereas the increased size of the cardiomyocytes and the decreased capillary density is anticipated to diminish the ratio oxygen supply/demand.[115] Therefore, an imbalance between oxygen/energy supply and demand possibly contributes to the transition to the decompensated, failing heart. In PAH, the RV undergoes several metabolic changes, including an increased reliance on glycolysis, not coupled with total glucose oxidation, which yields lactate.[116] The lactate produced promotes acidosis, leading to a reduction of contractility determined by the fall of intracellular pH, which affects the organelles' activity within the cardiac cell, impairing RV function.[117]

Recently, data from human RV tissue demonstrated an increase in lipid accumulation and decrease of FAO in PAH. As a result of lipid accumulation, lipotoxic metabolites and RONS accumulate and initiate apoptosis in the surrounding tissues.[118] The PAH-related elevated levels of ET-1 (**Figure 2**) are responsible for the stimulation of the classical signal transduction of G_q protein which leads to cardiomyocyte apoptosis, facilitating the opening of the mitochondrial membrane transition pores is promoted by Bax activation.[60]Previously, the increased expression of caspase 3[119] and Bax was observed in the RV of MCT-treated rats, while the expression of Bcl-2 was decreased. *Li et al*. reported downregulated levels of Bcl-2 protein, while Bax and activated caspase 3 were significantly upregulated in the RV of

MCT-induced rats.[120,121] Moreover, the cellular stress induced by RONS can start the activation of mitochondrial proteins that interact with domains of the Bax and Bcl-2 proteins. Hence, that interaction could open the transition pores in the outer mitochondrial membrane, helping the relocation of cytochrome c, that is released by the mitochondria. The release of cytochrome c is essential to the formation of apoptosomes and activation of pro-caspase 3, a protein directly involved in cellular apoptosis.[60,122]

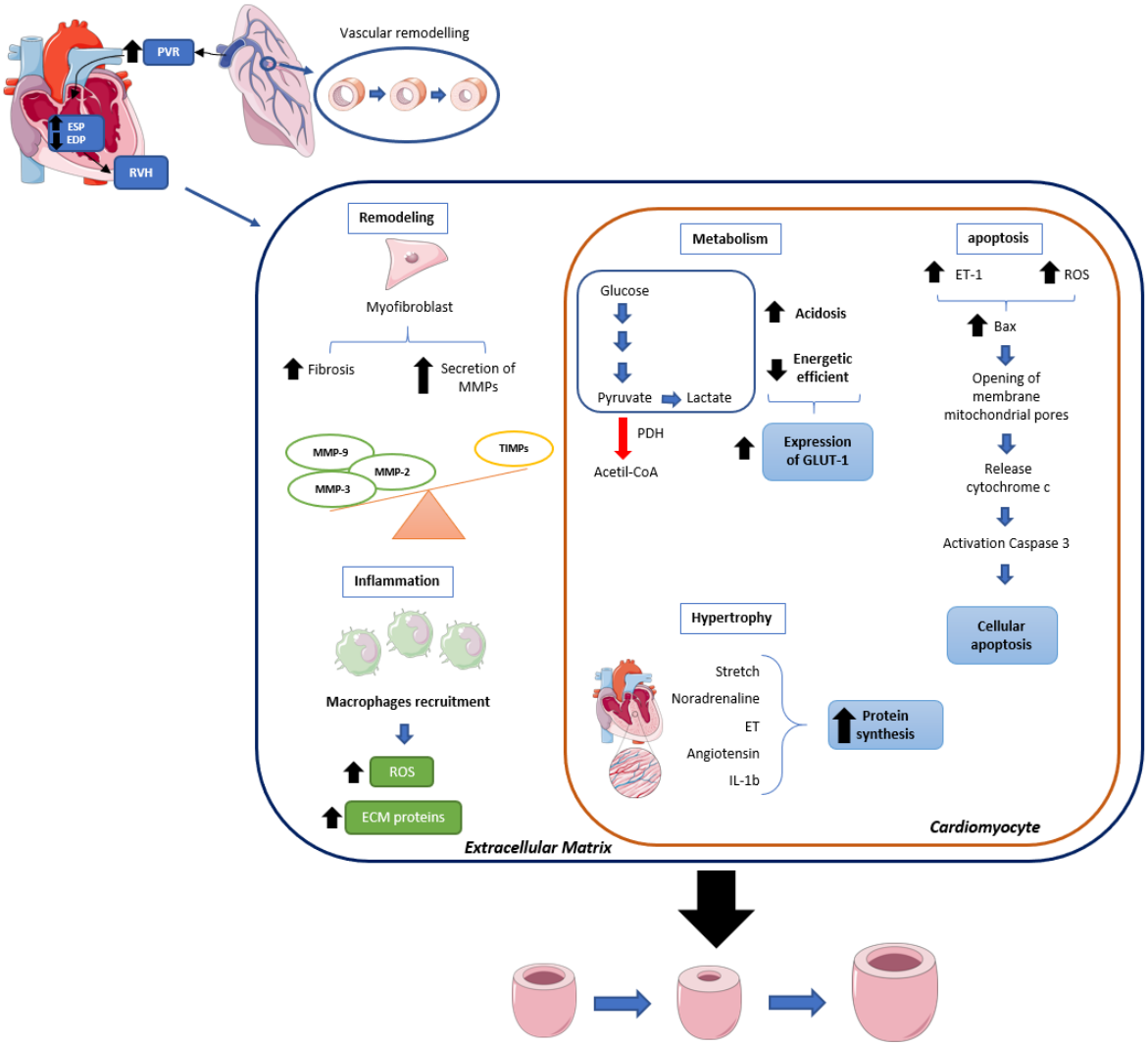

Figure 2 – **Cellular and molecular alterations promoted by PAH in the cardiomyocyte.** Activation of myofibroblast leads to an increase in the levels of MMPs promoting an imbalance between MMPs and TIMPs which leads to an increase ECM remodelling. The increased protein synthesis stimulated by stretch, noradrenaline, ET, angiotensin and interleukin 1b results in hypertrophy of right ventricle. In the cardiomyocyte, a metabolic switch is present, with an increased reliance on glycolysis, not coupled with total glucose oxidation, which yields lactate. Elevated levels of ET and ROS are responsible for the development of apoptosis events. Figure made using Servier Medical Art.by Servier (https://smart.servier.com/) and modified by the author under the Creative Commons Attribution 3.0 Unported License (CCBY 3.0) Abbreviations: ECM: extracellular matrix; EDP, end-diastolic pressure; ESP, end-systolic pressure; ET: endothelin; MMPs: metalloproteinases; ROS: reactive oxygen species; TIMPs: tissue inhibitors of MMPs

3. The protective effect of exercise in pulmonary arterial hypertension

Exercise training in PH was initially contraindicated due to safety concerns. However, an increasing number of studies have pointed out its benefits in exercise capacity, peak oxygen consumption and quality of life of patients with PH[123,124], leading the European Society of Cardiology to adopt guidelines in 2016 that recommend considering supervised exercise training in patients with PAH that are clinically stable and exhibit an optimal pharmacological treatment. Nevertheless, there are some conditions to the training that are still very much unknown, such as the optimal duration, intensity, frequency and type of exercise.[1] The overall positive results observed in human studies have translated into the improvement of global cardiac function, arterial and myocardial elasticity, muscle metabolism in opposition to the oxidative stress, and inflammation process and adverse cardiopulmonary remodelling present in PAH.[62,125,126] Considering the aforementioned benefits of exercise training, it is important to understand the mechanisms underlying those changes. It has been proposed that both central (pulmonary and cardiac) and peripheral (skeletal muscle) adaptations could contribute to the exercise-induced positive outcomes.[62] Given that cardiac function is the main prognostic outcome in PAH patients[127], we will review the main cardiac alterations promoted by exercise.

3.1. Functional and structural modifications

Regarding the chronic effects of exercise on the cardiac hemodynamic of preclinical models of PAH, different benefits, such as normalization of the pulmonary artery flow velocity[64], maintenance of RV systolic pressure[63] and attenuation of the RV systolic and diastolic dysfunction were observed.[65] Furthermore, evidences of the reestablishment of the resting heart rate, reduction of changes in the myocardial acceleration during isovolumetric contraction and relaxation time, as well as inhibition of changes in the maximum systolic velocity of the tricuspid annular plane were detected.[62] Additionally, when performed prior to PAH induction, exercise training normalized end-diastolic pressure and diastolic function.[58] Exercise preconditioning was able to prevent RV diastolic dysfunction, which was the main alteration observed in the cardiac function of sedentary[128] Monocrotaline (MCT)-treated animals. Heart rate (HR) was found to be impaired in these animals, having

been previously related with reduced responsiveness to sympathetic stimulation. Exercised MCT animals also showed decreased HR but this was probably a training effect since no differences were noted in comparison to its corresponding control group. Part of this cardioprotective effect can be attributed to the prevention of RV overload, which is supported by the fact that exercised MCT animals showed a reduction of RV Pmax and a significant reduction in the pulmonary artery remodelling.[58]

Structurally, a reduction in pulmonary artery tunica media thickness is present in MCT trained models. This change in exercised models could contribute to an attenuation of pulmonary vascular resistance and may lead to a reduction in RV afterload. Exercise training is responsible for a decrease in interstitial volume and an increase in intramyocardial vessels' volume when compared with sedentary MCT models.[57,63] These adaptive structural changes in the pulmonary artery and the RV in response to exercise may have contributed to a reduced right ventricular end-diastolic pressure (RVEDP)[61], reduced right ventricle hypertrophy[58–60,62,64,125,129], and reduced pulmonary artery thickness[63] in the MCT trained models. In contrast, exercise training did not modify cardiac output[57], or right ventricle systolic pressure (RVSP).[63,65]However, some data have suggested the heart hypertrophy found in MCT trained models could be induced by MCT and not by exercise.[63]

3.2. Cellular and molecular modifications in cardiac remodelling

Exercise preconditioning provides protection against RV dysfunction, remodelling, muscle wasting, hypertrophy and fibrosis in MCT models of PAH (**Figure 3**). The exercise-derived benefits to cardiac microcirculation might be present in MCT models submitted to exercise since there is a direct link between angiogenesis, cardiac hypertrophy and cardiac function.[128] For instance, insufficient cardiac microvascular growth has recently been identified as an important underlying mechanism in the transition from compensatory hypertrophy to heart failure.[106]

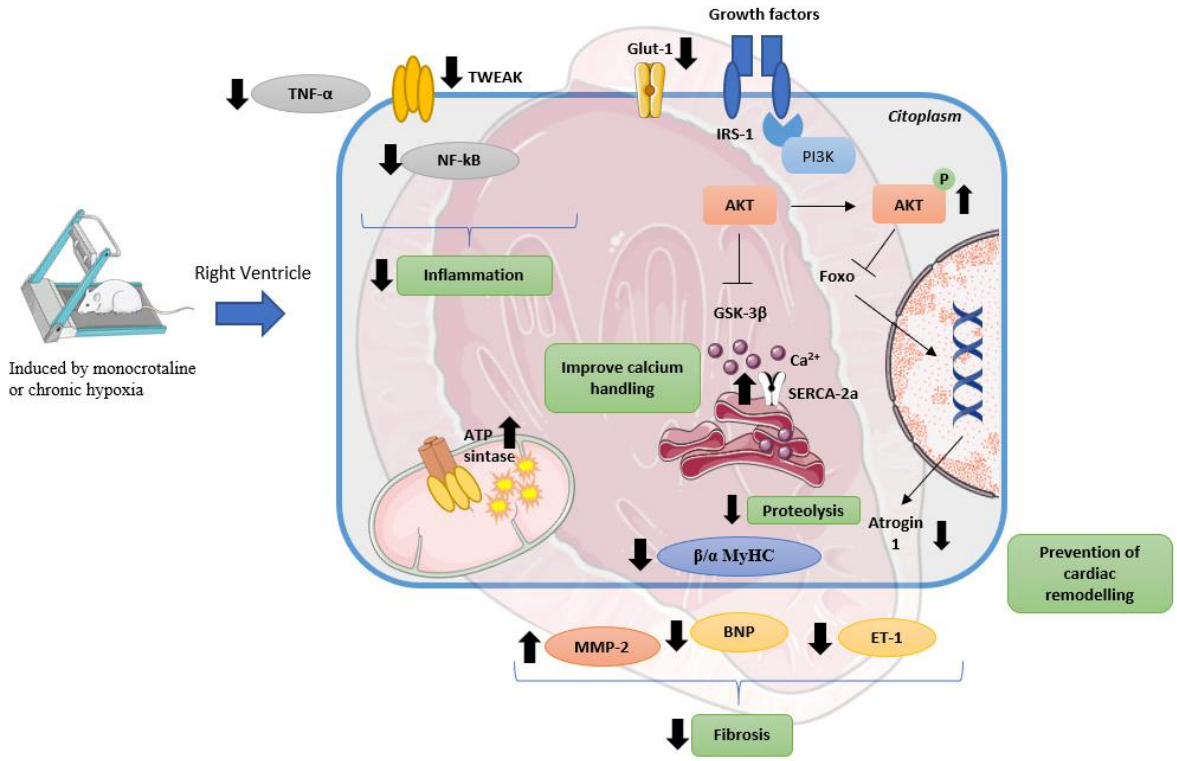

Figure 3 – **Mechanisms underlying the impact of exercise training in MCT/hypoxia models**. Figure made using Servier Medical Art.by Servier (https://smart.servier.com/) and modified by the author under the Creative Commons Attribution 3.0 Unported License (CCBY 3.0). Abbreviations: ATP: Adenosine Triphosphate; BNP: Brain Natriuretic Peptide; ET-1: Endothelin 1; GSK; Glycogen Synthase Kinase; IRS: Insulin Receptor Substrate; MHC: Myosin Heavy Chain; PI3K: Phosphoinositide 3-kinase; SERCA-2a: Sarco(endo)plasmatic reticulum calcium ATPase; TNF-α: Tumor necrosis factor α; TWEAK: TNF-related weak inducer of apoptosis. Figure was made using Servier Medical Art.

Few studies have addressed the impact of exercise training on the metabolic modifications promoted by PAH in the heart. Brown and co-workers investigated the changes in substrate utilization induced by exercise in myocardium in order to evaluate the metabolic shift. Data from immunofluorescence staining of RV for glucose transporter (GLUT-1) assessment evidenced a higher content of this glucose transporter in untreated MCT rats when compared with untreated healthy animals, suggesting a shift toward glycolytic metabolism in cardiac muscle. In their study, both high intensity interval trained and continuous exercise trained animals showed evidence of reduced levels of GLUT-1 in the RV (**Figure 3**) when compared with the MCT sedentary animals.[130] Consistent with these findings, is the fact that in both cardiac and skeletal muscle, mitochondrial substrate utilization from aerobic to anaerobic metabolism contributes to diminished exercise tolerance. Therefore, the impact of training on indicators of glycolytic *versus* oxidative metabolism is particularly relevant in

PAH, since this pathology has been associated with an inefficient metabolic shift, as previously mentioned in this review.[131,132]

Exercise training modulates the glycogen synthase kinase (GSK) signalling pathway (**Figure 3**) in the RV. In exercise trained MCT rats, a decrease in the ratio between the phosphorylated (inactive protein with pro-hypertrophic properties) and dephosphorylated (active protein with anti-hypertrophic properties) forms of the protein GSK-3β is observed. GSK-3β is implicated in cardiac growth and regulates the calcineurin/nuclear factor of activated T cells (NFAT) signalling pathway through phosphorylation of NFAT, opposing the role of the phosphatase calcineurin. NFAT phosphorylation prevents its translocation to the nucleus where it activates the transcription of hypertrophic response genes.[128]

The inflammatory response in the RV of MCT models (**Figure 3**) is also changed. Animals submitted to four weeks of moderate-intensity exercise training showed a reduction in the expression of tumour necrosis factor-related weak inducer of apoptosis (TWEAK) receptors, compared to the control group.[82] TWEAK is a member of the TNF receptor superfamily, a cell surface-associated transmembrane protein linked to the stimulation of cell growth, induction of inflammatory cytokines and stimulation of apoptosis.[128,133] This cytokine acts on the RV through the activation of NF-κB signalling.[89] However, exercise prevented the PAH-related overexpression of p100/p52 and Rel B subunits from this pathway, and diminished the expression of TWEAK receptors in the heart of an MCT rat model.[58] The anti-inflammatory effect of exercise training was also related with increased NO bioavailability[130], and induced a shift of MMPs activity from MMP-9 to MMP-2 activity promoting a decrease in deposition of collagen in the heart.[128]

The effect of moderate-intensity exercise training prior to and following the development of PAH on the MHC isoform profile of the RV has also been evaluated in two preclinical models (**Figure 3**). Exercise was shown to significantly prevent increases in total MHC content and β/α MHC ratio in sedentary PAH animals.[58,62] The PAH-related increase in β/α MHC ratio lead to reduced activity of the myosin ATPase enzyme, and consequently to slowing of myocyte contraction.[62] The switch from alpha to beta-MHC is widely used as an indicator of maladaptive cardiac remodelling. These benefits of exercise training in RV remodelling were also associated with increased SERCA2a content.[58,62]

Modifications in the ubiquitin/proteasome system are crucial for cardiac remodelling in response to pathological stresses. For instance, the upregulation of muscle RING-finger protein-1 (MuRF1), atrogin-1 and TRAF6 E3 ligases have been shown to promote cardiac dysfunction.[128] The E3 ligase atrogin-1 is also elevated in the RV of rats with PAH.[58] The transcription of atrogin-1 can be directly induced by FoxO3 through the IG-1/PI3K/Akt signalling axis and its inhibition has been related with prevention of pathological cardiac remodelling.[134,135] Concerning the effect of exercise training, four weeks of moderate-intensity treadmill running prior to PAH induction prevented the increase in atrogin-1 expression in the heart of animals with PAH.[58]

Finally, exercise training promotes an improvement in the aerobic capacity of the heart, which is directly associated with the cellular adaptations, namely mitochondrial biogenesis, improved efficiency of OXPHOS, reduction in the transition pore permeability of mitochondria and increased antioxidant activity.[136] Moreover, as previously mentioned, it is well known that apoptosis is an event associated with various features of cardiac pathology. The expression of proteins involved in apoptosis in the RV is influenced by aerobic exercise.[133] For example, five weeks of treadmill aerobic exercise was able to decrease caspase 3 expression in MCT-treated rats when compared with sedentary MCT rats. Since caspase 3 mediates mitochondrial permeability, its downregulation will lead to a reduction in permeability, and thus a reduction in apoptotic signalling mediated by Akt.[60] These findings suggest exercise plays an antiapoptotic role in this model of PAH.

4. Final remarks

Despite major discoveries have been made these past years concerning the mechanisms underlying the development of PAH, mortality rates remain high and available treatments mainly focus the alterations presents in the lung, excluding the heart as possible therapeutic target.[1] The awareness of the importance of the RV in PAH has increased considerably. Nowadays, there is a clear view of the RV and the pulmonary circulation as a functional unit. In this perspective, it is important to understand the cooperation between this functional unit. Currently, several studies already have characterized the cellular and molecular alterations in the RV of MCT models, allowing the identification of potential new therapeutic approaches targeting the heart.[52,70,95,137] Exercise training is widely known for its

preventive and therapeutic properties in several chronic diseases.[138] In patients with PAH, exercise training improves exercise tolerance, functional capacity and quality of life.[123,126,139] However, there is still a glaring lack of information regarding the direct effect of exercise training in PAH, specifically in the mechanism underlying such changes, and several potentially difficult crossroads continue to be the target of studies. Is it better to use exercise training as a preventive or therapeutic approach? Which type of exercise produces more benefit? What duration is recommended? In this way, a more comprehensive set of information could be developed about the effects of exercise training in PAH.

From this point of view, the work that we are current developing aims at understanding the impact of treadmill exercise training in the modulation of the molecular pathways underlying right ventricle dysfunction in pulmonary arterial hypertension, in a monocrotaline rat model, specifically focusing on cardiac regeneration (C-Kit, neuregulin-1), extracellular matrix remodelling (fibrosis, collagen; MMPs/TIMPs activity) and metabolism (GLUT-1 expression, glyceraldehyde 3 phosphate dehydrogenase expression, citrate synthase activity; ATP synthase expression; PGC-1α).

5. References

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Suplemmentary Table 1 - Animal models, drug dosage, exercise protocol and main physiological effects and molecular modifications in heart and lungs

Study	Animal strain and BW	Drug dosage	Exercise protocols	Physiological modifications	Molecular modification
Souza-Rabbo et al., 2008[125]	Male Wistar rats (160-180g)	Monocrotaline (60 mg/Kg BW)	Treadmill running (50 min, 5 d/wk, 5wk) at 0.6-0.9 km/h	↓ RVH ↑ Survival	↑ Erythrocyte superoxide dismutase and glutathione S-transferase activities ↓ Erythrocyte lipid peroxidation and catalase activity ↔ Erythrocyte glutathione peroxidase activity
Handoko et al., 2009[57]	Male Wistar rats (150-175g)	Monocrotaline (60 mg/Kg BW)	Treadmill running (30 min, 5 d/wk, 4wk) at 50% VO_2 max	↔ CO ↑ RV capilarization ↓ Survival in progressive PAH ↑ Exercise capacity in stable PAH	↓ Collagen deposition
Colombo et al., 2013[63]	Male Wistar rats (139±15g)	Monocrotaline (60 mg/Kg BW)	Treadmill running (60 min, 5 d/wk, 3wk) at 60% VO_2 max	↓ PA thickness ↓ RV interstitial volume ↑ Myocardial capillarity ↓ EDP	↓ *p*-GSK-3β/GSK-3β protein expression in RV
Colombo et al., 2015[60]	Male Wistar rats (146±16g)	Monocrotaline (60 mg/kg BW)	Treadmill running (60 min, 5 d/wk, 3wk) at 60% VO_2 max	↔ AT/ET ↓ RVH	↓ Hydrogen peroxide concentration in RV ↑ RV *p*-Akt ↓ Caspase 3 protein expression ↔ RV PI3K, Akt and Bax/Bcl-2 protein expression
Brown et al., 2015[61]	Male Sprague-Dawley rats (300g)	Monocrotaline (50 mg/kg BW)	Treadmill running (45 min at 75% reserve VO_2)	↓ RVSP	↓ eNOS protein expression and activity
Hargett et al., 2015[139]	Male Sprague-Dawley rats (160-200g)	Sugen 5416 (20 mg/kg BW)	Treadmill running (3 sessions)	↔ Exercise capacity	
Natali et al., 2015[129]	Male Wistar rats (200g)	Monocrotaline (60mg/kg BW)	Voluntary running (3 to 4 weeks)	↓ RVH ↑Survival	↑ myocyte contractility ↓ cardiac remodelling

Moreira-Gonçalves et al., 2015[62]	Male Wistar rats (150 g)	Monocrotaline (60mg/kg BW)	Treadmill running (60 min, 5 d/wk, 2 or 4wk) at 30 m/min	↓ RVH Restoring RHR Restore EDP and TAU Restore MAICR ↑ TAPSE ↑ Survival	↓ BNP mRNA expression ↑ SERCA 2a protein expression ↑ VEGF mRNA expression ↑ Mitochondrial complex V activity ↓Collagen ↓ β/α MyHC protein expression ↓ ET-1 mRNA expression ↓ serum levels of lactate ↓ mitochondria complex V nitration ↔ Atrogin-1 ↔ RV ATP synthase subunit β protein expression ↓ TNF-α/IL-10
Nogueira-Ferreira et al., 2016[58]	Male Wistar rats (150 g)	Monocrotaline (60mg/kg BW)	Treadmill running (60 min, 5 d/wk, 4wk) at 25 m/min	Prevent BW decrease ↓ RVH Restored EDP and TAU ↓ PA hypertrophy	↓ β/αMHC ↓ Inflammation ↓ Fibrosis ↔ Atrogin-1
Colombo et al., 2016[65]	Male Wistar (315 ± 34g)	Monocrotaline (60mg/kg BW)	Treadmill running (60 min, 5 d/wk, 2 or 4 wk) at 60% VO_2 max	↔ RV SF ↔ RV DF ↑ FAC ↑ RV FS	↑ lung hydrogen peroxide levels ↑ VEGF ↑ PTEN ↑ *p*-Akt protein expression ↔ lung lipid peroxidation ↔ superoxide dismutase ↔ catalase activity ↔ glutathione peroxidase activity ↔ peroxiredoxin ↔ PI3K ↔ Akt protein expression
Pacagnelli et al., 2016[64]	Male Wistar (206 ± 16g)	Monocrotaline (60mg/kg BW)	Treadmill running (60 min, 5 d/wk, 11 wk) blood lactate < 1 mmol/L	Restore PA flow ↓ RVH	↑ SOD activity ↓ LPO
Enache et al., 2017[59]	Male Wistar (224 ± 8g)		Downhill (15°) Treadmill running (33 min, 5 d/wk, 4 wk) at 65% VO_2 max	↓ RVH ↔ Survival	

Brown et al., 2017[130]	Male Sprague-Dawley rats	Monocrotaline (40 mg/kg)	Treadmill running (5 d/wk, 6 wk) HIIT: 6 min warm up at 50% VO_2R + 5 min cycles of alternating high and low intensity intervals as follows: 2 min at 85–90% VO2R and 3 min at 30% VO_2R, totaling 30 min CExT: 60 min of uninterrupted steady state running at 50% VO_2R	↓ RV hypertrophy ↑ RV function	HIIT: ↑ lung eNOS protein expression HIIT and CExT: ↓ RV Glut-1 ↔ RV cytochrome IV protein expression
Zimmer et al., 2017[24]	Male Wistar rats	Monocrotaline (60 mg/kg)	Treadmill running (5 d/wk, 60 min/session during the 1st and 2nd week and 50 min/session during 3rd week) at 60% VO_2 max		↑ lung superoxide anion concentration ↔ lung NO_2^- concentration ↔ NOS activity ↔ eNOS protein expression ↔ ET-1 receptor type A and B protein expression

[a]↑ increase; ↔ maintenance; ↓ decrease

[b] AT/ET, acceleration time to ejection time ratio; BNP, brain natriuretic peptide; BW, body weight; CExT, Continuous exercise training; CO, cardiac output; DF, diastolic function; EDP, end-diastolic function; eNOS, endothelial nitric oxide synthase; ET-1, Endothelin 1; FAC, fractional area change; FS, fractional shortening; GSK, Glycogen synthase kinase; HIIT, High-intensity interval training; IL-10, interleukin 10; LPO, lipid peroxidation; MHC, myosin heavy chain; MAICR, myocardial acceleration in isovolumetric contraction and relaxation; NO^{2-}, nitrogen dioxide; NOS, nitric oxide synthase; PA, pulmonary artery; PAH, pulmonary arterial hypertension; RHR, resting heart rate; RV, right ventricle; RVH, right ventricle hypertrophy; RVFS, right ventricle fractional shortening; RVSP, right ventricle systolic pressure; SERCA2a, Sarco(endo)plasmatic reticulum calcium ATPase; SOD, superoxide dismutase; SP, systolic pressure; SF, systolic function; TAPSE, Tricuspid annular plane systolic excursion; TAU, time constant of isovolumetric relaxation; TNF-α, tumour necrosis factor alpha; PVR, peripheral vascular resistance; VEGF, vascular endothelial growth factor.

CHAPTER III

Therapeutic effect of exercise training in the metabolic remodelling of right ventricle in an experimental animal model of pulmonary arterial hypertension

1. Introduction

Pulmonary arterial hypertension (PAH) is a devastating disease characterized by progressive obliteration of the pulmonary vasculature, right heart failure and premature death.[1] Despite the central role displayed by right ventricular (RV) function in terms of patients prognosis,[2,3] little is known about the pathophysiology of RV failure.[4,5] It has been suggested that metabolic remodelling in the RV plays a central role in the progression to RV failure.[6] Whereas in the adult healthy heart fatty acid oxidation (FAO) is the predominant energy source[7,8], the severely hypertrophic RV of PAH patients relies on glucose oxidation to support contraction .[9,10] Indeed, the shift in substrate utilization from FAO to glycolysis has been shown in both PAH animal models and patients, which was paralleled with RV dysfunction.[11] The right ventricle undergoes pathological changes in order to guarantee homeostasis of the heart-lung axis. In the hypertrophied and failing heart the increased systolic wall stress in the ventricular wall will lead to an increase in the energy demand, whereas the increased size of the cardiomyocytes and the decreased capillary density is anticipated to diminish the ratio oxygen supply/demand, thus contributing to insufficient oxygen delivery to the RV and promoting ischemia.[12] Thus, the RV is obligated to switch to a more efficient source of ATP, glycolysis.[13] Glycolysis is a common response to ischemia, which when sufficiently severe, impairs energetics and reduces RV function, creating a vicious cycle.[14] The lower formation of ATP due to the switch from FAO to glycolysis results in a lower energy supply which may originate functional impairment to the overloaded heart.[15] For the heart to work efficiently, ATPases like sarco/endoplasmatic reticulum Ca^{2+} ATPase (SERCA) and myosin ATPase require a high free energy of ATP hydrolysis. Those mechanisms are guaranteed by cytosolic creatine kinase (CK) which reversibly catalyses the transfer of high-energy phosphate from phosphocreatine (PCr) to adenosine diphosphate (ADP) producing ATP and creatine (Cr).[16] In PAH, a reduction in expression of CK, low levels of ATP and PCr are associated with worse outcomes.[17]

Exercise training promotes a wide range of health benefits such as improving the function and health of cardiovascular tissues, as well as the greater tolerance of the heart to injury.[18] In the setting of PAH, exercise training is now considered to be safe in stable patients and it was shown to effectively improve exercise tolerance, pulmonary arterial pressure and quality of life.[19] Moreover, pre-clinical data supports that the benefits provided by exercise training could be due to its effects on the modulation of several PAH-related changes at the level of the RV, lungs and skeletal muscle.[20,21] Specifically regarding the RV,

our group showed that exercise preconditioning prevented cardiac hypertrophy and RV diastolic dysfunction. At a molecular level, exercise modulated the TWEAK/NF-κB signalling axis and prevented the shift in MHC isoforms towards an increased expression of beta-MHC. Exercise preconditioning also prevented the increase of atrogin-1 expression, and induced a shift of MMP activity from MMP-9 to MMP-2 activity.[22] In addition, we also showed that early and late aerobic exercise training were capable to improve cardiac function despite persistent right pressure-overload, and to increase exercise tolerance and survival, with greater benefits when exercise was started earlier in the course of PAH. This was accompanied by improvements in the markers of cardiac remodelling (SERCA2a), neurohumoral activation (lower endothelin-1, brain natriuretic peptide and preserved vascular endothelial growth factor mRNA), and mitochondrial oxidative capacity.[23]

Despite this knowledge, little is known about the impact of exercise on RV metabolic changes occurring in the setting of PAH. During exercise, the heart has a greater energy demand, which translates into a greater continuous generation of ATP in order to sustain the contractile function.[13] Most of the ATP used by cardiomyocyte is originated in the oxidative phosphorylation (OXPHOS) system, harboured in mitochondria. This system is manly fuelled by fatty acid oxidation, also located at this organelle. Therefore, it is not surprising that the density of mitochondria in cardiomyocytes is highly correlated with the workload of the heart.[15] However, the metabolic adaptations promoted by exercise training in the RV of PAH subjects is poorly comprehended.

In order to add new insights on the impact of exercise training on PAH-induced cardiac remodelling, we explored the therapeutic effect of two weeks of treadmill exercise in the metabolic adaptation of RV using an animal model of chemically induced PAH.

2. Material and Methods

2.1. Animals

Male Wistar rats with 4 weeks of age were obtained from Charles River Laboratories (Barcelona). After arrival, animals were housed in groups of five rats *per* cage, in a controlled environment at a room temperature of 22ºC, with inverted 12:12 hours light-dark cycle, in order to match animals handling and training with their most active period and have free access to food and water. The experimental protocol was performed 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).

Animals were divided into sedentary injected with monocrotaline (MCT) or vehicle (MCT+SED, n=25 and V+SED, n=10, respectively) and 2-weeks exercise training 2 weeks after being injected with MCT (MCT+EX, n=15). The number of animals per group was determined based on the expected mortality and on the analysis (hemodynamic, histology and biochemistry) predicted. MCT animals received a subcutaneous injection of monocrotaline (MCT; 60 mg/kg, Sigma, Barcelona, Spain) or equal volume of vehicle (1mL/kg of NaCl 0.9%) at 8-weeks of age. Regarding the exercise training protocol, after 1 week of habituation, animals were exercised for 60 min/session with a running speed of 25 m/min, no grade, 5 days/week. The habituation period consisted of a first interaction of the animal with the treadmill in which a controlled increase of time and intensity was applied until the values of the defined protocol were reached. At day 28-29 after MCT or vehicle administration, animals were prepared for hemodynamic evaluation with pressure-volume catheters. At the end of the experiments, samples from right ventricle were collected and stored accordingly biochemical analysis. **Figure 1** summarizes the experimental protocol that was followed.

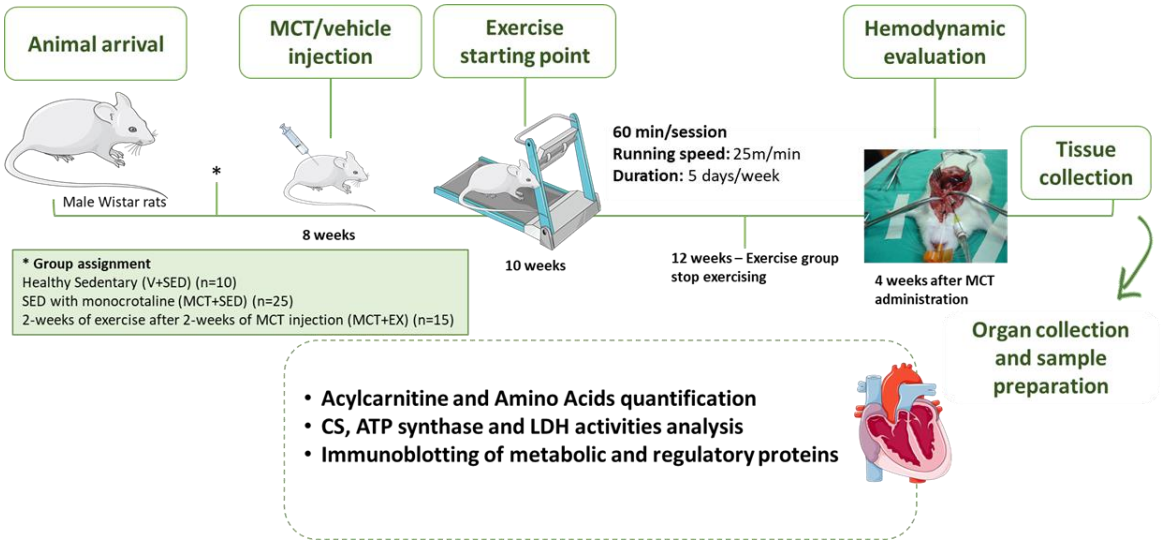

Figure 1. Overview of the experimental protocol followed in this study. After arrival, animals spent one week in habituation protocol to the treadmill, after which they were randomly divided into three experimental groups: healthy sedentary (V+SED, n=10), sedentary injected with monocrotaline (MCT+SED, n=25) and two weeks of exercise training two weeks after monocrotaline injection (MCT+EX, n=15). Animals from MCT+EX group started the exercise-training programme with 8 weeks of age. It consisted of treadmill running 60 min/day, 25 m/min, 5 days/week for 2 weeks. Twenty-eight days after monocrotaline injection hemodynamic evaluation was performed and samples were collected.

2.2. Hemodynamic evaluation

Twenty-four hours after ending their respective protocols, rats were anaesthetised by inhalation with a mixture of 4% sevoflurane with oxygen, intubated for mechanical ventilation (respiratory frequency 100 min^{-1} and weight adjusted tidal volume; Harvard Small Animal Ventilator Model 683) and placed over a heating pad (37ºC). The right jugular vein was cannulated for fluid administration (prewarned 0.9% NaCl solution) to compensate for perioperative fluid losses. A median sternotomy was performed to expose the heart and the pericardium was widely opened. One 1.9 F microtip pressure-volume conductance catheters (FTS-1912B-8018, Scisense) was inserted by apical puncture on the RV cavity, along the ventricular long axis. The catheter was connected to MVP-300 conductance system through interface cable (PCU-2000 MPVS, FC-MR-4, Scisense), coupled to PowerLab 16/30 converter (AD Instruments) and personal computer for data acquisitions. After complete instrumentation, the animal preparation was allowed to stabilize for 15 min. Hemodynamic recordings were made with respiration suspended at the end of expiration under steady-state conditions or during preload reductions (inferior vena cava occlusion).

Parameters from conductance catheter were recorded at a sampling rate of 1000 Hz, in order to accurately capture all the features of the pressure-volume waveforms produced by the fast-beating rat hearts. Data were stored and analysed with Millar conductance data acquisition and analysis software (PVAN3.5).

2.3. Biochemical analysis of cardiac muscle

2.3.1. Cardiac muscle preparation

Portions of RV cardiac muscle of approximately 50 mg were homogenised in 1 mL of 100 mM phosphate buffer (50 mM KH_2PO_4, 50 mM $Na_2HPO_4.H_20$ pH 7.4, 0.1% Triton-X100, with 200 mM PMSF (1:1000) and phosphatase inhibitor cocktail (P0044 and P5726, Sigma 1:1000)). The protein content of the cardiac muscle homogenates was assayed with RC-DC™ Protein Assay (Bio-Rad, CA, USA), following the manufacturer's instructions and using BSA as standard. Absorbance was measured at 750 nm in a Multiskan GO microplate spectrophotometer (Thermo Scientific, MA, USA).

2.3.2. Citrate synthase, ATP synthase and lactate dehydrogenase activities

Citrate synthase (CS) activity was measured according to Coore *et al.* [25]. In brief, the CoASH released from the reaction of acetyl-CoA with oxaloacetate was measured by its reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) at 412 nm (molar extinction coefficient of 13.6 $mM^{-1}cm^{-1}$) in a Multiskan GO microplate spectrophotometer (Thermo Scientific).

ATP synthase activity was using a commercia kit (ab109714). The ATP synthase enzyme is immunocaptured within the wells of the microplate and the enzyme is measured by monitoring the decrease in absorbance at 340 nm. The conversion of ATP to ADP by ATP synthase is coupled to the oxidation reaction of NADH to NAD^+ by ATP synthase is coupled to the oxidation reaction of NADH to NAD+ with a reduction in absorbance at 340 nm.

Serum levels of lactate were measured using a commercial kit (Randox) used according to the manufacturer's instructions.

2.3.3. Acylcarnitine and amino acid quantitation

Acylcarnitine (AC) and amino acid (AA) quantification was performed using the mass spectrometry (MS)-based methodology described by Petucci et al.[26] In brief, 100 µL

of cardiac muscle homogenate of each sample were added to 360 µL of methanol, followed by vortexing and centrifugation at 14000 rpm (10ºC, 5 minutes). Afterwards, 140 µL of supernatant were collected to a 96-well plate and 100 µL of methanol containing deuterated acylcarnitine internal standard solutions (Cambridge Isotope labs, MA, USA) was added to each well. The mixture was subsequently dried using nitrogen at 45ºC and shortly thereafter derivatised to the corresponding methyl esters by incubation with 95 µL of 3N methanolic HCl at 50ºC for 15 minutes. The samples were then dried once again using nitrogen at 45ºC and reconstituted with 200 µL of 80% methanol for flow injection MS/MS in an API 4000 QTRAP (Sciex, Washington, D.C., USA).

2.3.4. Immunoblotting analysis

Equivalent amounts of protein of each RV homogenate were electrophoresed in 12.5% SDS-PAGE gels as described by Laemmli.[27] Gels were blotted onto a nitrocellulose membrane (Amersham™ Protan™, GE Healthcare Lifesciences) in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 2 hours at 200 mA. Non-specific binding was blocked for 1 hour in 5% (w/v) non-fat dry milk prepared in TBS-T (100 mM Tris, 1.5 mM NaCl, 0.5% Tween 20). Then, membranes were incubated with primary antibody [diluted 1:1000 in 5% (w/v) non-fat dry milk in TBS-T; mouse monoclonal anti-ATP synthase subunit β, ab14730, Abcam; rabbit polyclonal anti-GAPDH, ab9485, Abcam; mouse polyclonal anti- GLUT4, ab48547, Abcam; rabbit polyclonal anti-PGC-1α, ab191838, Abcam; rabbit polyclonal anti-ETFDH, ab91508, Abcam; rabbit polyclonal anti-PPARα, ab24509, Abcam; mouse ab9485 anti-PPARγ, ab41928, Abcam; Total OXPHOS Rodent WB antibody cocktail, ab110413] for 1h at room temperature or overnight at 4ºC. Thereafter, membranes were washed and incubated with secondary HRP-conjugated anti-mouse or anti-rabbit (GE Healthcare Life Sciences; diluted 1:1000 in 5% (w/v) non-fat dry milk in TBS-T) for 2 hours at room temperature. Immunoreactive bands were detected by enhanced chemiluminescence (WesternBright™ ECL, Advansta) according to the manufacturer's procedure. Images were recorded using a ChemiDoc™ Imaging System (Bio-Rad) and analysed with Image Lab (version 5.0, Bio-Rad). The optical densities obtained were expressed in arbitrary units. Protein loading was confirmed by Ponceau S staining.

2.4. Statistical analysis

Values are presented as mean ± standard deviation for all variables. The Kolmogorov-Smirnov test was performed to check the normality of the data. Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by the Tukey multiple comparisons post hoc test (parametric data). Results were considered significantly different when $p < 0.05$. GraphPad Prism (version 7.0) was the software used.

3. Results

3.1. Effect of MCT-induced PAH and exercise training on morphometric and morphological parameters

Significant body weight loss was observed in MCT+SED rats ($p < 0.05$ vs. V+SED) (**Table 1**). Exercise training did not counteract this decrease since no differences where noticed between the exercised and sedentary monocrotaline groups. Reduction of body weight was related to the loss of skeletal muscle mass once a significant decrease of *gastrocnemius* mass was observed in trained and sedentary MCT-treated rats ($p<0.05$ MCT+SED *vs* V+SED; $p<0.01$ MCT+EX vs. V+SED) (**Table 1**). Regarding heart mass, no significant differences were found among the different groups. However, when heart mass was adjusted to body weight, a significant 13% increase of this ratio was found between MCT+SED with V+SED groups ($p < 0.05$), suggestive of cardiac hypertrophy. Exercise training attenuated the increase of heart mass-to-body weight, though without reaching statistical significance ($p=0.6$). No differences between groups were noticed for RV mass and RV mass-to-body weight. The lung mass was significantly increased in MCT groups, either sedentary or exercised ones ($p< 0.01$ *vs.* V+SED) (**Table 1**). These differences were also observed when lung mass was adjusted to body weight, tibia length and right atrium.

Table 1. Effect of MCT and exercise training on morphometric characteristics.

	Experimental groups		
	V+SED	**MCT+SED**	**MCT+EX**
Body Weight (g)	349±10	294±30*	284±20**
Heart (g)	0.858±0.050	0.944±0.090	0.851±0.080
Right Ventricle (g)	0.181±0.010	0.242±0.050	0.206±0.070
Right Atrium (g)	0.029±0.010	0.051±0.050	0.034±0.010
Lungs (g)	1.348±0.080	2.175±0.180**	2.037±0.460**
Gastrocnemius mass (g)	2.182±0.180	1.842±0.190*	1.713±0.170**
Heart/Body Weight (g/g)	2.458±0.120	3.271±0.690*	3.009±0.340
Right Ventricle/Body Weight (g/g)	0.518±0.040	0.851±0.300	0.734±0.280
Right Atrium/Body Weight (g/g)	0.082±0.015	0.095±0.027	0.119±0.025*
Lung/Body Weight (g/g)	3.866±0.250	7.532±1.330**	7.158±1.410**
Gastrocnemius/Body Weight (g/g)	0.0063±0.0004	0.0063±0.0002	0.0060±0.0003
Body Weight/ Tibia length (g/cm)	92.92±2.79	76.82±9.76**	72.88±5.98**
Heart/ Tibia length (g/cm)	0.228±0.012	0.247±0.019	0.218±0.018
Right Ventricle/Tibia length (g/cm)	0.0482±0.0048	0.0632±0.0130	0.0529±0.0174
Right Atrium/Tibia length (g/cm)	0.0076±0.0015	0.0076±0.0016	0.0086±0.0019
Lung/Tibia length (g/cm)	0.359±0.03	0.569±0.05**	0.522±0.11*
Gastrocnemius/Tibia length (g/cm)	0.580±0.04	0.482±0.05*	0.439±0.04***

Values are expressed as mean ± standard derivation (*p < 0.05 *vs.* V+SED; **p < 0.01 *vs.* V+SED; ***p < 0.001 *vs.* V+SED).

3.2. Effect of MCT-induced PAH and exercise training on cardiac hemodynamics

Results from RV hemodynamic evaluation are shown in **Table 2**. Heart rate was significantly reduced in both MCT-treated groups ($p<0.05$ vs. V+SED). Right ventricular overload was increased in both MCT-treated groups, as suggested by the higher maximum pressure and end-systolic pressure ($p<0.05$ vs. V+SED). Pulmonary arterial elastance, a marker of pulmonary vascular resistance, was also increased in both groups but significantly only in MCT-SED ($p<0.05$ vs. V+SED). Regarding parameters of systolic function, we found that cardiac output and stroke volume were preserved, while dP/dtmax and maximal power were increased in MCT-treated groups. On its turn, diastolic function was significantly compromised in MCT+SED, as suggested by their increased end-diastolic volume (suggestive of dilation), end-diastolic pressure and tau ($p<0.05$ vs. V+SED). Of note, exercise training was capable to prevent diastolic dysfunction.

Table 2. Hemodynamic evaluation of systolic and diastolic function.

	Experimental groups		
	V+SED	**MCT+SED**	**MCT+EX**
Heart Rate (bpm)	416.4±14.2	353.3±34.4*	348.0±55.6*
Arterial Elastance (Ea) (mmHg/μL)	0.2077±0.0225	0.3812±0.1115*	0.2768±0.1120
Stroke Volume (μL)	113.5±5.8	125.0±32.4	189.9±88.0
End-systolic Volume (μL)	224.8±25.0	330.2±67.6*	212.2±76.2§§
Minimum Volume (μL)	201.8±22.1	313.7±58.3**	197.0±68.2§§
dP/dt max (mmHg/sec)	1795±175	2461±505*	2841±387***
End-systolic Pressure (mmHg)	23.5±2.3	44.9±10.4**	46.7±11.9**
Maximum Pressure (mmHg)	24.9±1.9	45.3±10.4**	47.3±11.8**
Cardiac Output (μL/min)	47220±2133	44868±14391	65292±24905
Maximal Power (mWatts)	0.73±0.17	1.00±0.35	1.38±0.53*
End-diastolic Volume (μL)	307.6±24.4	432.3±63.3*	349.1±108.8
Maximum Volume (μL)	315.5±21.5	438.8±62.4*	353.7±108.9
dPdt min (mmHg/sec)	-1332±111	-2196±520**	-2301±409**
Minimum Pressure (mmHg)	0.70±0.50	2.83±1.98*	0.21±0.03§§
End-diastolic Pressure (mmHg)	3.8±1.1	6.1±1.9*	3.2±0.8§§
Tau_w (msec)	11.1±1.4	15.1±1.5*	11.1±3.6§
Preload adjusted maximal power (mWatts/μL²)	12.3±4.4	16.4±6.1	16.9±7.1

Values are expressed as mean ± standard deviation (*p < 0.05 *vs.* V+SED; **p < 0.01 *vs.* SED; ***p < 0.001 *vs.* V+SED § $p < 0.05$ vs MCT+SED; §§ $p < 0.01$ vs MCT+SED)

3.3. Effect of MCT-induced PAH and exercise training on cardiac metabolism

To unravel the RV metabolic adaptations of cardiac muscle in MCT-induced PAH, specific molecular players from the glycolytic and oxidative pathways were assessed. As depicted in **Figure 2**, MCT+SED showed a reduction of glucose transporter 4 (GLUT4; $p < 0.05$ *vs.* V+SED) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; $p < 0.05$ *vs.* V+SED) content in RV as well as of the electron transferring-flavoprotein-dehydrogenase (ETFDH; $p < 0.0001$ *vs.* V+SED) that is involved in the transference of electrons resultant from fatty acid oxidation to the electron transporter chain,[28] and also of ATP synthase β ($p < 0.001$ *vs.* V+SED). However, no differences of ATP synthase activity were noticed (**Figure 2, L**). Regarding the ratio GAPDH-to-ATP synthase β, a rough marker of the metabolic status, no significant changes were detected between groups; however, the ratio ETFDH-to-ATP synthase β was significantly reduced in MCT rats ($p<0.0001$ vs. V+SED), suggesting a decreased reliance on fatty acid oxidation for energetic purposes. A significant increase of LDH activity was noticed in RV of MCT-treated rats ($p<0.01$ vs. V+SED).

Exercise training counteracted the MCT-induced decrease of GLUT4 ($p<0.01$ *vs.* MCT+SED). The levels of GAPDH, ETFDH and ATP synthase β, and the ratios GAPDH-to-ATP synthase β and ETFDH-to-ATP synthase β were not modulated by exercise training in MCT rats; however, the activity of LDH was also increased in trained MCT rats compared to the sedentary ones ($p<0.05$ vs. MCT+SED; **Figure 2, M**).

To better explore the effect of exercise training on MCT-induced OXPHOS adaptations, the content of subunits from each OXPHOS complex was assessed. A trend similar to the one observed for the other metabolic proteins was noticed. Indeed, exercise training did not revert the MCT-induced diminishing of subunits from complex I ($p < 0.001$ *vs.* V+SED), II ($p < 0.01$ *vs.* V+SED), III ($p < 0.0001$ *vs.* V+SED) and V ($p < 0.0001$ *vs.* V+SED). A lower ability to produce ATP was noticed in the RV of trained MCT rats ($p<0.05$ vs. MCT+SED; **Figure 2, L**). These metabolic adaptations did not seem to be associated with changes in mitochondrial density since no differences of citrate synthase activity were observed (**Figure 2, K**).

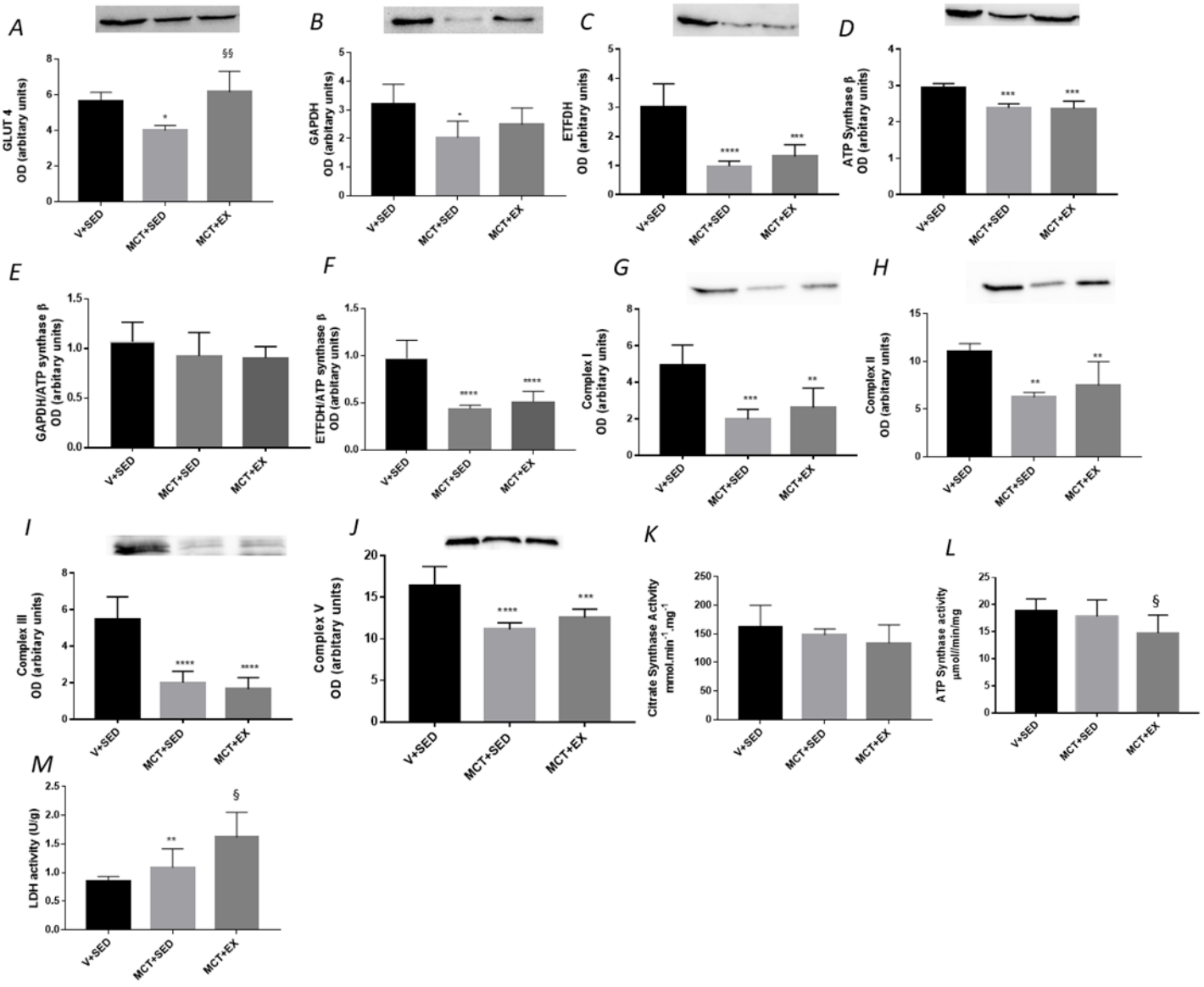

Figure 2. Effects of MCT-induced PAH and exercise training on right ventricle metabolism. *A*, GLUT4; *B*, GAPDH; *C*, ETFDH; *D*, ATP synthase β; *E*, GAPDH/ATP synthase β ratio; *F*, ETFDH/ATP synthase β; *G*, complex I, CI-NDUFB8; *H*, complex II, CII-SDHB; *I*, complex III, CIII-UQCRC2; *J*, complex V, CV-ATP5A *K*, Citrate synthase activity; *L*, ATP synthase activity; *M*, Lactate dehydrogenase activity. Representative blots are shown above the corresponding graphic – samples were loaded in the gel one *per* group side-by-side. Values are expressed as mean ± standard deviation of n=4-6 (*p < 0.05 *vs.* V+SED; ** p < 0.01 *vs.* V+SED; ***p < 0.001 *vs.* V+SED; ****p < 0.0001 *vs.* V+SED; §p < 0.05 *vs.* MCT+SED; §§ $p < 0.01$ vs MCT+SED).

Despite no apparent alterations in the mitochondrial density of RV between groups, a significant decrease of the content of the transcription factor involved in the regulation of mitochondrial biogenesis, peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1α),[29] was observed in MCT+SED rats (p < 0.05 *vs.* V+SED; **Figure 3 A**). Exercise

training reverted this decrease ($p < 0.05$ *vs.* MCT+SED) to values in the range of the ones observed in V+SED group. Moreover, the content of the transcription factors involved in the regulation of cardiac metabolism, PPAR-α and PPAR-γ, was also significantly decreased in MCT+SED group ($p < 0.05$ and $p<0.01$ *vs.* V+SED, respectively). Exercise training only reverted MCT-induced decrease of PPAR-γ ($p < 0.05$ *vs.* MCT+SED) (**Figure 3B and 3C**).

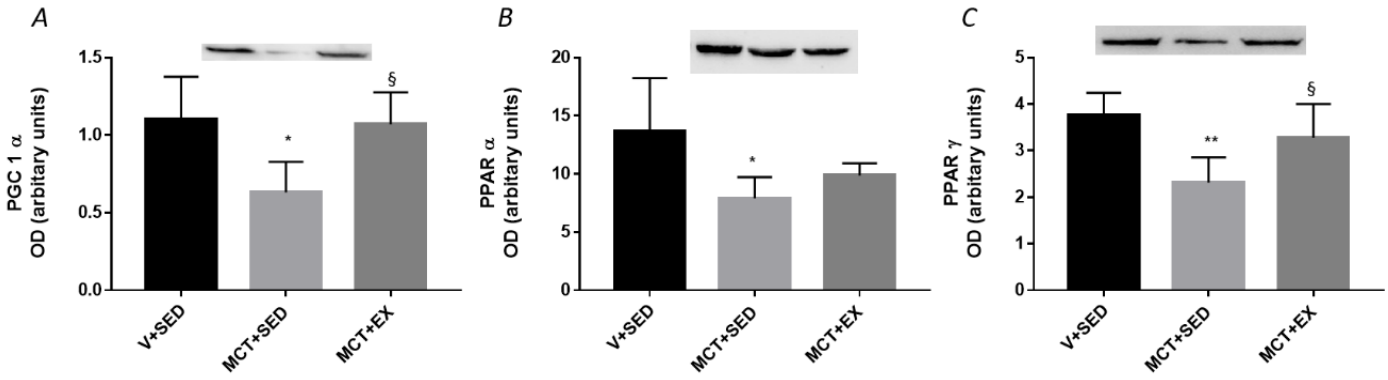

Figure 3. Effects of MCT-induced PAH and exercise training on the metabolic regulation of RV. *A*, PGC1-α; *B*, PPAR α; *C*, PPAR **γ.** Representative blots are shown above the corresponding graphic – samples were loaded in the gel one per group side-by-side. Values are expressed as mean ± standard deviation of n=4-6 (*$p < 0.05$ *vs.* V+SED; ** $p < 0.01$ *vs.* V+SED; §$p < 0.05$ *vs.* MCT+SED).

To better comprehend the effect of MCT-induced PAH and exercise training on the remodelling of cardiac metabolism, metabolite profiling was performed by MRM MS/MS. Regarding the acylcarnitine profile, a significant decrease of β-oxidation intermediates concentration was observed in both MCT-treated groups (**Figure 4**). Notably, no changes were detected between groups in the content of 3-hydroxybutyrilcarnitine (C4-OH; data not shown), a metabolite from ketone bodies metabolism. Exercise training did not revert this trend. Indeed, a higher decrease of C16, C14 and C6 metabolites levels were noticed in MCT+EX rats. So, data suggest a lower reliance of RV muscle on fatty acid oxidation for energy supply and two weeks of exercise training did not revert this trend.

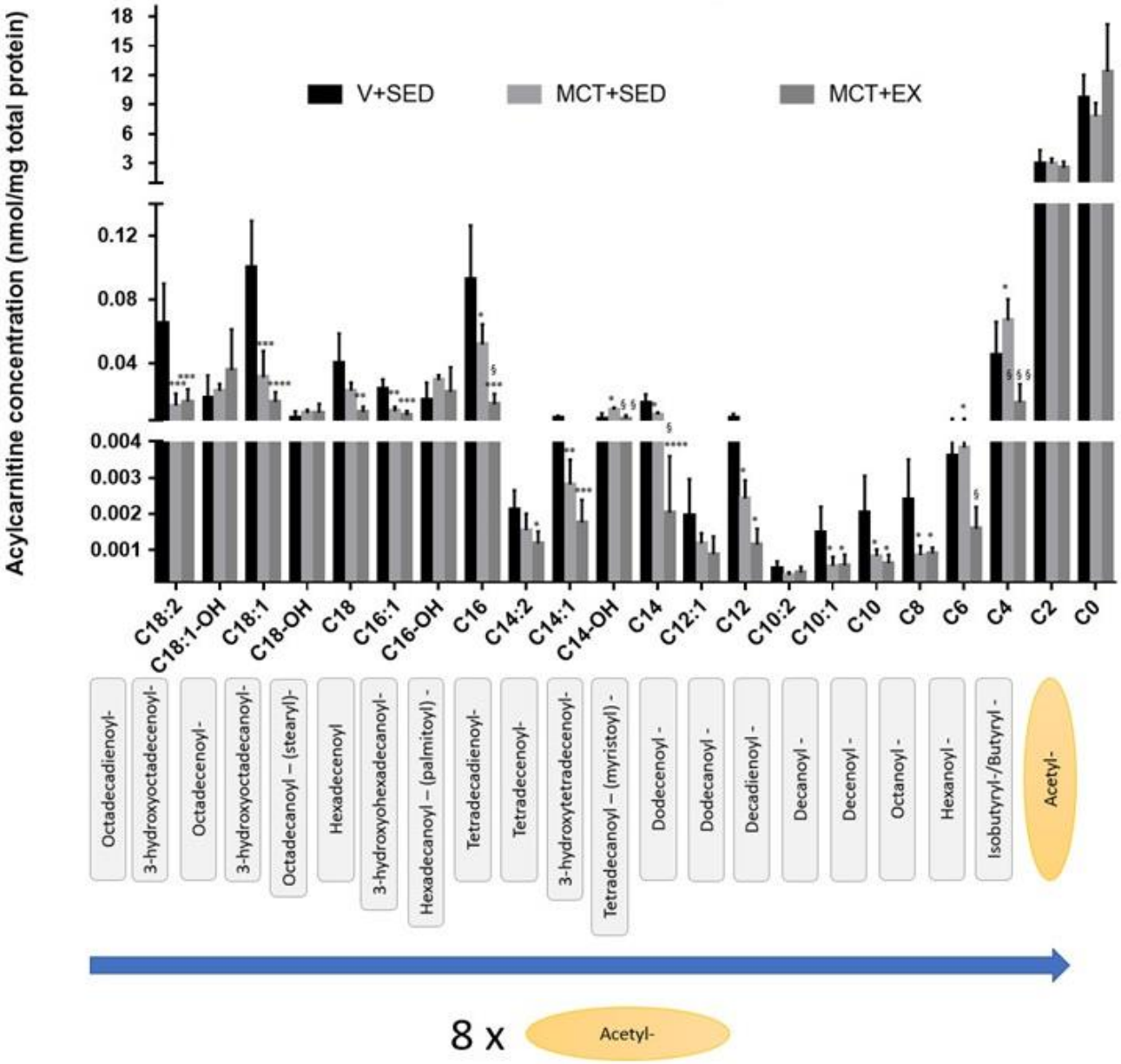

Figure 4. Effects of MCT-induced PAH and exercise training on the RV's acylcarnitine profile. Bellow the graphic, the complete sequence of oxidation of an octadecadienoyl fatty acid (C18:2) is shown; in total, nine acetyl carnitine molecules are yielded. Values are expressed as mean ± standard deviation of n=4-6 (*p < 0.05 *vs.* V+SED; ** p < 0.01 *vs.* V+SED; ***p < 0.001 *vs.* V+SED; ****p < 0.0001 *vs.* V+SED; §p < 0.05 *vs.* MCT+SED ; §§p < 0.01 *vs.* MCT+SED; §§§p < 0.001 *vs.* MCT+SED).

The contribution of amino acids metabolism to right ventricle metabolic remodelling was also assessed by MRM MS/MS (**Figure 5**). Data highlight a significant increase of leucine/isoleucine, alanine ($p < 0.05$ *vs.* V+SED), valine and glutamate ($p < 0.01$ *vs.* V+SED) concentrations in the RV of MCT+SED rats. The branched amino acids leucine, isoleucine and valine may be a source of acetyl-CoA and succinyl-CoA in the heart, supporting the tricarboxylic acid cycle after transamination.[30] However, the higher levels of these amino acids observed in MCT+SED rats does not support their metabolic utilization to fulfil the energetic needs of the RV. Exercise training did not revert MCT-induced increase of glutamate and valine ($p < 0.05$ *vs.* V+SED), though a trend to lower concentrations of these amino acids and of leucine/isoleucine was noticed.

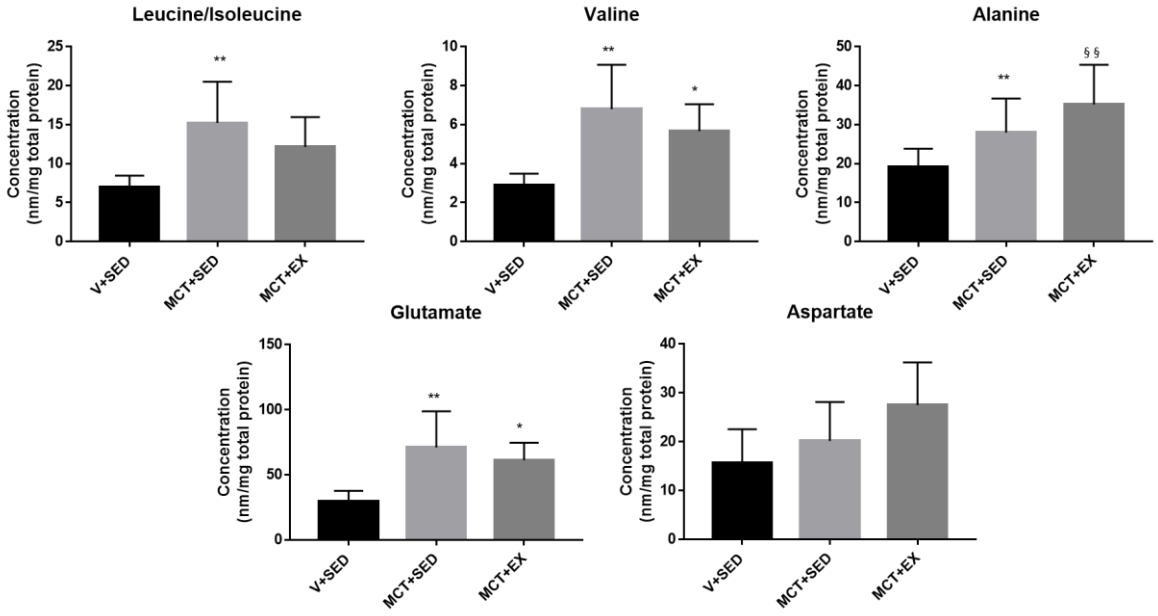

Figure 5. Effect of MCT-induced PAH and exercise training on the RV muscle content of the amino acids Leu/Ile, Val, Ala, Glu and Asp. Values are expressed as mean ± standard deviation of n=4-6 (*$p < 0.05$ *vs.* V+SED; ** $p < 0.01$ *vs.* V+SED; §§$p < 0.01$ *vs.* MCT+SED)

4. Discussion

The present study demonstrates that exercise training counteracts cardiac dysfunction and modulates the metabolic remodelling secondary to MCT-induced PAH. The improved cardiac phenotype promoted by two weeks of treadmill exercise was related to an increased uptake of glucose to cardiomyocytes through GLUT4 followed by its oxidation to lactate, and increased PGC1α and PPARγ protein expression.

The MCT animal model of PAH was chosen to study the therapeutic effect of exercise training. Although there are some differences described between this animal model and human PAH, the MCT animal model reasonably mimics some features of human PAH including vascular remodelling, proliferation of smooth muscle cells, endothelial dysfunction, upregulation of inflammatory cytokines, and right ventricle failure. [31] Our data added new molecular insights on this animal model, mostly at the metabolic level. Experimental evidences point to a decreased uptake of glucose in the RV of sedentary MCT-treated rats, given by the decreased content of GLUT4 transporter. However, a higher efficiency on this metabolite oxidation seems to occur, once increased LDH activity was observed. No differences of GAPDH content and GAPDH-to-ATP synthase beta ratio were noticed; however, protein levels not always reflect the activity of a metabolic pathway due to regulatory mechanisms such as posttranslational modifications.[13] This increase in the use of glycolysis to obtain energy reflects an attempt to compensate the decrease of fatty acid oxidation, given by the content of acylcarnitines and ETFDH, the enzyme that transfer the reducing equivalents from fatty acid oxidation to the electron transport chain[28], and of OXPHOS complexes subunits (**Figure 2**). In the setting of sustained increase in myocardial energetic requirements, transcriptional adaptations occur to adjust to metabolic needs.[6] The human heart responds to chronic stress, such as ischemia as well as pressure and volume overload, by switching the preferential substrate to metabolically support cardiac function. PAH-induced metabolic remodelling in the RV is characterized by a higher reliance on glycolysis, which requires less oxygen consumption for an equivalent amount of ATP synthesis.[13] Downregulation of PGC-1α and PPARs target metabolic genes is a hallmark of heart failure, contributing to insufficient energy production and further progression of the pathology.[32] The decreased expression of these transcription factors observed in the RV of MCT rats (**Figure 3**), together with the acylcarnitine profile (**Figure 4**) confirms the lower contribution of fatty acid oxidation and OXPHOS for energy suppling. To make it possible

for long chain fatty acids to cross the mitochondrial membrane to undergo fatty acid oxidation, they must be converted into acylcarnitines. Once in the mitochondrial matrix, fatty acids are oxidized with the formation of reducing equivalents that support OXPHOS.[33] Thus, acylcarnitines profile is probably the most appropriate marker of FAO activity.[34] On the other hand, the MS analysis of the amino acids profile highlights a significant increase of amino acids content, particularly of branched amino acids like leucine/isoleucine (not distinguished by MS) and valine, in the RV of MCT-treated rats, compared with the V+SED ones (**Figure 5).** Branched chain amino acids are major amino acids taken up by the heart and might energetically support this organ when necessary.[30] The increase of amino acids pool seem to reflect an imbalance between protein degradation and biosynthesis.[35] Histological findings do not support cardiomyocytes proteolysis since evidences of cardiomyocytes hypertrophy were previously reported.[23] Thus, data suggest a higher uptake of amino acids to the RV of MCT-treated rats. Three amino acids are more abundant in blood, namely, glycine, alanine and glutamine, which are substrates of Na^+-neutral amino acid symporters, such as SNAT1. These amino acids, particularly alanine, might result from skeletal muscle proteolysis, the largest reservoir of amino acids from mammals.[35] The decrease of *gastrocnemius* mass observed in MCT-treated rats (**Table 1**) is suggestive of muscle proteolysis,[36] which may feed other organs besides liver where it is usually used to support gluconeogenesis.[37]

The benefits of exercise training were morphologically associated to the reduction of fibrosis[23] and the improved influx of glucose via GLUT 4 transporter and oxidation through glycolysis. Insulin signalling has a central role in the regulation of myocardial metabolism by stimulating glucose transport into cardiomyocytes. Therefore, data support the effect of exercise training in improving insulin sensitivity in RV.[38] This data was further supported by the increase of PPARγ. The upregulation of this transcription factor may also explain the decreased cardiac fibrosis observed in trained MCT rats, as previously reported.[39]. Glucose oxidation with lactate formation was increased in the RV of trained MCT rats, assuring a higher energetic supply to RV. Despite experimental evidences of increased mitochondrial biogenesis, the contribution of OXPHOS to the metabolic remodelling of RV from these animals was discrete. Moreover, the content of free amino acids only slightly decreased in MCT-trained rats compared to the MCT -sedentary ones, which may reflect a trend to increase its oxidation for energetic purposes or its incorporation in muscle proteins, since no

alterations of skeletal muscle mass (**Table 1**) or cardiomyocytes cross-sectional area[23] were observed. Moreover, the remodelling of ECM did not seem to contribute to the amino acid pool of RV, despite increased MMPs activity previously reported.[22] Exercise training-induced upregulation of insulin signaling, through PI3K/Akt signalling, is expected to promote amino acids uptake and incorporation in muscle proteins.[35] To the best of our knowledge, this is the first study characterizing amino acid profile in the set of PAH.

5. Conclusion

In conclusion, our study shows that exercise training performed after disease diagnosis, slows the progression of the disease by modulating the energetic substrate of the heart. Future studies should explore the metabolic remodelling of RV and the functional adaptations promoted by long-term exercise training, envisioning the prescription of tailored exercise programs to PAH patients.

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CHAPTER IV

Final Remarks

Aiming to add new insights on the therapeutic effect of exercise training in the set of PAH, we performed a narrative review of the literature and an experimental study with the monocrotaline (MCT) animal model submitted to two weeks of treadmill exercise training after disease diagnosis. Data obtained allowed us to conclude that:

I. The integrative analysis of data from the literature supports the recommendation of exercise training for the management of PAH once it promotes the remodelling of ECM (decrease of fibrosis and modulation of MMPs/TIMPs), stimulates angiogenesis, reduces inflammation (decreased cell infiltration and levels of TNF-α and IL-6), neurohormonal activation/decreased BNP and ET-1 expression) and oxidative stress;
II. Experimental findings showed that exercise training improves cardiac tolerance to sustained pressure overload promoted by PAH by restoring diastolic function;
III. The cardioprotective phenotype provided by exercise training was related to the modulation of MCT-induced metabolic remodelling as shown by increased glucose uptake through GLUT4 and LDH activity. Exercise training did not counteract the downregulation of fatty acid oxidation, OXPHOS activity and amino acids uptake;
IV. The metabolic effect of exercise training in the RV of MCT rats was mediated by the transcription factors PPARγ and PGC-1α, despite no apparent association with increased mitochondrial density.

Taking together, data suggest that exercise training slows the progression to RV failure by modulating the energetic substrate of the heart towards glucose utilization, further supporting the adjunctive role that exercise training may have in the PAH setting.