Universidade de Aveiro Departamento de Biologia
Ano 2017

Stephanie Ribeiro Salgado

Efeito do tratamento crónico com Neuregulina-1 na tolerância à glicose na Hipertensão Arterial Pulmonar

Effect of chronic Neuregulin-1 treatment in glucose tolerance in Pulmonary Arterial Hypertension

DECLARAÇÃO

Declaro que este relatório é integralmente da minha autoria, estando devidamente referenciadas as fontes e obras consultadas, bem como identificadas de modo claro as citações dessas obras. Não contém, por isso, qualquer tipo de plágio quer de textos publicados, qualquer que seja o meio dessa publicação, incluindo meios eletrónicos, quer de trabalhos académicos.

Universidade de Aveiro Departamento de Biologia
Ano 2017

Stephanie Ribeiro Salgado

Efeito do tratamento crónico com Neuregulina-1 na tolerância à glicose na Hipertensão Arterial Pulmonar

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob orientação científica da Professora Carmen Dulce Silveira Brás Silva Ribeiro, professora auxiliar da Faculdade de Medicina da Universidade do Porto e da Professora Maria Paula Polónia Gonçalves, professora associada do Departamento de Biologia da Universidade de Aveiro.

o júri

Presidente

Professora Doutora Maria Helena Abreu Silva
professora auxiliar do Departamento de Biologia da Universidade de Aveiro

Professora Doutora Ana Patrícia Fontes de Sousa
professora auxiliar do Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto

Professora Doutora Carmen Dulce Silveira Brás Silva Ribeiro
professora auxiliar da Faculdade de Medicina da Universidade do Porto

agradecimentos

Um ano pode ser muito tempo para uns, um flash para outros, ou ainda uma eternidade para muitos. E no meio de todas as provações desses "never ending years" a ajuda e a força vem de onde menos se espera bem e por isso deixo aqui enaltecido agradecimento á professora Carmen Brás Silva, que no seu jeito impar me deu a mão quando mais nada parecia possível fazer para a entrega deste trabalho.

Queria ainda agradecer à professora Paula (não menos importante, mas tinha que começar por algum lado) pela sua preocupação constante e assistência naquilo que lhe era possível e impossível fazer, portanto às duas um eterno obrigado.

Ao Professor Doutor Adelino Leite Moreira por disponibilizar os meios logístico e humanos, sem os quais esta investigação não teria sido possível. Pelo incentivo e disponibilidade sempre demonstrada ao longo deste ano.

Deixo também um agradecimento especial aos meus colegas de laboratório, Doutor Pedro Ferreira, Mestre Rui Adão e Mestre Carolina Rocha, que se mostraram companheiros e amigos desde o primeiro dia com uma grande entre ajuda por todos, e acima de tudo compreensão nos momentos que cada um passou nesta caminhada. E claro por todo o conhecimento que me transmitiram ao longo deste ano, e que me ajudará na minha carreira profissional.

Deixo ainda um agradecimento especial para o meu namorado, Dinis Faustino, pela paciência e compreensão em todas as fases da minha vida, mas principalmente nesta que passei. Obrigada por me acompanhares ao longo destes e sido incansável e paciente.

Aos pais do meu namorado, Sameiro Faustino e António Faustino, pela preocupação e cuidado que me deram, não só agora, mas desde sempre.

Mãe, Mana dedico-vos esta minha conquista que seja, uma amostra daquilo que juntas e unidas podemos fazer, hoje e sempre. Obrigada por me apoiarem sempre, quaisquer que sejam as minhas escolhas!

palavras-chave

Hipertensão Arterial Pulmonar, hipertrofia do ventriculo direito, alterações metabólicas, transportadores de glicose 1 e 4, tolerância à glicose, Neuregulina-1

resumo

O coração saudável gera até 30% do seu ATP a partir da glicose. Em condições de lesão cardíaca e stress, o coração depende ainda mais da glicose como fonte de energia. A glicose é transportada para os cadiomiócitos por proteínas da família dos transportadores de glicose (GLUTs). As isoformas predominantes no miocárdio são GLUT1 e GLUT4. GLUT4 é a isoforma predominante no coração adulto e a sua deleção genética no coração está associada ao desenvolvimento de hipertrofia cardíaca. No coração fetal, a isoforma predominante é GLUT1, cuja expressão diminui no coração adulto em resposta a lesões do miocárdio e a stress. A neuregulina-1 (NRG1) é uma proteína envolvida no metabolismo da glicose e tem um papel benéfico na Hipertensão Arterial Pulmonar (HAP) e na hipertrofia do ventrículo direito (VD). Neste estudo, o principal objetivo foi estudar a correlação entre a expressão de GLUT1 e GLUT4 e de marcadores de HAP no VD, com recurso a um modelo animal de HAP. Também se avaliou o efeito do tratamento crónico com NRG1 na expressão de GLUT1 e GLUT4 no VD de animais com insuficiência cardíaca (IC) associada a HAP.

Ratos Wistar receberam aleatoriamente monocrotalina (MCT; 60mg /kg de peso corporal) ou veículo. Após 14 dias, os animais foram tratados aleatoriamente com NRG1 recombinante humana (rhNRG1; 40µg/kg de peso corporal/dia) ou com veículo. Durante o estudo 4 grupos foram formados: control (CTRL); CTRL+rhNRG1; MCT and MCT+rhNRG1. Decorridos 21 a 24 dias e após avaliação hemodinâmica dos animais experimentais, procedeu-se à sua eutanásia e recolha de amostras tecidulares.

Neste modelo animal de HAP, verificámos que a diminuição da fração de ejeção (FE) se correlaciona positivamente com o aumento da expressão de GLUT1 ($p=0,0005$) e com a diminuição da expressão de GLUT4 ($p=0,0167$) no VD. Também observámos correlação positiva ($p<0,0001$) entre o aumento da expressão de GLUT1 e do fator induzido por hipoxia-1 (HIF-1α). A redução da expressão de GLUT4 está correlacionada com o aumento da expressão do péptido natriurético cerebral (BNP) ($p=0,0032$) e da endotelina-1 (ET-1) ($p=0,0006$), dois marcadores de sobrecarga e hipertrofia. Verificámos o aumento da expressão de GLUT1 no VE do grupo MCT relativamente à sua expressão no grupo CTRL; no grupo MCT+rhNRG1 esses valores foram completamente revertidos para os valores do controlo. A expressão de GLUT4 aumentou em todos os grupos de animais tratados com rhNRG1.

No presente estudo, observámos que a expressão de GLUT1 está associada desenvolvimento de doença e que a expressão de GLUT4 é afetada pelo tratamento crónico com rhNRG1. A expressão de GLUT1 e GLUT4 está correlacionada com parâmetros de função cardíaca e marcadores de doença. O tratamento crónico com rhNRG1 atenua as alterações nos GLUTs observadas na HAP induzida por MCT. Assim, podemos concluir que os efeitos terapêuticos da rhNRG1 na HAP podem ser devidos, pelo menos em parte, à regulação da expressão de GLUT1 e GLUT4.

keywords **Pulmonary arterial hypertension, right ventricle hypertrophy, metabolic changes, glucose transporters 1 and 4, glicose tolerance, neuregulin-1**

abstract

The healthy heart generates up to 30% of its ATP from glucose. Under conditions of cardiac injury or stress, the heart relies even more heavily on glucose as a source of energy. Glucose is transported into the heart by members of the family of facilitative glucose transporters (GLUTs). The two major isoforms of glucose transporters in the myocardium are GLUT1 and 4. GLUT4 is the predominant GLUT expressed in the adult heart and its genetic ablation in the heart has been shown to result in marked cardiac hypertrophy. GLUT1 is a major GLUT expressed in the fetal heart but it decreases in the adult heart in response to myocardial injury or stress. Neuregulin-1 (NRG1), a protein that has been shown to play beneficial effects on Pulmonary Arterial Hypertension (PAH) and right ventricle (RV) hypertrophy, has also been associated with the regulation of glucose metabolism.

In this study, we aimed to investigate the correlation between disease markers and the GLUT1 and 4 expressions in RV, in an animal model of PAH. We also aimed to evaluate the effect of chronic treatment with NRG1 on GLUT1 and 4 expressions in the RV of animals with HF associated with PAH.

Wistar rats randomly received 60mg/kg of monocrotaline (MCT) or vehicle. After 14 days, they were randomly treated with rhNRG1 (40µg/kg/day) or vehicle. The study resulted in 4 groups: control (CTRL); CTRL+rhNRG1; MCT and MCT+rhNRG1. Between the 21st and 24th days after administration of MCT, hemodynamic studies and sample collection were performed.

In this animal model of PAH, we found that the decrease in ejection fraction (EF) correlates with increased GLUT1 expression ($p=0.0005$) and decrease in GLUT4 expression ($p=0.0167$) in RV. We also observed that increased GLUT1 expression correlates with increased hypoxia-inducible factor 1-alpha (HIF1a) expression ($p<0.0001$). The decrease in GLUT4 expression was shown to correlate with increased brain natriuretic peptide (BNP) ($p=0.0032$) and endothelin 1 (ET1) ($p=0.0006$) expression, two markers of overload and hypertrophy. We observed an increase of GLUT1 RV expression in the MCT group compared to the CTRL group, in the MCT+rhNRG1 group these values were completely reverted. GLUT4 increased in all groups of animals treated with rhNRG1.

In the present study, we observed that the expression of GLUT1 is associated with the development of the disease whereas GLUT4 is affected by chronic treatment with rhNRG1. The expression of GLUT1 and 4 correlates with parameters of cardiac function and with disease markers and chronic treatment with rhNRG1 attenuates the changes in GLUTs induced by MCT. So, we can conclude that the therapeutic effects of rhNRG1 in PAH might be due in part to the regulation of GLUT1 and 4 expressions.

Index

Index of figures

Index of tables

Abbreviations

5-HT	Hydroxytryptamine (Serotonin)
5-HTT	5-Hydroxytryptamine Transporter
Akt	Protein Kinase B
ALK1	Activin-Like Kinase Type I
ATP	Adenosine Triphosphate
BCL-1	B-Cell Lymphoma 2
BMPR2	Bone morphogenetic protein receptor type 2
BNP	Brain Natriuretic Peptide
BSA	Bovine Serum Albumin
BWT	Body Weight
Ca^{2+}	Calcium Ion
CGF2	Cimaglermin Alpha
CO	Cardiac Output
CRV	Compensated Right Ventricle
DCM	Diabetic Cardiomyopathy
Dox	Doxorubicin
DRP-1	Dynamic Related-Protein 1
DRV	Decompensated Right Ventricle
EC	Endothelial Cell
ERK	Extracellular Signal-Regulated Kinase
EGF	Epidermal Growth Factor
ErbB	Erythroblastic Leukaemia Viral Oncogene Homolog
ET-1	Endothelin-1
ETC	Electron Transport Chain
btw	Body Weight
FAO	Fatty Acid Oxidation
GLUT1	Glucose Transporter 1
GLUT4	Glucose Transporter 4
HF	Heart Failure
HIF-1	Hypoxia-Inducible Factor 1
HIF-1α	Hypoxia-Inducible Factor-1 Alpha
iPAH	idiopathic PAH
KCNK3	Potassium Channel Subfamily K Member 3
KCNK5	Potassium Channel Subfamily K Member 5
KO	Knockout
Kv1.5	Voltage-Gated Potassium Channel
LV	Left Ventricle
MAPK	Mitogen-Activated Protein Kinase
MCT	Monocrotaline
mPAP	mean Pulmonary Arterial Pressure
NFAT	Nuclear Factor of Activated T Cells
NFATc2	Nuclear Factor of Activated T Cells Isoform 2
NFDM	Non-fat Dried Milk
NO	Nitric Oxide
NRG	Neuregulin
NRG1	Neuregulin-1
OGTT	Oral Glucose Tolerance Test

OPA-1	Optic Atrophy 1
PAEC	Pulmonary Arterial Endothelial Cell
PAH	Pulmonary Arterial Hypertension
PASMC	Pulmonary Arterial Smooth Muscle Cell
PDE	Phosphodiesterase
PDGF	Platelet-Derived Growth Factor
PDGFR	Platelet-Derived Growth Factor Receptor
PDK1	Dehydrogenase Kinase 1
PH	Pulmonary Hypertension
PI3K	Phosphoinositide-3- Kinase
PIM1	Moloney Murine Leukemia 1
PTP	Tyrosine Phosphatase
rhNRG1	Recombinant Human Neuregulin 1
ROS	Reactive Oxygen Species
RV	Right Ventricle
RVH	Right Ventricle Hypertrophy
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SMAD	Small Mothers Against Decapentaplegic
SOD1	Superoxide Dismutase 1
SOD2	Superoxide Dismutase 2
STAT3	Signal Transducer and Activator of Transcription 3
TBS	Tris-Buffered Saline
TBS-T	Tris-Buffered Saline with Tween solution
TCA	Tricarboxylic Acid
TGF-β	Transforming Growth Factor Beta
VEGF	Vascular Endothelial Growth Factor

I - INTRODUCTION

I - INTRODUCTION

1. Pulmonary arterial hypertension

1.1. Etiology and definition

Pulmonary arterial hypertension (PAH) is classified as an uncommon subgroup of pulmonary hypertension (PH) and it's characterized by excessive vascular remodeling related to high arterial pressure and progressive overload of the right ventricle (RV), eventually leading to RV dysfunction and heart failure (HF)[1]. PAH can be an idiopathic (IPAH) disease, when no causes are determined, or hereditable (HPAH). However, PAH can also be induced by drugs or toxins or connected to other conditions, like connective tissue disease or congenital heart disease, and others[2].

PAH diagnosis is made by a right heart catheterization (RHC) exhibiting precapillary PH with a mean pulmonary arterial pressure (mPAP) of 25 mmHg or higher, a pulmonary capillary wedge pressure (PCWP) less than or equal to 15 mmHg and a pulmonary vascular resistance (PVR) higher than 3 Wood units[3].

Patients with PAH can present several symptoms including shortness of breath, fatigue, a non-productive cough, angina pectoris, syncope and peripheral oedema[4]. These symptoms are usually detected in late stage of PAH. To achieve an early diagnosis and treatment a better understanding of PAH is required[3].

1.2. Epidemiology and survival

PAH is a rare disease with an estimated prevalence of 15–50 cases per million individuals[5-8]. The first systematic analysis of PAH epidemiology was performed by the NIH in 1981 and involved 32 medical centers in the USA, with data on 187 patients with "primary pulmonary hypertension" (corresponding to idiopathic, heritable, and anorexigen-induced PAH in current classification)[9,10]. Since the original NIH registry in the USA, national and multinational PAH registries have reported baseline characteristics and outcome data on >10,000 patients with PAH[5-7]. These registries provide important insights into the evolving epidemiology of PAH[11].

The principal aim of the registries is the survival analysis. Before the era of targeted PAH therapy, data from the NIH registry showed a miserable prognosis for primary PH, with a median survival of 2.8 years and estimated 1-year, 3-year and 5-year survival rates of 68%, 48% and 34%, respectively[9]. With the advances in PAH therapy beginning in the 1990s (initially with intravenous epoprostenol and later with oral therapies), data registered between 2002 and 2003 from the French registry showed 1-year and 3-year survival rates of 86% and 55%, respectively, in patients with incident idiopathic, heritable and anorexigen-induced PAH[12]. These patients were a homogeneous population, and had similar characteristics to those of patients with primary PH reported in the NIH registry. To date, the US-REVEAL registry is the largest PAH registry reporting survival data. A total of 2,635 patients with all subtypes of PAH registered between 2006 and 2009 were involved in the primary survival analysis, with 1-year, 3-year, 5-year and 7-year survival rates from time of diagnostic right-sided heart catheterization of 85%, 68%, 57% and 49%, respectively[13]. Data from the European COMPERA registry showed estimated 1-year, 2-year and 3-year survival rates of 92%,

83% and 74%, respectively, for patients with iPAH diagnosed between 2007 and 2011[14]. Finally, data from the French registry showed that in patients with PAH diagnosed between 2007 and 2013, and who were treated with a strategy of upfront dual combination therapy (endothelin receptor antagonist and phosphodiesterase type-5 inhibitor), had 1-year, 2-year, and 3-year survival rates of 97%, 94%, and 83%, respectively[15].

1.3. Classification

The clinical classification of PH categorize several clinical conditions into five groups according to their similar clinical presentation, pathological findings, hemodynamic characteristics and treatment strategy[16]. The clinical classification may be updated when new data are available on the above features or when additional clinical entities are considered. A comprehensive version of the clinical classification is presented in Table 1[16]. This new version of clinical classification presents some alterations. The first one is that new conditions, frequently found in children, have been included in different clinical groups, providing a comprehensive classification appropriate to both adult and pediatric patients. Also, recently identified gene mutations have been included in the HPAH subgroup of clinical group 1. The new mutations are rarer than the mutations in bone morphogenetic protein receptor 2 (BMPR2) gene. In addition, pre-capillary PH associated with chronic hemolytic anemia appears to be significantly different from other forms of PAH regarding pathological findings (absence of plexiform lesions), hemodynamic characteristics (PVR and high cardiac output (CO)) and response to PAH-specific therapies (no demonstration of efficacy). Consequently, these clinical conditions have been moved from group 1 (PAH) to group 5 (unclear and/or multifactorial mechanisms). Finally, the group 1' [pulmonary veno-occlusive disease and/or pulmonary capillary hemangiomatosis] has been expanded and includes idiopathic, heritable, drug-, toxin- and radiation-induced and associated forms[17].

Table 1 - Classification of Pulmonary Arterial Hypertension

1. **Pulmonary arterial hypertension (PAH)**
   - 1.1. Idiopathic PAH
   - 1.2. Heritable PAH
     - 1.2.1.*BMPR2* mutations
     - 1.2.2.Other mutations
   - 1.3. Drug-induced or toxin-induced
   - 1.4. Associated with:
     - 1.4.1.Connective tissue diseases
     - 1.4.2.HIV infection
     - 1.4.3.Portal hypertension
     - 1.4.4.Congenital heart disease
     - 1.4.5.Schistosomiasis

1' Pulmonary veno-occlusive disease and/or pulmonary capillary haemangiomatosis

1'' Persistent pulmonary hypertension of the newborn

2. **Pulmonary hypertension due to left heart disease**
   - 2.1. Left ventricular systolic dysfunction
   - 2.2. Left ventricular diastolic dysfunction
   - 2.3. Valvular heart disease
   - 2.4. Congenital/acquired left heart inflow/outflow obstruction and congenital cardiomyopathies
3. **Pulmonary hypertension due to lung disease and/or hypoxia**
   - 3.1. Chronic obstructive pulmonary disease
   - 3.2. Interstitial lung disease
   - 3.3. Other pulmonary disease 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. Developmental lung disease
4. **Chronic thromboembolic pulmonary hypertension and other pulmonary artery obstructions**
   - 4.1. Chronic thromboembolic pulmonary hypertension
   - 4.2. Other pulmonary artery obstructions
5. **Pulmonary hypertension with unclear multifactorial mechanisms**
   - 5.1. Haematologic disorders
   - 5.2. Systemic disorders
   - 5.3. Metabolic disorders
   - 5.4. Others

Reproduced from Galiè, N. *et al*. 2015 ESC/ERS guidelines for the diagnosis and treatment of pulmonary hypertension. The Joint Task Force for the Diagnosis and Treatment of Pulmonary Hypertension of the European Society of Cardiology (ESC) and the European Respiratory Society (ERS). *Eur. Respir. J.* **46**, 903–975 (2015), with permission from the European Society of Cardiology & European Respiratory Society.

1.4. Pathophysiology

1.4.1. Histopathology

PAH is an arteriopathy of small- to medium-sized pulmonary arteries which undergo remodeling and reduction in luminal cross-sectional area. All these pulmonary arteries layers are affected, occurring medial hypertrophy, intimal and adventitial thickening[18,19]. Medial hypertrophy is characterized by a proliferation of PASMCs within medial tunica, that corresponds to a hyperplasia (i.e., increase in number) and hypertrophy (i.e., increase in volume) of these cells. The diameter, from the intern to extern elastic lamina, is the measure used to define medial hypertrophy, which must exceed 10% cross-section diameter of the normal arteries. Typically, local hypertrophy is

considered reversible and an early event of PAH. Intima and adventitial thickening is a consequence of fibroblast, myofibroblast and other conjunctive tissue cells proliferation and/or recruitment and collagen deposition, causing fibrosis. The intima thickness can be classified in uniform and concentric or focal and eccentric. Eccentric and focal intima thickening is associated to thrombotic events (also known as, *in situ thrombosis*)[18,20].

Besides, PAH patients can exhibit various and complex lesions in the pulmonary arteries, such as plexiform lesions, dilated lesions and arteritis. Plexiform lesions are frequently observed in PAH patients. These lesions are defined by an intimal increase of small pulmonary arteries accompanied with endothelial cells (ECs) proliferation, leading to the formation of capillary-like channels within the arterial lumen. Plexiform lesions are responsible for the decrease of lumen space because of arterial wall extension and obliteration. Dilated lesions appear to be associated with plexiform lesions, and are observed as a consequence of pulmonary arterial wall thickness. Arteritis is the less frequent event of the complex lesions. This lesion is an inflammatory infiltrate of lymphocytes T, macrophages and scattered mast cells and an accumulation of necrotic and fibrotic tissue in the arteries wall[20,21].

These phenomena are responsible for the pulmonary arteries obstruction that leads to the increase of their resistance and decreasing of the blood flow, leading to RV overload and hypertrophy and, consequently to HF and ultimately to death[22].

1.4.2. Cellular changes

The main mechanisms responsible for pulmonary vascular dysfunction are the abnormal proliferation of pulmonary arterial smooth muscle cells (PASMCs) and pulmonary arterial endothelial cells (PAECs), infiltration of inflammatory cells and fibrosis[23].

PAH is not only associated with cell proliferation, but also with apoptotic processes, as the imbalance between these two events is the major cause of the narrowing of the pulmonary arteries in PAH[24].

Smooth muscles cells and fibroblasts

A typical characteristic of PAH remodeling is the distal extension of smooth muscle into small peripheral, usually nonmuscular, pulmonary arteries within the respiratory acinus. The cellular processes underlying muscularization of this distal part of the pulmonary arterial tree are incompletely understood. This uncontrolled proliferation of PASMCs eventually leads to hypertrophy of the media and contributes to the thickening of the intima and adventitia of the arterial wall. These modifications in the vascular wall lead to the decrease of the vessel lumen, consequently contributing to increased arterial pressure[25].

Another feature observed in muscularized arteries affected by PAH is the development of an extracellular matrix and myofibroblast layer between the endothelium and internal elastic lamina, named *neointima*[25]. In some model systems, mostly in hypoxia models, the first activated cell to proliferate and to synthesize matrix proteins in response to the pulmonary hypertensive stimulus appears to be the adventitial fibroblast. The mechanisms that allow the adventitial fibroblast to migrate into the media, and ultimately into the intima, are unclear. It has been suggested that

upregulation of matrix metalloproteinases (MMP2 and MMP9), which are involved in cell migration, plays a role in fibroblast migration. These proteins are enzymes with proteolytic activity, that degrade extracellular matrix proteins during invasive events. The MMP2 and MMP9 can also degrade type IV collagen in the basal lamina, which is probably responsible for the invasive process[26].

In the different forms of PAH, as the vessel wall thickens, a concurrent increase occurs in neovascularization of the vasa vasorum. This progression affects primarily the adventitia but can extend up to the media[27].

Endothelial cells

The endothelium plays an important role in the regulation of pulmonary blood flow and vascular resistance. In many cases of PAH, it is described that disorganized proliferation of ECs results in plexiform lesions development, but it is unknown the cause of this phenomena[28]. Hypoxia, shear stress, inflammation, viral infections or response to drugs or toxins on a background of genetic susceptibility is suggested to be the initial stimulus for this abnormal proliferation of endothelial cells[3].

In response to stimulation, endothelial cells undergo changes not only at the proliferative and apoptotic levels, but also at the functional level. The impaired functional capacity of ECs can result in an imbalance between the synthesis of vasoconstrictors/vasodilators, activating/inhibitory growth and migration of PASMCs, prothrombotic/antithrombotic mediators and pro-inflammatory/anti-inflammatory signals[29].

Inflammatory cells

Inflammatory mechanisms appear to play an important role in PAH. Infiltration of various inflammatory cells and the increased expression of some cytokines chemokines (interleukin IL-1β, IL-6, IL-8, monocyte chemoattractant protein-1, fractalkine, CCL5/RANTES and tumor necrosis factor) occurs in remodeled pulmonary vessel, leading to an inflammatory mechanism deregulation[30]. Various types of PAH, including iPAH and PAH associated with connective tissue diseases or with infectious etiologies such as HIV, are characterized by accumulation of macrophages, T and B lymphocytes and dendritic cells in vascular lesions[31].

Additionally, other alterations can directly contribute to the recruitment of inflammatory cells to the pulmonary vascular remodeling process, which includes increased expression of growth factors (e.g., endothelial growth factor and platelet-derived growth factor), transcriptional factors (e.g., nuclear factor of activated T cells), and viral protein components (e.g., HIV-1 negative regulatory factor)[32].

Platelets and thrombosis

Patients with PAH often present thrombotic lesions and platelet dysfunction. In pulmonary vasculature, the decrease of blood flow leads to an increased risk of thrombosis *in situ*[33]. In the response to injury or endothelial dysfunction, activation of circulating platelets occurs[22]. Activated platelets can release vital vasoconstrictors, such as thromboxane A2, platelet activating factors,

serotonin, as well as important growth factors, such as epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF). These factors will promote the worsening of vascular remodeling. Another important biological evidence of continuous intravascular coagulation processes and changed fibrinolytic activity of vascular endothelium of patients with PAH is the increase of levels of D-dimer, fibrin (fibrinopeptide A), von Willebrand factor, and plasminogen activator inhibitor (type I) in the plasma of these patients[34].

1.4.3. Molecular abnormalities and therapeutic targets

Pulmonary vasoconstriction is supposed to be an early event of the pulmonary hypertensive process. Excessive vasoconstriction has been connected to abnormal function or expression of potassium channels, as well as to endothelial dysfunction. Endothelial dysfunction leads to recurrent impaired production of vasodilators (nitric oxide and prostacyclin), along with prolonged overexpression of vasoconstrictors (endothelin). These signaling molecules not only affect vascular tone, but also promote vascular remodeling, and, therefore, are key therapeutic targets for the management of PAH[25] (Fig. 1).

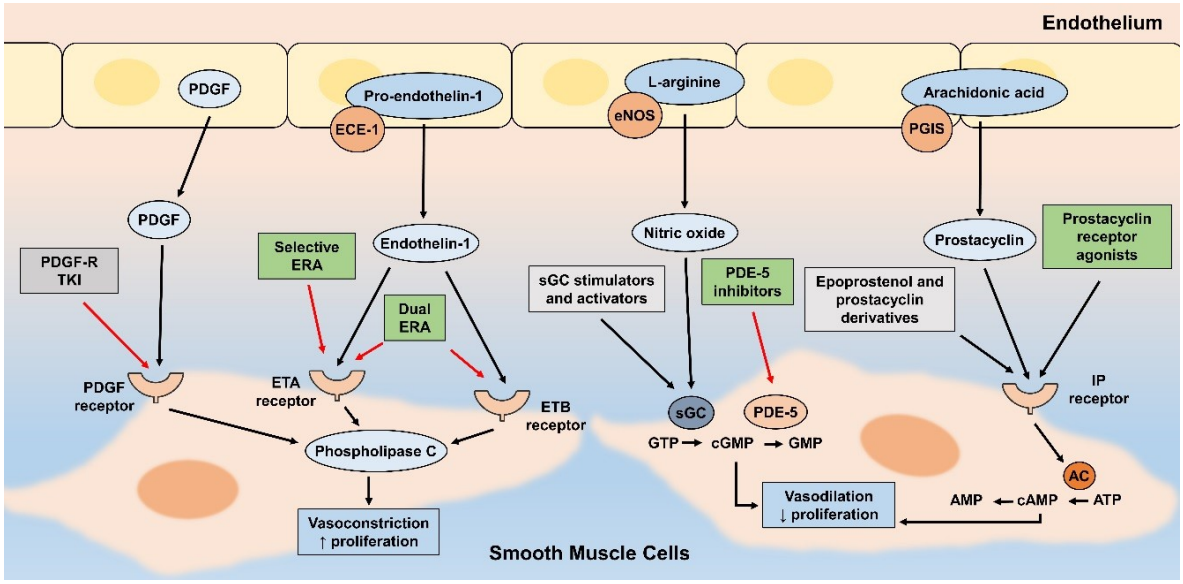

Figure 1 - Current therapies for PAH. Currently approved therapeutic options (green boxes) and new therapies under development (grey boxes). Black arrows represent stimulation, while red arrows represent inhibition of targeted pathways. Abbreviations: **AC**, adenylate cyclase; **cAMP**, cyclic AMP; **cGMP**, cyclic GMP; **ECE-1**, endothelin converting enzyme 1; **eNOS**, endothelial nitric oxide synthase; **ETA** endothelin receptor type A; **ETB**, endothelin receptor type B; **ERA**, endothelin receptor antagonists; **IP**, prostaglandin I2; **PDE-5**, phosphodiesterase type 5; **PDGF**, platelet derived growth factor; **PDGF-R TKI**, PDGF receptor tyrosine kinase inhibitors; **PGIS**, prostaglandin I synthase; **sGC**, soluble guanylate cyclase. Adapted from O'Callaghan *et al.*[35].

Prostanoids

Prostacyclin (or prostaglandin I2) and thromboxane A2 belong to the prostanoid family, and are produced from arachidonic acid metabolites. Prostacyclin is a potent vasodilator and inhibitor of platelet activation, while thromboxane A2 has opposite effects[36]. The vascular ECs produce

prostacyclin, which acts on both systemic and pulmonary vascular smooth muscle cells, as well as on circulating platelets and other cells, via the cyclic adenosine monophosphate (cAMP) pathway[37].

Prostacyclin plays a significant role in antiproliferative, antithrombotic, antimitogenic and immunomodulatory activity[37]. In PAH patients, the expression of prostacyclin synthase in the pulmonary arteries is reduced, and therefore the production of prostacyclin in ECs is decreased. In fact, patients with PAH have reduced endogenous prostacyclin[38].

Therefore, prostacyclin analogues are an established PAH therapy, which mimic the prostacyclin signaling pathway and are able to induce vasodilation while inhibiting platelet activation[37].

Endothelin-1

Endothelin-1 (ET-1), a 21-amino acid vasoactive peptide, is expressed in numerous mammalian tissues in different types of cells. ET-1 regulates the vascular tone and acts through interaction with two different types of receptor: ET receptor type A (ET_A) and ET receptor type B (ET_B). These receptors belong to the G-protein-coupled receptor family.

Both receptors have a vasoconstrictor effect when activated in PASMC. However, PAECs don't express ET_A, and ET_B activation leads to vasodilatation[39]. Overexpression of ET-1 is observed in endothelial cell dysfunction, which contributes to decrease the synthesis of NO and prostacyclin, and consequent worsening of the vasoconstriction response. Inflammatory responses and increased fibrosis are also the result of an upregulation of ET-1. In patients with PAH, ET-1 clearance by the pulmonary vasculature is decreased, and its plasma concentrations are raised and relate with PAH harshness and prognosis[40,41]. Nowadays, ET receptor antagonists are used as treatment for PAH, differing in their selectivity for ET_A and ET_B receptors. The therapies targeting this pathway can be dual, if they block both receptors, or selective if they only block ET_A[39,42].

Endothelial nitric oxide

Nitric oxide, a gaseous molecule, can be produced from L-arginine in mammalian tissues via activation of one of the NO synthase isoforms. NO is a vasodilator that controls several physiological processes, and is also able of preventing leukocyte adhesion, platelet aggregation, thrombus formation and vascular proliferation[37]. Endothelial NO synthase can be activated either by G-protein-coupled receptor signal transduction, Akt signaling, VEGF and hormonal stimuli (e.g. estrogen and insulin)[39].

In both PAH animal models and humans, pulmonary vascular endothelial NO synthase activity is decreased, along with NO bioavailability decay. The latter is connected to impaired endothelium-dependent and -independent vasodilatation, improved PASMCs mitogenesis and platelet aggregation[39,43].

Phosphodiesterases

Cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) are degraded by phosphodiesterases (PDE). Nowadays, eleven isoforms of PDE are known in mammalian tissues[44]. PDE-5 was found in high concentrations in PASMCs, platelets and myocytes, gathering interest to PAH studies.

PDE-5 regulates cGMP bioactivity via hydrolysis of cGMP to GMP. The allosteric binding of cGMP to PDE-5 induces a conformational modification to the enzyme structure, and positively feeds back to promote metabolism of cGMP[39]. In PAH, the expression of PDE-5 is increased in PASMCs and RV myocytes[45,46], as a result of decreased levels of NO, pulmonary vascular dysfunction and impaired RV lusitropy[47]. As the vasodilatory activity of NO in PASMCs is accomplished via cGMP signaling pathway, PDE inhibitors are used in the treatment of PAH, since they can delay the enzymatic breakdown of cGMP, and thus have vasodilator effects[37].

Mitochondrial dysfunction and voltage-gated potassium channels (Kv channels)

In PAH, likewise to cancer, smooth muscle cell mitochondria have repressed glucose oxidation and increased cytoplasmic glycolysis, in line with inhibition of pyruvate dehydrogenase[48]. The decreased pyruvate metabolism slows down the Krebs cycle and the electron transport chain (ETC), which is accompanied by reduction of reactive oxygen species (ROS) and α-ketoglutarate. The decreased synthesis of ROS inhibits membrane Kv channels, resulting in the increase of intracellular calcium[49]. These changes are caused by various stimuli, such hypoxia or anorexigens[49], and contribute to the activation of the hypoxia-inducible factor-1 alpha (HIF-1α)[50] and the NFAT[51]. HIF-1α and NFAT contribute to the downregulation of Kv channel expression and to the reduction of mitochondrial factors and enzymes, leading to the suppression of glycolysis and the remodeling process in smooth muscle cells that is typical in PAH. Some Kv channels are downregulated in the smooth muscle cells of PAH patients[52], and consequently gene transfer of these types of channels has been used in animal models, as an experimental strategy to avoid and/or attenuate PAH[53]. Furthermore, the inhibition of pyruvate dehydrogenase through dichloroacetate administration seems to reverse mitochondrial irregularities and Kv channel dysfunction, leading to reversion of pulmonary vascular remodeling[48,54].

Serotonin and Rho proteins

Serotonin (5-hydroxytryptamine, 5-HT) is a vasoconstrictor that can induce hypertrophy and hyperplasia in PASMC[36]. The 5-HT transporter (5-HTT) enables the induction of proliferation by carrying 5-HT into PASMCs, while the 5-HT_{1B} receptor mediates vasoconstriction. Both processes contribute to PAH pathogenesis[55]. Patients with PAH usually present increased plasmatic concentrations of 5-HT[56].

Rho proteins, especially Rho protein A, regulate cellular functions, such as contraction, migration, proliferation and apoptosis. Rho kinases have been implicated in PAH vasoconstriction and vascular remodeling[57]. During the PAH progression, 5-HTT-mediated PASMC proliferation and platelet activation involve the signaling pathway of these proteins[58]. 5-HT-induced proliferation re relies on the activation of Rho kinase by $5\text{-HT}_{1B/D}$ receptors, triggering nuclear translocation of extracellular signal-regulated kinases (ERK) and the activation of GATA4-dependent transcriptional pathway that is involved in cell proliferation[59].

Genetic mutations

If not associated with other clinical condition or induced by toxins, PAH can be either idiopathic or heritable. This disease segregates an autosomal dominant trait with a markedly reduced penetrance, since only 10-20% of individuals that carry the mutation will develop PAH[60]. The bone

morphogenetic protein receptor type 2 (BMPR2) gene encodes for a serine/threonine receptor kinase that belongs to the family of transforming growth factor beta (TGF-β). It is considered the major responsible for the development of 75% of heritable PAH and approximately 25% of iPAH[61,62]. Mutations in BMPR2 cause an aberrant signal transduction in PASMC, resulting in an imbalance between apoptosis and proliferation in favor of the latter[63]. The pathway downstream of BMPR2 activation is tightly regulated by the small mothers against decapentaplegic (SMAD) cascade, which begins with the phosphorylation of SMAD1, 5 and/or 8 that bind to SMAD 4 and enter the nucleus to drive transcription[64]. SMAD proteins also can regulate microRNA splicing through a non-transcriptional mechanism[65]. Thus, this signaling pathway regulates smooth muscle differentiation state by either direct transcriptional regulation of genes involved in the maintenance of a differentiated and contractile state or by regulation of miRNAs[66].

Other two PAH predisposing genes are activin-like kinase type I (ALK1) that codes for ALK1 receptor, present in ECs, and endoglin, and are most common in patients displaying hereditary hemorrhagic telangiectasia[67,68].

Interestingly, all genes mentioned above encode proteins involved in the TGF-β signaling pathway, that controls growth, differentiation and apoptosis in different cell types and may be a trigger for pulmonary vascular remodeling[69].

Recently, Ma *et al.*[70] identified the potassium channel subfamily K member 3 (KCNK3) as a novel gene in PAH pathogenesis. KCNK3 codes for a distinct family of mammalian potassium channels, non-voltage-dependent, that seems to have a crucial role in the regulation of resting membrane potential and pulmonary vascular tone. In PAH, KCNK3 is important in the vascular remodeling process, since it prevents or attenuates apoptosis. Moreover, the presence of loss-of-function mutations can lead to vasoconstriction, through the depolarization of the resting membrane potential.

1.4.4. Right ventricle

The integrity of RV function, rather than the degree of vascular injury, is the major determinant of prognosis in PAH[71]. The abnormal changes that occur in the pulmonary arteries of PAH patients, at first lead to vessel narrowing and/or obstruction, which then results in a progressive increase in PVR and mPAP[72].

In a healthy heart, the RV, which differs anatomically from the left ventricle (LV), can adapt and respond to an increase in load with an increase in contractility since its thin wall, crescent shape and greater compliance give the RV the ability to adapt rapidly to changes in volume and pressure load[71].

In PAH patients, the RV copes with increased afterload and with an enhanced contraction and a concentric RV remodeling, while the right atrium pressure remains normal. The rise in ventricular pressures increases diastolic and systolic stretch on the RV wall, which firstly leads to an increase in muscle mass – adaptive hypertrophy – due to increased protein synthesis and cardiomyocyte size. However, if the pressure overload is maintained, the RV cannot sustain the adaptive

hypertrophy and eventually dilates, without any increase in RV contractility, despite further increases in load, reaching a state called uncoupling of the RV[73].

The mechanisms involved in further adaptation of the RV and decline of its contractility are poorly understood. Apparently, it is associated with an imbalance between oxygen supply and demand[74], increased chronic sympathetic activation[75], oxidative and nitrosative stress, immune activation and cardiomyocyte apoptosis[73].

The increase in ventricular volume may also lead to tricuspid regurgitation, resulting in further RV volume overload and functional decline. The latter is accompanied by an increase in RV contraction time and ventricular asynchrony, together with a reduction in RV stroke volume, leading to underfilling of the left ventricle[76]. Impaired left ventricular filling in concert with RV dysfunction contributes to the evident decline in CO seen in severe cases of PAH and, if not interrupted, this circle of events ends in right HF and death[72].

1.5. Experimental animal models in PAH.

To better understand the pathophysiological mechanism and remodeling process behind PAH, and discovery of novel therapeutic agents, a variety of animal models have been developed and characterized. These experimental *in vivo* models mimic certain histological and molecular characteristics seen in PAH pathophysiology in humans. These include endothelial dysfunction, muscularization of previously non-muscular arterioles and increased medial thickness of normally muscularized arterioles, *in situ thrombosis* and plexiform lesions appearance[77]. Currently, there are several techniques available to induce PAH-associated alterations in animals, such as chemical agents[78,79], genetic manipulation[80,81], environmental factors[82] and surgical procedures[83] (Table 2). Currently, monocrotaline (MCT) administration and chronic exposure to hypoxia are the most widely used models of PH in translational research, due to good reproducibility and well described histopathology.

Although no animal model completely presents all the features of human disease, they may correlate with milder forms of human PH, a phase that is frequently missed at the time of diagnosis[84]. Nevertheless, animal models have experienced major developments and improvements over the years, and multiple-pathological-insult models appear to correlate better with PH in humans. All the models have contributed to a better understanding of PAH and development of novel therapies[84,85].

Table 2 - *Experimental animal models of pulmonary arterial hypertension*

Animal model	Species	Histological Features	Advantages	Disadvantages
MCT	Sheep; dog; rat	Medial hypertrophy; muscularization of non-muscular arteries; vascular inflammation	Severe PH; RV failure; predictable and reproducible	Toxic stimulus; no plexiform lesions
MCT + pneumectomy	Rat	Medial hypertrophy; muscularization of non-muscular arteries; neointima formation	Severe PH; RV failure; proliferation of endothelial cells	Toxic stimulus; difficult manipulation
***BMPR2* knockout**	Mouse	↑ Muscularization	Well-suited to study the genetic factors that contribute to PH	Homozygous knockouts die *in utero*
Fawn-hooded rat	Rat	↑ Muscularization	Well-suited to study the genetic factors that contribute to PH; Presence of plexiform lesions	Presence of systemic hypertension
Overexpression of *S100A4*	Mouse	Plexiform lesions	Presence of plexiform lesions	Only 5% of S100A4 overexpressing mice develop PH
Chronic hypoxia + SU5416	Rat	Medial hypertrophy; muscularization of non-muscular arteries; neointima formation; plexiform lesions	Physiological stimulus; proliferation of endothelial	Not clear which group it mimics
Schistosomiasis	Mouse	Perivascular inflammation; medial thickening; formation of plexiform-like lesions	Comparable to human schistosomiasis associated with PAH	Not significant PH
Left-to-right shunt	Sheep; pig; dog; rat	Medial hypertrophy; ↑ VSMC proliferation; intimal proliferation; plexiform lesions can be seen	Imitate the formation of plexiform lesions in human severe PAH	Sophisticated surgical approaches; pathological alterations can appear at a late stage
Closure of the ductus arteriosus	Fetal and newborn lambs	Medial hypertrophy; muscularization of non-muscular arteries; adventitial fibrosis	Relatively large size of the fetal lamb; uterine surgical intervention is well tolerated	Sophisticated surgical approaches; pathological alterations can appear at a late stage

BMPR2: bone morphogenetic protein receptor type 2 gene; **PAH**: pulmonary arterial hypertension; **PH**: pulmonary hypertension; **RV**: right ventricle; **S100A4**: S100 calcium-binding protein A4 gene; **SU5416**: vascular endothelial growth factor receptor inhibitor; **VSMC**: vascular smooth muscle cells. Adapted from Santos-Ribeiro *et al.*[21].

2. Metabolic abnormalities in PAH

As previous mentioned, PAH is a highly morbid and fatal disease and it's considered an arteriopathy of small-to-medium-sized pulmonary arteries[16,18,86,87]. However, PAH is progressively being recognized as a systemic disease with a preference for the pulmonary vasculature and RV[88].
Besides that, iPAH has been associated to various systemic metabolic imbalances. Previous studies emphasized the role of insulin resistance, glucose intolerance, and the metabolic syndrome in PAH[89-92]. The main alterations are in aerobic glycolysis, fatty acid oxidation (FAO), and the tricarboxylic acid (TCA) cycle[93]. These metabolic changes lead to the progression of PAH and development of lipotoxicity in the RV[94] (Fig. 2).

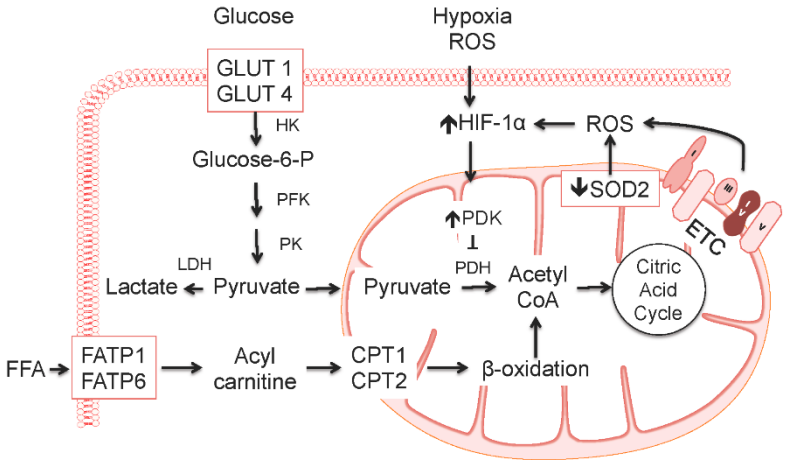

Figure 2 - Metabolism in PAH. Metabolism in PAH is perturbed like what is observed in cancer. Glycolysis occurs when glucose is taken up by the glucose transporters 1 (GLUT1) and 4 (GLUT4), gets phosphorylated by hexokinase (HK), and goes through a series of reactions to produce pyruvate. Pyruvate is the substrate for pyruvate dehydrogenase (PDH) in the mitochondria to support glucose oxidation. Free fatty acids (FFA) are taken up by fatty acid transport protein-1 (FATP-1) and -6 (FATP-6) and transformed to acyl carnitines that are shuttled across the mitochondrial membrane by carnitine palmitoyltransferase-1 (CPT1) and transformed to acyl CoA by carnitine palmitoyltransferase-2 (CPT2). Acyl CoA is converted to acetyl CoA during β-oxidation. In PAH, aerobic glycolysis is intensified, due to normoxic upregulation of HIF-1α, which upregulates pyruvate dehydrogenase kinase (PDK) to inhibit pyruvate dehydrogenase, and epigenetic regulation of the superoxide dismutase 2 (SOD2) gene. PFK, phosphofructokinase; PK, pyruvate kinase; LDH, lactate dehydrogenase; ROS, reactive oxygen species; ETC, electron transport chain. Adapted from: *Molecular Mechanisms of Pulmonary Vascular Remodeling in Pulmonary Arterial Hypertension*.

2.1. Metabolic changes in the pulmonary vasculature

In 1924, Otto Warburg discovered that tumor cells convert glucose to lactate even in sufficiently oxygen, sustaining the mitochondrial oxidative phosphorylation. Since then, this metabolic abnormality is called Warburg effect[95]. Various studies discovered that PAH and cancer have multiple similarities, namely dysregulated angiogenesis, development of antiapoptotic activity and, primarily, the Warburg effect[96,97]. Recently, the Warburg effect was purposed to be responsible for mitochondrial changes in PAH, in which mitochondria play an important role in adenosine triphosphate (ATP) production through oxidative phosphorylation[98]. In addition, the Warburg effect plays a significant role in PAH, due to the contribution in the abnormal proliferation of PASMCs and endothelial dysfunction[99]. However, it isn't known if this effect is involved in the genesis or progress of PAH.

2.1.1. Warburg effect in PASMCs

In PAH occurs up-regulation of growth factors[100], as well as a metabolic shift[101], which involves the recruitment of inflammatory and immune cells, increasing the expression of cytokine and chemokines[102]. This increase causes mitochondrial membrane hyperpolarization and subsequent hyperproliferation of PASMCs and apoptosis resistance. Some studies haves shown that both SIRT3/STAT3/NFAc2/Kv and PDGF/HIF-1α signaling pathways are involved in the Warburg effect in PASMCs hyperproliferation[103].

Via SIRT3/STAT3/NFATc2/Kv signaling pathway

Sirtuin3 (SIRT3) is the most mitochondrial sirtuin characterized so far[104]. SIRT3 belongs to NAD^+-dependent histone deacetylase family[105], which regulates the mitochondrial function through deacetylation activity that activates multiple ETC complexes and enzymes. SIRT3 compromises oxidative phosphorylation and stimulates glucose uptake and glycolysis (Warburg effect)[104]. SIRT3 is also an important regulator of metabolic homeostasis in PASMCs, since a lack of SIRT3 promotes the development of PAH through the increase of acetylation of ETC complexes and enzymes, suppressing the mitochondrial oxidative phosphorylation[106] and inhibiting mitochondria-dependent apoptosis[104]. Downstream mitochondrial signaling it promotes the activation of proliferative and antiapoptotic transcription factors that are vital in human and animal PAH, including the signal transducer and activator of transcription 3 (STAT3)[107] and the NFAT[108]. STAT3 is one of the seven STAT family isoforms and the most important in cardiovascular disease[109]. Mitochondrial STAT3 increases the activity of complexes I and II of the respiratory chain in a transcription-independent manner[110,111]. The STAT3 target gene is the provirus integration site for Moloney murine leukemia (PIM1). Curiously, PIM1 activation enhances the NFAT isoform 2 (NFATc2) [109]. NFATc2 is a master activator of T cells and stimulates the transcription of several inflammatory mediators in T and B cells[112]. NFATc2 plays a crucial role in promoting proliferation and apoptosis inhibition in PASMCs of PAH, due to its role in glycolysis and regulation of glycolytic enzymes transcription[104]. Besides, the NFATc2 target gene KCNA5 encodes the voltage-gated potassium channel (Kv1.5), and KCNA5 mRNA levels are reduced in SIRT3-KO PASMCs[104]. As Kv1.5 regulates the resting plasma membrane potential in PASMC, its loss or inhibition causes plasma membrane depolarization and Ca^{2+} influx, which lead to an increase in Ca^{2+} that further enhances NFATc2 activation within a positive feedback loop, contributing to its sustained activation[113].

Via PDGF/HIF-1α signaling pathway

Platelet-derived growth factor (PDGF) acts as a chemokine via activation of two PDGF receptor (PDGFR) subunits, PDGFRα and PDGFRβ, which promotes several cellular responses, such as proliferation, migration, cellular survival and transformation, in various types of cells[114,115]. PDGF binds to its receptor, leading to the dimerization of two receptor subunits and, consequently, increases the activity of receptor's tyrosine kinase, which results in a receptor trans- or auto-phosphorylation. The complex formed by PDGF and PDGFRs leads to activation of receptor-associated signaling molecules and triggers downstream signal cascades, facilitating PDGF-dependent cellular responses, such as protein synthesis, proliferation, migration, apoptosis resistance and cellular transformation[114-116].

The lungs of both MCT- and hypoxia-induced experimental PAH animals and PAH patients revealed that PDGF and its receptor are up-regulated[116,117]. This feature was also observed in PASMCs[117,118]. Persistent activation of PDGF causes pulmonary vascular remodeling and PAH in mice with chronic hypoxia-induced PAH[119]. PDGFRβ signaling enhancement and PDGF-dependent proliferation caused by chronic hypoxia-induced by PDGF signaling have been observed in human PASMCs [120]. Frequently, hypoxia is present within lungs of PAH patients and it's a modulator of RTK signaling, activating hypoxia-inducible factor 1 (HIF-1). This transcriptional factor is an important modulator of hypoxia transcriptional responses, having a crucial role in the progression of PAH[121,122]. HIF-1 is a heterodimeric protein with two subunits, HIF-1α and HIF-1β[122,123]. The subunit HIF-1α is a prolyl hydroxylase that bind to von Hippel-Lindau tumor suppressor protein (VHL), under normoxia conditions. The complex HIF-1α/VHL promotes the HIF's ubiquitination and degradation[120,122]. It is known that HIF-1α controls the proliferation of PASMCs via two signaling pathways, such as PDGF signaling or via Warburg Effect[120]. In the first pathway, the PASMC hyperproliferation verified, occurs as consequence of hypoxia, which increase the PDGF signaling in PA by HIF-1α- dependent down-regulation of protein tyrosine phosphatases (PTPs) expression and activity. PTP, a PDGF antagonist, promote the constant activation and phosphorylation of PDGF, leading to proliferation of PASMC[120]. The other pathway implicates the Warburg Effect and activation of glycolytic genes through HIF-1α, increasing the conversion of glucose to pyruvate and consequently in lactate as an important hypoxia adaptor. HIF-1α also suppress actively the metabolism of TCA cycle, directly activating the gene encoding pyruvate dehydrogenase kinase 1 (PDK1). PDK1 inhibits pyruvate dehydrogenase (PDH), an enzyme of TCA cycle, leading to the interruption of mitochondrial oxidative phosphorylation. This increase of PDK1 expression leads to an enhancement of ATP levels and decreases ROS synthesis by attenuating cells oxidative metabolism. These decrease leads to an inhibition of Kv channels, promoting potassium accumulation[124,125]. High concentrations of intracellular potassium inhibit caspases and apoptosis, along with cellular depolarization. Membrane depolarization enhances L-type calcium channels, permitting calcium influx. The increased calcium levels trigger calcineurin, which dephosphorylates NFATs. Dephosphorylated NFATs are active and works as transcriptional factors inhibiting Kv channels and increasing mitochondrial B-cell lymphoma 2 (BCL-2) levels. BCL-2 causes hydrogen ions efflux from the mitochondria, resulting in its hyperpolarization, impeding pro-apoptotic molecules release and promoting cell hyperpolarization[98].

2.1.2. Warburg effect in endothelial dysfunction

The pulmonary vascular remodeling verified in PAH is mainly caused by excessive proliferation of ECs and PAEC dysfunction[126]. Recently, it has been confirmed that the Warburg effect is present in PAH, and studies in PAH patients and laboratory PAH animal models confirm that PAECs also switch glucose metabolism from glycolytic metabolism to aerobic glycolysis in PAH[127], and the metabolic shifts are analogous to cancer cell metabolism[128]. However, whether or how the Warburg effect mediates PAEC dysfunction in PAH is unknown. It has been shown that PAEC dysfunction involves HIF-1α, BMPR2 and caveolin-1 mediated via the Warburg effect.

BMPR2

BMPR2 is a member of the TGF-β superfamily of transmembrane serine/threonine kinase receptors. Signal transduction through the BMP pathway involves heterodimerization of BMPR2 with BMPR1, resulting in BMPR1 phosphorylation and activating signal transduction[129]. Newly, mutations in BMPR2 have been documented in patients with iPAH. BMPR2 signaling promotes survival in PAECs, and the lack of BMPR2 signaling improves PAECs apoptosis in PAH[127]. Some animal models of PAH show a shift towards aerobic glycolysis, modifications in glucose uptake and utilization, and changes in mitochondrial oxidative phosphorylation. The same observations were also seen and confirmed in the PAECs from patients with PAH[130]. Furthermore, during hypoxia-reoxygenation in PAH, reduced BMPR2 decreases mitochondrial biogenesis and ATP production and promotes mitochondrial deoxyribonucleic acid injury and PAEC apoptosis[131]. Interestingly, this feature has been observed in PAECs from PAH patients with BMPR2 mutations. BMPR2 gene mutations leads to an increase activity of isocitrate dehydrogenase, an enzyme of TCA cycle, in pulmonary microvascular ECs and serum samples of patients with PAH. These results indicate that BMPR2 mutations promote pulmonary microvascular ECs dysfunction via reprogramming cell metabolism in PAH[93].

Caveolin-1

Caveolin-1 gene belongs to the caveolin gene family and is extremely expressed in adipocytes, epithelial cells, fibroblasts and myocytes[132]. Caveolin-1 is a protein that interacts and negatively regulates other proteins, such as G-proteins and G-protein-coupled receptors, endothelial nitric oxide synthases (eNOS) and several growth factor receptors[132], Actually, caveolin-1 is an anchor within caveolae holding numerous proteins to regulate their function through its scaffolding domain[133]. Caveolin-1 deficiency plays a serious role in the pathogenesis of PAH. The lungs of caveolin-1 deficient mice exhibit PH and the hearts show right ventricle hypertrophy (RVH). Hemodynamic measurements also exhibit a significant increase in right ventricular contractility and diastolic function in the lungs of caveolin-1 knockdown mice[134]. Furthermore, lack of endothelial caveolin-1 has been described in clinical PAH patients and experimental PAH mouse models, resulting in ECs damage and dysfunction. Curiously, one study has reported that caveolin-1 inhibits eNOS by its interaction, and eNOS is predominantly expressed in endothelial cells[135]. The loss of caveolin-1 results in persistent eNOS activation and increased ROS production, further impairing PAECS in PAH[134].

Moreover, lack of caveolin-1 causes mitochondrial dysfunction, membrane hyperpolarization, and mitochondrial synthesize of oxidant species, which is considered the underlying cause of the metabolic switch or the Warburg effect in PAECs. The precise signaling pathway responsible for the loss of caveolin-1, which mediates PAEC dysfunction through the Warburg effect, has not been established. Though, absence of caveolin-1 in PAH activates eNOS, increases ROS, impairs mitochondrial function with a metabolic shift towards the Warburg effect, and ultimately results in PAEC hyperproliferative or dysfunction[136].

HIF-1α

HIF-1α is an oxygen-sensitive alpha subunit of HIFs, which regulate genes that control energy metabolism, vasomotor tone and angiogenesis[121]. Moreover, HIF-1α plays a pathologic role in PAH[122] and is directly involved in the metabolic shift of PAECs towards the Warburg effect[127]. Unusually, it has been demonstrated that HIF-1α expression and the downstream target gene VEGF play an important role in endothelial plexiform lesions, which suggests that HIF-dependent signaling contributes to the hyperproliferative of PAECs and metabolic abnormalities in PAH[137].

Curiously, irregularities of NO that are verified in PAECs with PAH may further contribute to the activation of HIF-1α in PAH and activate various intracellular signaling pathways[127]. Under hypoxia, HIF-1α is destabilized by low levels of NO, and the low concentrations of NO inhibit mitochondrial oxidative metabolism, which contributes to a cellular metabolic shift towards the Warburg effect[138]. In addition, cellular metabolism is inhibited by NO under hypoxia, which increases intracellular O2 availability and promotes degradation of HIF-1α. It is appealing to hypothesize that the lack of NO synthesis may promote HIF activation in PAH endothelial cells, which are likely all involved in the development of PAEC dysfunction via the Warburg effect[50,139].

2.2. Metabolic changes in the RV

While pathological changes in the pulmonary vasculature have been the outdated subject of most intensive study in PAH, structural remodeling of the RV means a much worse prognosis, thus suggesting its distinct importance to this disease[140]. As the disease syndrome progresses, the RV is forced to maintain CO in the face of mounting vascular resistance. This leads to RVH that, in turn, carries a greater oxygen requirement that cannot be met by coronary blood flow[140]. In cases of adaptive remodeling, RVH does not lead to RV failure; but in many PH patients, termed maladaptive remodelers, RVH is closely followed by a loss of RV function[140]. Current understanding of the mechanisms involved in RVH remains limited and, perhaps erroneously, relies heavily on data extrapolated from studies of the LV. Evidence of a Warburg phenotype specifically in the RV, however, has emerged and is consistent with prior knowledge of suppressed oxidative phosphorylation during LV overload[141]. Thus, these molecular events represent the first available clues that may help to elucidate the potential importance of metabolic dysfunction in the RV as it controls the clinical course of PH. In contrast with the pulmonary vasculature, 60–90% of ATP produced in the myocardium is derived from FAO. The other 10–40% is accounted for by glucose oxidation[140]. FAO and glucose oxidation share a mutually inhibitory relationship in tissue, as defined by the Randle cycle. This relationship is common to most muscle and adipose tissues and occurs without any form of hormonal mediation[142]. Confronted with an increased oxygen requirement and a decreased oxygen supply, it is thought that hypertrophic RV tissue experiences higher degrees of hypoxia, resulting in the activation of transcription factors HIF-1 and Myc[143,144]. As in the pulmonary vasculature, the activation of HIF-1 produces a glycolytic phenotype via up-regulation of glycolytic enzymes and suppression of glucose oxidation. This has been demonstrated by evidence of a glycolytic shift in several experimental models of RV dysfunction. For example, using positron emission tomography (PET), Oikawa *et al.*[145] reported that glucose uptake is unusually high in the RV in both human RVH patients and in animal disease models. It has also been demonstrated that

the glucose transporters 1 (GLUT1) and 4 (GLUT4), as well as the rate-limiting glycolytic enzyme HK-II, are all up-regulated in a variety of RVH models[140,146].

Moreover, Piao *et al.*[140] confirmed that rats experiencing MCT-induced RVH display increased activation of PDK1. FAO has also been shown to be impaired in severe cases of hypoxic PH and PAH, although this effect was not seen in milder forms of either disease. Importantly, FAO requires more oxygen per unit of ATP than glucose oxidation by about 12%[140]. It is likely that myocardial FAO is unable to compensate for the ATP production lost when glucose oxidation is suppressed, even in cases where FAO itself is relatively spared. Because anaerobic glycolysis is inherently less efficient for ATP generation than oxidative phosphorylation, it has been proposed that insufficient energy production induces RV hibernation as seen in late-stage RVH[140]. This idea is supported by studies that contrast adaptive and maladaptive RV remodeling, such as those demonstrating that p38-MAPK, a promoter of glycolysis, is increasingly activated by maladaptive, but not the adaptive, RV alterations[147].

3. Neuregulin-1

3.1. Neuregulin and their receptor

Neuregulins (NRGs) are a subclass of transmembrane polypeptide growth factors belonging to the EGF family. Normally, these proteins are expressed in the nervous system, the cardiovascular system, mammary glands, the intestine and kidneys[148]. It exists four different genes, NRG1 to NRG4, which codes for distinct NRG proteins. Every gene contains various promotors and, along with alternative splicing sites, it results in more NRG protein variants. In this thesis, NRG1 will be the focus due to its recognized effects in the cardiovascular system.

NRG1 release occurs at the plasma membrane when the N-terminal ectodomain of pro-NRG1 undergoes proteolytic cleavage by specific proteases, namely b-secretase 1, and a disintegrin and metalloproteinase domain containing protein. In addition, NRG1 mRNA can be alternatively spliced, resulting in many different isoforms[149]. The final biological activity of these protein isoforms is defined by functional differences and actions through paracrine or juxtacrine communication pathways[150]. The NRG1 mRNA expression and protein synthesis are mediated by neurohormones (e.g., angiotensin II and phenylephrine inhibit and ET-1 stimulates NRG1 mRNA expression) and by mechanical strain, which rises NRG1 expression[151].

NRGs bind to erythroblastic leukaemia viral oncogene homolog (ErbB) monomeric receptors, specifically to ErbB3 and ErbB4 (also known as human EGF receptor, HER3 and HER4). Ligand binding triggers these receptors and results in their dimerization. The receptor dimerization results in homodimers or heterodimers (with ErbB2), which activates signaling cascades, like the pathways of the mitogen-activated protein kinase (MAPK) and of the phosphatidylinositol-3-OH kinase (PI3K)/Akt. These pathways affect cell survival strategies and migration, proliferation, adhesion and differentiation properties[150,152].

3.2. NRG1 and its receptors in the cardiovascular system

In the heart, NRG1 is expressed and released by the endocardial and microvasculature endothelium. ErbB4 and ErbB2 are expressed in cardiomyocytes, while ErbB3 is expressed in mesenchymal cells of the endocardial cushion[151,153,154]. The differential expression of the solubilized ligand and receptors involves the paracrine transduction pathway associated with this signaling axis in the heart[151]. The presence of different receptors in distinct cell types regulates the phenotypes observed in the various knockout (KO) models. The paracrine pathway through which NRG1 acts between cardiac EC and cardiomyocytes has been confirmed in co-culture studies[151], pointing to the fact that NRG1 is a newly discovered contributor in cardiac endothelial–myocardial signaling, regulating the cardiovascular function[155].

3.2.1. NRG1/ErbB signaling and cardiac development

The gene deletion studies in mice have demonstrate the role of the NRG1/ErbB system in cardiac trabelucation and myocardial development. NRG1 KO mice die during mid-embryogenesis as a consequence of the absence of normal trabeculation of the ventricles[154]. Also, ErbB2 or ErbB4 KO mice have a cardiac phenotype identical to NRG1 KO mice[156,157]. This cardiac phenotype seen in these gene mutants suggests the direct connection between ligand and receptor in the heart. It is suggested that NRG1 signaling needs ErbB2-ErbB4 heterodimers due to neither ErbB2 or ErbB4 alone compensate the loss of the other receptor in the heart[158]. Moreover, ErbB3 KO mice exhibit cardiac cushion abnormalities, causing the reflux of blood through defective valves. This leads to embryo death, as shown for NRG1 KO and ErbB2 KO mice[159]. Besides that, *in vitro* NRG1 converts embryonic cardiomyocytes into cells of the conduction system[160,161] and induces differentiation and survival of cardiomyocytes derived from embryonic stem cells[162]. Additionally, NRG1β/ErbB signaling regulates the ratio of nodal- to working-type cells in differentiating hESC-CM (human embryonic stem cell-derived cardiomyocytes) cultures and presumably functions equally through early human heart development[163].

3.2.2. NRG1/ErbB signaling in cardiac cellular responses

Some *in vitro* studies performed with postnatal and adult cardiomyocytes have demonstrated that recombinant NRG1 administration promotes cell survival regulation[164,165], hypertrophy, proliferation[165,166], myofibrillar organization[167] and cell-to-cell contact among cardiomyocytes[168]. NRG1 released from ECs protects cultured cardiomyocytes from apoptotic cell death induced by oxidative stress and anthracyclines[164,169,170]. NRG1 prevents cardiomyocyte apoptosis by inhibiting the release of cytochrome c and the activation of caspase-3 through a PI3K/Akt-dependent signaling pathway[164,170]. Furthermore, inhibition of ErbB2 and ErbB4 receptors leads to induction of Bcl-x splicing to its pro-apoptotic protein beta-adrenergic receptor, resulting in mitochondrial dysfunction and apoptosis[171]. Treatment of cultured adult rat ventricular myocytes with NRG1 increases cardiomyocyte proliferation through ErbB4 signaling [172]. In addition, NRG1 induces myocyte hypertrophy in an ErbB2-, 70-kDa protein kinase (p70S6K) and MAPK-dependent manner[151,166]. The NRG1 can also modulate the structure organization of cardiomyocyte. NRG1 treatment induces cytoskeletal and sarcomeric organization[166,168] and prevents myofibrillar disorganization in response to anthracycline damage[167] (Fig. 3).

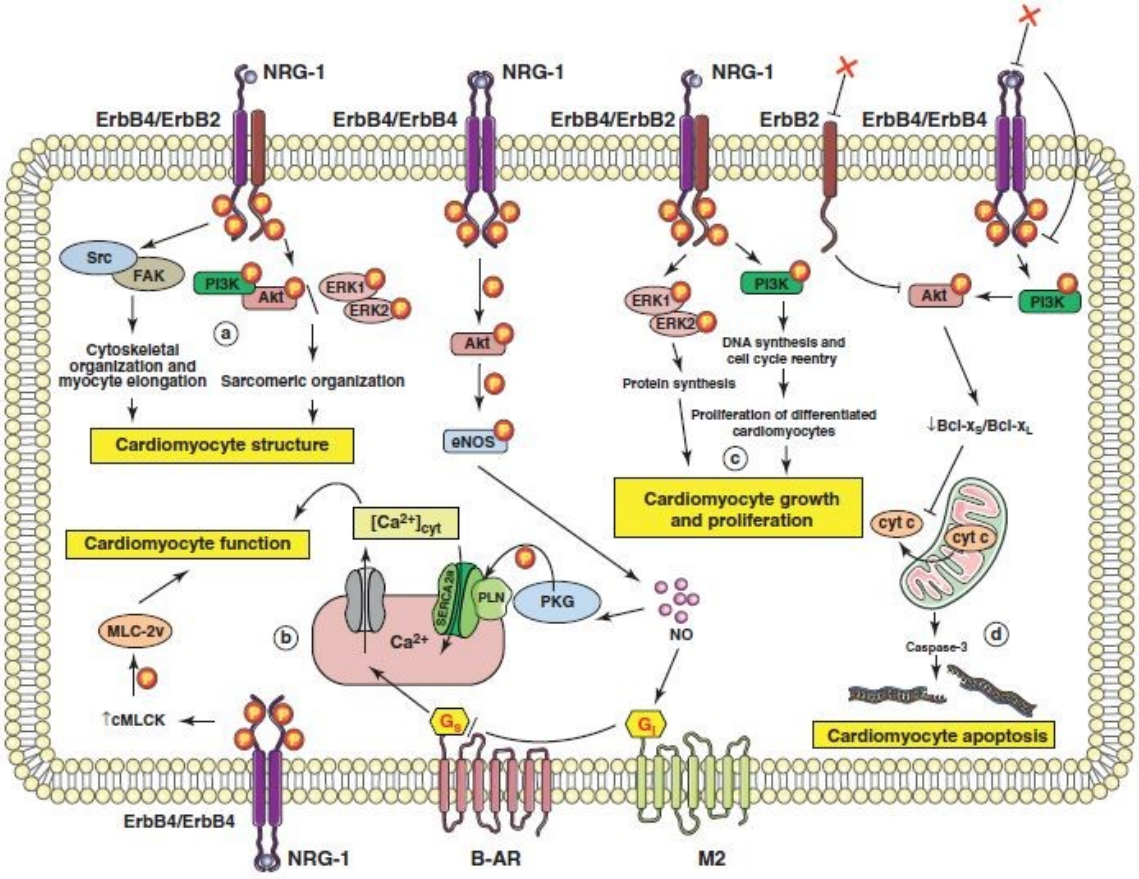

Figure 3 - NRG1/ErbB signaling in the cardiomyocyte. NRG1 regulates cardiomyocyte structure (a), function (b), growth, proliferation (c) and survival (d). (a) NRG1 stimulates myocyte lamellipodia formation and longitudinal elongation, in an ErbB2/FAK/Src-dependent way, enabling cellular adhesion and cell-to-cell contact between cardiomyocytes[168]. PI3K/Akt and ERK1/2 activation occur adjacently to the NRG1-stimulated sarcomeric organization, in healthy[166] and cardiotoxic[167] situations. (b) eNOS activation, through NRG1/ErbB4-mediated Akt phosphorylation leads to an increase in NO production. NO can modulate exacerbated adrenergic stimulation (enhancing muscarinic receptor antiadrenergic actions)124 and activate PKG, which in turn phosphorylates PLN leading to increased SERCA2a activity and calcium uptake by the sarcoplasmic reticulum[173]. This contributes to improved cardiomyocyte function in healthy conditions, and in conditions with excessive b-adrenergic stimulation. In response to ischemic injury NRG1 leads to increased cMLCK expression and MLC-2v phosphorylation, improving cardiomyocyte functional recovery[174]. (c) NRG1/ErbB signaling induces protein synthesis and cardiomyocyte growth through ERK1/2 signaling [166]. Differentiated, mononucleated cardiomyocytes can re-enter the cell cycle and proliferate in response to NRG1 stimulation[172]. Moreover, in conditions of ischemic injury, NRG1 can improve myocardial structure by decreasing hypertrophy[172]. (d) NRG1 protects cardiomyocytes from apoptosis in an ErbB/PI3K/Akt- dependent manner, by regulating Bcl-x splicing, cytochrome c release and capase-3 activation[164,170,171]. Abbreviations: Akt, protein kinase B; B-AR, beta-adrenergic receptor; Bcl-xS/L, b-cell lymphoma xS/xL; cMLCK, cardiac myosin light chain kinase; cyt c, cytochrome c; eNOS, endothelial nitric oxide synthase; ErbB2/4, erythroblastic leukemia viral oncogene homolog 2/4; ERK1/2, extracellular signal-regulated kinases1/2; FAK, focal adhesion kinase; Gi, inhibitory guanine nucleotide binding protein; Gs, stimulatory guanine nucleotide binding protein; M2, muscarinic receptor; MLC-2v, myosin light chain 2v; PI3K, phosphoinositide-3- kinase; PKG, protein kinase G; PLN, phospholamban; SERCA2a, sarcoplasmic reticulum calcium ATPase; Src, proto-oncogene tyrosine-protein kinase. Adapted from Mendes-Ferreira *et al.*[175].

3.2.3. NRG1/ErbB signaling in cardiac and vascular function

Up-to-date experimental evidence reveals that NRG1 regulates myocardial performance and sympathovagal balance, suggesting that it is involved in hemodynamic homeostasis of the cardiovascular system. NRG1 desensitizes the cardiac muscle for the inotropic action of isoproterenol, providing modulatory feedback for the autonomic imbalance present in acute cardiac stress and in chronic HF[176]. This effect is dependent upon the activation of eNOS[176]. eNOS and β-adrenergic receptors are translocated to the caveolae, where eNOS attenuates adrenergic stimulation while increasing excitation–contraction coupling. Interestingly, ErbB receptors are co-localized with eNOS in the caveolae[177], allowing a better spatial relationship between NRG1 activation of ErbB receptors, and a downstream signaling leading to its NO-mediated, anti-adrenergic cardioprotective effects. Additionally, cardiomyocytes deficient in NRG1 signaling are unable to adequately counterbalance β-adrenergic activation by inhibitory parasympathetic activity[178]. This mechanism might contribute to the known increased risk of HF in injured human hearts where NRG1/ErbB signaling is suppressed. NRG1 acutely enhances NO production in adult ventricular myocytes through the activation of the PI3K/Akt pathway. The increase in NO leads to cGMP-dependent protein kinase (PKG) activation with the phosphorylation of phospholamban, which increases calcium uptake by the sarcoplasmic reticulum. This effect reveals an additional protective role of NRG1, through diastolic calcium handling[173] (Fig. 3).
Recent studies support a role for NRG1 in vascular function (Fig. 4).

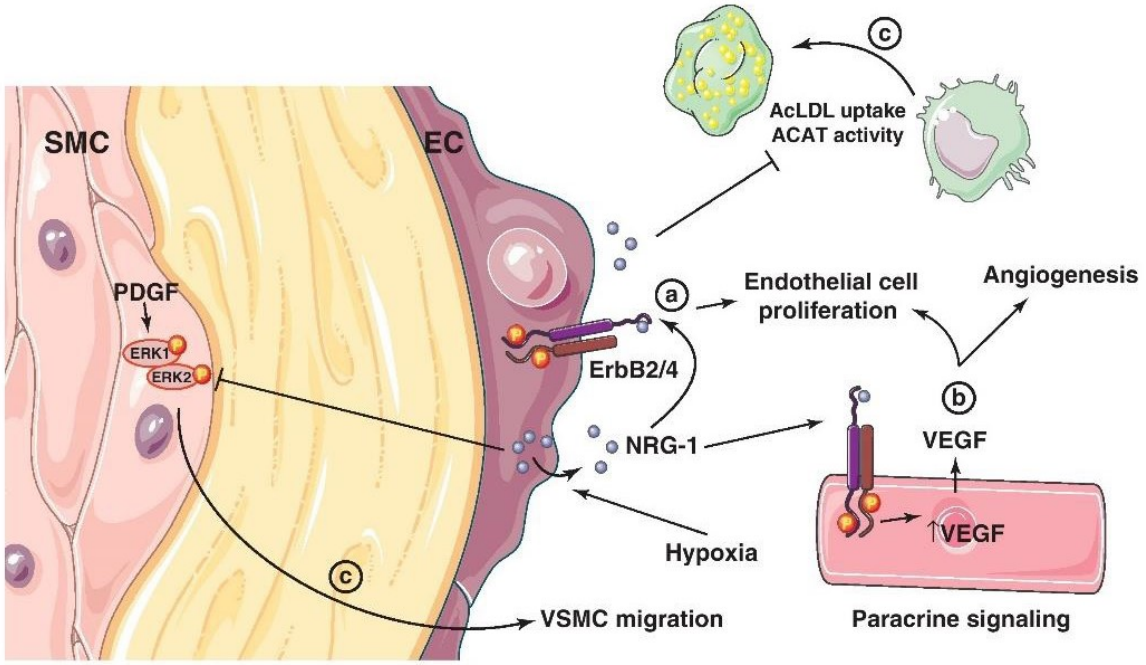

Figure 4 - ***NRG1 signaling in vessels.*** NRG1 is present in ECs and its release is increased in response to hypoxia133. However, there are contradictory results regarding the presence of ErbB receptors in ECs. Autocrine signaling (a) induces endothelial cell proliferation[179]. By contrast, later studies suggest that the effects of NRG1 on endothelial cell proliferation and angiogenesis are mediated through paracrine actions (b), via VEGF production[180,181]. Besides inducing EC proliferation and new vessel formation, NRG1 inhibits pathologic VSMC migration[182] and foam cell formation, through decreased uptake of AcLDL and ACAT activity[183], important mediators in the atherogenic process (c). Abbreviations: ACAT, acyl-coenzyme A: cholesterol acyltransferase; AcLDL, acetylated low-density lipoprotein; EC, endothelial cell; ErbB2/4, erythroblastic leukemia viral oncogene homolog 2/4; ERK1/2, extracellular signal-regulated kinases1/2; PDGF, platelet-

derived growth factor; SMC, smooth muscle cell; VEGF, vascular endothelial growth factor; VSMC, vascular smooth muscle cell. Adapted from Mendes-Ferreira *et al.*[175].

NRG1 is expressed in vascular ECs[180] and stimulates angiogenesis *in vitro* (collagen gel tube formation) and *in vivo* (rat corneal angiogenesis and chick embryo chorioallantoic membrane models)[179,181]. NRG1 has a role in angiogenesis not only in normal physiologic conditions but also in response to tissue damage. NRG1 is required for proper angiogenesis and arteriogenesis during ischemic injury[182]. Additionally, NRG1 is expressed in atherosclerotic lesions[184] and is associated with the inhibition of neointimal formation and mitogen-induced VSMC proliferation and migration after vascular injury[185]. These studies suggest that NRG1 could be a novel therapeutic candidate for the prevention of neointimal formation in diseases such as atherosclerosis and restenosis.

3.2.4. NRG1/ErbB signaling in cardiovascular disease

In vitro studies have demonstrated a central role for NRG1 in the modulation of cell survival, growth and integrity, as well as intrinsic myocardial performance. *In vivo* experiments emphasized the integrative role of NRG1/ErbB signaling in the response to several physiologic and pathophysiologic stimuli[186]. The premature development of dilated cardiomyopathy by pressure overload in NRG1/ErbB deficient mice[187-189] suggest that, *in vivo,* NRG1 plays a protective function in the adult heart. Subsequent studies have reinforced the hypothesis that the NRG1/ErbB system is involved in the pathophysiology of several cardiac diseases, involving acute and/or chronic cardiac stresses. In response to acute ischemic damage, ventricular levels of NRG1 mRNA and protein, as well as phosphorylated ErbB4 receptors, are increased[190]. In endothelial cells, the deletion of NRG1 gene worsens ischemic injuries. The protective role of NRG1 in these conditions seems to be mediated by activation of cardiomyocyte eNOS[176] through MAPK and Akt-dependent signaling [191]. The activation of ErbB2 is required due to its role on cardiomyocyte proliferation and heart regeneration following myocardial infarction[192]. NRG1 also protects against doxorubicin (Dox)-induced cardiac toxicity. NRG1 and ErbB4 receptor knockouts show high sensibility to Dox treatment[193,194], and Dox-associated cardiomyocyte myofilament injury is increased when ErbB2 is inactivated[151,167]. NRG1 also reduced Dox-induced alterations of excitation–contraction coupling and attenuated oxidative stress in adult rat cardiomyocytes[195].

NRG1/ErbB signaling could also have a function during chronic cardiac metabolic stress in diabetes and during development of diabetic cardiomyopathy (DCM). In rats with type 2 diabetes, ErbB receptor activation in the LV and delayed cardiac muscle relaxation is observed. These cardiac muscles perturbations are reversed when treated chronically with NRG, but without insulin[196]. Thereby, in DCM, it is observed the reduction of ErbB2 and ErbB4 receptor expression and phosphorylation, as well as of NRG1 protein synthesis[197].

During the development of HF induction occurs an increase of ErbB2 and ErbB4 receptor phosphorylation concurrently with an enhancement of NRG1 and metallopeptidase domain 19 expression[198]. Also, an increased myocardial expression of NRG1 and ErbB4 phosphorylation is observed in the chronically stressed heart[199]. After experimental myocardial infarction, exercise training also results in increased NRG1 expression, as well as its downstream signaling [200].

In left ventricular hypertrophy secondary to aortic stenosis, ErbB2 and ErbB4 levels are downregulated at the stage where the LV begins to fail[201]. NRG1 expression is increased during the

initial concentric hypertrophic phase. NRG1 levels decrease upon development of left ventricular dysfunction and eccentric left ventricular hypertrophy. These changes are possibly explained by increased neurohormonal activation[151], which might be associated with decreased expression of both NRG1 and ErbB2 in the brainstem in animals with LV dysfunction secondary to aortic banding[202]. Administration of NRG1 directly into the brain prevented development of LV dysfunction and decreased noradrenaline excretion[202].

The animal studies propose that, in conditions of ventricular injury and dysfunction, activation of NRG1/ErbB signaling is part of an adaptive compensatory program that preserves cardiac function. In advanced stages of ventricular dysfunction, the activity of the NRG1/ErbB system is decreased. This maladaptive change is the result of reduced ventricular expression of NRG1, reduced expression of ErbB receptors and perhaps dysfunctional downstream ErbB signalling, Attenuation of compensatory NRG1/ErbB signaling could thus be an important event in the progression of HF.

Various studies suggest that, as in animal models, NRG1 signaling has a protective function in the human heart, and loss of this pathway might induce HF. These observations have been derived from the cardiotoxic side effects of the chemotherapeutic agent trastuzumab (Herceptin1), a monoclonal antibody directed against ErbB2 used to treat receptor-positive breast carcinoma. Exposure to trastuzumab can induce a clinically significant cardiomyopathy that frequently reverses after therapy interruption[203]. These discoveries propose the possible interaction of the NRG1 pathway with the cardiovascular system and represent the vulnerability of heart to unexpected modulations of such signaling cascades[204]. In addition, NRG1 levels are increased and expression of ErbB2/ErbB4 is decreased in ventricular tissue from patients with advanced HF. Curiously, these changes are reversed in patients with left ventricular unloading, due to a ventricular assist device[205].

A cohort of 899 patients with chronic HF showed an increase of NRG1 levels in serum, which correlates with disease severity (I–IV class of the New York Heart Association; NYHA), as well as risk of death or cardiac transplantation, identifying NRG1 as a possible biomarker of HF[206]. In addition, NRG1 expression by human macrophage foam cells was correlated with the progression of the atherosclerotic plaque[183]. Perik *et al*.[207], in a study with 50 patients, found that serum levels of ErbB2 were increased in patients with higher NYHA classes, lower left ventricular ejection fraction and higher levels of apoptosis-related cytokines. However, in a larger study, including 765 patients, ErbB2 serum levels were not associated with left ventricular end-diastolic pressure or ejection fraction[208].

3.2.5. NRG1/ErbB signaling in PAH glucose metabolism

NRG1 pathway is also involved in the regulation of glucose metabolism. Indeed, acute treatment with NRG1 induces translocation of the GLUT4 to the plasma membrane and stimulates glucose uptake in L6E9 muscle cells and rat soleus[209]. NRG1 action on glucose uptake is mediated by a signaling cascade involving the PI3K-PDK1-PKCz pathway, and this effect is additive to that of insulin[210]. NRG1-induced glucose uptake has also been observed in adult rat ventricular myocytes, and it was associated with phosphorylation of Thr202/Tyr204 by ERK1/2 and of Ser473 by AKT[153]. The finding that NRG1-induced AKT phosphorylation at Ser473 can be blunted by pretreating cardiac myocytes with palmitate[211] suggests that over-nutrition or hyperlipidemia could lead to

NRG1 resistance. Besides these acute short-term effects, incubation with NRG1 for 48h increases GLUT4 content in L6E9 muscle cells, suggesting that chronic NRG1 treatment could regulate muscle glucose metabolism and consequently systemic glucose homeostasis[212]. Conversely, NRG1 effects in liver, which also has a key role in glucose metabolism regulation[213], have been less investigated. It has been reported that ERBB3 and, to a less extent, ERBB4 are expressed in liver of adult rats[214-216]. Acute exposure of rat hepatocytes to NRG1 induces ERBB3 phosphorylation and activation of DNA synthesis[217]. Insulin seems to inhibit NRG1 action in rat hepatocytes. In two animal models of insulin deficiency (type I diabetes and fasting), liver ERBB3 expression is repressed following insulin treatment, suggesting an interaction between insulin and the NRG1/ERBB pathway[216].

Some studies evaluated the impact of NRG1 on glucose metabolism, *in vivo. In vivo* studies, a single injection of NRG1β was found to reduce blood glucose concentrations over 30–60 min and to increase blood glucose disposal over 60–90 min after treatment, as compared to vehicle treatment in diabetic mice. Glucose disposal was also increased after the NRG1β administration in diabetic mice[218]. Similarly, NRG1β was found to promote glucose disposal in normal and diabetic rats[219]. Consistent with these previous findings, Huang *et al.*[220] revealed that cimaglermin alpha (CGF2), a full-length variant of NRG1β, reduces blood glucose concentrations in pigs and mice and increases blood glucose disposal in mice. These data are consistent with and further support the previous reports on NRG effects on glucose uptake, and clearly indicate that systemic administration of pharmacologic doses of NRG1β affect blood glucose handling in mice and pigs.

The metabolic effects of NRG1 have not been completely clarified in the context of cardiovascular disease. So, further investigation of NRG1 effects on RV heart failure in PAH is deserved.

3.2.6. Preclinical studies with NRG1 as a therapy for heart failure

Several animal studies have shown the therapeutic effect of NRG1 in HF, with an improvement of cardiac performance, attenuation of disease markers and prolonged animal survival [172,174,190,221-224] (Table 3). These positive effects of NRG1 treatment have been related with the following mechanisms: (i) cardiomyocyte replacement[172]; (ii) limitation of myocardial cell damage[224]; (iii) organization of sarcomeric structure[174]; (iv) reversal of ventricular remodeling[223]; (v) promotion of angiogenesis[225]; (vi) attenuation of mitochondrial dysfunction[222]; (vii) reduction of oxidative stress[222]; (viii) prevention of apoptosis[225]; (ix) modulation of myocardial calcium homeostasis[195], and (x) attenuation of myocardial endoplasmic reticulum stress[190].

NRG1 attenuated pathologic alterations in experimental HF associated with numerous cardiovascular disorders[224] by promoting sarcomere organization, decreasing myocardial cell damage and, thus, improving cardiomyocyte integrity and performance, leading to improved left ventricular function and decreased mortality[224]. Cardiomyocyte mitosis might play an important role in these structural changes once NRG1 induces mononucleated cardiomyocytes to divide[172]. Additionally, *in vivo* genetic inactivation of ErbB4 decreases cardiomyocyte proliferation, while increasing of ErbB4 expression enhances it. Injecting NRG1 in adult mice induces cardiomyocyte cell-cycle activity and promotes myocardial regeneration, causing the function improvement after myocardial infarction[172], and lentiviral delivery carrying the human NRG1 gene injected into infarcted myocardium of rats, promoted angiogenesis and prevented apoptosis[225]. Encapsulation

of NRG1 in hydrogel administered directly to the infarcted myocardium border zone resulted in sustained and localized distribution, improving LV function through increased cardiomyocyte proliferation and decreased apoptosis[226]. Otherwise, association of NRG1 with microparticles results in a bioactivity increase for up to 12 weeks in rats with myocardial infarction[227], and increases protein stability[228], and administering these microparticles together with adipocyte-derived stem cells resulted in decreased infarct size and improved LV function and remodeling[229].

Table 3 *- Preclinical studies with NRG1 as a therapy for heart failure.*

Ref	Model	Treatment	Outcome
224	MI, rat	10 mg/kg/day IV for 5 or 10 days, 1 week or 2 months after LAD ligation	Improved LV structure and function Decreased neurohumoral activation Improved survival
172	MI, mouse	2.5 mg/mouse IP for 12 weeks, 1 week after LAD	Improved LV structure and function
190	MI–I/R, rat	1, 2, 4 or 8 mg/kg IV for 20 minutes prior to I/R	Improved LV structure Decreased release of ischemia injury markers Decreased apoptosis
174	MI, rat	5 mg/kg/h IV for 7 days, 8 weeks after LAD ligation	Improved LV structure and function through cMLCK upregulation
222	MI, rat	10 mg/kg/d IV for 10 days, 4 weeks after LAD ligation	Improved LV structure and function Attenuation of mitochondrial dysfunction
225	MI, rat	Infarcted area injected with rhNRG1 carrying lentivirus	Decreased apoptosis and increased revascularization
230	MI, pig	0.67 – 2 mg/kg for 7 days after MI, and every 3 – 4 days, for a total of 8 doses (from day 7 to 35)	Increased LV fractional shortening Decreased LV end-diastolic dimension Decreased fibrosis ultrastructure
226	MI, mouse	2.5 µg through 3 peri-infarct intramyocardial injections	Increased LVEF Decreased LV area
231	MI, rat	0.65 – 3.25 mg/kg 2 weeks after MI for 10-20 days with a washout period between 1 and 4 weeks	Increased LVEF
190	MI-I/R, rat	4 µg/kg IV 20 minutes before I/R	Decreased endoplasmic reticulum stress Decreased infarct size and apoptosis
191	MI-I/R, mouse	80 ng/kg IP 5 minutes before I/R	Decreased infarct size
224	Dox-induced CM, rat	20 mg/kg/day IV for 5 days, 4 weeks after first doxorubicin administration	Improved cardiac function and survival Decreased necrosis
221	Dox-induced CM, mouse	0.75 mg/kg/day SC for 3–5 days (beginning 1 day prior to dox administration)	Improved cardiac function and survival Decreased myocardial injury markers
223	DCM, rat	10 mg/kg IV every 2 days for 2 weeks, 12 weeks after streptozotocin injection	Improved LV function Decreased apoptosis and fibrosis
196	DCM, apoE(-/-) mice	20 µg/kg/day, 5 days/ week for 14 weeks in a preventive approach	Improved LV function and remodeling Decreased the development of diabetic nephropathy
224	Myocarditis, mouse	30 mg/kg/day IV for 5 days	Improved LV structure, function and improved survival Decreased myocardial necrosis
224	CRP, dog	mg/kg/day IV for 5 days with continuous pacing, 3 weeks after the beginning of rapid pacing	Improved cardiac function
232	CLP, rat	10 µg/kg 30 minutes before CLP	Improved survival Improved LV function Decreased myocardial inflammation Decreased expression of cardiac injury markers
233	RIHD, rat	15 µg/kg, 3 days before and 7 days after irradiation	Decreased myocardial injury Improved LV function Decreased energetic dysfunction Improved cardiac structure

Abbreviations: apoE(-/-), apolipoprotein E knock-out; CM, cardiomyopathy; cMLCK, myosin light chain kinase; CLP, cecal ligation and puncture; CRP, chronic rapid pacing; DCM, diabetic cardiomyopathy; Dox, doxorubicin; IP, intraperitoneal; IV, intravenous; I/R, ischemia/reperfusion; LAD, left anterior descending artery; LV, left ventricular; LVEF, left ventricle ejection fraction; MI, myocardial infarction; rhNRG1, recombinant human neuregulin-1; RIHD, radiation induced heart dysfunction; SC, subcutaneous.

The same is observed when differentiating fibroblast-derived induced pluripotent stem cells into cardiomyocytes with NRG1[234], or overexpressing ErbB4 in mesenchymal stem cells[235], and delivering them into the infarcted or peri-infarct zones.

Recently, administration of GGF2, which binds to ErbB4 receptor with high-affinity, increased LV function in animals with myocardial infarction, for up to a month after treatment withdrawal[231]. Probably, it represents a significant advantage when compared to the commonly used NRG1 isoform containing the EGF domain. Other animal models also showed significant improvements using this isoform, suggesting that treatment with GGF2 might result in better outcomes[230].

Despite very promising, NRG1 stimulation of cardiomyocyte proliferation might be time-dependent, where treatment with NRG1 at an early stage in mice with cryoinjury resulted in increased myocardial function and cardiomyocyte proliferation, compared to a late administration[236].

In a rat model of HF, NRG1 improved left ventricular remodeling and cardiac function in the failing heart, through the reduction of mitochondrial dysfunction, myocyte apoptosis and oxidative stress. In a DCM animal model, treatment with NRG1 also improved heart function, decreased apoptosis and reversed cardiac remodeling through decreased fibrotic tissue deposition[223], and is able to decrease the development of diabetic nephropathy[196].

Interestingly, treating mice with rhNRG1 at a dose high enough to stimulate cardiac regeneration, for long periods of time (up to 211 days), did not result in any somatic, organ or neoplastic growth[237], confirming the safety of this treatment. In addition, radiation induced myocardial injury, a secondary effect of antineoplastic treatment, is reduced by NRG1 treatment that is able to attenuate nuclear damage and maintain energetic homeostasis and cardiac function[233]. These findings validate the observation that inhibiting this pathway in patients undergoing chemotherapy might lead to cardiac dysfunction[203].

3.2.7. Clinical trials with recombinant NRG1 as a therapy for heart failure

Recently reported clinical trials have demonstrated the effects of recombinant human NRG1 (rhNRG1) in patients with stable chronic HF on optimal medical therapy[238,239] . A Phase II trial, in which a daily intravenous infusion of rhNRG 1 was given to patients during 10 consecutive days, showed an increase in left ventricular function and structure observed at day 30 after the 10-day treatment. Interestingly, on day 90 structure and function were even further increased[238]. Another Phase II trial in which patients received a consecutive 12-hour intravenous infusion of rhNRG1 for 10 days showed an increase in CO of 30% and a decrease in pulmonary artery wedge pressure and systemic vascular resistance of 30% and 20%, respectively. Twelve weeks post the 11 days of infusion, left ventricular ejection fraction showed a 12% increase[239]. Importantly, in these studies, the short-term rhNRG1 administration was safe and well tolerated.

Larger trials are now ongoing. A Phase II interventional clinical trial has set out to determine, over a period of 12 months, the efficacy and safety of NRG1 as a treatment in 120 patients with chronic HF (NCT01251406; http://www.clinicaltrials.gov), and has been finished, but no results have been published yet. The same research group also planned Phase III clinical trials with the purpose of evaluating the ability of NRG1 in reducing the death rate (NCT01541202; http://www.clinicaltrials.gov, status unknown according to ClinicalTrials.gov), cardiac remodeling (NCT01439893; http://www.clinicaltrials.gov, replaced by a another study with different endpoints) and efficacy and safety of its subcutaneous administration in patients with chronic HF (NCT01214096; http://www.clinicaltrials.gov, patient recruitment has been suspended, but no results have been posted yet). A Phase I clinical trial with GGF2, a NRG1 isoform, set out to determine its effects on patients with left ventricular dysfunction and symptomatic HF (NCT01258387; http://www.clinicaltrials.gov). The results have been published recently, and have suggested that a single intravenous dose of GGF2 improved LV ejection fraction over 90 days, while being well tolerated[240].

In summary, rhNRG1 is emerging as a promising treatment option for cardiovascular disease and left ventricular dysfunction. Disabled NRG1/ErbB signaling is implicated in the transition from compensatory hypertrophy to failure, whereas ErbB receptor activation promotes cardiac protection during acute cardiac injury and chronic ventricular remodeling. Recent clinical trials have shown that brief administration of rhNRG1 to humans is safe, well tolerated and results in apparent acute and sustained improvements in cardiac function in patients with stable chronic HF. The potential development of cancer during prolonged administration is a concern and will require attention. Also, a better understanding of the mechanisms by which the NRG1/ErbB system exerts protective cardiac effects is needed.

II – AIM AND OBJECTIVES

II – AIM AND OBJECTIVES

In this thesis, we aimed to explore the metabolic mechanism underlying the beneficial effects of rhNRG1 in the treatment of PAH, in order to guide further molecular research in this context.

Our specific goals are to:

- Evaluate the gene expression of glucose transporters, specially GLUT1 and GLUT4 in the different degrees of the disease and correlate them with some PAH markers.

- Evaluate the effect of chronic treatment with NRG1 on the expression of the same glucose transporter genes and to compare the molecular changes between animals with PAH and animals with PAH treated with rhNRG1.

- Determine the effect of chronic treatment with NRG1 on the expression of some mitochondrial proteins, comparing the levels of these proteins in MCT animals and treated animals.

III – MATERIALS AND METHODS

III - MATERIALS AND METHODS

All the procedures in this work followed the recommendations of the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication No. 85-23, Revised 1996), and are accredited by the Portuguese Direção Geral de Alimentação e Veterinária (DGAV) and approved by Fundação para a Ciência e a Tecnologia (FCT PTDC/SAU-FCT/100442/2008).

1. Animal model

Wistar male rats (Charles River Laboratories; Barcelona, Spain), weighting 180-200 g, were randomly assigned to receive either a subcutaneous injection of 60mg/kg BWT of MCT (Sigma Chemical) or an equivalent volume of vehicle (0.9% NaCl). Fourteen days after the first injection, animals were again randomly assigned into 4 subgroups according to pharmacological treatment: CTRL (animals without PAH and without pharmacological treatment, n=8); MCT (animals with PAH and without pharmacological treatment n=8); MCT+rhNRG1 (animals with PAH and with pharmacological treatment n=8); CTRL+rhNRG1 (animals without PAH and with pharmacological treatment, n=8). Pharmacological treatment consisted on a daily intraperitoneal injection of 40µg/kg BWT of rhNRG1 (Peprotech, Germany) for 7 days, while the vehicle treatment consisted of 0.1% Bovine Serum Albumin (BSA, Sigma-Aldrich) intraperitoneal injection for 7 days.

Animals were grouped three per box, in a controlled environment, with a light-darkness cycle of 12:12h, controlled temperature at 22ºC, and water and food *ad libitum*. Before MCT administration, an echocardiography evaluation was performed, to allow a basal evaluation to compare experimental groups and to prevent the inclusion of potential ill animals (cardiac pathology or other) in the next step of the protocol. Between 21 and 24 days after MCT administration, the animals were submitted again to echocardiographic evaluation and to invasive hemodynamic evaluation. After the morphometric evaluation of animals, tissue samples from different organs were collected and processed to histological and molecular biology studies.

2. Protein expression

4.2.1. RT-PCR

Total RNA was extracted by using the method RNeasy Mini Kit (Quiagen, 74104) according to the instructions of the manufacturer. Concentration and purity of RNA were evaluated using the NanoDrop® ND-2000 spectrophotometer (Thermo Fisher Scientific). mRNA relative expression quantification was performed by two-step Real-Time Polymerase Chain Reaction (RT-PCR). Using RV samples from the CTRL group, standard curves were built to all the studied genes, correlating the initial total mRNA quantity and the threshold cycle. Reverse transcription was performed in a conventional thermocycler (Whatmann Biometra, 050-901) and consisted in 10 minutes at 22ºC, 50 minutes at 50ºC, and 10 minutes at 95ºC. Ten percent of the obtained cDNA was amplified and detected by RT-PCR (Step-One™ Applied Biosystems), using the probes SYBR Green (PerfeCta® SYBR Green FastMix, Rox, Kit, Quanta Biosciences) according to the manufacturer instructions.

Amplification curves were analyzed with the equipment software (v2.2.2), through absolute quantification. Melting curves of each PCR reaction were used to exclude the formation of primer-dimers and unspecific products, confirming the purity of the amplified product. GAPDH was chosen as reference gene, since no significant changes were observed in the different groups. Gene expression results were presented in Arbitrary Units (AU), being the CTRL group means values after GAPDH normalization correspondent to 1AU. All assays were performed twice. The primers used in the molecular analysis (see Table 4) were designed in-house with the appropriate software (DNAstar™).

Table 4 - List of primers

Gene	Primers sequences	
	Forward	Reverse
GAPDH	5'TGG CCT TCC GTG TTC CTA CCC3'	5'CCG CCT GCT TCA CCA CCT TCT3'
ET-1	5'CGG GGC TCT GTA GTC AAT GTG3'	5'CCA TGC AGA AAG GCG AAT GTG3'
BNP	5'CAG AGC TGG GGA AAG AAG AG3'	5'GGA CCA AGG CCC TAC AAA AGA3'
HIF-1α	5'TCA TAG GCG GTT TCT TGT AGC3'	5'CTA ACA AGC CGG AGG AC3'
GLUT1	5'TCC TTA TTG CCC AGG TGT TC3'	5'GCA GAA GGG CAA CAG GAT AC3'
GLUT4	5'ATA GCC CTT TTC CTT CCC AA3'	5'AGG CAC CCT CAC TAC CCT TT3'

4.2.2. Western Blotting

Frozen samples of RV were homogenized, on ice, in 1 mL RIPA lysis buffer (ThermoFisher Scientific), containing protease inhibitors (protease inhibitor cocktail, Sigma Chemical, St. Louis, MO), and phosphatase inhibitors (phosphatase inhibitor cocktail, Sigma Chemical, St. Louis, MO). Homogenates were centrifuged at 12,000 *x g* for 20 min at 4°C. Supernatants were collected, and total protein concentration was determined using the Bradford assay (Bio-Rad Laboratories, CA, USA). Protein aliquots (15 µg), diluted with Laemmli loading buffer (Cell Signaling Technology) containing β-mercaptoethanol (4:1), were loaded onto a 10% SDS-PAGE gel, run and electroblotted onto a 0,2 µm nitrocellulose membrane (Bio-Rad Laboratories, CA, USA). Pre-stained molecular weight marker proteins (Precision Plus Protein™ Standards, Bio-Rad Laboratories, CA, USA) were used as size standards for the SDS-PAGE. Ponceau staining was performed to verify the quality of the transfer. Blots were blocked in 5% NFDM (Non-fat dry milk, Bio-Rad Laboratories, CA, USA) for 1 hour at room temperature, and incubated overnight at 4ºC with primary antibody (Table 5) at a dilution of 1:500.

Table 5 - List of primary antibodies

Protein	Antibody's Identification
GLUT4	GLUT4 (sc-53566), Mouse mAb, Santa Cruz Biotecnology, Inc.
DRP-1	DRP-1 (sc-101270), Mouse mAb, Santa Cruz Biotecnology, Inc.
OPA-1	OPA-1 (sc-393296), Mouse mAb, Santa Cruz Biotecnology, Inc.
SOD1	SOD1 (sc-101523), Mouse mAb, Santa Cruz Biotecnology, Inc.
SOD2	SOD2 (sc-130345), Mouse mAb, Santa Cruz Biotecnology, Inc.
GAPDH	GAPDH (0411), Mouse mAb, Santa Cruz Biotechnology, Inc.

Prior to incubation with secondary antibodies, nitrocellulose membranes were washed with TBS-T, containing Tris-buffered saline (Cell Signaling Technology) and 0.01% Tween solution (Sigma-Aldrich, St. Louis, USA). All secondary antibodies (IRDye 680LT, Goat-anti-Mouse Ab and 800CW, Goat-anti-Mouse Ab, LI-COR Biosciences, Lincoln, USA) were applied in 5% NFDM at a 1:10000 dilution for 1 hour at room temperature. After washing the membranes with TBS-T, membranes were scanned using an Odyssey scanner (infrared imaging system, LI-COR Biosciences, Lincoln, USA) and analyzed using the Odyssey provided software (version 3.0). Protein expression data are presented in Band Intensity (B.I.), being the CTRL group means values after GAPDH normalization correspondent to 1B.I. All assays were performed twice.

3. Oral glucose tolerance tests and metabolic measurements

Oral glucose tolerance tests (OGTTs) were performed at the first day of NRG1 injection and at the end of protocol. Rats were fasted overnight (12 hours) and OGTTs was performed glucose loading by gastric gavage (2 g/kg BWT). Blood glucose levels were determined, using a glucometer at the time of glucose loading (0), and then at 15, 30, 60, 90 and 120 min after loading (FreeStyle Precision Neo). OGTTS were repeated in the day of animal's hemodynamic evaluation.

4. Statistical analysis

Statistical analysis was performed using *GraphPad Software* (vs.7). One-way ANOVA was used to statistically analysis these parameters. Group data are presented as means ± SEM. Differences with $p<0.05$ were considered statistically significant.

IV - RESULTS

IV -RESULTS

1. Characterization of GLUT1 and GLUT4 expression in the MCT model

In order to characterize the GLUT1 and GLUT4 expression in MCT model, we divided the MCT-induced PAH animals group in two subgroups, according to their RV ejection fraction (EF): compensated RV (CRV) and decompensated RV (DRV), and compared their mRNA levels with those of the control group (Fig. 5).

The ejection fraction is an important measurement in determining how well heart is pumping out blood and in diagnosing and tracking HF (Fig. 5A). The CRV subgroup of MCT-induced PAH animals displayed an EF above of 35%, while the DRV subgroup was characterized by exhibiting EF values below 35%. We've found a significant difference ($p<0.05$) between the control group and both compensated and decompensated RV. Also, a significant difference between compensated and decompensated RV groups was verified.

The analysis of the gene expression of ET-1 and brain natriuretic peptide (BNP) (Fig. 5B and 5C) gave us an indication of the state of overload and ventricular hypertrophy. The expression of HIF-1α (Fig. 5D) gene was studied, once it's involved in PAH metabolic dysfunction and RVH. In the DRV animals we've observed an increase in mRNA levels of ET-1 (Fig. 5B), BNP (Fig. 5C) and HIF-1α (Fig. 5D), when compared with the control group. However, no difference was observed between the CRV and control group.

As described above, PAH has been associated with signaling pathways of the energy metabolism. Thereby, we've quantified the mRNA levels of the glucose transporters, GLUT1 and GLUT4. We verified an increase of GLUT1 gene expression in both MCT groups (Fig.5E). Conversely, the expression of GLUT4 gene was decreased in the DRV group(Fig.5F).

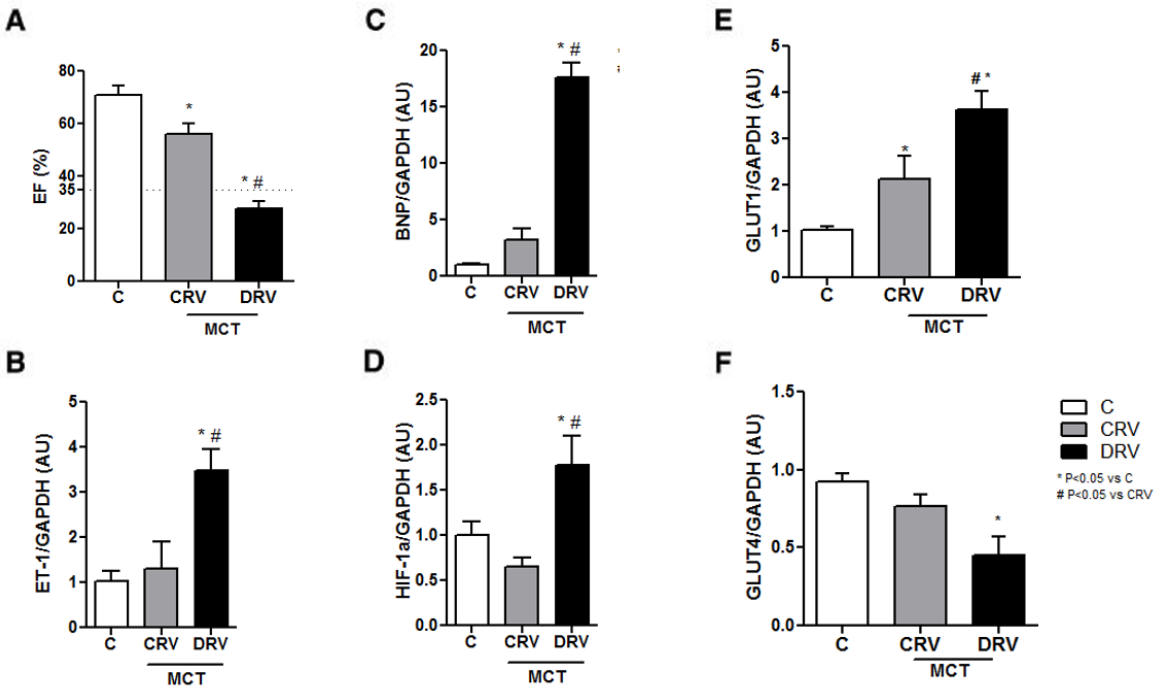

Figure 5 - Characterization of GLUT1 and GLUT4 expression in the MCT model. The healthy animals were included in the control group (C) and MCT-induced PAH animals (MCT) were divided into compensated right ventricle group (CRV) and

decompensated right ventricle group (DRV). The percentage (%) of ejection fraction (EF) was evaluated by invasive hemodynamics (Panel A) and mRNA levels were determined by RT-PCR (Panels B-F). The dashed line in panel A assigns for 35% of EF. The expression levels of endothelin-1 (ET-1), brain natriuretic peptide (BNP), hypoxia-inducible factor 1-alpha (HIF-1α), glucose transporter 1 (GLUT1) and glucose transporter 4 (GLUT4) are expressed in arbitrary units (AU), using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as reference gene. Bars represent mean+SEM of 8 rats per group. *P<0.05 *vs*. Control, #P<0.005 *vs*. CRV. One-way ANOVA was used for all the parameters presented.

2. GLUT1 and GLUT 4 correlation with markers of disease

After the characterization of GLUT1 and GLUT4 expression in the MCT model, we've determined the correlation between the expression of these genes and those of markers of disease (Fig. 6).
We observed that EF values correlate negatively with mRNA levels of GLUT1 (Fig. 6A) and positively with mRNA levels of GLUT4 (Fig. 6B).
As previous mentioned, HIF-1α has been associated with the induction of GLUT1 expression. Accordingly, when we compared GLUT1 expression with HIF-1α expression, we verified that exists a positive correlation between them, as shown in Fig. 6C.
Conversely, when we related the expression of GLUT4 gene with the expression of both BNP and ET-1 genes we've found a negative correlation (Fig.6D and 6E, respectively).

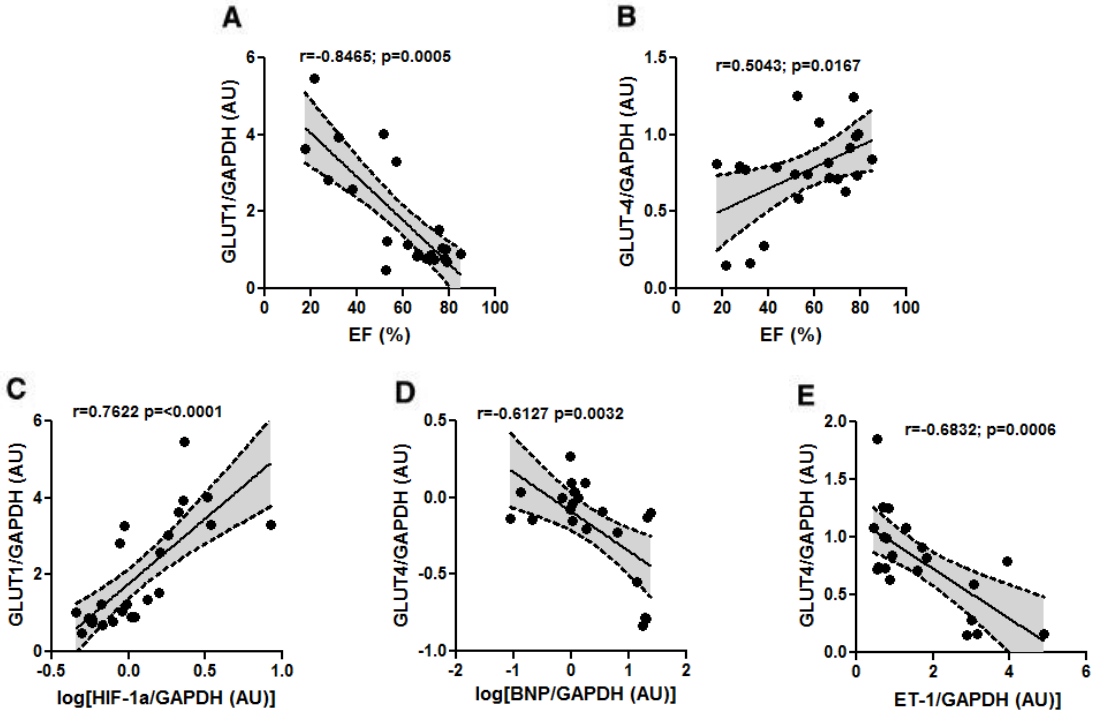

Figure 6 - GLUT1 and GLUT4 correlation with markers of disease. The correlation analysis was performed using the data displayed in Fig.5, which correspond to data obtained from animals that had mRNA quantification (AU, arbitrary units), using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as reference gene, and percentage (%) of ejection fraction (EF) analysis (•). Statistical analysis reveals significant correlation (r>0.05; p<0.005) between the expression of glucose transporter 1 (GLUT1) with EF (panel A) and the expression of hypoxia-inducible factor 1-alfa (HIF-1α) (panel C), as well as between the expression of glucose transporter 4 (GLUT4) with EF (panel B) and the expression of brain natriuretic peptide (BNP) (panel D) and endothelin-1 (ET-1) (panel E).

3. Effect of chronic treatment with rhNRG1 on the expression of GLUT1 and GLUT4

In Fig. 7, we show the effect of chronic treatment with rhNRG1 on the expression of GLUT1 and GLUT4 in RV samples from the several experimental groups. We observed that GLUT4 gene expression was increased in all rhNRG1-treated animals when compared with the control group, but only the MCT+rhNRG1 (MN) group presents a significant difference. Although we observed a decreased expression of GLUT4 gene in the MCT group, this decline was not significant when compared with both control groups (Fig. 7A).

In addition, we also noticed that the expression of GLUT1 gene was significantly increased on the MCT group, and the treatment with rhNRG1 attenuated the expression of this gene, as seen in Fig. 7B.

In the Fig. 7C, we show the results of grouping all the animals that belong to the MCT group. Thereby, we can observe that the ejection fraction was above 35%. In Fig. 7C, we can also observe that the treatment with rhNRG1 improved the ejection fraction of MCT animals significantly when compared with the MCT group.

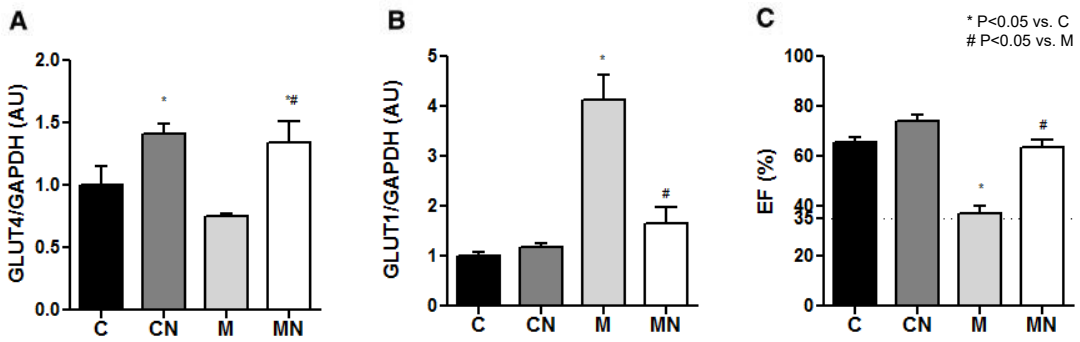

Figure 7 - Effect of chronic treatment with NRG1 on the expression of GLUT1 and GLUT4. The experimental animals were divided in control group (C), control group treated with rhNRG1 (CN), MCT-induced PAH group (M) and M group treated with rhNRG1 (MN). The mRNA levels of glucose transporter 4 (GLUT4) and glucose transporter 1 (GLUT1) were determined by RT-PCR (Panels A and B) and the percentage (%) of ejection fraction (EF) was evaluated by invasive hemodynamics (Panel C). The dashed line in panel C assigns for 35% of EF. mRNA levels of GLUT4 and GLUT1 are expressed in arbitrary units (AU), using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as reference gene. *P<0.05 *vs.* Control, #P<0.005 *vs*. M. One-way ANOVA was used for all the parameters presented.

In Fig. 8, we showed that GLUT4 protein levels were greatly reduced in the diseased group relative to the other groups. Moreover, it was found that chronic administration of rhNRG1 to MCT animals induces a significant increase of the GLUT4 protein levels. Actually, the expression of GLUT4 gene at protein level in MN group and in control group are similar.

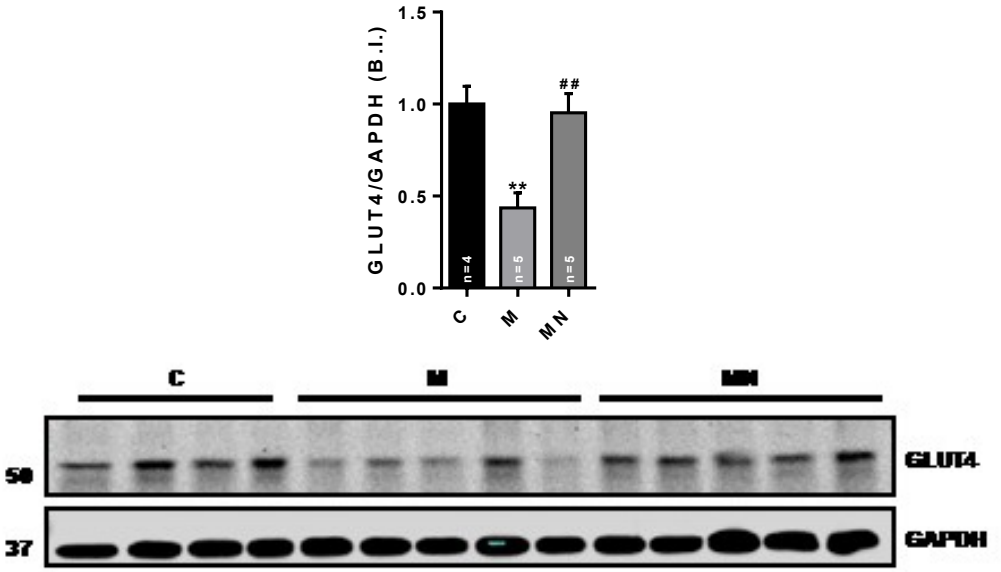

Figure 8 - ***Effect of NRG1 in the GLUT4 protein.*** The experimental animals were divided in control group (C), MCT-induced PAH group (M) and M group treated with rhNRG1 (MN). The protein levels of glucose transporter 4 (GLUT4) was quantified by immunoblotting. The GLUT4 protein levels are expressed in band intensity (B.I.). Bars represent mean+SEM of 4-5 rats per group. **P<0.05 *vs.* Control, ##P<0.005 *vs*. M. One-way ANOVA was used for all the parameters presented. The numbers on the left side of the representative gel assign to the molecular weights, in kilodaltons, of GLUT4 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), used as reference protein.

4. Effect of chronic treatment with rhNRG1 on glucose tolerance

As we demonstrated, the glucose transporters expression was altered in the diseased group, and the treatment with rhNRG1 attenuated the changes in their expression, *in vitro*. So, we decided to evaluate the impact of rhNRG1 on glucose metabolism, *in vivo*. For that, we performed OGTT. However, we did not find differences in glucose tolerance between study groups, as showed in Fig. 9.

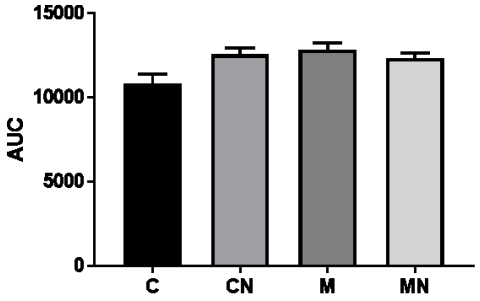

Figure 9 - ***Effect of chronic treatment with rhNRG1 in glucose tolerance.*** Blood samples were collected from animals of control group (C; n=8), control group treated with rhNRG1 (CN; n=8), MCT-induced PAH group (M; n=8) and M group treated with rhNRG1 (MN; n=8), at different time points for measuring concentrations of glucose. Oral glucose tolerance is expressed as area under the curve (AUC) of oral glucose tolerance tests (OGTTs), in the four animal groups after subtracting baseline concentrations. Bars represent mean+SEM.

5. Effect of chronic treatment with NRG1 on the expression of mitochondrial proteins.

To understand the effect of rhNRG1 treatment in metabolic dysfunction of the RV, and knowing that mitochondria play an important role in cells metabolism, we evaluated the protein levels of superoxide dismutase 2 (SOD2), once epigenetic silence of SOD2 results in HIF-1α activation and triggers redox changes in PAH. However, we found no differences in our study. In addition, we've also studied the protein levels of SOD1, a cytosolic superoxide dismutase which expression and activity are decreased in rats with MCT-induced PH. Once again, no significant differences in this protein was observed between studied groups.

Finally, we evaluated dynamic related-protein 1 (DRP-1), the key mediator of mitochondrial fission, and optic atrophy 1 (OPA-1), an inner mitochondrion membrane GTPase, involved in mitochondrial fusion. Again, we've not found statistically significant differences between the experimental groups.

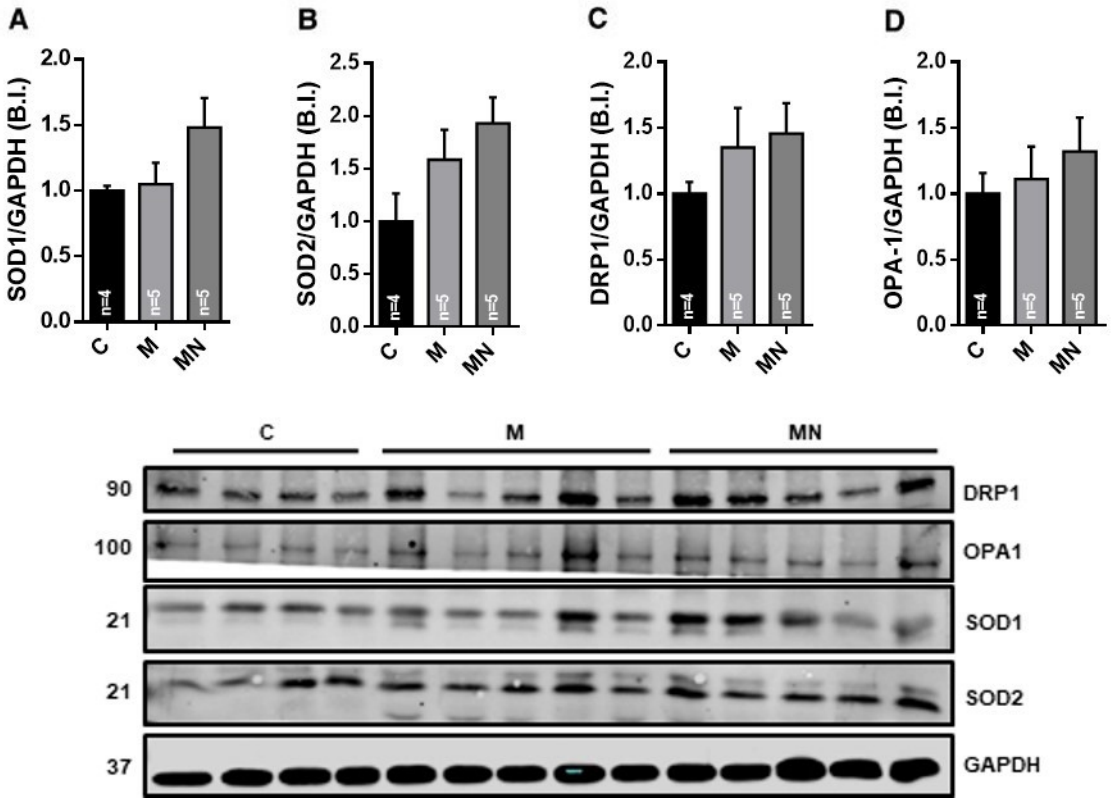

Figure 110 - ***Effect of chronic treatment with rhNRG1 on the expression of mitochondrial proteins.*** The experimental animals were divided in control group (C), MCT-induced PAH group (M) and M group treated with rhNRG1 (MN). The protein levels of superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2), dynamic related-protein 1 (DRP1) and optic atrophy-1 (OPA1) were quantified by immunoblotting (Panels A-D). The protein levels are expressed in units of band intensity (B.I.). Bars represent mean+SEM of 4-5 rats per group. The numbers on the left side of the representative gel assign to the molecular weights, in kilodaltons, of DRP1, OPA1, SOD1, SOD2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), used as reference protein.

V - DICUSSION

V -DISCUSSION

1. PAH markers and glucose transporters expression are changed in the progression of RV hypertrophy.

As known, right ventricular failure is the leading cause of death in patients with PAH[140]. Myocardial hypertrophy is a compensatory mechanism whereby cardiac tissue adapts to increased workload. Depending on the degree or duration of increased workload, ventricular hypertrophy may progress from a compensatory state to impaired systolic or diastolic function and HF. This transition is characterized by alterations in extracellular matrix composition, energy metabolism, β-adrenergic responsiveness, myofilament proteins, Ca^{2+} handling, signal transduction pathways, and gene expression profiles[241].

In this study, we divided MCT animals in two groups: (i) with an ejection fraction above 35% that presented a compensated RVH and (ii) MCT animals with an EF below 35% that showed a decompensate RVH. These findings are also concordant with the study of Hessel *et al.*[241], where they characterized the right ventricular function after MCT-induced PAH in the rat with two different doses of MCT. They conclude that in the lower dose of MCT (30 mg/kg BWT), rats showed a compensatory RVH and a significant reduction of the ejection fraction, but when rats were treated with the higher dose (80 mg/kg BWT) presented a decompensated RVH and an EF much lower than the controls and rats injected with 30 mg/kg BWT of MCT.

We've also analyzed cardiac overload and hypertrophy markers in PAH, such as ET-1 and BNP-1. BNP levels are associated with PAH[242], its production is stimulated in response to pressure-overload[243] and is associated with the extent of RV dysfunction[244], being increased in MCT-injected animals[245]. Plasma BNP is secreted mainly from the ventricles and correlates with cardiac indices in heart disease, showing a markedly increasing in decompensated HF[246]. Langenickel *et al.*[247] evaluated BNP mRNA in compensated and overt HF in both ventricles, concluding that ventricular BNP mRNA expression was not upregulated in any of the compensated HF models, whereas it increased in overt HF. These data indicate that cardiac BNP mRNA expression might be induced specifically in overt HF, pointing toward the possible role of BNP as a marker of the transition from compensated to overt HF. In our study we verified the same, an extreme increase of BNP gene expression in DRV group, but not in CRV group when both compared with control group. ET-1 plasmatic levels are elevated in PAH, and associated with disease severity[41]. ET-1 is associated with the development of RVH in MCT-induced PAH[248,249] and its expression is increased in this experimental model[250,251]. In our study, only the DRV group demonstrated an increase of ET-1 gene expression when compared with the study groups.

As mentioned before, the RV when confronted with an increased oxygen requirement and/or a decreased oxygen supply, it is thought that hypertrophic RV tissue experiences higher degrees of hypoxia, resulting in the activation of transcription factors HIF-1[143,144]. As in the pulmonary vasculature, the activation of HIF-1 produces a glycolytic phenotype via up-regulation of glycolytic enzymes and suppression of glucose oxidation. Again, our studies are in agreement with the literature once we found that HIF-1α mRNA levels were only increased in the DRV group.

Hypertrophied hearts exhibit gene expression and metabolic profiles similar to that in fetal hearts, namely, increased reliance on glucose for fuel metabolism[252]. Since glucose has a higher oxygen efficiency for ATP production, the shift of substrate preference from fatty acid to glucose is therefore considered beneficial in the hypertrophied and failing myocardium[253,254]. However, "glucotoxicity" is observed in cardiomyocytes cultured in high glucose medium[255-257], raising concerns whether adult cardiomyocytes in hypertrophied hearts can tolerate enhanced glucose uptake. In transgenic mice overexpressing insulin-independent glucose transporter GLUT1 in the heart (GLUT1-TG), the hearts have adapted to long-term increases in glucose usage without developing dysfunction even in the old age[258]. Moreover, the GLUT1-TG mice are protected from developing HF in pressure overload induced hypertrophy model as well as from ischemia/reperfusion injury[258,259]. Clinical studies [260,261] suggested that promoting glucose utilization in the hypertrophied and failing heart could be beneficial. However, a short-term induction of GLUT1 in cardiomyocytes at the onset of pressure overload-induced hypertrophy failed to improve contractile function despite the beneficial effects on mitochondrial function. Furthermore, researches have suggested that altered glucose metabolism in cardiac hypertrophy affects biological processes beyond energy generation[259,262,263]. A major change of glucose metabolism during cardiac hypertrophy is accelerated glycolysis. Increased rate of glucose uptake and glycolysis has been reported in multiple animal models of cardiac hypertrophy[264-266]. Upregulations of GLUT1 expression have been observed in hypertrophied hearts both *in vitro* and *in vivo*. This finding coincided with our results, once we found that GLUT1 mRNA levels were increased in both stages of RVH when compared with control animals[267]. Although, the same was not observed for GLUT4 mRNA levels. The GLUT4 gene expression was reduced only in a late phase of RV, corresponding to our DRV group. It was described that mRNA and protein levels of GLUT4 are decreased in hypertrophied hearts of both animal models and human patients and insulin stimulated glucose and uptake is only moderately affected, which it agreed with our findings[267-270]. Concluding it seems that the increase of glycolytic flux in cardiac hypertrophy is largely dependent upon a higher rate of insulin-independent glucose uptake. It has been proposed that activation of AMPK in the hypertrophied heart, as the consequence of impaired energetics, is responsible for promoting GLUTs translocation, enhancing glucose uptake and stimulating glycolysis by phosphorylation/activation of PFK2[241,265,271,272].

2. GLUT1 and GLUT4 correlate with markers of disease

As explained before, GLUT1 and GLUT4 cardiac expression are related to the stage of RVH. The hypertrophied hearts act like a fetal heart, metabolically[252]. In embryonic and neonatal heart, GLUT1 is the predominant glucose transporter[268]. Our results showed a negative correlation between the EF and the expression of GLUT1 gene. However, the same was not verified for GLUT4 gene expression, being the previous proportional to the EF. This result was concordant with literature in other pathophysiology contexts, where it was suggested that GLUT4 is rapidly upregulated only after birth while GLUT1 is downregulated. So, GLUT4 is the primary glucose transporter GLUT4 expressed in the postnatal and adult heart[273,274]. Also, GLUT4 has the same behavior with the EF and with the markers of hypertrophy and RV overload, being these three parameters indicators of RVH.

In our study, we concluded that GLUT1 gene expression was also positively correlated with RV HIF-1α expression. Several studies, related to cancer[275,276], have shown the same correlation between HIF-1α and GLUT1. They showed that tumor cells, like what happens to PAH lungs cells, shift of glucose metabolism from the more efficient oxidative phosphorylation to the less efficient glycolytic pathway, to maintain the energy production (the Warburg effect). For this reason, hypoxic cells tend to consume more glucose to meet their energy needs. HIF-1α mediates this metabolic conversion through the induction of enzymes involved in the glycolysis pathway and overexpression of GLUT1 which increase glucose import into tumor cells[275,276].

3. NRG1 treatment reverses changes in glucose transporters

In the present study, RV GLUT1 mRNA expression was significantly increased in MCT animals, but the GLUT4 expression seems to be affected with the disease, although it was increased in all rhNRG1 animals. These results agree with previous studies[146,209].

Sivitz *et al.*[277] investigated the mechanism by which cardiac glucose utilization increases during hypoxia and increased workload. They studied the effect of 2 and 14 days of hypobaric hypoxia on the cardiac expression of two subtypes of the facilitative D-glucose transporter, the GLUT4 or "insulin-regulatable" isoforms and the GLUT1 isoforms thought to mediate basal transport. Rats lost weight when exposed to hypobaric hypoxia, and developed RVH. RV GLUT1 mRNA levels increased compared with normal controls rats and RV GLUT4 mRNA decreased after hypobaric hypoxia. They conclude that hypobaric hypoxia increases cardiac GLUT1 expression and reduces GLUT4 expression early in the development of RVH.

GLUT4 plays a key role in glucose uptake and metabolism in insulin target tissues. Being a rate-limiting step in glucose metabolism, the expression and function of the GLUT4 isoform has been extensively studied and found to be tightly regulated at both mRNA and protein levels. Adaptation to states of enhanced metabolic demand is associated with increased glucose metabolism and GLUT4 gene expression[278]. In patients with HF, GLUT4 protein reduction is present and is related to disease severity, contributing to insulin resistance and energy metabolism modification. The reduction in GLUT4 protein may contribute to metabolic imbalance in this disease[279].

As mentioned, *in vivo* studies showed that a single injection of NRG1β was found to reduce blood glucose concentrations over 30–60 min and to increase blood glucose disposal over 60–90 min after treatment, as compared to vehicle treatment in diabetic mice. Glucose disposal was also increased after the NRG1β administration in diabetic mice[218]. In this work, we also tried to show the influence of the NRG1 treatment on glucose metabolism, *in vivo*, in blood samples of animal with PAH induced by MCT. Unfortunately, we did not find differences in glucose tolerance in all study groups. Possible explanations for these results are the heterogeneity of MCT model and the small number of experimental animals.

4. NRG1 treatment on the expression of mitochondrial proteins

In the vasculature, it was observed that metabolic changes appear to be triggered by changes in redox state that activate transcription factors, such as HIF-1α. These redox changes in PAH include epigenetic silencing of SOD2 and/or changes in mitochondrial fission/fusion. Mitochondria in PAH

are deficient in complex I expression and SOD2 and their membrane potential is hyperpolarized[50]. These changes in the mitochondria create a "pseudohypoxic" redox milieu PASMC's, in which local PO_2 and the production of ROS are dissociated[280]. The consequence of this redox disorder is normoxic activation of HIF-1α, both in experimental[50], and human PAH[50,281,282]. This pathological HIF-1α activation contributes to the aerobic glycolysis. HIF-1α activation can result from the redox changes initiated by epigenetic silencing of SOD2 in PAH[283], consistent with prior descriptions of redox-regulation of HIF-1α[284,285]. Activated HIF-1α suppresses mitochondrial oxidative metabolism (by increasing the expression of pyruvate dehydrogenase kinases, PDKs, thereby blocking pyruvate uptake into the Krebs cycle)[286] while simultaneously upregulating enzymes and transporters that favor glycolysis (hexokinase-2 and GLUT1). Targeting these mitochondrial metabolic abnormalities in PAH, using the PDK inhibitor dichloroacetate, reduces glycolysis, restores oxidative metabolism and regresses PAH in several experimental models[287].
In addition, SOD1 expression and activity are decreased in rat lungs with MCT-induced PAH, which is associated with increased markers of oxidative stress and decreased total antioxidant capacity despite enhanced SOD2 expression[288].
As mentioned the redox changes in PAH include alterations in mitochondrial fission, consequently DRP-1 expression is altered, since it is the key mediator of mitochondrial fission[287]. The expression and activity of DRP-1 is increased in human PAH. Inhibition of mitochondrial fission, using the small molecular inhibitor of DRP1 arrests PAH PASMC in the G2/M phase of the cell cycle. This indicates that mitochondrial fission is a mitotic checkpoint. In human and experimental PAH, excessive activation of the GTPase DRP-1 promotes excessive fission. HIF-1α promotes fission by activating DRP-1. HIF-1α activation can be stimulated by administration of cobalt, and within two hours this fragments the mitochondrial network through a DRP-1-dependent mechanism. *In vivo*, administration of cobalt leads to a form of PAH[287]. Mitochondrial fusion is decreased in PAH, and is mediated by large GTPases, mitofusin-1 and mitofusin-2, which reside in the outer mitochondrial membrane, and a GTPase called optic atrophy-1 (OPA-1), in the inner mitochondria membrane[289].

However, our study didn't show any significant difference between experimental groups concerning the expression of these mitochondrial proteins. Once again, these results may be caused by a small sample number, being necessary to increase the number of study animals. Also, these results can reflect the variability of the MCT model.

VI - CONCLUSION

VI-CONCLUSION

In the present study, we observed that the expression of GLUT1 is associated with the development of the disease whereas GLUT4 is affected by chronic treatment with rhNRG1. The expression of GLUT1 and 4 correlates with parameters of cardiac function and with disease markers and chronic treatment with rhNRG1 attenuates the changes in GLUTs observed in MCT-induced PAH. This was to our knowledge the first study exploring the effects of NRG1 treatment in the expression of glucose transporters and in glucose tolerance in the context of PAH. We conclude that the therapeutic effects of rhNRG1 in PAH might be due, at least in part, to the regulation of GLUT1 and 4 expressions.

VII - REFERENCES

VII -REFERENCES

1. Galie N, Simonneau G. The Fifth World Symposium on Pulmonary Hypertension. *J Am Coll Cardiol.* Dec 24 2013;62(25 Suppl):D1-3.

2. Lau EM, Humbert M, Celermajer DS. Early detection of pulmonary arterial hypertension. *Nat Rev Cardiol.* Mar 2015;12(3):143-155.

3. Montani D, Günther S, Dorfmüller P, et al. Pulmonary arterial hypertension. *Orphanet Journal of Rare Diseases.* 2013;8:97.

4. Chester AH, Yacoub MH. The role of endothelin-1 in pulmonary arterial hypertension. *Glob Cardiol Sci Pract.* 2014;2014(2):62-78.

5. Humbert M, Sitbon O, Chaouat A, et al. Pulmonary arterial hypertension in France: results from a national registry. *Am J Respir Crit Care Med.* May 01 2006;173(9):1023-1030.

6. Ling Y, Johnson MK, Kiely DG, et al. Changing demographics, epidemiology, and survival of incident pulmonary arterial hypertension: results from the pulmonary hypertension registry of the United Kingdom and Ireland. *Am J Respir Crit Care Med.* Oct 15 2012;186(8):790-796.

7. Peacock AJ, Murphy NF, McMurray JJ, Caballero L, Stewart S. An epidemiological study of pulmonary arterial hypertension. *Eur Respir J.* Jul 2007;30(1):104-109.

8. Strange G, Playford D, Stewart S, et al. Pulmonary hypertension: prevalence and mortality in the Armadale echocardiography cohort. *Heart.* Dec 2012;98(24):1805-1811.

9. D'Alonzo GEea. Survival in patients with primary pulmonary hypertension. Results from a national prospective registry. *Ann. Intern. Med. .* 1991;115: 343–349.

10. Rich Sea. Primary pulmonary hypertension. A national prospective study. *Ann. Intern. Med.* 1987;107:216–223.

11. Lau EMT, Giannoulatou E, Celermajer DS, Humbert M. Epidemiology and treatment of pulmonary arterial hypertension. *Nat Rev Cardiol.* Oct 2017;14(10):603-614.

12. Humbert Mea. Survival in patients with idiopathic, familial, and anorexigen-associated pulmonary arterial hypertension in the modern management era. *Circulation* 2010;122:156–163.

13. Benza RL, Miller DP, Barst RJ, Badesch DB, Frost AE, McGoon MD. An evaluation of long-term survival from time of diagnosis in pulmonary arterial hypertension from the REVEAL Registry. *Chest.* Aug 2012;142(2):448-456.

14. Hoeper MM, Huscher D, Ghofrani HA, et al. Elderly patients diagnosed with idiopathic pulmonary arterial hypertension: Results from the COMPERA registry. *International Journal of Cardiology.* 2013;168(2):871-880.

15. Sitbon O, Sattler C, Bertoletti L, et al. Initial dual oral combination therapy in pulmonary arterial hypertension. *European Respiratory Journal.* 2016;47(6):1727-1736.

16. Simonneau G, Gatzoulis MA, Adatia I, et al. Updated clinical classification of pulmonary hypertension. *J Am Coll Cardiol.* Dec 24 2013;62(25 Suppl):D34-41.

17. Galie N, Humbert M, Vachiery JL, et al. 2015 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension: The Joint Task Force for the Diagnosis and Treatment of Pulmonary Hypertension of the European Society of Cardiology (ESC) and the European Respiratory Society (ERS): Endorsed by: Association for European Paediatric and Congenital Cardiology (AEPC), International Society for Heart and Lung Transplantation (ISHLT). *Eur Heart J.* Jan 01 2016;37(1):67-119.

18. Pietra GG, Capron F, Stewart S, et al. Pathologic assessment of vasculopathies in pulmonary hypertension. *J Am Coll Cardiol.* Jun 16 2004;43(12 Suppl S):25S-32S.

19. Prins KW, Thenappan T. World Health Organization Group I Pulmonary Hypertension: Epidemiology and Pathophysiology. *Cardiol Clin.* Aug 2016;34(3):363-374.

20. Dorfmüller P. Pathology of Pulmonary Arterial Hypertension. *Pulmonary Vascular Disorders.* 2012;41(Karger Publishers):14-22.

21. Santos-Ribeiro D, Mendes-Ferreira P, Maia-Rocha C, Adao R, Leite-Moreira AF, Bras-Silva C. Pulmonary arterial hypertension: Basic knowledge for clinicians. *Arch Cardiovasc Dis.* Oct 2016;109(10):550-561.

22. Montani D, et al. Pulmonary arterial hypertension. *Orphanet journal of rare diseases* 2013;8(1):97.

23. Waxman A, Zamanian R. Pulmonary Arterial Hypertension: New Insights Into the Optimal Role of Current and Emerging Prostacyclin Therapies. *The American Journal of Cardiology.* 2013;111(5):17A-19A.

24. Humbert M, Morrell NW, Archer SL, et al. Cellular and molecular pathobiology of pulmonary arterial hypertension. *J Am Coll Cardiol.* Jun 16 2004;43(12 Suppl S):13S-24S.

25. Jeffery TK, Morrell NW. Molecular and cellular basis of pulmonary vascular remodeling in pulmonary hypertension. *Prog Cardiovasc Dis.* Nov-Dec 2002;45(3):173-202.

26. Kerkela E, Saarialho-Kere U. Matrix metalloproteinases in tumor progression: focus on basal and squamous cell skin cancer. *Experimental Dermatology* 2003;12:109-125.

27. Neil J. Davie, T. J, Crossno J, et al. Hypoxia-induced pulmonary artery adventitial remodeling and neovascularization: contribution of progenitor cells. *Am J Physiol Lung Cell Mol Physiol.* 2003;286: 668–678.

28. Budhiraja R, Tuder RM, Hassoun PM. Endothelial dysfunction in pulmonary hypertension. *Circulation.* Jan 20 2004;109(2):159-165.

29. Caraballo Fonseca JC, Martínez Balzano CD, Sánchez de León R. Endothelial Dysfunction in Pulmonary Hypertension. *Archivos de Bronconeumología ((English Edition)).* 2005;41(7):389-392.

30. Rabinovitch M, Guignabert C, Humbert M, Nicolls MR. Inflammation and immunity in the pathogenesis of pulmonary arterial hypertension. *Circ Res.* Jun 20 2014;115(1):165-175.

31. El Chami H, Hassoun PM. Inflammatory mechanisms in the pathogenesis of pulmonary arterial hypertension. *Compr Physiol.* Oct 2011;1(4):1929-1941.

32. El Chami H, Hassoun PM. Immune and inflammatory mechanisms in pulmonary arterial hypertension. *Prog Cardiovasc Dis.* Sep-Oct 2012;55(2):218-228.

33. Tuder RM, Cool CD, Yeager M, Taraseviciene-Stewart L, Bull TM, Voelkel NF. The pathobiology of pulmonary hypertension. *Clinics in chest medicine.* 2001;22(3):405-418.

34. Philippe Herve, Marc Humbert, Olivier Sitbon, et al. Pathobiology of pulmonary hypertension. The role of platelets and thrombosis. *Clin Chest Med.* 2001;22:451-458.

35. O'Callaghan DS, Savale L, Montani D, et al. Treatment of pulmonary arterial hypertension with targeted therapies. *Nat Rev Cardiol.* Jul 19 2011;8(9):526-538.

36. McLaughlin VV, Archer SL, Badesch DB, et al. ACCF/AHA 2009 expert consensus document on pulmonary hypertension a report of the American College of Cardiology Foundation Task Force on Expert Consensus Documents and the American Heart Association developed in collaboration with the American College of Chest Physicians; American Thoracic Society, Inc.; and the Pulmonary Hypertension Association. *J Am Coll Cardiol.* Apr 28 2009;53(17):1573-1619.

37. Montani D, Chaumais MC, Guignabert C, et al. Targeted therapies in pulmonary arterial hypertension. *Pharmacol Ther.* Feb 2014;141(2):172-191.

38. Tuder RM CC, Geraci MW, et al. Prostacyclin syn-thase expression is decreased in lungs from patients withsevere pulmonary hypertension. *Am J Respir Crit Care Med.* 1999;159:1925-1932.

39. Maron BA, Loscalzo J. Pulmonary hypertension: pathophysiology and signaling pathways. *Handb Exp Pharmacol.* 2013;218:31-58.

40. Giaid A YM, Langleben D, et al. Expression ofendothelin-1 in the lungs of patients with pulmonary hyper-tension. *N Engl J Med.* 1993;328:1732-1739.

41. Rubens C, Ewert R, Halank M, et al. Big Endothelin-1 and Endothelin-1 Plasma Levels Are Correlated With the Severity of Primary Pulmonary Hypertension. *Chest.* 2001;120(5):1562-1569.

42. Liu C CJ, Gao Y, Deng B, Liu K. Endothelin receptor antag-onists for pulmonary arterial hypertension. *Cochrane DatabaseSyst Rev.* 2013;2:CD004434.

43. Gangopahyay A, Oran M, Bauer EM, et al. Bone morphogenetic protein receptor II is a novel mediator of endothelial nitric-oxide synthase activation. *J Biol Chem.* Sep 23 2011;286(38):33134-33140.

44. Francis SH, Busch JL, Corbin JD, Sibley D. cGMP-dependent protein kinases and cGMP phosphodiesterases in nitric oxide and cGMP action. *Pharmacol Rev.* Sep 2010;62(3):525-563.

45. Nagendran J, Archer SL, Soliman D, et al. Phosphodiesterase type 5 is highly expressed in the hypertrophied human right ventricle, and acute inhibition of phosphodiesterase type 5 improves contractility. *Circulation.* Jul 17 2007;116(3):238-248.

46. Wharton J, Strange JW, Moller GM, et al. Antiproliferative effects of phosphodiesterase type 5 inhibition in human pulmonary artery cells. *Am J Respir Crit Care Med.* Jul 01 2005;172(1):105-113.

47. Waxman AB. Pulmonary hypertension in heart failure with preserved ejection fraction: a target for therapy? *Circulation.* Jul 12 2011;124(2):133-135.

48. Sutendra G, Dromparis P, Bonnet S, et al. Pyruvate dehydrogenase inhibition by the inflammatory cytokine TNFalpha contributes to the pathogenesis of pulmonary arterial hypertension. *J Mol Med (Berl).* Aug 2011;89(8):771-783.

49. Weir EK L-BJ, Buckler KJ, Archer SL. Acute oxygen-sensing mechanisms. *N Engl J Med.* 2005;353:2042-2055.

50. Bonnet S, Michelakis ED, Porter CJ, et al. An abnormal mitochondrial-hypoxia inducible factor-1alpha-Kv channel pathway disrupts oxygen sensing and triggers pulmonary arterial hypertension in fawn hooded rats: similarities to human pulmonary arterial hypertension. *Circulation.* Jun 06 2006;113(22):2630-2641.

51. Bonnet S RG, Sutendra G, et al. The nuclear fac-tor of activated T cells in pulmonary arterial hypertensioncan be therapeutically targeted. *Proc Natl Acad Sci U S A.* 2007;104:11418—11423.

52. Yuan JX AA, Juhaszova M, et al. Dysfunctionalvoltage-gated K+ channels in pulmonary artery smooth musclecells of patients with primary pulmonary hypertension. *Circulation.* 1998;98:1400-1406.

53. Pozeg ZI, Michelakis ED, McMurtry MS, et al. In vivo gene transfer of the O2-sensitive potassium channel Kv1.5 reduces pulmonary hypertension and restores hypoxic pulmonary vasoconstriction in chronically hypoxic rats. *Circulation.* Apr 22 2003;107(15):2037-2044.

54. Bonnet S, Archer SL, Allalunis-Turner J, et al. A mitochondria-K+ channel axis is suppressed in cancer and its normalization promotes apoptosis and inhibits cancer growth. *Cancer Cell.* Jan 2007;11(1):37-51.

55. MacLean MR HP, Eddahibi S, Adnot S. 5-hydroxytryptamine and the pulmonary circulation: receptors,transporters and relevance to pulmonary arterial hypertension. *Br J Pharmacol.* 2000;131:161-168.

56. Herve P LJ, Scrobohaci ML, et al. Increased plasmaserotonin in primary pulmonary hypertension. *Am J Med.* 1995;99:249-254.

57. Guilluy C, Sauzeau V, Rolli-Derkinderen M, et al. Inhibition of RhoA/Rho kinase pathway is involved in the beneficial effect of sildenafil on pulmonary hypertension. *Br J Pharmacol.* Dec 2005;146(7):1010-1018.

58. Guilluy C, Eddahibi S, Agard C, et al. RhoA and Rho kinase activation in human pulmonary hypertension: role of 5-HT signaling. *Am J Respir Crit Care Med.* Jun 15 2009;179(12):1151-1158.

59. Suzuki YJ, Day RM, Tan CC, et al. Activation of GATA-4 by serotonin in pulmonary artery smooth muscle cells. *J Biol Chem.* May 09 2003;278(19):17525-17531.

60. Loyd JE PR, Newman JH. Familial primary pulmonary hypertension: Clinical patterns. *The American review of respiratory disease.* 1984(129):194-197.

61. Machado RD, Southgate L, Eichstaedt CA, et al. Pulmonary Arterial Hypertension: A Current Perspective on Established and Emerging Molecular Genetic Defects. *Hum Mutat.* Dec 2015;36(12):1113-1127.

62. Soubrier F, Chung WK, Machado R, et al. Genetics and Genomics of Pulmonary Arterial Hypertension. *Journal of the American College of Cardiology.* 2013;62(25):D13-D21.

63. Machado RD, Aldred MA, James V, et al. Mutations of the TGF-β type II receptorBMPR2 in pulmonary arterial hypertension. *Human Mutation.* 2006;27(2):121-132.

64. Marom B, Heining E, Knaus P, Henis YI. Formation of stable homomeric and transient heteromeric bone morphogenetic protein (BMP) receptor complexes regulates Smad protein signaling. *J Biol Chem.* Jun 03 2011;286(22):19287-19296.

65. Davis BN, Hilyard AC, Lagna G, Hata A. SMAD proteins control DROSHA-mediated microRNA maturation. *Nature.* Jul 03 2008;454(7200):56-61.

66. King KE, Iyemere VP, Weissberg PL, Shanahan CM. Kruppel-like factor 4 (KLF4/GKLF) is a target of bone morphogenetic proteins and transforming growth factor beta 1 in the regulation of vascular smooth muscle cell phenotype. *J Biol Chem.* Mar 28 2003;278(13):11661-11669.

67. Girerd B, Montani D, Coulet F, et al. Clinical outcomes of pulmonary arterial hypertension in patients carrying an ACVRL1 (ALK1) mutation. *Am J Respir Crit Care Med.* Apr 15 2010;181(8):851-861.

68. Harrison RE FJ, Sankelo M, Abdalla SA, Rowell J, Machado RD, Elliott CG, Robbins IM, Olschewski H, McLaughlin V, Gruenig E, Kermeen F, Halme M, Raisanen-Sokolowski A, Laitinen T, Morrell NW, Trembath RC. Molecular and functional analysis identifies alk-1 as the predominant cause of pulmonary hypertension related to hereditary haemorrhagic telangiectasia. *Journal of medical genetics.* 2003;40:865-871.

69. Morrell NW YX, Upton PD, Jourdan KB, Morgan N, Sheares KK, Trembath RC. Altered growth responses of pulmonary artery smooth muscle cells from patients with primary pulmonary hypertension to transforming growth factor-beta(1) and bone morphogenetic proteins. *Circulation.* 2001;104:790-795.

70. Ma L, Roman-Campos D, Austin ED, et al. A novel channelopathy in pulmonary arterial hypertension. *N Engl J Med.* Jul 25 2013;369(4):351-361.

71. Chin K, Kim N, Rubin L. The right ventricle in pulmonaryhypertension. *Coron Artery Dis* 2005;16:13-18.

72. Vonk Noordegraaf A, Galie N. The role of the right ventricle in pulmonary arterial hypertension. *Eur Respir Rev.* Dec 2011;20(122):243-253.

73. Bogaard HJ, Abe K, Vonk Noordegraaf A, Voelkel NF. The right ventricle under pressure: cellular and molecular mechanisms of right-heart failure in pulmonary hypertension. *Chest.* Mar 2009;135(3):794-804.

74. Sano M, Minamino T, Toko H, et al. p53-induced inhibition of Hif-1 causes cardiac dysfunction during pressure overload. *Nature.* Mar 22 2007;446(7134):444-448.

75. Velez-Roa S, Ciarka A, Najem B, Vachiery JL, Naeije R, van de Borne P. Increased sympathetic nerve activity in pulmonary artery hypertension. *Circulation.* Sep 07 2004;110(10):1308-1312.

76. Gan C, Lankhaar JW, Marcus JT, et al. Impaired left ventricular filling due to right-to-left ventricular interaction in patients with pulmonary arterial hypertension. *Am J Physiol Heart Circ Physiol.* Apr 2006;290(4):H1528-1533.

77. Marsboom GR, Janssens SP. Models for pulmonary hypertension. *Drug Discovery Today: Disease Models.* 2004;1(3):289-296.

78. Mendes-Ferreira P, Maia-Rocha C, Adao R, et al. Neuregulin-1 improves right ventricular function and attenuates experimental pulmonary arterial hypertension. *Cardiovasc Res.* Jan 01 2016;109(1):44-54.

79. Schroll S, Arzt M, Sebah D, Nuchterlein M, Blumberg F, Pfeifer M. Improvement of bleomycin-induced pulmonary hypertension and pulmonary fibrosis by the endothelin receptor antagonist Bosentan. *Respir Physiol Neurobiol.* Jan 31 2010;170(1):32-36.

80. Sato K WS, Tucker A, Rabinovitch M, O'Brien RF, McMurtry IF, Stelzner TJ. Factors influencing the idiopathic development of pulmonary hypertension in the fawn hooded rat. *American review of respiratory disease.* 1992;145:793-797.

81. West J, Fagan K, Steudel W, et al. Pulmonary hypertension in transgenic mice expressing a dominant-negative BMPRII gene in smooth muscle. *Circ Res.* Apr 30 2004;94(8):1109-1114.

82. Schnader J SB, Anderson W, Stephenson LW, Fishman AP. Chronic pulmonary hypertension in sheep: Temporal progression of lesions. *The Journal of surgical research.* 1996;62:243-250.

83. Zagorski J, Debelak J, Gellar M, Watts JA, Kline JA. Chemokines Accumulate in the Lungs of Rats with Severe Pulmonary Embolism Induced by Polystyrene Microspheres. *The Journal of Immunology.* 2003;171(10):5529-5536.

84. Benisty JI. Pulmonary Hypertension. *Circulation.* 2002;106(24):192e-194.

85. Stenmark KR, Meyrick B, Galie N, Mooi WJ, McMurtry IF. Animal models of pulmonary arterial hypertension: the hope for etiological discovery and pharmacological cure. *Am J Physiol Lung Cell Mol Physiol.* Dec 2009;297(6):L1013-1032.

86. Benza RL, Miller DP, Gomberg-Maitland M, et al. Predicting survival in pulmonary arterial hypertension: insights from the Registry to Evaluate Early and Long-Term Pulmonary Arterial Hypertension Disease Management (REVEAL). *Circulation.* Jul 13 2010;122(2):164-172.

87. Farber HW LJ. Pulmonary arterial hypertension. *N Engl J Med. .* 2004;351(16):1655–1665.

88. Austin ED, Loyd JE. The genetics of pulmonary arterial hypertension. *Circ Res.* Jun 20 2014;115(1):189-202.

89. Hansmann G, Wagner RA, Schellong S, et al. Pulmonary arterial hypertension is linked to insulin resistance and reversed by peroxisome proliferator-activated receptor-gamma activation. *Circulation.* Mar 13 2007;115(10):1275-1284.
90. Zamanian RT, Hansmann G, Snook S, et al. Insulin resistance in pulmonary arterial hypertension. *Eur Respir J.* Feb 2009;33(2):318-324.
91. Pugh ME, Robbins IM, Rice TW, West J, Newman JH, Hemnes AR. Unrecognized glucose intolerance is common in pulmonary arterial hypertension. *J Heart Lung Transplant.* Aug 2011;30(8):904-911.
92. Belly MJ, Tiede H, Morty RE, et al. HbA1c in pulmonary arterial hypertension: a marker of prognostic relevance? *J Heart Lung Transplant.* Oct 2012;31(10):1109-1114.
93. Fessel JP, Hamid R, Wittmann BM, et al. Metabolomic analysis of bone morphogenetic protein receptor type 2 mutations in human pulmonary endothelium reveals widespread metabolic reprogramming. *Pulm Circ.* Apr-Jun 2012;2(2):201-213.
94. Hemnes AR, Brittain EL, Trammell AW, et al. Evidence for right ventricular lipotoxicity in heritable pulmonary arterial hypertension. *Am J Respir Crit Care Med.* Feb 01 2014;189(3):325-334.
95. Chung WJ, Park YB, Jeon CH, et al. Baseline Characteristics of the Korean Registry of Pulmonary Arterial Hypertension. *J Korean Med Sci.* Oct 2015;30(10):1429-1438.
96. R.M. Tuder BG, D.B. Badesch, N.F. Voelkel. Exuberant endothelial cell growth and elements of inflammation are present in plexiform lesions of pulmonary hypertension. *Am. J. Pathol.* 1994;144:275–285.
97. L. Taraseviciene-Stewart YK, L. Alger, P. Hirth, G. Mc Mahon, J., Waltenberger NFV, R.M. Tuder. Inhibition of the VEGF receptor 2 combined with chronic hypoxia causes cell death-dependent pulmonary endothelial cell proliferation and severe pulmonary hypertension. *FASEB J.* 2001;15 427–438.
98. Liu N, Parry S, Xiao Y, Zhou S, Liu Q. Molecular targets of the Warburg effect and inflammatory cytokines in the pathogenesis of pulmonary artery hypertension. *Clin Chim Acta.* Mar 2017;466:98-104.
99. Paulin R, Michelakis ED. The metabolic theory of pulmonary arterial hypertension. *Circ Res.* Jun 20 2014;115(1):148-164.
100. Lee M, Yoon JH. Metabolic interplay between glycolysis and mitochondrial oxidation: The reverse Warburg effect and its therapeutic implication. *World J Biol Chem.* Aug 26 2015;6(3):148-161.
101. Cottrill KA, Chan SY. Metabolic dysfunction in pulmonary hypertension: the expanding relevance of the Warburg effect. *Eur J Clin Invest.* Aug 2013;43(8):855-865.
102. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell.* Mar 04 2011;144(5):646-674.
103. Peng H, Xiao Y, Deng X, Luo J, Hong C, Qin X. The Warburg effect: A new story in pulmonary arterial hypertension. *Clin Chim Acta.* Oct 01 2016;461:53-58.
104. Cui Y, Qin L, Wu J, et al. SIRT3 Enhances Glycolysis and Proliferation in SIRT3-Expressing Gastric Cancer Cells. *PLoS One.* 2015;10(6):e0129834.
105. Price LC, Wort SJ, Perros F, et al. Inflammation in pulmonary arterial hypertension. *Chest.* Jan 2012;141(1):210-221.
106. Finley LW, Haas W, Desquiret-Dumas V, et al. Succinate dehydrogenase is a direct target of sirtuin 3 deacetylase activity. *PLoS One.* 2011;6(8):e23295.
107. Paulin R, Dromparis P, Sutendra G, et al. Sirtuin 3 deficiency is associated with inhibited mitochondrial function and pulmonary arterial hypertension in rodents and humans. *Cell Metab.* Nov 04 2014;20(5):827-839.
108. He W, Newman JC, Wang MZ, Ho L, Verdin E. Mitochondrial sirtuins: regulators of protein acylation and metabolism. *Trends Endocrinol Metab.* Sep 2012;23(9):467-476.
109. Paulin R, Courboulin A, Meloche J, et al. Signal transducers and activators of transcription-3/pim1 axis plays a critical role in the pathogenesis of human pulmonary arterial hypertension. *Circulation.* Mar 22 2011;123(11):1205-1215.
110. B. Sebastien RG, S. Gopinath, et al. The nuclear factor of activated T cells in pulmonary arterial hypertension can be therapeutically targeted. *Proc. Natl. Acad. Sci.* 2007;104 11418–11423.
111. Boengler K, Hilfiker-Kleiner D, Drexler H, Heusch G, Schulz R. The myocardial JAK/STAT pathway: from protection to failure. *Pharmacol Ther.* Nov 2008;120(2):172-185.

112. Gough DJ, Corlett A, Schlessinger K, Wegrzyn J, Larner AC, Levy DE. Mitochondrial STAT3 supports Ras-dependent oncogenic transformation. *Science.* Jun 26 2009;324(5935):1713-1716.
113. Wang AP, Li XH, Gong SX, et al. miR-100 suppresses mTOR signaling in hypoxia-induced pulmonary hypertension in rats. *Eur J Pharmacol.* Oct 15 2015;765:565-573.
114. E. Berghausen HTF, S. Rosenkranz. Targeting of platelet-derived growth factor signaling in pulmonary arterial hypertension. *Handb. Exp. Pharmacol.* 2013;218:381–408.
115. Theisen CS, Wahl JK, 3rd, Johnson KR, Wheelock MJ. NHERF links the N-cadherin/catenin complex to the platelet-derived growth factor receptor to modulate the actin cytoskeleton and regulate cell motility. *Mol Biol Cell.* Apr 2007;18(4):1220-1232.
116. Andrae J, Gallini R, Betsholtz C. Role of platelet-derived growth factors in physiology and medicine. *Genes Dev.* May 15 2008;22(10):1276-1312.
117. Perros F, Montani D, Dorfmuller P, et al. Platelet-derived growth factor expression and function in idiopathic pulmonary arterial hypertension. *Am J Respir Crit Care Med.* Jul 01 2008;178(1):81-88.
118. Overbeek MJ, Boonstra A, Voskuyl AE, et al. Platelet-derived growth factor receptor-beta and epidermal growth factor receptor in pulmonary vasculature of systemic sclerosis-associated pulmonary arterial hypertension versus idiopathic pulmonary arterial hypertension and pulmonary veno-occlusive disease: a case-control study. *Arthritis Res Ther.* Apr 14 2011;13(2):R61.
119. Dahal BK, Heuchel R, Pullamsetti SS, et al. Hypoxic pulmonary hypertension in mice with constitutively active platelet-derived growth factor receptor-beta. *Pulm Circ.* Apr-Jun 2011;1(2):259-268.
120. Perez J, Hill BG, Benavides GA, Dranka BP, Darley-Usmar VM. Role of cellular bioenergetics in smooth muscle cell proliferation induced by platelet-derived growth factor. *Biochem J.* May 13 2010;428(2):255-267.
121. Werle M, Kreuzer J, Hofele J, et al. Metabolic control analysis of the Warburg-effect in proliferating vascular smooth muscle cells. *J Biomed Sci.* Oct 2005;12(5):827-834.
122. Ten Freyhaus H, Dagnell M, Leuchs M, et al. Hypoxia enhances platelet-derived growth factor signaling in the pulmonary vasculature by down-regulation of protein tyrosine phosphatases. *Am J Respir Crit Care Med.* Apr 15 2011;183(8):1092-1102.
123. N.V. Iyer LEK, F. Agani, S.W. Leung, E. Laughner, R.H. Wenger, M. Gassmann, J.D. Gearhart, A.M. Lawler, A.Y. Yu, G.L. Semenza. Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha. *Genes Dev.* 1998;12 149–162.
124. Luo W, Hu H, Chang R, et al. Pyruvate kinase M2 is a PHD3-stimulated coactivator for hypoxia-inducible factor 1. *Cell.* May 27 2011;145(5):732-744.
125. Semenza GL. Hypoxia-inducible factor 1: regulator of mitochondrial metabolism and mediator of ischemic preconditioning. *Biochim Biophys Acta.* Jul 2011;1813(7):1263-1268.
126. Xu W, Erzurum SC. Endothelial cell energy metabolism, proliferation, and apoptosis in pulmonary hypertension. *Compr Physiol.* Jan 2011;1(1):357-372.
127. W. Xu TK, A.R. Lara, et al. Alterations of cellular bioenergetics in pulmonary artery endothelial cells. *Proc. Natl. Acad. Sci. .* 2007;104 1342–1347.
128. Garber K. Energy deregulation: licensing tumors to grow. *Science* 2006;312 1158–1159.
129. Wang RN, Green J, Wang Z, et al. Bone Morphogenetic Protein (BMP) signaling in development and human diseases. *Genes Dis.* Sep 2014;1(1):87-105.
130. Langleben D, Orfanos SE, Giovinazzo M, et al. Pulmonary capillary endothelial metabolic dysfunction: severity in pulmonary arterial hypertension related to connective tissue disease versus idiopathic pulmonary arterial hypertension. *Arthritis Rheum.* Apr 2008;58(4):1156-1164.
131. Diebold I, Hennigs JK, Miyagawa K, et al. BMPR2 preserves mitochondrial function and DNA during reoxygenation to promote endothelial cell survival and reverse pulmonary hypertension. *Cell Metab.* Apr 07 2015;21(4):596-608.
132. Mathew R. Pulmonary hypertension and metabolic syndrome: Possible connection, PPARgamma and Caveolin-1. *World J Cardiol.* Aug 26 2014;6(8):692-705.
133. Zhao YY, Malik AB. A novel insight into the mechanism of pulmonary hypertension involving caveolin-1 deficiency and endothelial nitric oxide synthase activation. *Trends Cardiovasc Med.* Oct 2009;19(7):238-242.

134. Y.Y. Zhao YL, R.V. Stan, et al. Defects in caveolin-1 cause dilated cardiomyopathy and pulmonary hypertension in knockout mice. *Proc. Natl. Acad. Sci. .* 2002;99 11375–11380.
135. Mathew R. Cell-specific dual role of caveolin-1 in pulmonary hypertension. *Pulm Med.* 2011;2011:573432.
136. Shiroto T, Romero N, Sugiyama T, et al. Caveolin-1 is a critical determinant of autophagy, metabolic switching, and oxidative stress in vascular endothelium. *PLoS One.* 2014;9(2):e87871.
137. R.M. Tuder MC, L. Alger, et al. Expression of angiogenesis-related molecules in plexiform lesions in severe pulmonary hypertension: evidence for a process of disordered angiogenesis. *J. Pathol.* 2001;195 367–374.
138. J. Mateo MG-L, S. Cadenas, et al. Regulation of hypoxia-inducible factor-1alpha by nitric oxide through mitochondria-dependent and -independent pathways. *Biochem. J.* 2003;376 537–544.
139. Hagen T, Taylor CT, Lam F, Moncada S. Redistribution of intracellular oxygen in hypoxia by nitric oxide: effect on HIF1alpha. *Science.* Dec 12 2003;302(5652):1975-1978.
140. Piao L, Marsboom G, Archer SL. Mitochondrial metabolic adaptation in right ventricular hypertrophy and failure. *J Mol Med (Berl).* Oct 2010;88(10):1011-1020.
141. Kleiman RB HS. Outward currents in normal and hypertrophied feline ventricular monocytes. *Am J Physiol* 1989;256:H1450–1461.
142. Tuder RM, Davis LA, Graham BB. Targeting energetic metabolism: a new frontier in the pathogenesis and treatment of pulmonary hypertension. *Am J Respir Crit Care Med.* Feb 01 2012;185(3):260-266.
143. Bogaard HJ, Natarajan R, Henderson SC, et al. Chronic pulmonary artery pressure elevation is insufficient to explain right heart failure. *Circulation.* Nov 17 2009;120(20):1951-1960.
144. Redout EM, Wagner MJ, Zuidwijk MJ, et al. Right-ventricular failure is associated with increased mitochondrial complex II activity and production of reactive oxygen species. *Cardiovasc Res.* Sep 01 2007;75(4):770-781.
145. Oikawa M, Kagaya Y, Otani H, et al. Increased [18F]fluorodeoxyglucose accumulation in right ventricular free wall in patients with pulmonary hypertension and the effect of epoprostenol. *J Am Coll Cardiol.* Jun 07 2005;45(11):1849-1855.
146. Piao L, Fang YH, Cadete VJ, et al. The inhibition of pyruvate dehydrogenase kinase improves impaired cardiac function and electrical remodeling in two models of right ventricular hypertrophy: resuscitating the hibernating right ventricle. *J Mol Med (Berl).* Jan 2010;88(1):47-60.
147. Buermans HP, Redout EM, Schiel AE, et al. Microarray analysis reveals pivotal divergent mRNA expression profiles early in the development of either compensated ventricular hypertrophy or heart failure. *Physiol Genomics.* May 11 2005;21(3):314-323.
148. S. B. The neuregulin-i/erbb signaling system in development and disease. *Advances in anatomy, embryology, and cell biology.* 2007;190:1-65.
149. Luo X, Prior M, He W, et al. Cleavage of neuregulin-1 by BACE1 or ADAM10 protein produces differential effects on myelination. *J Biol Chem.* Jul 08 2011;286(27):23967-23974.
150. Falls D. Neuregulins: functions, forms, and signaling strategies. *Experimental Cell Research.* 2003;284(1):14-30.
151. Lemmens K, Segers VF, Demolder M, De Keulenaer GW. Role of neuregulin-1/ErbB2 signaling in endothelium-cardiomyocyte cross-talk. *J Biol Chem.* Jul 14 2006;281(28):19469-19477.
152. Yarden Y SM. Untangling the erbb signalling network. *Nature reviews. Molecular cell biology.* 2001;2:127-137.
153. Cote GM, Miller TA, Lebrasseur NK, Kuramochi Y, Sawyer DB. Neuregulin-1alpha and beta isoform expression in cardiac microvascular endothelial cells and function in cardiac myocytes in vitro. *Exp Cell Res.* Nov 15 2005;311(1):135-146.
154. Meyer D BC. Multiple essential functions of neuregulin in development. *Nature.* 1995;378:386-390.
155. DL. B. Cardiac endothelial-myocardial signaling: Its role in cardiac growth, contractile performance, and rhythmicity. *Physiological reviews.* 2003;83:59-115.
156. Gassmann M CF, Orioli D, et al. Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. *Nature.* 1995;378(6555):390-394.

157. Lee KF SH, Chen H, et al. Requirement for neuregulin receptor erbB2 in neural and cardiac development. *Nature.* 1995;378(6555):119-124.

158. Lemmens K, Doggen, K., & Keulenaer, G. W. Neuregulin-1 and its potential role in the control of cardiac function. *Heart failure monitor.* 2008;5(4):119.

159. Erickson SL OSK, Ghaboosi N, et al. ErbB3 is required for normal cerebellar and cardiac development: a comparison with ErbB2-and heregulin-deficient mice. *Development.* 1997;124(24):4999-5011.

160. Patel R, Kos L. Endothelin-1 and Neuregulin-1 convert embryonic cardiomyocytes into cells of the conduction system in the mouse. *Dev Dyn.* May 2005;233(1):20-28.

161. Rentschler S ZJ, Meyers K, et al. Neuregulin-1 promotes formation of the murine cardiac conduction system. *Proc Natl Acad Sci U S A.* 2002;99(16):10464-10469.

162. Suk Kim H, Hidaka K, Morisaki T. Expression of ErbB receptors in ES cell-derived cardiomyocytes. *Biochemical and Biophysical Research Communications.* 2003;309(1):241-246.

163. Zhu WZ, Xie Y, Moyes KW, Gold JD, Askari B, Laflamme MA. Neuregulin/ErbB signaling regulates cardiac subtype specification in differentiating human embryonic stem cells. *Circ Res.* Sep 17 2010;107(6):776-786.

164. Fukazawa R. Neuregulin-1 protects ventricular myocytes from anthracycline-induced apoptosis via erbB4-dependent activation of PI3-kinase/Akt. *Journal of Molecular and Cellular Cardiology.* 2003;35(12):1473-1479.

165. Zhao YY SD, Baliga RR, et al. Neuregulins promote survival and growth of cardiac myocytes. Persistence of ErbB2 and ErbB4 expression in neonatal and adult ventricular myocytes. *J Biol Chem.* 1998;273(17):10261-10269.

166. Baliga RR PD, Zhao YY, et al. NRG-1-induced cardiomyocyte hypertrophy. Role of PI-3-kinase, p70(S6K), and MEK-MAPK-RSK. *Am J Physiol.* 1999;277(5 Pt 2):H2026-2037.

167. Sawyer DB. Modulation of Anthracycline-Induced Myofibrillar Disarray in Rat Ventricular Myocytes by Neuregulin-1beta and Anti-erbB2: Potential Mechanism for Trastuzumab-Induced Cardiotoxicity. *Circulation.* 2002;105(13):1551-1554.

168. Kuramochi Y, Guo X, Sawyer DB. Neuregulin activates erbB2-dependent src/FAK signaling and cytoskeletal remodeling in isolated adult rat cardiac myocytes. *J Mol Cell Cardiol.* Aug 2006;41(2):228-235.

169. Kuramochi Y, Cote GM, Guo X, et al. Cardiac endothelial cells regulate reactive oxygen species-induced cardiomyocyte apoptosis through neuregulin-1beta/erbB4 signaling. *J Biol Chem.* Dec 03 2004;279(49):51141-51147.

170. Kuramochi Yea. Myocyte contractile activity modulates norepinephrine cytotoxicity and survival effects of neuregulin-1beta. *Am. J. Physiol. Cell Physiol.* 2004;286:222-229.

171. Rohrbach S, Muller-Werdan U, Werdan K, Koch S, Gellerich NF, Holtz J. Apoptosis-modulating interaction of the neuregulin/erbB pathway with anthracyclines in regulating Bcl-xS and Bcl-xL in cardiomyocytes. *J Mol Cell Cardiol.* Mar 2005;38(3):485-493.

172. Bersell K, Arab S, Haring B, Kuhn B. Neuregulin1/ErbB4 signaling induces cardiomyocyte proliferation and repair of heart injury. *Cell.* Jul 23 2009;138(2):257-270.

173. Brero A, Ramella R, Fitou A, et al. Neuregulin-1beta1 rapidly modulates nitric oxide synthesis and calcium handling in rat cardiomyocytes. *Cardiovasc Res.* Dec 01 2010;88(3):443-452.

174. Gu X, Liu X, Xu D, et al. Cardiac functional improvement in rats with myocardial infarction by up-regulating cardiac myosin light chain kinase with neuregulin. *Cardiovasc Res.* Nov 01 2010;88(2):334-343.

175. Mendes-Ferreira P, De Keulenaer GW, Leite-Moreira AF, Bras-Silva C. Therapeutic potential of neuregulin-1 in cardiovascular disease. *Drug Discov Today.* Sep 2013;18(17-18):836-842.

176. Lemmens K, Fransen P, Sys SU, Brutsaert DL, De Keulenaer GW. Neuregulin-1 induces a negative inotropic effect in cardiac muscle: role of nitric oxide synthase. *Circulation.* Jan 27 2004;109(3):324-326.

177. Zhao YY FO, Dessy C, Han X, Marchionni MA, Kelly RA. . Neuregulin signaling in the heart. Dynamic targeting of erbb4 to caveolar microdomains in cardiac myocytes. *Circulation research.* 1999;84:1380-1387.

178. Okoshi K, Nakayama M, Yan X, et al. Neuregulins regulate cardiac parasympathetic activity: muscarinic modulation of beta-adrenergic activity in myocytes from mice with neuregulin-1 gene deletion. *Circulation.* Aug 10 2004;110(6):713-717.
179. Russell KS SD, Polverini PJ, Bender JR. . Neuregulin activation of erbb receptors in vascular endothelium leads to angiogenesis. *The American journal of physiology.* 1999;277:H2205-2211.
180. Iivanainen E, Paatero I, Heikkinen SM, et al. Intra- and extracellular signaling by endothelial neuregulin-1. *Exp Cell Res.* Aug 01 2007;313(13):2896-2909.
181. Yen L YX, Al Moustafa AE, Batist G, Hynes NE, Mader S, Meloche S, Alaoui-Jamali MA. Heregulin selectively upregulates vascular endothelial growth factor secretion in cancer cells and stimulates angiogenesis. *Oncogene.* 2000;19:3460-3469.
182. Hedhli N, Dobrucki LW, Kalinowski A, et al. Endothelial-derived neuregulin is an important mediator of ischaemia-induced angiogenesis and arteriogenesis. *Cardiovasc Res.* Mar 01 2012;93(3):516-524.
183. Xu G, Watanabe T, Iso Y, et al. Preventive effects of heregulin-beta1 on macrophage foam cell formation and atherosclerosis. *Circ Res.* Aug 28 2009;105(5):500-510.
184. Panutsopulos D, Arvanitis DL, Tsatsanis C, Papalambros E, Sigala F, Spandidos DA. Expression of heregulin in human coronary atherosclerotic lesions. *J Vasc Res.* Nov-Dec 2005;42(6):463-474.
185. Clement CM, Thomas LK, Mou Y, Croslan DR, Gibbons GH, Ford BD. Neuregulin-1 attenuates neointimal formation following vascular injury and inhibits the proliferation of vascular smooth muscle cells. *J Vasc Res.* 2007;44(4):303-312.
186. Xu Y, Li X, Zhou M. Neuregulin-1/ErbB signaling: a druggable target for treating heart failure. *Curr Opin Pharmacol.* Apr 2009;9(2):214-219.
187. Crone SA ZY, Fan L, Gu Y, Minamisawa S, Liu Y, Peterson KL, Chen J, Kahn R, Condorelli G, Ross J, Jr., Chien KR, Lee KF. . Erbb2 is essential in the prevention of dilated cardiomyopathy. *Nature medicine.* 2002;8:459-465.
188. Garcia-Rivello H, Taranda J, Said M, et al. Dilated cardiomyopathy in Erb-b4-deficient ventricular muscle. *Am J Physiol Heart Circ Physiol.* Sep 2005;289(3):H1153-1160.
189. Ozcelik C EB, Pilz B, Wettschureck N, Britsch S, Hubner N, Chien KR, Birchmeier C, Garratt AN. Conditional mutation of the erbb2 (her2) receptor in cardiomyocytes leads to dilated cardiomyopathy. *Proceedings of the National Academy of Sciences of the United States of America.* 2002;99:8880-8885.
190. Fang SJ WX, Han ZH, Zhang XX, Wang CM, Li XY, Lu LQ, Zhang JL. . Neuregulin-1 preconditioning protects the heart against ischemia/reperfusion injury through a pi3k/akt-dependent mechanism. *Chinese medical journal.* 2010;123:3597-3604.
191. Ebner B, Lange SA, Hollenbach D, et al. In situ postconditioning with neuregulin-1beta is mediated by a PI3K/Akt-dependent pathway. *Can J Cardiol.* Jan 2015;31(1):76-83.
192. D'Uva G, Aharonov A, Lauriola M, et al. ERBB2 triggers mammalian heart regeneration by promoting cardiomyocyte dedifferentiation and proliferation. *Nat Cell Biol.* May 2015;17(5):627-638.
193. Liu FF, Stone JR, Schuldt AJ, et al. Heterozygous knockout of neuregulin-1 gene in mice exacerbates doxorubicin-induced heart failure. *Am J Physiol Heart Circ Physiol.* Aug 2005;289(2):H660-666.
194. Vasti C, Witt H, Said M, et al. Doxorubicin and NRG-1/erbB4-Deficiency Affect Gene Expression Profile: Involving Protein Homeostasis in Mouse. *ISRN Cardiol.* 2012;2012:745185.
195. Timolati F, Ott D, Pentassuglia L, et al. Neuregulin-1 beta attenuates doxorubicin-induced alterations of excitation-contraction coupling and reduces oxidative stress in adult rat cardiomyocytes. *J Mol Cell Cardiol.* Nov 2006;41(5):845-854.
196. Vandekerckhove L, Vermeulen Z, Liu ZZ, et al. Neuregulin-1 attenuates development of nephropathy in a type 1 diabetes mouse model with high cardiovascular risk. *Am J Physiol Endocrinol Metab.* Apr 01 2016;310(7):E495-504.
197. Gui C, Zhu L, Hu M, Lei L, Long Q. Neuregulin-1/ErbB signaling is impaired in the rat model of diabetic cardiomyopathy. *Cardiovasc Pathol.* Sep-Oct 2012;21(5):414-420.
198. Doggen K, Ray L, Mathieu M, Mc Entee K, Lemmens K, De Keulenaer GW. Ventricular ErbB2/ErbB4 activation and downstream signaling in pacing-induced heart failure. *J Mol Cell Cardiol.* Jan 2009;46(1):33-38.

199. Dang R, Guo Y, Zhang L, Chen L, Yang R, Jiang P. Chronic stress and excessive glucocorticoid exposure both lead to altered Neuregulin-1/ErbB signaling in rat myocardium. *Steroids.* Aug 2016;112:47-53.
200. Cai MX, Shi XC, Chen T, et al. Exercise training activates neuregulin 1/ErbB signaling and promotes cardiac repair in a rat myocardial infarction model. *Life Sci.* Mar 15 2016;149:1-9.
201. Rohrbach S YX, Weinberg EO, Hasan F, Bartunek J, Marchionni MA, Lorell BH. . Neuregulin in cardiac hypertrophy in rats with aortic stenosis. Differential expression of erbb2 and erbb4 receptors. *Circulation.* 1999;100:407-412.
202. Matsukawa R, Hirooka Y, Ito K, Honda N, Sunagawa K. Central neuregulin-1/ErbB signaling modulates cardiac function via sympathetic activity in pressure overload-induced heart failure. *J Hypertens.* Apr 2014;32(4):817-825.
203. Seidman A HC, Pierri MK, Shak S, Paton V, Ashby M, Murphy M, Stewart SJ, Keefe D. Cardiac dysfunction in the trastuzumab clinical trials experience. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology.* 2002;20:1215-1221.
204. De Keulenaer GW, Doggen K, Lemmens K. The vulnerability of the heart as a pluricellular paracrine organ: lessons from unexpected triggers of heart failure in targeted ErbB2 anticancer therapy. *Circ Res.* Jan 08 2010;106(1):35-46.
205. Rohrbach S, Niemann B, Silber RE, Holtz J. Neuregulin receptors erbB2 and erbB4 in failing human myocardium -- depressed expression and attenuated activation. *Basic Res Cardiol.* May 2005;100(3):240-249.
206. Ky B, Kimmel SE, Safa RN, et al. Neuregulin-1 beta is associated with disease severity and adverse outcomes in chronic heart failure. *Circulation.* Jul 28 2009;120(4):310-317.
207. Perik PJ, de Vries EG, Gietema JA, et al. Serum HER2 levels are increased in patients with chronic heart failure. *Eur J Heart Fail.* Feb 2007;9(2):173-177.
208. Posch MG PA, Kersten A, Ghadge SK, Geier C, Richter S, Perrot A, Gailani M, Dietz R, Luftner D, Ozcelik C. . Plasma her2 levels are not associated with cardiac function or hypertrophy in control subjects and heart failure patients. . *International journal of cardiology.* Nov 05 2010;145:105-106.
209. Suarez E, Bach D, Cadefau J, Palacin M, Zorzano A, Guma A. A novel role of neuregulin in skeletal muscle. Neuregulin stimulates glucose uptake, glucose transporter translocation, and transporter expression in muscle cells. *J Biol Chem.* May 25 2001;276(21):18257-18264.
210. Canto C, Suarez E, Lizcano JM, et al. Neuregulin signaling on glucose transport in muscle cells. *J Biol Chem.* Mar 26 2004;279(13):12260-12268.
211. Miller TA, Icli B, Cote GM, et al. Palmitate alters neuregulin signaling and biology in cardiac myocytes. *Biochem Biophys Res Commun.* Jan 30 2009;379(1):32-37.
212. Canto C, Pich S, Paz JC, et al. Neuregulins increase mitochondrial oxidative capacity and insulin sensitivity in skeletal muscle cells. *Diabetes.* Sep 2007;56(9):2185-2193.
213. Sherwin R. Role of the liver in glucose homeostasis. *Diabetes Care.* 1980;3(2).
214. Carver RS, Stevenson MC, Scheving LA, Russell WE. Diverse expression of ErbB receptor proteins during rat liver development and regeneration. *Gastroenterology.* Dec 2002;123(6):2017-2027.
215. Camprecios G LJ, Pardina E,Peinado-Onsurbe J,Soley M,RamirezI. Expression, localization, and regulation of the neuregulin receptor ErbB3 in mouse heart . *J Cell Physiol.* 2011;226(2):50–55.
216. Carver RS MP, Russell WE. . Hepatic expression of ErbB3 is repressed by insulin in a pathway sensitive to PI-3 kinase inhibitors. *Endocrinology.* 1997;138(12).
217. Carver RS SM, Sitaric S, Russell WE. . Insulin regulates heregulin binding and ErbB3 expression in rat hepatocytes. *J Biol Chem.* 1996;271(23):13491–13496.
218. Ennequin G, Boisseau N, Caillaud K, et al. Neuregulin 1 Improves Glucose Tolerance in db/db Mice. *PLoS One.* 2015;10(7):e0130568.
219. Lopez-Soldado I, Niisuke K, Veiga C, et al. Neuregulin improves response to glucose tolerance test in control and diabetic rats. *Am J Physiol Endocrinol Metab.* Mar 15 2016;310(6):E440-451.
220. Huang Z, Sawyer DB, Troy EL, et al. Species-specific effects of neuregulin-1beta (cimaglermin alfa) on glucose handling in animal models and humans with heart failure. *Toxicol Appl Pharmacol.* Oct 01 2017;332:92-99.
221. Bian Y, Sun M, Silver M, et al. Neuregulin-1 attenuated doxorubicin-induced decrease in cardiac troponins. *Am J Physiol Heart Circ Physiol.* Dec 2009;297(6):H1974-1983.

222. Guo YF ZX, Liu Y, Duan HY, Jie BZ, Wu XS. . Neuregulin-1 attenuates mitochondrial dysfunction in a rat model of heart failure. *Chinese medical journal.* 2012;125:807-814.
223. Li B ZZ, Wei Y, Wang M, Peng J, Kang T, Huang X, Xiao J, Li Y, Li Z. . Therapeutic effects of neuregulin-1 in diabetic cardiomyopathy rats.;. *Cardiovascular diabetology.* 2011;10:69.
224. Liu X, Gu X, Li Z, et al. Neuregulin-1/erbB-activation improves cardiac function and survival in models of ischemic, dilated, and viral cardiomyopathy. *J Am Coll Cardiol.* Oct 03 2006;48(7):1438-1447.
225. Xiao J, Li B, Zheng Z, et al. Therapeutic effects of neuregulin-1 gene transduction in rats with myocardial infarction. *Coron Artery Dis.* Nov 2012;23(7):460-468.
226. Cohen JE PB, MacArthur JW, Jr., Mu A, Shudo Y, Patel JB, Brusalis CM, Trubelja A, Fairman AS, Edwards BB, Davis MS, Hung G, Hiesinger W, Atluri P, Margulies KB, Burdick JA, Woo YJ. A bioengineered hydrogel system enables targeted and sustained intramyocardial delivery of neuregulin, activating the cardiomyocyte cell cycle and enhancing ventricular function in a murine model of ischemic cardiomyopathy. *Circulation. Heart failure.* 2014;7:619-626.
227. Pascual-Gil S, Simon-Yarza T, Garbayo E, Prosper F, Blanco-Prieto MJ. Tracking the in vivo release of bioactive NRG from PLGA and PEG-PLGA microparticles in infarcted hearts. *J Control Release.* Dec 28 2015;220(Pt A):388-396.
228. Formiga FR, Pelacho B, Garbayo E, et al. Controlled delivery of fibroblast growth factor-1 and neuregulin-1 from biodegradable microparticles promotes cardiac repair in a rat myocardial infarction model through activation of endogenous regeneration. *J Control Release.* Jan 10 2014;173:132-139.
229. Diaz-Herraez P, Saludas L, Pascual-Gil S, et al. Transplantation of adipose-derived stem cells combined with neuregulin-microparticles promotes efficient cardiac repair in a rat myocardial infarction model. *J Control Release.* Mar 10 2017;249:23-31.
230. Galindo CL, Kasasbeh E, Murphy A, et al. Anti-remodeling and anti-fibrotic effects of the neuregulin-1beta glial growth factor 2 in a large animal model of heart failure. *J Am Heart Assoc.* Oct 23 2014;3(5):e000773.
231. Parry TJ, Ganguly A, Troy EL, et al. Effects of neuregulin GGF2 (cimaglermin alfa) dose and treatment frequency on left ventricular function in rats following myocardial infarction. *Eur J Pharmacol.* Feb 05 2017;796:76-89.
232. Zhou Q, Pan X, Wang L, Wang X, Xiong D. The protective role of neuregulin-1: A potential therapy for sepsis-induced cardiomyopathy. *Eur J Pharmacol.* Oct 05 2016;788:234-240.
233. Gu A, Jie Y, Sun L, Zhao S, E M, You Q. RhNRG-1beta Protects the Myocardium against Irradiation-Induced Damage via the ErbB2-ERK-SIRT1 Signaling Pathway. *PLoS One.* 2015;10(9):e0137337.
234. Iglesias-Garcia O, Baumgartner S, Macri-Pellizzeri L, et al. Neuregulin-1beta induces mature ventricular cardiac differentiation from induced pluripotent stem cells contributing to cardiac tissue repair. *Stem Cells Dev.* Feb 15 2015;24(4):484-496.
235. Liang X, Ding Y, Zhang Y, et al. Activation of NRG1-ERBB4 signaling potentiates mesenchymal stem cell-mediated myocardial repairs following myocardial infarction. *Cell Death Dis.* May 21 2015;6:e1765.
236. Polizzotti BD GB, Walsh S, Choudhury S, Ammanamanchi N, Bennett DG, dos Remedios CG, Haubner BJ, Penninger JM, Kuhn B. . Neuregulin stimulation of cardiomyocyte regeneration in mice and human myocardium reveals a therapeutic window. *Science translational medicine.* 2015;7:281ra245.
237. Ganapathy B, Nandhagopal N, Polizzotti BD, et al. Neuregulin-1 Administration Protocols Sufficient for Stimulating Cardiac Regeneration in Young Mice Do Not Induce Somatic, Organ, or Neoplastic Growth. *PLoS One.* 2016;11(5):e0155456.
238. Gao R, Zhang J, Cheng L, et al. A Phase II, randomized, double-blind, multicenter, based on standard therapy, placebo-controlled study of the efficacy and safety of recombinant human neuregulin-1 in patients with chronic heart failure. *J Am Coll Cardiol.* May 04 2010;55(18):1907-1914.
239. Jabbour A, Hayward CS, Keogh AM, et al. Parenteral administration of recombinant human neuregulin-1 to patients with stable chronic heart failure produces favourable acute and chronic haemodynamic responses. *Eur J Heart Fail.* Jan 2011;13(1):83-92.

240. Lenihan DJ, Anderson SA, Lenneman CG, et al. A Phase I, Single Ascending Dose Study of Cimaglermin Alfa (Neuregulin 1β3) in Patients With Systolic Dysfunction and Heart Failure. *JACC: Basic to Translational Science.* 2016;1(7):576-586.
241. Hessel MH, Steendijk P, den Adel B, Schutte CI, van der Laarse A. Characterization of right ventricular function after monocrotaline-induced pulmonary hypertension in the intact rat. *Am J Physiol Heart Circ Physiol.* Nov 2006;291(5):H2424-2430.
242. Leuchte HH, Holzapfel M, Baumgartner RA, et al. Clinical significance of brain natriuretic peptide in primary pulmonary hypertension. *J Am Coll Cardiol.* Mar 03 2004;43(5):764-770.
243. King LW, M.R. . Natriuretic peptide receptors and the heart. *Heart* 2002;87:314-315.
244. Nagaya N, Nishikimi T, Okano Y, et al. Plasma Brain Natriuretic Peptide Levels Increase in Proportion to the Extent of Right Ventricular Dysfunction in Pulmonary Hypertension. *Journal of the American College of Cardiology.* 1998;31(1):202-208.
245. Lourenco AP, Roncon-Albuquerque R, Jr., Bras-Silva C, et al. Myocardial dysfunction and neurohumoral activation without remodeling in left ventricle of monocrotaline-induced pulmonary hypertensive rats. *Am J Physiol Heart Circ Physiol.* Oct 2006;291(4):H1587-1594.
246. Cheng V, Kazanagra R, Garcia A, et al. A rapid bedside test for B-type peptide predicts treatment outcomes in patients admitted for decompensated heart failure: a pilot study. *Journal of the American College of Cardiology.* 2001;37(2):386-391.
247. Langenickel T, Pagel, I., Höhnel, K., Dietz, R., & Willenbrock, R. Differential regulation of cardiac ANP and BNP mRNA in different stages of experimental heart failure. *American Journal of Physiology-Heart and Circulatory Physiology.* 2000;278(5):H1500-H1506.
248. Ichikawa KI, et al. . Endogenous endothelin-1 mediates cardiac hypertrophy and switching of myosin heavy chain gene expression in rat ventricular myocardium. *J Am Coll Cardiol.* 1996;27:1286-1291
249. Miyauchi T, et al. . Contribution of endogenous endothelin-1 to the progression of cardiopulmonary alterations in rats with monocrotaline-induced pulmonary hypertension. *Circ Res.* 1993;73:887-897.
250. Jasmin JF, Cernacek, P. & Dupuis. Activation of the right ventricular endothelin (ET) system in the monocrotaline model of pulmonary hypertension: response to chronic ETA receptor blockade. . *J. Clin Sci (Lond).* 2003;105:647-653.
251. Lourenco AP, Fontoura D, Henriques-Coelho T, Leite-Moreira AF. Current pathophysiological concepts and management of pulmonary hypertension. *Int J Cardiol.* Mar 22 2012;155(3):350-361.
252. Razeghi P YM, Alcorn JL, Moravec CS, Frazier OH, Taegtmeyer H. . Metabolic gene expression in fetal and failing human heart. *Circulation.* 2001;104:2923–2931.
253. Korvald C EO, Myrmel T. . Myocardial substrate metabolism influences left ventricular energetics in vivo. *Am J Physiol Heart Circ Physiol.* 2000;278:H1345–H1351.
254. Opie LH. The metabolic vicious cycle in heart failure. *The Lancet.* 2004;364(9447):1733-1734.
255. Ren J GG, Miller RE, Davidoff AJ. High extracellular glucose impairs cardiac E-C coupling in a glycosylation-dependent manner. *Am J Physiol.* . 1997;273:H2876–H2883.
256. Suarez J, Hu Y, Makino A, Fricovsky E, Wang H, Dillmann WH. Alterations in mitochondrial function and cytosolic calcium induced by hyperglycemia are restored by mitochondrial transcription factor A in cardiomyocytes. *Am J Physiol Cell Physiol.* Dec 2008;295(6):C1561-1568.
257. Toth PP, Raghavan VA. Glucolipotoxicity and the heart. *Heart Fail Clin.* Oct 2012;8(4):xvii-xviii.
258. Luptak I, Yan J, Cui L, Jain M, Liao R, Tian R. Long-term effects of increased glucose entry on mouse hearts during normal aging and ischemic stress. *Circulation.* Aug 21 2007;116(8):901-909.
259. Liao R. Cardiac-Specific Overexpression of GLUT1 Prevents the Development of Heart Failure Attributable to Pressure Overload in Mice. *Circulation.* 2002;106(16):2125-2131.
260. Lee L, Campbell R, Scheuermann-Freestone M, et al. Metabolic modulation with perhexiline in chronic heart failure: a randomized, controlled trial of short-term use of a novel treatment. *Circulation.* Nov 22 2005;112(21):3280-3288.
261. Schmidt-Schweda S HC. First clinical trial with etomoxir in patients with chronic congestive heart failure. *Clin Sci.* 2000;99:27–35.
262. Pereira RO, Wende AR, Olsen C, et al. Inducible overexpression of GLUT1 prevents mitochondrial dysfunction and attenuates structural remodeling in pressure overload but does not prevent left ventricular dysfunction. *J Am Heart Assoc.* Sep 19 2013;2(5):e000301.

263. Kolwicz SC, Jr., Olson DP, Marney LC, Garcia-Menendez L, Synovec RE, Tian R. Cardiac-specific deletion of acetyl CoA carboxylase 2 prevents metabolic remodeling during pressure-overload hypertrophy. *Circ Res.* Aug 31 2012;111(6):728-738.

264. Kagaya Y KY, Takeyama D, Ishide N, Maruyama Y, Takahashi T, Ido T, Takishima T. Effects of long-term pressure overload on regional myocardial glucose and free fatty acid uptake in rats. A quantitative autoradiographic study. *Circulation.* 1990;81:1353–1361.

265. Nascimben L, Ingwall JS, Lorell BH, et al. Mechanisms for increased glycolysis in the hypertrophied rat heart. *Hypertension.* Nov 2004;44(5):662-667.

266. R. T. Transcriptional regulation of energy substrate metabolism in normal and hypertrophied heart. *Curr Hypertens Rep.* 2003;5:454–458.

267. Tian R MN, D'Agostino J, Hirshman MF, Goodyear LJ. Increased adenosine monophosphate-activated protein kinase activity in rat hearts with pressure-overload hypertrophy. *Circulation.* 2001;104:1664–1669.

268. Montessuit C TA. Transcriptional activation of the glucose transporter GLUT1 in ventricular cardiac myocytes by hypertrophic agonists. *J Biol Chem.* 1999;274:9006–9012.

269. Paternostro G CK, Heath J, Seymour AM, Radda GK. . Decreased GLUT-4 mRNA content and insulin-sensitive deoxyglucose uptake show insulin resistance in the hypertensive rat heart. *Cardiovasc Res.* 1995;30:205–211.

270. Paternostro G PD, Gnecchi-Ruscone T, Bonser RS, Camici PG. Insulin resistance in patients with cardiac hypertrophy. *Cardiovasc Res.* 1999;42:246–253.

271. Russell RR III BR, Shulman GI, Young LH. . Translocation of myocardial GLUT-4 and increased glucose uptake through activation of AMPK by AICAR. *Am J Physiol.* 1999;277:H643–H649.

272. Xing Y, Musi N, Fujii N, et al. Glucose metabolism and energy homeostasis in mouse hearts overexpressing dominant negative alpha2 subunit of AMP-activated protein kinase. *J Biol Chem.* Aug 01 2003;278(31):28372-28377.

273. Santalucia T BK, Brand NJ, Sahye U, Fandos C, Vinals F, Ferre J, Testar X, Palacin M, Zorzano A. . Factors involved in GLUT-1 glucose transporter gene transcription in cardiac muscle. *J Biol Chem.* 1999;274:17626–17634.

274. Liu ML OA, Moye-Rowley WS, Buse JB, Bell GI, Pessin JE. Expression and regulation of the human GLUT4/muscle-fat facilitative glucose transporter gene in transgenic mice. *J Biol Chem.* 1992;267:11673–11676.

275. Masoud GN, Li W. HIF-1alpha pathway: role, regulation and intervention for cancer therapy. *Acta Pharm Sin B.* Sep 2015;5(5):378-389.

276. Yu XJ, Song, J. C., Du, J., Shi, Y. Q., Liu, Y. X., & Shen, Y. . GLUT-1 and its regulating factor HIF-1 alpha expression in epithelial ovarian tumors: GLUT-1 is associated with molecular typing and grade of epithelial ovarian cancer. . *International Journal of Clinical and Experimental Pathology.* 2017;10(4):4479-4487.

277. Sivitz WI, Lund, D.D., Yorek, B., Grover-McKay, M. & Schmid, P.G. . Pretranslational regulation of two cardiac glucose transporters in rats exposed to hypobaric hypoxia. *The American journal of physiology* 1992;263:E562-569.

278. Karnieli E, Armoni M. Transcriptional regulation of the insulin-responsive glucose transporter GLUT4 gene: from physiology to pathology. *Am J Physiol Endocrinol Metab.* Jul 2008;295(1):E38-45.

279. Doehner W, Gathercole D, Cicoira M, et al. Reduced glucose transporter GLUT4 in skeletal muscle predicts insulin resistance in non-diabetic chronic heart failure patients independently of body composition. *International journal of cardiology.* 2010;138(1):19-24.

280. Weir EK LBJ, Buckler KJ, Archer SL. . Acute oxygen-sensing mechanisms. *N Engl J Med.* 2005;353:2042-2055.

281. Tuder RM CM, Alger L, Wang J, Taraseviciene-Stewart L, Kasahara Y,, al. e. Expression of angiogenesis-related molecules in plexiform lesions in severe pulmonary hypertension: Evidence for a process of disordered angiogenesis. *J Pathol.* 2001;195:367-374.

282. Fijalkowska I, Xu W, Comhair SA, et al. Hypoxia inducible-factor1alpha regulates the metabolic shift of pulmonary hypertensive endothelial cells. *Am J Pathol.* Mar 2010;176(3):1130-1138.

283. Archer SL, Marsboom G, Kim GH, et al. Epigenetic attenuation of mitochondrial superoxide dismutase 2 in pulmonary arterial hypertension: a basis for excessive cell proliferation and a new therapeutic target. *Circulation.* Jun 22 2010;121(24):2661-2671.

284. Wang GL JB, Semenza GL. Effect of altered redox states on expression and DNA-binding activity of hypoxia-inducible factor 1. *Biochem Biophys Res Commun.* 1995;212:550–556.

285. Huang LE AZ, Livingston DM, Bunn HF. . Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit. *J Biol Chem.* 1996;271:32253–32259.

286. Kim JW, Tchernyshyov I, Semenza GL, Dang CV. HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. *Cell Metab.* Mar 2006;3(3):177-185.

287. Marsboom G, Toth PT, Ryan JJ, et al. Dynamin-related protein 1-mediated mitochondrial mitotic fission permits hyperproliferation of vascular smooth muscle cells and offers a novel therapeutic target in pulmonary hypertension. *Circ Res.* May 25 2012;110(11):1484-1497.

288. Ramiro-Diaz JM, Nitta CH, Maston LD, et al. NFAT is required for spontaneous pulmonary hypertension in superoxide dismutase 1 knockout mice. *Am J Physiol Lung Cell Mol Physiol.* May 01 2013;304(9):L613-625.

289. Archer SL. Mitochondrial dynamics--mitochondrial fission and fusion in human diseases. *N Engl J Med.* Dec 05 2013;369(23):2236-2251.

VIII - APPENDIX

VIII -APPENDIX

The results of this master thesis and other works in which I have participated were presented at several national scientific meetings and published as abstracts in scientific journals.

Publications

As abstract:

Salgado S, Adão R, Maia-Rocha C, Mendes-Ferreira P, Pinto C, Pimentel L, Falcão-Pires I, Leite-Moreira AF, Brás-Silva C. Modulação da função diastólica do ventrículo direito pela neuregulina-1 na hipertensão arterial pulmonar. Revista Portuguesa de Cardiologia 2017; vol. 36 (Espec Congr):116.

Maia-Rocha C, Adão R, Mendes-Ferreira P, **Salgado S**, Leite-Moreira AF, Brás-Silva C. Efeito do tratamento crónico com neuregulina-1 na expressão génica dos transportadores da glucose na hipertensão arterial pulmonar. Revista Portuguesa de Cardiologia 2017; vol. 36 (Espec Congr):117.

Pimentel L, Mendes-Ferreira P, Maia-Rocha C, Adão R, Santos-Ribeiro D, **Salgado S**, Pinto C, Potus F, Provencher S, Bonnet S, Leite-Moreira AF, Brás-Silva C. O papel do MicroRNA-146a na hipertensão arterial pulmonar. Revista Portuguesa de Cardiologia 2017; vol. 36 (Espec Congr):12.

Pinto C, Adão R, Mendes-Ferreira P, Maia-Rocha C, Santos-Ribeiro D, Pimentel L, **Salgado S**, Potus F, Rademaker MT, Bonnet S, Leite-Moreira AF, Brás-Silva C. Urocortin-2 improves right ventricular function and attenuates pulmonary arterial hypertension. Revista Portuguesa de Cardiologia 2017; vol. 36 (Espec Congr):218.

Communications at Scientific Meetings

Poster communications:

Salgado S, Maia-Rocha C, Adão R, Mendes-Ferreira P., Leite-Moreira AF, Brás-Silva C. Efeito do tratamento crónico com neuregulina-1 na expressão génica dos transportadores da glucose na hipertensão arterial pulmonar. Congresso Português de Cardiologia 2017. 22 - 25 de abril, 2017; Albufeira, Portugal.

Salgado S, Adão R, Maia-Rocha C, Mendes-Ferreira P, Pinto C, Pimentel L, Falcão-Pires I, Leite-Moreira AF, Brás-Silva C. Modulação da função diastólica do ventrículo direito pela neuregulina-1 na hipertensão arterial pulmonar. Congresso Português de Cardiologia 2017. 22 - 25 de abril, 2017; Albufeira, Portugal.

Pinto C, Adão R, Mendes-Ferreira P, Maia-Rocha C, Santos-Ribeiro D, Pimentel L, **Salgado S**, Potus F, Rademaker MT, Bonnet S, Leite-Moreira AF, Brás-Silva C. Urocortin-2 improves right ventricular function and attenuates pulmonary arterial hypertension Congresso Português de Cardiologia 2017. 22 - 25 de abril, 2017; Albufeira, Portugal.

Oral communications:

Pimentel L, Mendes-Ferreira P, Maia-Rocha C, Adão R, Santos-Ribeiro D, **Salgado S**, Pinto C, Potus F, Provencher S, Bonnet S, Leite-Moreira AF, Brás-Silva C. O papel do MicroRNA-146a na hipertensão arterial pulmonar. Congresso Português de Cardiologia 2017. 22 - 25 de abril, 2017; Albufeira, Portugal.

Scientific Awards

Thomé Villar / Boehringer Ingelheim Prize 2017.Mendes-Ferreira P, Santos-Ribeiro D, Maia-Rocha C, Adão R, **Salgado S**, Monteiro Pinto C, Provencher S, Bonnet S, Leite-Moreira AF, Brás-Silva C. MicroRNA-146a is implicated in the development of pulmonary hypertension – human and experimental insight.

Dissemination Activities

Participation in the UA **Open Campus 2017** of the University of Aveiro with the activity Cardioscience, with the purpose of divulging the scientific research of translation that is performed in UnIC, FMUP to students of the several Cycles of Studies of the University of Aveiro. 10th May 2017.