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Functional and metabolic characterization of endothelial cells in chronic thromboembolic pulmonary hypertension

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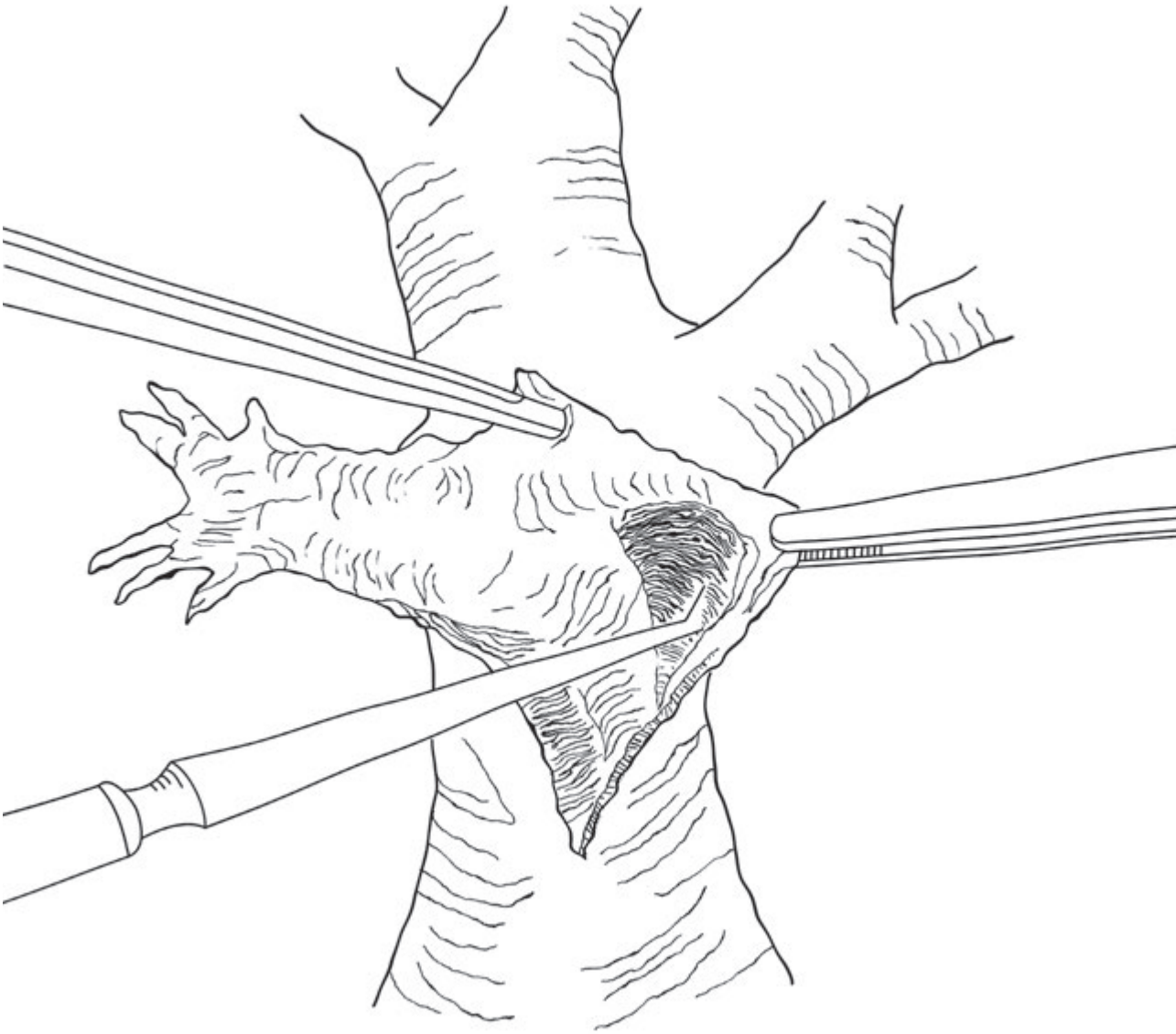

Valérie F.E.D. Smolders

FUNCTIONAL AND METABOLIC CHARACTERIZATION OF ENDOTHELIAL CELLS IN CHRONIC THROMBOEMBOLIC PULMONARY HYPERTENSION

VALÉRIE FRANÇOISE ELISABETH DENISE SMOLDERS

Functional and Metabolic Characterization of Endothelial Cells in Chronic Thromboembolic Pulmonary Hypertension.

Cover: The removal of obstructive material from a pulmonary artery during pulmonary thromboendarterectomy. Design by Joaquim Bobi (adjusted from Surgical Management of Chronic Pulmonary Embolism, Fabian A.G. Vallejo, 2017).

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FUNCTIONAL AND METABOLIC CHARACTERIZATION OF ENDOTHELIAL CELLS IN CHRONIC THROMBOEMBOLIC PULMONARY HYPERTENSION

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Possibility

"Soar, eat ether, see what has never been seen;
depart, be lost
but climb"

- Edna St. Vincent Millay (1892-1950)

THESIS CONTENT

CHAPTER 1

GENERAL INTRODUCTION AND THESIS OUTLINE

GENERAL CARDIOVASCULAR PHYSIOLOGY

Almost all tissues in the body depend on a blood supply, and the blood supply depends on endothelial cells, which form the lining of the blood vessels of the entire vascular system. The blood is responsible for transportation, delivery and elimination of materials such as oxygen, nutrients and carbon dioxide through the body as part of the circulatory system. The circulatory system is composed of two circulations: the pulmonary circulation and the systemic circulation. The movement (flow) of the blood through these circulations is driven by the heart. The heart consists of four chambers: two blood receiving chambers, the atria, and two ejecting chambers, the ventricles. In short, the right atrium receives low-oxygen blood from the body and sends it via the right ventricle into the pulmonary circulation for oxygenation. Blood is oxygenated in the lungs where after it enters the left atrium. The left atrium pushes the high-oxygen blood into the left ventricle which subsequently pumps it to rest of the body via the aorta. In the organs oxygen is absorbed and low-oxygen blood returns to the right atrium where the cycle starts again. The endothelium maintains a stable environment and controls the passage of materials and cells into and out of the bloodstream [1,2].

Pulmonary circulation

The pulmonary circulation is essential for the body to ensure a continuous supply of high-oxygen blood. It carries low-oxygen blood away from the right ventricle through the pulmonary arteries, arterioles and capillaries. Oxygen is supplied by the airways (bronchi) which branch repeatedly into progressively smaller bronchi. The smallest bronchi branch becomes bronchioles which ultimately branch into the smallest air sacs (alveoli) where gas exchange occurs with the pulmonary capillaries. Following oxygenation in the lungs, blood is returned to the left atrium via the pulmonary veins. A mean pulmonary arterial pressure (mPAP) of approximately 14mmHg at rest makes the pulmonary circulation a relative low pressure system compared to the systemic circulation. Since the total pressure drop from pulmonary artery to left atrium is about 10mmHg (100mmHg in the systemic circulation), a low pulmonary vascular resistance (about ten times less than the systemic circulation) enables high blood flow through the lungs. This low resistance relies on thinner, less muscularized vessels as compared to their systemic counterparts and allows for optimal exchange of gasses [1,3].

PULMONARY HYPERTENSION

In pulmonary hypertension (PH) patients, the pulmonary vascular resistance is strongly increased due to vasoconstriction, remodeling or obstruction, resulting in an elevated pressure (mPAP ≥ 25mmHg) to maintain the blood flow through the vasculature bed. This increase in pressure impairs gas exchange and if prolonged it can lead to compensatory dilatation and/or hypertrophy of the heart and can lead eventually to right heart failure. PH affects approximately 1% of the global population [4,5]. It embraces several diseases and therefor based on pathogenesis, clinical symptoms, haemodynamic characteristics and therapeutic management it is divided into the following five groups: Group 1 PH, pulmonary arterial hypertension (PAH); group 2 PH, PH due to left heart disease (PH-LHD); group 3 PH, PH associated with lung disease and/or hypoxia; Group 4 PH, chronic thromboembolic pulmonary hypertension (CTEPH) and group 5 PH, PH with unclear multifactorial mechanisms [4,6]. Symptoms of PH are often non-specific and include dyspnoea, fatigue, exercise intolerance, chest pain, syncope or oedema. All these symptoms strongly impair the quality of life of patients with PH [6]. The non-specific nature of symptoms significantly delays diagnosis of PH, or in some cases patients even remain undiagnosed. This also negatively impacts the determination of the true incidence and prevalence of PH worldwide.

Group 1 pulmonary arterial hypertension

The incidence of PAH ranges from 2.0 to 7.6 cases per million adults per year [7]. PAH affects mainly the younger population and is mostly diagnosed in females, however due to aging of the population it is also increasingly diagnosed in elderly people [8]. The increased vascular resistance in PAH is caused by vascular proliferation and remodelling of medial and intimal layers of the vessel wall, compromising the arterial lumen (**Figure 1**) [8,9]. In addition, complex structures, such as plexiform lesions, are also observed in PAH patients. Plexiform lesions are well-organised structures composed of vascular channels that result from misguided neoangiogenesis with a disbalance in apoptosis and subsequent increase in proliferation of endothelial cells [10,11]. These remodelling processes mainly take place in distal arteries and arterioles with a vessel diameter smaller than 0.5 mm, the so-called microvasculature [12]. The underlying pathophysiologic processes driving these structural changes in PAH are not clear, but endothelial dysfunction has been considered an important driver. Pulmonary endothelial dysfunction has been associated with impairment of endothelial-dependent vasodilatation in

favor of vasoconstriction, but it also refers to reduced anticoagulant properties, metabolic changes, increased oxidative stress and inflammation, and increased release of growth factors. All these changes results in impairments in angiogenesis and repair mechanisms that play an important role in vascular remodeling [12].

Current treatment strategies for PAH mainly promote vasodilation by normalizing the imbalance in vasoactive factors. These therapies aim to stimulate the nitric oxide (NO) and prostacyclin pathway, and inhibit the endothelin (ET-1) pathway in order to promote vasodilation and decrease vasoconstriction, respectively. Despite increased survival upon treatment (compared to untreated patients), PAH remains a progressive disease with fatal outcome due to limited effect of current therapies on endothelial dysfunction and pulmonary vascular remodelling [6,13,14].

Group 4 chronic thromboembolic pulmonary hypertension

A pulmonary embolism (PE) is a blood clot that gets trapped in the lungs. People who have had PEs are at greater risk to develop more clots, which can obstruct pulmonary arteries. This causes high blood pressure in the lungs or in rare cases causes the development of chronic thromboembolic pulmonary hypertension (CTEPH). The incidence of CTEPH after symptomatic acute PE is estimated to range from 0.9 to 5 per million adults per year [15-17]. CTEPH is characterised by residual remodeled clots that remain attached to the vessel walls of large and/or middle-sized pulmonary arteries [18]. These unresolved clots result in high pressure and shear stress in nonoccluded pulmonary arteries which triggers endothelial dysfunction, pulmonary vascular constriction and pulmonary vascular remodeling of also more distal arteries (0.1-0.5mm in diameter), similar to the remodeling observed in PAH (**Figure 1**) [18-21]. These changes progressively cause an increase in the vascular resistance and pulmonary artery pressure which ultimately leads to symptomatic CTEPH [18,21]. Why some patients fail to resolve acute PE and develop CTEPH remains to be resolved but endothelial dysfunction is suggested as one of the explanations.

In CTEPH patients, the gold standard therapy with possible curative outcome is pulmonary thrombo-endarterectomy (PEA), the surgical removal of thrombotic material from the pulmonary arteries to restore pulmonary flow [22]. PEA has shown to improve haemodynamic characteristics, exercise capacity and survival of CTEPH patients [22,23].

Although positive outcome associated with PEA surgery, 40% of CTEPH patients are not operable and up to one-third has persistent or recurrent PH [22,24]. Recently, balloon pulmonary angioplasty has emerged as an alternative approach for blood flow restoration in CTEPH patients with lesions that are not reachable with PEA. However, the use of this technique is still limited and associated with severe complications [25]. Parallels in clinical symptoms and pathogenesis between PAH and CTEPH have led to the off-label use of PAH based pharmacological therapies in CTEPH patients that are non-candidates for surgery or present recurrent PH [24,26]. Up to now, the only PAH based medical therapy approved for CTEPH is the soluble guanylate cyclase stimulator riociguat, which promotes relaxation of the vascular wall [24,27]. A deeper understanding of cellular changes and molecular mechanisms leading to CTEPH is crucial to improve insights in disease pathology and eventually will contribute to the development of novel therapeutic interventions.

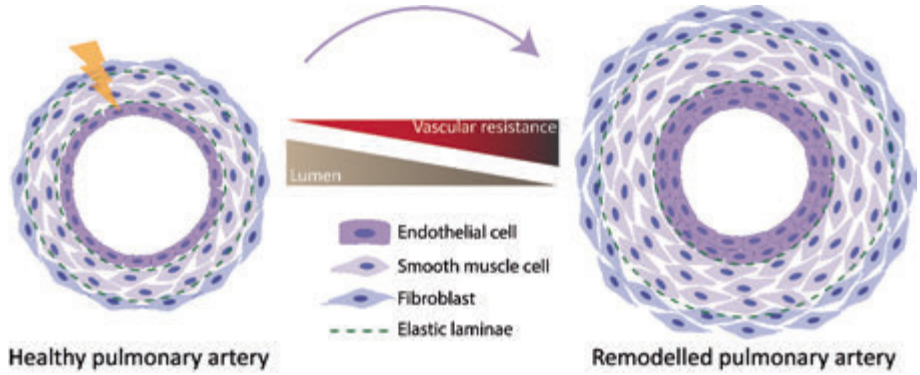

Figure 1. Pulmonary vascular remodeling. Vascular remodeling is a hallmark of PH that involves structural changes in all three layers of the vessel wall that impair blood flow. On the left a healthy pulmonary artery and on the right a remodeled artery. The decrease in lumen size because of remodeling causes a progressive increase in the pulmonary vascular resistance and cause an irreversible increase in pulmonary artery pressure.

ENDOTHELIAL DYSFUNCTION – THE VASCULAR CRIMINAL

The endothelium is a monolayer of cells that line the entire vascular system, from the heart to the smallest capillary. The pulmonary vascular endothelium, at the interface between the blood stream and lung tissue, presents an important mechanical barrier between the blood and the lungs and plays key roles in optimizing gas exchange. Besides those functions, the endothelium is also actively involved in various other functions. It regulates the vascular tone through the production and release of vasoactive substances such as NO, prostacyclin, ET-1

and serotonin, but also controls inflammation, wound repair and angiogenesis, and therefore, is crucial to maintain vascular-tissue homeostasis. While adult endothelial cells are considered as a quiescent (resting) layer of cells, they are highly metabolically active and respond to different chemical, physical or mechanical (activating) stimuli from the extracellular environment by the release of physiologically active substances that benefit the host. By doing so the endothelium maintains a thrombosis-free surface, controls inflammatory cell adhesion and trafficking, assures normal angiogenesis/proliferation and vessel wall integrity [3,28,29]. Failure of endothelial cells to adequately perform any of these basal functions, is referred to as endothelial cell dysfunction [29].

Endothelial dysfunction results from the prolonged exposure of ECs to environmental changes such as oxidative stress, shear stress, inflammatory factors, hypoxia and toxins which results in cell injury or death [9,28,30-32]. The main characteristics of endothelial dysfunction are increased vasoconstriction, acquisition of a pro-inflammatory and pro-thrombotic surface, and an imbalance between proliferation and apoptosis [3,28]. Endothelial dysfunction is thought to be key in the vascular remodelling observed in PAH [28]. Markers of endothelial dysfunction are observed patients with PAH and CTEPH [26,28,33] and causal involvement of endothelial cell abnormalities in PAH development and progression has been extensively studied and reviewed [12,33]. However, the mechanisms and consequences of endothelial dysfunction in CTEPH are less investigated. Studying ECs from CTEPH patients will contribute to an improved understanding of CTEPH pathology. In this thesis we mainly focus on the angiogenic capacity and the inflammatory status of the pulmonary endothelium.

Angiogenesis

Endothelial cells not only repair the lining of blood vessels, they also create new blood vessels from existing small vessels in response to hypoxia (low oxygen) and factors such as vascular endothelial growth factor (VEGF). In order to form a new vessel ECs adopt either a tip (migratory) cell phenotype or a stalk (proliferative) phenotype to elongate the sprout. This specification into tip or stalk cells depends on the VEGF／DLL4-Notch signaling pathway [34]. In the presence of VEGF, Notch ligand DLL4 is upregulated in tip cells whereby JAGGED1 is expressed by stalk cells promoting EC proliferation [34,35]. This balanced specification between tip and stalk cells is critical in the formation of functional new sprouts. Once the sprout is formed, vascular remodeling takes place which allows maturation of the newly formed vessel

loop including adaptation of a quiescent phenotype, basement membrane deposition and pericyte coverage. However, both insufficient and uncontrolled vessel growth are related to cardiovascular diseases, cancer and PAH. These conditions are often characterized by alterations in VEGF production and/or response as a result of endothelial dysfunction [34,36].
Interestingly, ECs isolated in culture still show the capacity to spontaneously form capillary structures when grown in culture medium containing correct growth factors [2]. Knowing this, culturing ECs from CTEPH patients will help to improve the knowledge on the angiogenic capacity of diseased ECs and its role in disease pathogenesis.

Inflammation

Another special feature of the endothelium is the control of the inflammatory response. The quiescent endothelium maintains an anti-inflammatory surface that blocks adhesion and infiltration of immune cells. Upon activation, the acquisition of a new endothelial function that benefits the host, the endothelium is known to be an essential contributor to transient inflammatory processes that promote tissue repair [3]. Endothelial dysfunction on the other hand is associated with a continuously activated endothelium that expresses several adhesion molecules such as selectins, vascular cell adhesion protein-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1) and platelet endothelial cells adhesion molecule-1 (PECAM-1) to prolong adhesion of leukocytes. In addition, the endothelium excessively releases various cytokines and chemokines such as tumor necrosis factor alpha (TNFα), interleukin-6 (IL-6), IL-8 , IL-1B, chemokine ligand-5 (CCL5) and CCL2 [37,38]. This pro-inflammatory gene expression is largely mediated by the activation of nuclear factor (NF)-κB, a heterodimer composed of the subunits p65 (RelA) and p50/p52. Under basal conditions NF-κB is inhibited by IκB (inhibitor of κB) and is found in the cell cytoplasm. Upon activation, IκB is phosphorylated and degraded which allows release and translocation of NF-κB to the nucleus where it can activate target gene expression [39]. As transcription factor, NF-κB promotes the transcription of genes involved in inflammatory response such as TNFα, IL-1, IL-8, E-selectin, VCAM-1 and ICAM-1 but also upregulates the expression of VEGF. The NF-κB pathway can be activated by various stimuli such as EC injury, shear stress, reactive oxygen species (ROS) and viral infection but also by pro-inflammatory cytokines themselves such as TNFα, IL-1β and IL-8 [39,40].
An example of beneficial inflammation, relevant to CTEPH pathology, is the involvement in thrombus recanalization and resolution via the recruitment of leukocytes and through

promoting angiogenesis into the affected area (in addition to plasma driven fibrinolysis) [41]. The central role of inflammation in thrombus recanalization and resolution has been shown by an animal study where endothelial specific deletion of angiogenic factor VEGF-receptor-2 (VEGFR2) shows to delay thrombus resolution. This delay has been attributed to a reduction in the formation of neovessels and subsequent diminished leukocyte recruitment to the affected area [42]. Although inflammation seems indispensable for thrombus recanalization and resolution, conditions of sustained inflammation, whether or not induced by endothelial dysfunction, are thought to be involved in thrombus nonresolution through increased collagen production and fibrosis [41]. Based on this knowledge, studying the role of inflammation and NF-κB signaling in CTEPH-EC will be interesting to better understand mechanisms that contribute to the lack of thrombus resolution and vascular remodeling in CTEPH.

ENDOTHELIAL CELL METABOLISM: SIMILAR, YET DIFFERENT

In order to survive, cells rely on metabolic pathways that break down glucose, fatty acids and amino acids as primary source of energy for the synthesis of adenosine triphosphate (ATP). This process is oxygen dependent and called aerobic cell respiration.

Aerobic cell respiration

Aerobic cell respiration can be divided into 3 processes: 1) glycolysis, 2) tricarboxylic acid (TCA) cycle, and 3) the electron transport chain (**Figure 2**). Aerobic glycolysis starts with the uptake of glucose from the cell cytoplasm by glucose transporter-1 (GLUT1) and next glucose becomes phosphorylated by hexokinase-2 (HK2), an important rate-limiting enzyme of the glycolytic pathway. Glycolysis is under control of master glycolytic regulator 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-3 (PFKFB3). This latter enzyme produces fructose-2,6-bisphosphate, a glycolytic intermediate that is a strong allosteric activator of the second rate-limiting enzyme phosphofructokinase-1 (PFK1) [43]. The net result of glycolysis is 2 pyruvate and 2 ATP molecules [43-46]. Next, pyruvate is transported into the mitochondria and converted into acetyl-coenzyme-A (CoA) where it is further catabolized through the TCA cycle. Pyruvate entry in the TCA cycle depends on its conversion into acetyl-CoA by the pyruvate dehydrogenase (PDH) complex which links glycolysis to the TCA cycle. Enzyme activity of PDH is controlled by the expression of pyruvate dehydrogenase kinase (PDK), which can block PDH activity *via*

phosphorylation. Inside the mitochondrial matrix, acetyl-CoA undergoes a cycle of reactions catalyzed by mitochondrial enzymes such as isocitrate dehydrogenase and succinate dehydrogenase which generates ATP, NADH and $FADH_2$. The electrons from these 2 latter molecules are transferred to the electron transport chain which uses the movement of electrons to facilitate ATP production through oxidative phosphorylation (OXPHOS) (**Figure 2-3**). Even though OXPHOS has the highest ATP yield (approximately 30 ATP per glucose), in moments of oxygen deficiency mitochondrial OXPHOS is repressed and cells shift to glycolysis for ATP production [44,47,48].

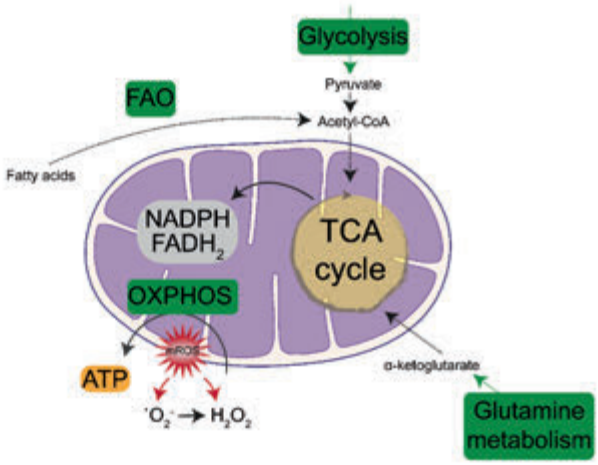

Figure 2. Aerobic cell respiration. Cells rely on glycolysis coupled with mitochondrial OXPHOS for the production of ATP. Once Pyruvate is converted into acetyl-coA it is metabolized in the TCA cycle with the production of NADPH and $FADH_2$. These molecules are essential to complete cell respiration with the production of ATP during OXPHOS. This process is associated with the production of mitochondrial ROS. (mROS) At last, cells can additionally use fatty acid oxidation (FAO) and glutamine metabolism to generate essential cellular building blocks.

Endothelial cell metabolism

ECs differ with other cell types in the fact that regardless of the oxygen supply, they predominantly use the aerobic glycolysis pathway with the production of lactate, instead of acetyl-CoA, for their ATP production. The use of glycolysis over OXPHOS renders ECs with several benefits such as less ROS production, survival in oxygen deficient environments and sustained macromolecule synthesis but also delivers ATP far more quickly than OXPHOS [44]. In response to growth factors such as VEGF, ECs even further increase the use of glycolysis to

support their highly proliferative and migratory state which is needed for processes such as angiogenesis [44].

Additionally, ECs also use a glycolytic side branch, namely the pentose phosphate pathway (PPP). This pathway uses glycolytic intermediates mainly for the production of nucleotides and to combat oxidative stress through the expression of rate-limiting enzymes glucose-6-phosphate dehydrogenase (G6PD) and transketolase (TKT) (**Figure 3**) [43,44]. Interestingly, the mitochondria of ECs serve alternative purposes to maintain proper cell function. Firstly, when low in glucose, ECs can easily switch back to OXPHOS to maintain viability, and secondly, mitochondria are an important source for the supply of building blocks for biosynthetic pathways such as nucleotide and amino acid synthesis. At last, ECs increase the use of carbon sources such as fatty acids (fatty acid oxidation) and amino acids (glutamine metabolism) via the regulation of the expression of carnitine palmitoyltransferase-1A (CPT1A), glutaminase-1 (GLS1) and glutamate dehydrogenase-1 (GLUD1) to support cell growth and proliferation, availability of TCA cycle intermediates, and to maintain redox homeostasis (**Figure 3**) [43,44,49].

EC metabolism is an important co-determinant in normal EC function. Silencing and pharmacological inhibition of PFKFB3 (glycolysis), CPT1A (FAO) and GLS1 (glutamine metabolism) in healthy ECs has shown to impair EC sprouting, proliferation and migration but also has shown to be involved in oxidative stress-induced EC dysfunction in quiescent ECs. Similar observations were obtained with EC-selective knockout studies in mice. Interestingly, blockage of these metabolic enzymes does not impair ATP production nor induce cell death but rather induces a hypometabolic state with a shift back to oxidative phosphorylation [49-53]. Alterations in the normal functioning of the EC metabolism favors excessive cellular proliferation, increased angiogenesis and a pro-survival cellular phenotype and has been found to contribute to several vascular diseases such atherosclerosis and diabetes but more importantly also PAH [43,44]. Taken together, endothelial cell metabolism plays important roles in the proper functioning of ECs and therefore, investigating EC metabolism in CTEPH could help to improve our understanding of thrombus nonresolution and vascular remodeling.

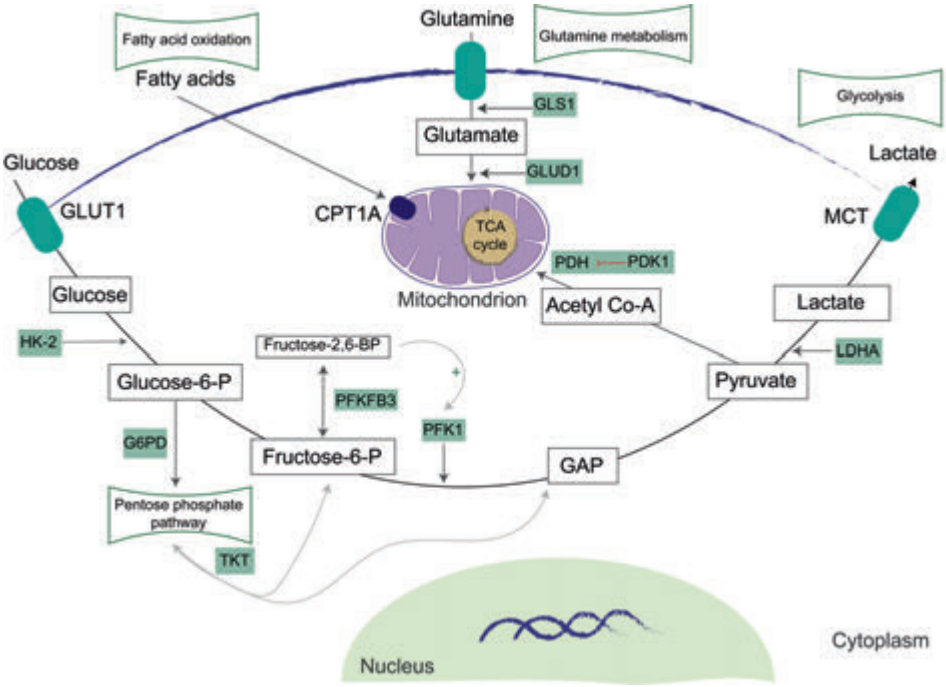

Figure 3. Endothelial cell metabolism. Cellular metabolism consists of aerobic glycolysis linked with oxidative phosphorylation in the mitochondria. In normal conditions, glucose is converted into pyruvate which is shuttled into the mitochondria where it participates in the TCA cycle. The TCA cycle is connected with the electron transport chain which facilitates ATP production. In endothelial cells however, oxidative phosphorylation is suppressed and lactate instead of pyruvate is produced. In addition, ECs rely also on metabolic pathways such as pentose phosphate pathway, fatty acid oxidation and glutamine metabolism to keep up with the metabolic needs of proliferating cells.

MITOCHONDRIA: MORE THAN ENERGY PRODUCERS

Mitochondria are double-membrane-bound organelles located in the cell's cytoplasm. The inner membrane is folded into cristae that contain oxidative phosphorylation enzyme complexes, whereas the outer membrane defines the mitochondrial shape [54]. The number of mitochondria in ECs is rather low, 2-6% of the cytoplasmic volume, compared to other cell types such as cardiac myocytes where mitochondria occupy approximately 32% of the cytoplasmic volume [55].

The mitochondrial life cycle

Mitochondrial biogenesis and mitophagy (selective autophagy of old and damaged mitochondria) in response to environmental stimuli such as hypoxia, allow the availability of functional mitochondria but the clearance of damage malfunctioning ones to assure proper cell function [55]. In order to remain functional, mitochondria undergo dynamic cycles of fusion and fission to assure mitochondrial integrity and connectivity. Fusion of the outer membranes is mediated by mitofusin-1 and -2 (MFN1 and MFN2) whereas fusion of the inner membranes is mediated by optic atrophy protein-1 (OPA1). Fission is mediated by dynamin-related protein-1 (DRP1) and fission-1 (FIS1) [56]. Fusion allows the exchange of mitochondrial DNA, proteins and lipids through mitochondrial networks in order to rescue mitochondria with loss-of-function mutations [54]. In growing and dividing cells, fission is important to populate newly generated cells with adequate numbers of mitochondria whereas in non-dividing cells mitochondrial fission is essential for maintaining mitochondrial health by segregating damaged parts of the mitochondria for elimination by mitophagy [54]. Elimination of damaged mitochondria is essential to avoid excessive amounts of ROS produced by dysfunctional mitochondria but also to prevent damage of the healthy mitochondrial network. Defective mitochondrial dynamics are thought to be important contributors to vascular disease and, therefore, interesting to investigate whether mitochondrial impairment is also present in CTEPH.

The importance of mitochondrial ROS

Mitochondrial ROS (mROS) are natural byproducts of cellular metabolism. Physiological (low) levels of ROS can act as signaling molecules that control a wide range of cellular functions, while accumulation of ROS cause oxidative stress contributing to endothelial cell dysfunction and damage. Because of that, the amount of cellular mROS is tightly regulated by ROS generating enzymes and antioxidant systems such as superoxide dismutases (SODs) [57].

Endothelial cell homeostasis is also tightly linked to the production of mROS. An increase in mROS (oxidative stress) influences vascular inflammation, angiogenesis, matrix remodeling, and proliferation and apoptosis through the effects on transcription factors (e.g. NF-κB and HIF-1), metalloproteases and signaling molecules (e.g. Akt, Scr, MAPK). More importantly, excessive ROS is also known to promote endothelial dysfunction and vascular diseases [55,57-60]. Therefore, it is interesting to study whether dysfunctional mitochondria and oxidative stress play a role in vascular changes observed in CTEPH.

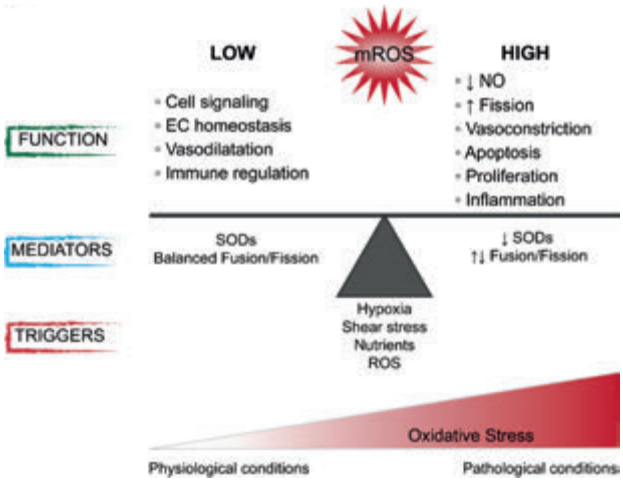

Figure 4. Mitochondria and ROS production. Mitochondrial reactive oxygen species (mROS) are natural byproducts of mitochondrial respiration. Low concentrations of mROS have several physiological functions, but excessive mROS on the other hand have several pathological functions that mediate EC dysfunction. The amount of mROS in a cell depends on the cell's antioxidant capacity and can be influenced by several environmental factors such as hypoxia and shear stress. A high amount of mROS results in oxidative stress which contributes to vascular disease development. SODs, super oxide dismutases; NO, nitric oxide

THESIS OUTLINE

The aim of this thesis is to improve our understanding of mechanisms underlying the development of endothelial dysfunction, thrombus nonresolution and vascular remodeling in CTEPH patients.

In the review in **chapter 2**, the latest advances on the role of EC dysfunction in the pathogenesis of all forms of PH are highlighted. We discuss the role of vasoactive regulators, inflammation, endothelial-to-mesenchymal transition, apoptosis and (epi)genetics in the process of EC dysfunction and subsequent vascular remodeling in PH. Finally, we address potential targets and pitfalls for restoring EC function in order to limit or reverse vascular remodeling in PH. The existence of EC dysfunction in PAH and the similar vascular remodeling observed between CTEPH and PAH has led to the question whether ECs from CTEPH patients might have EC abnormalities too. To date, the pathophysiology of CTEPH remains poorly understood and *in vitro* studies in EC from CTEPH patients would allow us to identify key targets and molecular pathways that might be altered in CTEPH. Therefore, in **Chapter 3** we aim to identify key EC abnormalities in CTEPH by studying endothelial and mitochondrial function. EC are isolated from thromboembolic material, removed during PEA surgery, from CTEPH patients. Before patient-ECs (CTEPH-ECs) are functionally characterized, the endothelial nature of these cells is confirmed by the use of immunocytochemistry and flow cytometry. CTEPH-EC functions are assessed by mean of proliferation, migration and angiogenic capacity. At last, mitochondrial function and oxidative stress, common factors in EC dysfunction, are studied.

Chapter 4 describes the role of metabolic alterations in cardiopulmonary vascular diseases such as PH. In this review, we discuss the growing evidence that endothelial dysfunction in cardiopulmonary vascular disorders is strongly associated with disease-specific metabolic changes in ECs. At last, we discuss targeting endothelial cell metabolism as potential strategy to restore normal endothelial functions. Therefore, **Chapter 5** focusses on the role of EC metabolism as possible contributor to CTEPH pathogenesis. Changes in endothelial metabolism have shown to fuel vascular remodeling by promoting EC proliferation, apoptosis and migration. Therefor we hypothesize a role for EC metabolism in CTEPH disease development. **Chapter 6** is a comparative study between ECs from CTEPH and PAH patients. Despite histological similarities between both forms of PH, CTEPH-EC metabolism is

characterized by a downregulation of glycolysis whereas PAH is characterized by an increase in glycolysis. In order to better understand different metabolic needs between CTEPH-EC and PAH-EC, ECs are incubated with several metabolic inhibitors and changes in viability are assessed. Differences in response of CTEPH-EC and PAH-EC to metabolic inhibitors will provide insight in the predominant reliance/dependency on certain metabolic pathways to maintain endothelial viability.

The vascular endothelium is not only an important mediator of the inflammatory response but also a target of an inflammatory environment which can trigger endothelial dysfunction and subsequent vascular remodeling. Therefore, **Chapter 7** describes the inflammatory status of CTEPH-patient derived ECs as possible driver in disease progression with specific attention to the NF-κB signaling pathway. This pathway has been associated with several vascular diseases, cancer and we show that it is also an interesting target in CTEPH.

The results described in this thesis are summarized and discussed in **Chapter 8.** This chapter also shares current challenges and future perspectives on the research described**.**

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CHAPTER 2

ENDOTHELIAL DYSFUNCTION IN PULMONARY HYPERTENSION: CAUSE OR CONSEQUENCE?

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Submitted

ABSTRACT

Pulmonary arterial hypertension (PAH) is a rare, complex, and progressive disease characterized by abnormal remodelling of the pulmonary arteries that leads to right ventricular failure and death. Although our understanding of the causes for abnormal vascular remodelling in PAH is limited, accumulating evidence indicates that endothelial cell (EC) dysfunction is one of the first triggers initiating this process. EC dysfunction leads to the activation of several cellular signalling pathways in the endothelium, resulting in uncontrolled proliferation of ECs, pulmonary artery smooth muscle cells and fibroblasts, and eventually leads to vascular remodelling and occlusion of the pulmonary blood vessels. Other factors that are related to EC dysfunction in PAH are an increase in endothelial to mesenchymal transition, inflammation, apoptosis, and thrombus formation. In this review, we outline the latest advances on the role of EC dysfunction in PAH and other forms of pulmonary hypertension. We also elaborate on the molecular signals that orchestrate EC dysfunction in PAH. Understanding the role and mechanisms of EC dysfunction will unravel the therapeutic potential of targeting this process in PAH.

Keywords: Pulmonary hypertension – endothelial cell dysfunction – vasoactive factors – TGF-β – EndoMT – epigenetics

INTRODUCTION

Pulmonary hypertension (PH) is a condition defined by a mean pulmonary arterial pressure of more than 20 mmHg at rest and 30 mmHg during exercise. The range of genetic, molecular, and humoral causes that can lead to this increase in pressure is extensive. Therefore, PH is grouped into different classes based on clinical and pathological findings as well as therapeutic interventions [1,2]. The World Health Organization (WHO) classifies PH into five groups, namely: 1. Pulmonary arterial hypertension (PAH), 2. Pulmonary hypertension due to left heart disease (PH-LHD), 3. Pulmonary hypertension due to lung disease (PH-LD), 4. Chronic thromboembolic pulmonary hypertension (CTEPH), 5. Pulmonary hypertension due to unclear and/or multifactorial mechanisms [1,3,4]. PH is becoming more and more a global health issue due to the ageing population. Although PH-LHD and PH-LD are the most prevalent PH groups, research and drug development focuses mainly on PAH and CTEPH, which are rarer diseases that affect mainly younger people [5]. Because of the amount of research conducted in PAH compared to the other four groups, this review will focus mostly on PAH.

PAH is characterized by remodelling of distal pulmonary arteries, causing a progressive increase in vascular resistance. Vascular remodelling is associated with alterations in vasoconstriction, pulmonary artery- endothelial cells (PAECs) and -smooth muscle cells (PASMCs) cell proliferation, inflammation, apoptosis, angiogenesis and thrombosis, which leads to muscularization and occlusion of the lumen of pulmonary arteries by formation of vascular lesions. Plexiform lesions are the most common lesions in PAH, characterized by deregulated endothelial cell (EC) proliferation. Other lesions in PH are thrombotic lesions and neointima formation, which form a layer of myofibroblasts and extracellular matrix between the endothelium and the external elastic lamina [6]. One of the first triggers for development of PAH is EC injury triggering the activation of cellular signalling pathways that are not yet completely understood.

In normal conditions the endothelium is in a quiescent and genetically stable state. However, different types of injury can activate the endothelium. When activated, the endothelium secretes different growth factors and cytokines that affect EC and SMC proliferation, apoptosis, coagulation, attract inflammatory cells or affect vasoactivity to restore homeostasis. EC dysfunction, the loss of cellular functions leading to pathological changes, is crucial in the development of cardiovascular diseases and so too in PAH [7,8]. Many different

factors have been suggested to be involved in the initiation of EC dysfunction in PAH, like shear stress, hypoxia, inflammation, cilia length, and genetic factors (**Figure 1**) [6,9-11]. In PAH the endothelium switches from a quiescent to an overactive state where it starts to secrete vasoconstrictive factors like endothelin-1 (ET-1) [12] and thromboxane [13], and proliferative factors like vascular endothelial growth factor (VEGF) and reduce the secretion of vasodilators like nitric oxide (NO) and prostacyclin, indicating that EC dysfunction might play an central role in the pathogenesis of PAH [7,14].

The purpose of this review is to provide a state-of-the-art overview on EC dysfunction in PAH and to highlight current progress made in understanding this phenomenon. At last, this review discusses several models for studying EC dysfunction in PH and explores possible molecular targets and drugs for restoring EC function in PH.

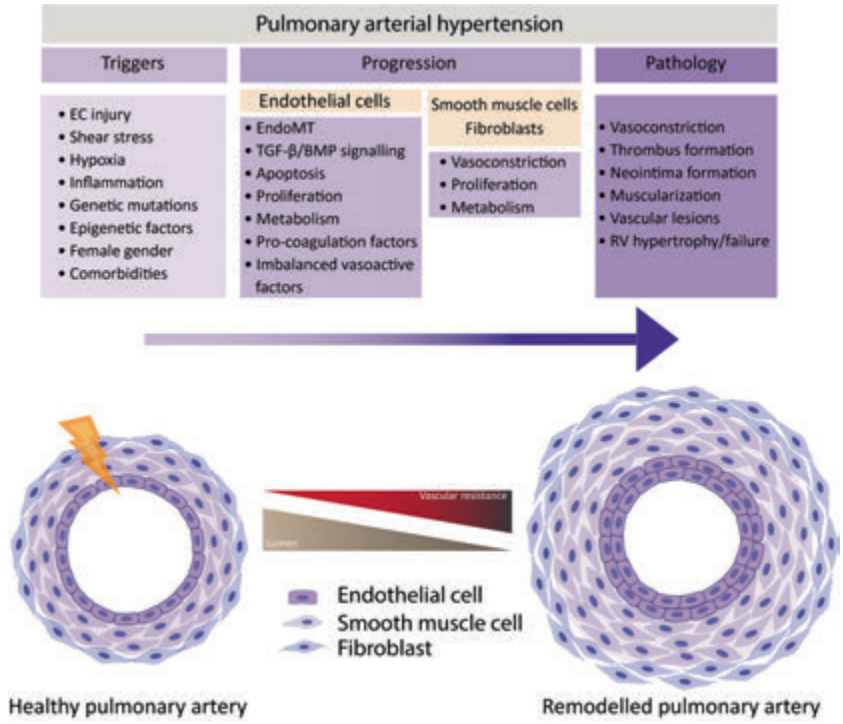

Figure 1. Pulmonary artery remodelling, vascular resistance and pulmonary arterial hypertension (PAH) development. PAH results from a progressive increase in vascular resistance caused by pulmonary vascular remodelling. Molecular mechanisms behind the process of vascular remodelling are still not fully elucidated but endothelial cell (EC) injury is thought to be one of the early triggers. EC injury can be caused by shear stress, hypoxia and inflammation. Host factors such as genetic mutations and gender but also epigenetic factors and comorbidities are thought to play an important role in EC dysfunction. EC dysfunction leads to altered cell signalling that induces cellular processes such as EndoMT, apoptosis and proliferation. In addition, changes are found in cell metabolism and in the secretion of vasoactive, coagulation and thrombotic factors. Also vascular smooth muscle cells and fibroblasts are found to display a diseased cellular phenotype. EC dysfunction eventually promotes vasoconstriction, thrombus formation, neointima formation, muscularization and development of vascular lesions. As lumen size decreases, pulmonary vascular resistance increases and induces right ventricle (RV) hypertrophy with eventually RV failure.

FACTORS CONTRIBUTING TO EC DYSFUNCTION IN PAH

Approximately 80% of familial PAH (hPAH) and 20% of idiopathic cases of PAH (iPAH) are associated with mutations in the bone morphogenic type 2 receptor (BMPR2) but a penetrance of 20-30% suggests secondary stimuli such as endothelial to mesenchymal transition (EndoMT), inflammation, thrombosis, apoptosis and perturbations in vasoactivity as important contributors to EC dysfunction and PAH development [15-17].

Bone morphogenic type 2 receptor

BMPR2 encodes for a transmembrane serine/threonine kinase receptor belonging to the transforming growth factor-β (TGFβ) family of signalling proteins (**Figure 2**) [18]. BMPR2 modulates cellular growth, apoptosis, inflammation and differentiation via binding of bone morphogenetic proteins (BMPs) to a heteromeric complex of a BMP type-I receptor and BMPR2, in a time, concentration and cell type dependent manner [19]. Depending on the localization in the vascular bed, BMPR2 promotes survival of PAECs, while it has an anti-proliferative effect on PASMCs [20-22].

To date over 380 PAH related mutations in *BMPR2* are known, mostly loss of function mutations [23,24]. Low penetrance of disease development associated with *BMPR2* mutations observed in humans has also been confirmed in experimental models of PH, where *BMPR2* deletion alone does not induce PAH in the majority of the cases [25-27]. Interestingly, reduced levels of BMPR2 have also been found in PH patients without *BMPR2* mutations, suggestion additional involvement of genetic modifiers or environmental factors reducing BMPR2 dependent signaling [28-31].

BMPR2 is predominantly present in ECs lining the vascular lumen in the lung and expression is reduced in ECs from PH lung. Therefore mutated *BMPR2* is postulated to play a significant role in EC dysfunction in PAH [24,28]. Association between endothelial BMPR2 and PAH development was further supported by the observation that mice with endothelial specific deletion of *BMPR2* were prone to develop PAH [32,33]. PAECs overexpressing a kinase-inactive BMPR2 mutant show increased susceptibility to apoptosis and conditioned medium from these PAECs stimulated proliferation of PASMCs via increased release of TGFβ1 and fibroblast growth factor (FGF)-2 [34]. BMP9 administration selectively enhanced endothelial *BMPR2* and reversed PH in rats [35]. In line with these findings, several compounds attenuated EC dysfunction via increased BMPR2 signalling and reduced abnormal remodelling in experimental PH[36-38]. Moreover, BMPR2 acts as a gatekeeper to protect ECs from increased TGFβ responses and integrin-mediated mechano-transduction [39].

Loss of endothelial BMPR2 promotes release of pro-inflammatory cytokines in a SOD3-dependent manner, allowing leukocyte transmigration to underlying tissues, causing further vascular remodelling *in vivo* [25,40,41]. Furthermore, loss of BMPR2 signalling in PAECs promotes a pro-inflammatory state during normoxia by enhancing mitochondrial biogenesis, mitochondrial potential and promoting glycolysis [42]. BMPR2 deficiency in iPAH PAECs lacking BMPR2 are associated with loss of DNA damage control via reduced DNA repair related genes such as BRCA1. Increased DNA damage reciprocally leads to further reduction of BMPR2 expression and EC dysfunction [43]. Transcriptome analysis of PAECs from iPAH patients revealed a correlation between reduced BMPR2 levels and downregulation of β-catenin, resulting in reduced Collagen-4 (COL4) and ephrinA1 (EFNA1) expression [44]. Both COL4 and EFNA1 perform intertwining roles in endothelium structure. siRNA mediated silencing of *BMPR2* in PAECs resulted in increased PAEC proliferation, migration, and disruption of cytoskeletal architecture. One of the changes observed was increase in Ras/Raf/ERK signalling, and Ras inhibitors, like nintedanib [45], reversed the enhanced proliferation and hypermotility of BMPR2 silencing in PAECs [46].

Carboxylesterase-1 (CES1) promotes BMP signalling by ensuring proper trafficking of BMPR2 from the endoplasmic reticulum (ER) to the plasma membrane [47]. CES1 is reduced in iPAH patients and impaired ER trafficking will result in decreased BMPR2 availability [47].

Pro-inflammatory cytokines, such as IL-6 and TNFα, have also been found to downregulate BMPR2 expression in PAECs via a STAT3-miR-(Cluster 17/92) and NF-κB-p65 pathway,

respectively [29,48]. Finally, miRNA-21, although primarily induced by BMPR2 signalling, negatively targets BMPR2 expression [49].

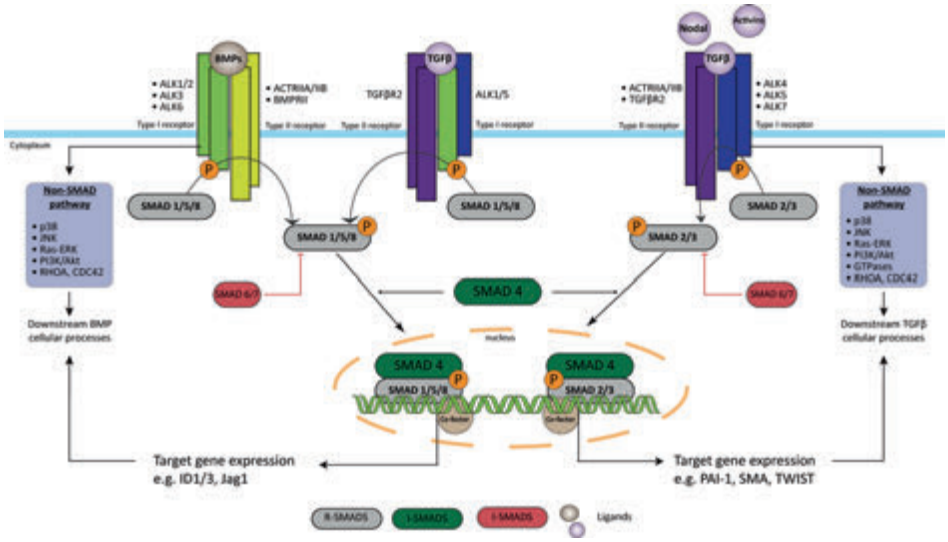

Figure 2. TGF-β superfamily signalling in PAH. The TGF-β superfamily is subdivided into the TGF-β group that include TGFβ, Nodal and activins and the BMP group that includes BMPs. Both groups signal through intracellular mediators, known as Smads. Receptor-regulated Smads (R-Smads) are phosphorylated by type-1 receptors (e.g. ALK1/2/3/6 and ALK4/5/7) and form complexes with a Common mediator Smad (Co-Smad). Subsequently, these complexes translocate into the nucleus where they induce transcriptional responses that alter gene expression of specific targets that influence apoptosis, cell differentiation, inflammation and proliferation. Inhibitory Smads (I-Smads) negatively regulate TGF-β and BMP signalling. Both TGF-β and BMP receptors can also signal independently from Smads and alter downstream cell-specific processes. It is know that TGF-β superfamily signalling plays an important role the initiation of EndoMT by triggering overexpression of genes like TWIST1, αSMA and phospho-vimentin.

Endothelial to mesenchymal transition

EndoMT is a phenomenon where ECs acquire a mesenchymal-like phenotype which is accompanied with loss of endothelial markers and gain of mesenchymal markers. In addition, ECs lose cell-cell contact, change their morphology and adopt a highly migratory and invasive phenotype (**Figure 3A**) [50,51]. In lungs of human PAH patients and monocrotaline (MCT) and Sugen/hypoxia (SuHx) experimental PH rat models, EndoMT was observed whereby cells express high levels of α-SMA and activated phospho-vimentin and VE-cadherin, indicating their endothelial origin [52-54]. Moreover, TWIST1, a key transcription factor in inducing EndoMT, is highly expressed in human PAH lungs compared to healthy lungs [52].

TGFβ treatment of PAECs induces expression of the EndoMT transcription factors TWIST1 and SNAIL1[50,55] and the mesenchymal markers α-SMA and phospho-vimentin[56]. TWIST1 increases

expression of TGFβ, leading to enhanced TGFβ signalling[57]. In addition, reduced BMPR2 signalling promotes EndoMT via upregulation of the High Mobility Group AT-hook 1 and its target gene SLUG, independently of TGFβ signalling [58]. More interestingly, BMP-7, a protein previously described as having anti-inflammatory and anti-tumour effects in several diseases, was attenuated hypoxia-induced EndoMT in PAECs both *in vivo* and *in vitro* by inhibiting the m-TORC1 signalling pathway[59]. BMPR2 favours EndoMT allowing cells of myo-fibroblastic character to create a vicious feed-forward process leading to hyperactivated TGFβ signalling[39]. In summary, alterations in TGFβ/BMP signalling are linked to the process of EndoMT observed in PAH [60].

Hypoxia is also an inducer of EndoMT through hypoxia-inducible transcription factor-1α (HIF-1α) and HIF-2α, and both transcription factors are increased in PAH [61,62]. PAH ECs display increased expression of HIF-2α, leading to SNAIL upregulation [54]. In addition, HIF-1α knockdown alone effectively blocks hypoxia-induced EndoMT but also knockdown of its downstream target gene TWIST1 showed effective blockage of hypoxia-induced EndoMT in microvascular ECs (MVECs), however less pronounced[63]. Nonetheless, microvascular endothelium may differ from arterial endothelial function.

Inflammation

Pulmonary arteries of PAH patients showed infiltration of macrophages, dendritic cells and lymphocytes into the plexiform lesions and an increased migration of monocytes[9,64]. Increased levels of pro-inflammatory cytokines and chemokines, such as IL-1β, TNFα and IL-6, known activators of vascular endothelium, were found (**Figure 3B**) [37,65,66]. IL-1β stimulates endothelial ET-1 production [67]. Administration of IL-6 to experimentally induced PAH in a rat model and overexpression of IL-6 in transgenic mice led to occlusion of pulmonary arteries and RV hypertrophy [68,69]. IL-33 has a dual role as cytokine and a role in the nucleus [70]. Nuclear IL-33 is expressed in nuclei of healthy ECs but is less expressed in nuclei of ECs from iPAH lungs. Nuclear IL-33 modulates gene expression of pro-inflammatory cytokines and IL-33 knock-down in PAECs upregulates expression of IL-6. Therefore, loss of nuclear IL-33 could contribute to EC dysfunction in PAH [70]. Additionally IL-33 may contribute to inflammatory activation of the endothelium by promoting endothelial production of granulocyte macrophage-colony stimulating factor (GM-CSF) and macrophage-CSF [71]. Hypoxia induces

expression of IL-33 and its receptor ST2 on ECs, leading to EC and SMC dysfunction with concomitant PH development [72].

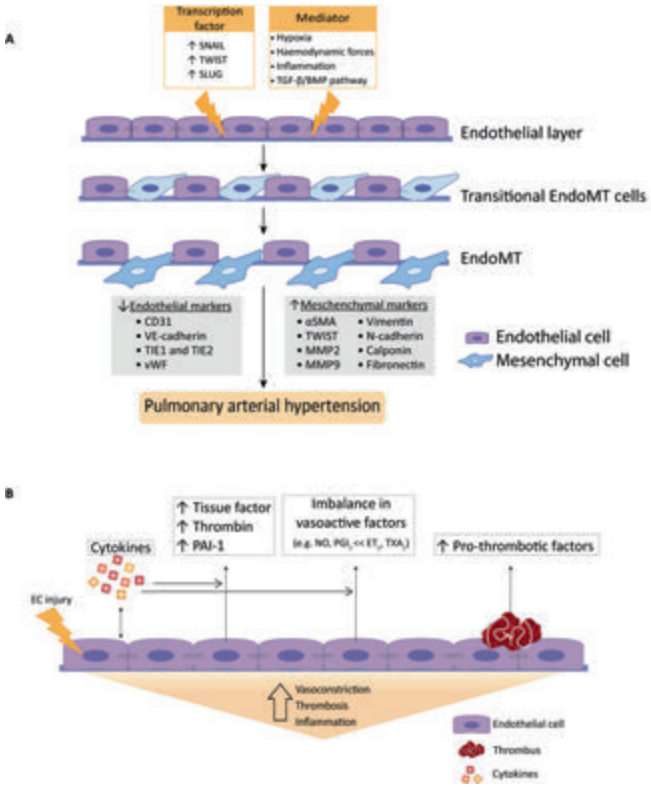

Figure 3. Endothelial to mesenchymal transition (EndoMT) and endothelial dysfunction in PAH. **A)** Upon activation by transcriptional factors, hypoxia, haemodynamic forces, inflammation and TGF-β/BMP pathway signaling pulmonary endothelial cells (PAECs) undergo cellular transition to a mesenchymal phenotype. During transition, PAECs lose endothelial markers and gain mesenchymal markers such as αSMA and TWIST. These mesenchymal-like cells also gain mesenchymal characteristics that trigger vascular remodeling and PAH pathogenesis. **B)** Upon endothelial cell injury, PAECs become dysfunctional and alter their secretion of cytokines and other factors that regulate coagulation, thrombosis and vascular tone. A failure of PAECs in maintaining vessel homeostasis promotes vasoconstriction, thrombosis and inflammation that initiate PAH disease progression.

Thrombosis and coagulation

In situ pulmonary artery thrombosis is regularly found in PAH. However, it remains unclear if thrombosis due to EC dysfunction causes progression of PAH or whether it forms as a result of it. Various factors, e.g. von Willebrand factor, plasminogen activator inhibitor-1 and tissue factor (TF), secreted by EC, have been implicated in coagulation and are found differently expressed in PAH (**Figure 3B**) [73-75].

Recently TF has emerged as an interesting target involved in the pathogenesis of PAH. TF is a glycoprotein expressed on the cell surface of SMCs, macrophages, monocytes and ECs [76]. It plays a role in initiation of coagulation, facilitation of angiogenesis and mediation of arterial injury in the circulation [77,78]. Interestingly, TF is rarely expressed in healthy cells, but is highly expressed in PAECs in PAH, predominantly in plexiform lesions [79]. In PAH animal models , increased TF expression correlates with formation of plexiform-lesions [79]. Furthermore, in PAH patients elevated levels of thrombin, a downstream target of TF and essential for clot formation, are detected [74,80]. Elevated levels of fibrinopeptide-A (FPA) which increases thrombin activity (downstream of TF) are observed in PAH patients [74]. PAH patients have lower thrombomodulin levels, consistent with the hypercoagulable state observed in PAH patients[81]. However, the role of EC dysfunction in this is still unclear.

Apoptosis

EC apoptosis may also play a role in PH development via vascular dropout and selection pressure on ECs, contributing to the apoptosis-resistant phenotype of ECs in vascular lesions [82]. Several attempts were made to elucidate the molecular pathways involved in regulation of PAEC apoptosis. The hypothesis is that disturbed responses to VEGF signalling in combination with hypoxia cause an initial increase in apoptosis in PAECs, leading to the emergence of aggressive apoptosis resistant and hyperproliferative ECs that cause formation of intimal lesions [83-85]. A possible explanation for the initial increase in apoptosis of PAECs is that loss of BMPR2 signalling promotes mitochondrial dysfunction and subsequent PAEC apoptosis [42]. White *et al*., interestingly, proposes a model in which the pro-apoptotic factor programmed cell death-4 (PDCD4) activates cleavage of caspase-3, inducing PAEC apoptosis. Interestingly, they show that reducing PDCD4 levels *in vivo* by overexpressing miRNA-21 prevents PH development in SuHx rats [86]. Besides an initial increase in apoptosis, PAH is also characterized by PAECs that are hyperproliferative and apoptosis resistant [85]. PAECs from iPAH patients showed increased expression of pro-survival factors IL-15, BCL-2 and Mcl-1, together with persistent activation of the pro-survival STAT3 signalling pathway [85]. Furthermore, in lungs from iPAH patients and from SuHx rats Notch1 was elevated. Notch1 contributes to PAH pathogenesis by increasing EC proliferation and inhibiting apoptosis via p21downregulation and regulating BCL-2 and survivin expression. Furthermore, HIF1α expression promotes Notch signalling human PAECs [87].

VASOACTIVE FACTORS CONTRIBUTING TO EC DYSFUNCTION

PAH is characterized by an activated endothelium of which the balance between vasodilation and vasoconstriction, but also growth factor production, is altered, causing perturbations in pulmonary vascular homeostasis that promote vascular remodelling (**Figure 3B**).

Nitric oxide

NO is a fast-reacting endogenous free radical produced by endothelial NO Synthase (eNOS). NO is essential for vasorelaxation via PASMCs but also has antithrombotic effects and controls EC differentiation and growth [88-90]. NO has long been implicated in the pathogenesis of PAH, and lungs of PAH patients have reduced NO expression [91]. However, other studies reported contradictory results and some PH patients even show an increase in eNOS expression [91,92]. Furthermore, $eNOS^{-/-}$ mice show reduced vascular remodelling after chronic hypoxia caused by reduced vascular proliferation [93].

Next to hypoxia as a known regulator of eNOS expression, increasing evidence supports the involvement of epigenetic regulations such as histone acetylation and DNA methylation in expression eNOS. This is independent of the initial hypoxic environment. Experimental models of persistent pulmonary hypertension of the new-born (PPHN) and PPHN PAECs showed that epigenetic modifications can contribute to reduced eNOS expression and subsequent PPHN pathogenesis [94,95]. However, it is unclear whether such mechanisms exist in PAH pathogenesis [96-100].

Reduced NO availability can also be caused by freely circulating endogenous eNOS inhibitors [101], such as asymmetric dimethylarginine (ADMA) [102]. The metabolism of this protein is facilitated by dimethylarginine dimethylaminohydrolase (DDAH) [103]. Increased levels of ADMA are associated with the pathogenesis of PH [101,103,104], and hypoxia-induced increase of miRNA-21 is found to reduce DDAH activity [49,104].

Prostacyclin

Prostacyclin is another important vasodilator produced by EC with additional antithrombotic and antiproliferative properties [7,105-107]. Prostacyclin is synthesized from arachidonic acid, by prostacyclin synthase and cyclo-oxygenase (COX) [108]. Decreased prostacyclin levels are measured in various patients with different forms of PAH, like iPAH and HIV-associated PAH [7,109] explaining in part the increase in pulmonary vasoconstriction, SMC proliferation and

coagulation occurring in these patients. In experimental PAH models, mice overexpressing prostacyclin synthase are protected from developing chronic hypoxia-induced PAH [110].

Endothelin-1

ET-1 is a potent vasoconstrictor, mainly synthesized in EC, but also in smaller amounts in vSMCs, macrophages, fibroblasts, myocytes and epithelial cells [7,111,112]. The lungs show the highest level of ET-1 in the entire body [113]. ET-1 stimulates vSMCs proliferation and platelet aggregation [7,106]. ET-1 exhibits its effects by binding to the ET_A and ET_B receptors, which activate signalling pathways in vSMCs regulating proliferation, vasorelaxation and vasoconstriction [107,113]. ET_A is predominantly expressed on vSMCs and is involved in vasoconstriction and proliferation of these cells, while ET_B is expressed on vSMCs and PAECs, and is involved in stimulating the release of vasodilators, like NO and prostacyclin, and inhibition of apoptosis [67,107,111,113,114]. Expression of ET-1 and its receptors is increased in lungs of PAH patients and experimental PH models [115-118]. Furthermore, a correlation exists between expression of ET-1 and increase in pulmonary resistance in PAH [117]. Multiple PAH associated factors are able to increase ET-1 expression including hypoxia, cytokines, growth factors, TGFβ/BMP signalling and shear stress [119-123]. Increased synthesis of endothelial ET-1, accompanied with an increase in expression of ET_A on PASMCs likely contributes to the increased vasoconstriction and vascular remodelling observed in PAH [106,118,124].

Thromboxane

Thromboxane A_2, produced by ECs and platelets, is a vasoconstrictor, inducer of platelet aggregation and a vSMCs mitogen [7,13]. Its production is increased by hypoxia and oxygen metabolites [125,126]. In PAH thromboxane A_2 is increased, creating an imbalance that might contribute to excessive platelet aggregation and vascular remodelling observed in PAH [13].

Vascular endothelial growth factor

VEGF is an angiogenic factor secreted by ECs. VEGF has multiple roles in maintaining lung structure and homeostasis but also is associated with several vascular disorders [7,127,128]. The pulmonary endothelium does not secrete VEGF during normal homeostasis but iPAH ECs from plexiform lesions show increased expression of VEGF and VEGF receptor 2 [129], and also VEGF plasma levels of PH patients are elevated [130]. The relation between PAH and increased VEGF expression is still poorly understood. It is suggested that VEGF levels in PAECs are elevated in early stages of PAH as a protective response, while during disease progression VEGF keeps

promoting growth of PAECs, causing the formation of plexiform lesions [7]. Rats treated with a VEGF receptor blocker in combination with hypoxia develop angio-obliterative PAH [131]. Furthermore, overexpression of VEGFA slows down the development of hypoxia-induced PAH, and improves endothelial function by increasing eNOS activity among others [132].

EPIGENETICS

In recent years epigenetics has become a growing field of interest in PAH research. Currently the main focus of study for targeting PAH are the following three mechanisms of epigenetic regulation; DNA methylation, histone modifications and RNA interference (**Figure 4**) [14].

DNA methylation profiling of PAECs from iPAH and hPAH patients revealed differences in expression of several genes involved in inflammatory processes, remodelling and lipid metabolism compared to controls [133]. Among those genes ABCA1 was found most differently methylated/ downregulated in the discrimination between PAH and controls. ABCA1 belongs to the family of ATP binding cassette (ABC) transporters that are important for pulmonary homeostasis [133]. Furthermore, ABCA1 is linked to PAH pathophysiology in a MCT animal model of PAH where activation of ABCA1 improved RV hypertrophy and pulmonary haemodynamics [14,133].

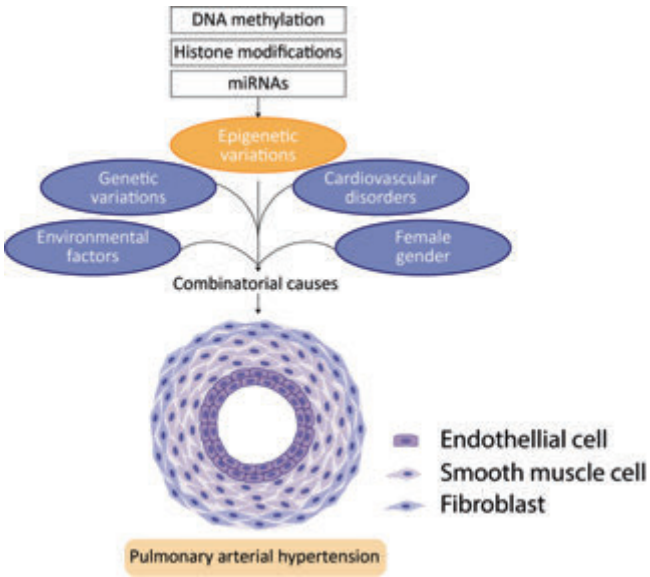

Figure 4. Epigenetics in PAH. In addition to genetic variations and other risk factors such as gender, comorbidities and environmental factors, epigenetic variations in PAH gain interest. Differences in DNA methylation profiles, increased histone acetylation and dysregulated miRNA expression in PAH patients point out a growing field in PAH research that provides better understanding of disease pathology.

Increased histone acetylation through histone-deacetylases (HDAC) is associated with vascular remodelling found in PAH [134,135]. In humans, HDAC enzymes are divided into 4 classes: class-1 HDACs (HDAC-1, -2, -3 and -8), class-2a HDACs (HDAC-4, -5, -7 and -9), class-2b HDACs (HDAC-6 and -10), class-3 HDACs (Sir2-like proteins) and class-4 HDACs (HDAC-11) [136]. HDAC-1 and -5 show increased expression in both lungs of iPAH patients and chronic hypoxic rats whereas HDAC-4 was only increased in human iPAH lungs [135]. More recently HDAC-6 is linked to PAH pathogenesis, possibly through upregulation of HSP90 [137]. HDAC-6 was overexpressed in PAECs and PASMCs of PAH patients and PH experimental models [138]. In the SuHx and MCT rat model pharmacological HDAC-6 inhibition improved PH [138]. Several other studies showed that class-1 HDAC inhibitors attenuate PAH by suppressing arterial remodelling in a chronic hypoxia model and by reducing inflammation in PH-fibroblasts [135,139,140]. In PAECs class-2a HDAC inhibitors restore the levels of myocyte-enhancer-factor-2 and attenuate PAH in both the MCT and SuHx PAH rat models [141].

The epigenetic regulator bromodomain-containing-protein-4 (BRD4) is linked to the pathogenesis of PAH [38]. BRD4 is a member of the Bromodomain and Extra-Terminal (BET) motif family that binds histones to influence gene expression [142]. BRD4 is overexpressed in lungs of PAH patients in a miR-204 dependent manner. It inhibits apoptosis by sending cell survival signals [38,143], and stimulates proliferation of PAEC and PASMC proliferation at these sites [14,143]. Selective inhibition of BRD4 with RVX-208 restored EC function, reversed PAH in the MCT and SuHx rat models, and supported the RV function in pulmonary artery banding model of PAH [38].

EC DYSFUNCTION IN OTHER PH GROUPS

Group 2 PH

Group 2 PH is due to a complication of left heart disease and is most common in patients with heart failure (HF). Therefore research in group 2 PH focuses mostly on left ventricular dysfunction and not so much the lung vasculature. However, EC dysfunction is also associated with PH-LHD [144]. An experimental model of chronic HF showed reduced NO activity and responsiveness to NO in pulmonary arteries [145]. Moreover, ET-1 is elevated in certain PH-LHD phenotypes and ET-1 activity is increased in plasma of patients with chronic HF. Blocking the ET_A receptor caused pulmonary vasodilation in these patients [146,147]. Furthermore, polymorphisms found eNOS also contribute to PH development in patients with LHD [148].

Unfortunately, treating PH-LHD patients with drugs used to treat PAH patients was not beneficial and even harmful [144,149] .

Group 3 PH

Chronic obstructive lung disease (COPD) associated PH is the best described form of PH in group 3. EC dysfunction is one of the causes for these patients to develop PH [150]. Cigarette smoke decreases eNOS and prostacyclin expression in PAECs [151,152]. COPD patients can show overexpression of VEGF and ET-1 in pulmonary arteries [153,154]. These findings have led to the hypothesis that cigarette smoke may be one of the initiating factors for PH in COPD [150]. A role for HIF1α and EndoMT has also been suggested in COPD [155,156]. Although there are similarities in EC dysfunction, drugs used to treat PAH are currently not recommend for group 3 PH, due to lack of evidence how these drugs may influence PH progression in combination with the underlying lung diseases [157].

Group 4 PH

CTEPH develops as a result of a pulmonary embolism (PE) that does not resolve [158]. These organized pulmonary thrombi in the lungs are associated with distal vascular remodelling of non-occluded vessels similar to the remodelling observed in PAH lungs [158]. Activated platelets with a hyper-responsiveness to thrombin are likely to contribute to the CTEPH pathogenesis and progression via enhancing inflammatory responses of pulmonary ECs [159]. EC dysfunction-associated vascular remodelling has been suggested as a common mechanism between CTEPH and PAH [158,160]. Primary cell cultures isolated from endarterectomized tissue co-expressed both EC and SMC markers, suggesting a role for EndoMT in intimal remodelling/lesion development in CTEPH [161]. The existence of endothelial dysfunction in CTEPH pathogenesis is further supported by the fact that conditioned medium from CTEPH derived PAECs, containing high levels of growth factors and inflammatory cytokines, increased PASMC proliferation and monocyte migration [162]. In addition, PAECs from CTEPH patients show an increased proliferation, altered angiogenic potential and metabolism, and apoptosis resistance [163-167]. Increased levels of soluble intracellular adhesion molecule-1 (ICAM1) in PAECs from CTEPH patients and in endarterectomy may contribute to EC proliferation and apoptosis resistance through its effect on cell survival pathways [166]. Also FoxO1, in a PI3K/Akt dependent manner, is a possible contributor to the loss of balance between cell survival and death and was downregulated after PE in a rat model of CTEPH [168].

At last, PAECs isolated from CTEPH patients showed a significant rise in basal calcium levels, which is an important regulatory molecule for EC function [169]. This imbalance in calcium homeostasis is caused by angiostatic factors such as PF4, IP-10 and collagen type 1, that are formed in the microenvironment created by the unresolved clot and eventually lead to EC dysfunction [169]. So far, a soluble guanylate cyclase stimulator (Riociguat) is the only PAH based therapy that has been approved in patients with CTEPH that are not eligible for surgery [170].

CURRENT AND FUTURE PERSPECTIVES

Although much progress has been made to understand EC dysfunction in PAH, to date there is still no definitive cure and patients only have a median survival rate of 2.8 years [171]. Current therapies for PAH, consisting of calcium channel blockers, ET-1 receptor antagonists, phosphodiesterase type 5 inhibitors, prostacyclin-derivatives and more recently also Riociguat, focus on SMC relaxation with limited or no effect on EC dysfunction and subsequent progressive pulmonary vascular remodelling [172-174]. The effects of EC dysfunction are neglected thus far. Therefore, research on EC dysfunction and its stimuli to target structural changes that narrow lumen size in PAH is vital to find a cure.

A first step towards reversing vascular remodelling in PAH is the use of apoptosis-inducing drugs, such as anthracyclines and proteasome inhibitors. They are already used in combination with cardio-protectants such as p53 inhibitors to reduce pulmonary pressure and restore blood flow in experimental models of PAH [175,176]. The combinatorial use is essential to circumvent the lack of cell-type/organ specificity of cell-killing drugs. Cancer patients but also experimental PAH animals treated with only cell-killing drugs show signs of cardiotoxicity which should be prevented in PAH patients that already suffer from reduced right heart function [175,177-179].

Another way to target progressive pulmonary vascular remodelling focuses on restoring signalling pathways and EC function, e.g. using TGFβ inhibitors, like kallistatin, known to inhibit EndoMT in HUVECs, stimulate eNOS expression and prevent TGFβ induced miRNA-21 synthesis [180]. Blocking inflammation to restore normal EC function in PAH, however, was not successful. One explanation might be the complexity of the immune system and by inhibiting the bad side, one also suppresses beneficial inflammatory pathways [181,182].

Modulating BMPR2 has been proposed as therapeutic approach to reverse endothelial dysfunction in PAH too. A recent study comparing human induced pluripotent stem cell-

derived ECs (iPSC-ECs) from unaffected BMPR2-mutation carriers with iPSC-ECs from BMPR2-mutation carriers that present PAH identified several BMPR2 modifiers and differentially expressed genes in unaffected iPSC-ECs. These BMPR2 modifiers exert a protective response against PAH by improving downstream signalling, which compensates against BMPR2 mutation-induced EC dysfunction and offer insights towards new strategies to rescue BMPR2 signalling [183]. A potential therapy for stimulating BMPR2 signalling is through pharmaceuticals [184]. Direct enhancement of endothelial BMPR2 signalling using recombinant BMP9 protein prevents and reverses established experimental PAH [35]. However, in contrast to Long *et al*, Tu *et al* (2019) showed that deletion or inhibition of BMP9, protects against experimental PH via its effect on endothelial production of ET-1, apelin and adrenomedullin [185]. These studies show the BMP receptor family complexity as therapeutics in PAH. More recently, ACTRIIA-Fc, an activin and growth and differentiation factor (GDF) ligand trap, prevented and reversed existing PH in experimental PAH models. ACTRIIA-Fc inhibited SMAD2/3 activation and restored a favourable balance of BMP signalling versus TGFB/activin/GDF signalling. ACTRIIA-Fc is currently tested in a phase-2 clinical trial for efficacy and safety in PAH patients (NCT03496207) [186]. Spiekerkoetter *et al*. uncovered a molecular mechanism where FK506 (tacrolimus) restores defective BMPR2 signalling in PAECs from iPAH patients, and reverses severe PAH in several rat models [184]. Based on improvements in clinical parameters and stabilization of cardiac function of end-stage PAH patients in a phase-2a clinical trial, low dose of FK506 was proposed as potential beneficial in the treatment of end-stage PAH [187].These findings open-up an area in which correcting BMPR2 mutations in combination with other therapies might be more successful in curing PAH. A proposed hypothesis to cure PAH describes collecting iPSCs from PAH patients, restoring the BMPR2 mutation with CRISPR/Cas9 and reinjecting those iPSCs in the patient to normalize EC function and signalling along with administration of drugs that could restore the protective gene expression profile of unaffected BMPR2 mutation carriers [188]. 6-Mercaptopurine (MP), a well-established immunosuppressive drug, inhibits EC dysfunction and reverses development of PH in the SuHx rat model by restoring BMP signalling through upregulation of nuclear receptor Nur77 [189]. A recent proof-of-concept study with MP in a small group of PAH patients showed a significant reduction pulmonary vascular resistance, accompanied by increased BMPR2 mRNA expression in the patients' peripheral blood mononuclear cells. However, unexpected severe side-effects require further dose optimisation and/or use of other thiopurine analogues [36].

Transplantation of mesenchymal cells in rats from the SuHx model improved haemodynamic parameters but more interestingly reduced EndoMT (partially) through modulation of HIF2α expression [190]. Furthermore, mesenchymal stem cells are also suggested to reduce inflammation through secretion of paracrine factors and to attenuate vascular remodelling by lowering collagen deposition [190-192]. However the underlying mechanisms for this observation remain unclear [190].

At last, epigenetic modulation has received growing interests as potential therapeutic intervention. Especially specific HDAC inhibition shows great promise in reversing pulmonary remodelling and pressure [135]. A problem with broad-spectrum HDAC drugs is that they show severe side effects on the right ventricle, which can have fatal consequences in PAH patients with RV failure [139,193,194]. Therefore, searches for more selective HDAC inhibitors that do not show cardiotoxicity are still being done. One example is MGCD0103, a HDAC inhibitor that selectively inhibits class-1 HDACs, which has been tested in a chronic hypoxia rat model. This inhibitor showed improved haemodynamics, reduced wall thickening while RV function was maintained [139]. Also BET inhibitors such as RVX208 seem promising in the treatment of PAH through its beneficial effect on reducing the apoptosis-resistant and pro-inflammatory phenotype in PASMCs and MVECs isolated from PAH patients but also on vascular remodelling and the RV in several experimental models of PH [38]. Finally, miRNA-21 has been associated with multiple pathogenic features, such as TGFB signalling, EndoMT and apoptosis, central to PAH. Therefore, therapeutic modulation of miRNA-21 may be an important issue for future research to restore pathogenic signalling.

CONCLUSION

To date we still do not fully understand what triggers the onset and progression of PAH. We do know that BMPR2 mutations, epigenetics, physiological conditions, and inflammation are important triggers. EC dysfunction plays a central role in all of this, through EC proliferation, EndoMT and a misbalanced production of vasoactive factors resulting in the disorganized growth of PASMCs. However, the question still remains whether EC dysfunction is a cause or consequence of PAH. Despite advancements made in treating this disease, no focus on targeting PAH at its core. A better understanding of the molecular mechanisms involved in EC dysfunction in PAH is of utmost importance for developing successful therapies to save the lung as well as the heart, and maybe cure PAH in the future.

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CHAPTER 3

ENDOTHELIAL DYSFUNCTION: A POTENTIAL NEW TARGET IN CHRONIC THROMBOEMBOLIC PULMONARY HYPERTENSION

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ABSTRACT

Pulmonary endarterectomy (PEA) resected material offers the unique opportunity to develop an *in vitro* endothelial cell model of chronic thromboembolic pulmonary hypertension (CTEPH) at the disease site. We aimed to comprehensively analyse the endothelial molecular signature, function and mitochondrial profile of CTEPH-derived endothelial cells (EC) to identify key targets and molecular pathways for the prevention and treatment of the disease.

Isolated cells from specimens obtained at PEA (CTEPH-EC), were characterized based on morphology, endothelial phenotype and functional analyses (*in vitro* and *in vivo* tubule formation, proliferation, apoptosis, and migration). Mitochondrial content, morphology, and dynamics, as well as high-resolution respirometry and oxidative stress, were studied. CTEPH-EC showed typical endothelial morphology and stained positive for endothelial markers. They displayed a hyperproliferative phenotype and an increased expression of adhesion molecules. Functionally, they showed decreased apoptosis, reduced eNOS activity and migration, and reduced angiogenic capacity *in vitro* and *in vivo*. CTEPH-EC presented altered mitochondrial dynamics, increased mitochondrial respiration and an unbalanced production of reactive oxygen species and antioxidants. Finally, eNOS and VCAM-1 levels were associated with clinical risk factors.

This study forms the largest comprehensive investigation of isolated CTEPH-EC. Our results show that CTEPH-EC have abnormal phenotype and function. CTEPH-EC present hyperproliferation, reduced angiogenesis, increased adhesion molecule expression, mitochondrial dysfunction, eNOS uncoupling, and unbalanced oxidative stress/antioxidant production. This study identifies novel molecular pathways involved in CTEPH endothelial pathology. Modulation of oxidative stress/antioxidant production, mitochondria homeostasis, and adhesion molecule overexpression arise as potential new targets and biomarkers in CTEPH.

Keywords: Endothelial dysfunction – chronic thromboembolic pulmonary hypertension – mitochondrial dynamics – adhesion molecules – oxidative stress

INTRODUCTION

Chronic thromboembolic pulmonary hypertension (CTEPH) is a major cause of severe pulmonary hypertension (PH)[1], defined by increased mean pulmonary artery pressure and the presence of non-resolved thrombotic lesions in pulmonary arteries after anticoagulant therapy[2]. It is a progressive disease with significant burden in both severity and prevalence[1,3]. CTEPH may develop in 3-4% of cases after an acute pulmonary embolism (PE) and is mostly underdiagnosed[4]. Chronic obliteration of pulmonary arteries by the presence of a intraluminal organized thrombi, produces a gradual increase in pulmonary vascular resistance (PVR), leading to right ventricular failure and death[5]. The pathogenesis of CTEPH, the mechanisms leading to the lack of thrombus resolution and the development of peripheral vasculopathy are still unknown. It has been suggested that local factors as a result of endothelial dysfunction similar to PAH, play a critical role in the development of CTEPH. This could explain why many patients diagnosed with CTEPH do not have a documented clinical history of PE[1]. A better understanding of how pulmonary endothelial dysfunction contributes to the pathogenesis of CTEPH will improve the therapeutic management of the disease.

Pulmonary endarterectomy (PEA) is the gold-standard procedure for patients with CTEPH[6], addressed to remove the occluding thromboembolic material from the pulmonary arteries. PEA provides symptomatic, hemodynamic and prognostic benefit[1,7]. The outcome of PEA is largely dependent on the accessibility of the thrombotic lesions and the presence of concomitant vasculopathy[7]. However, up to 50% of patients are not eligible for surgery and up to 35% of operated patients show persistent or residual PH[8].

Endothelial dysfunction is believed to be an initial and essential step in the onset and progression of PAH[9]. It remains elusive whether endothelial dysfunction exists in CTEPH. No well characterized biomarkers for endothelial dysfunction have been identified and/or translated into the clinical practice as useful tools to detect its presence and severity in CTEPH. In 1973 *Moser and Braunwald*[10], examining the histopathological features of specimens obtained from patients who had undergone PEA, found distinct vessel abnormalities such as media thickening and increased intimal cell proliferation[9-11]. Recently, it has been shown that CTEPH pulmonary arteries presented abnormal vascular responses to acetylcholine[12]. In this study, we hypothesized that CTEPH-pulmonary endothelial cells (ECs) are dysfunctional. It

remains unknown why in a group of patients, residual organized thrombi remain attached to the pulmonary vessel walls and fail to resolve.

Intravascular occluding material extracted during PEA offers the unique opportunity to evaluate ECs at the site of damaged pulmonary arteries of patients with CTEPH. Accordingly, we aimed to develop an *in vitro* EC model of CTEPH to determine endothelial function, molecular signature and mitochondrial profile of the CTEPH-derived ECs and to identify key targets and molecular pathways for CTEPH prevention and treatment.

MATERIALS AND METHODS

An expanded material and methods section is available in **Data Supplement**.

Subjects

Subjects with CTEPH diagnosed per current guidelines[13], who underwent PEA at the Hospital Clinic of Barcelona, Spain were enrolled. Patient characteristics are shown in **Table 1.**

Morphometric and histological assessments

PEA resected material was fixed and stained. Cellular markers were analysed by immunohistochemistry as described[14]. On the other hand, PEA material was digested, stained and analysed by flow cytometry[15]. The antibodies used are listed in **Supplementary Table 1.**

Primary cell cultures

Isolated ECs (CTEPH-EC) were obtained from PEA specimens. Human pulmonary artery smooth muscle cells (PASMCs), human lung microvascular ECs (HMVEC-L) and human pulmonary artery ECs (HPAE) were used as control cells (Lonza).

Cell characterization

Cells were directly analysed by flow cytometry and immunofluorescence for phenotypic expression and proliferation[14,15]. The antibodies used are listed in Supplementary Table 1. Total RNA and protein extraction were performed following manufacturer's instructions. Primer sequences and antibodies are listed in **Supplementary Table 1-2**.

Cell growth kinetics and cell viability

Fold expansion/day was measured as number of final cells divided by the number of seeded cells/days of culture. Cellular viability was determined using Vybrant® MTT Cell Proliferation Kit (ThermoFisher Scientific). For clonogenic assays, single cells were plated in a 96-well plate and cultured as previously described[16]. Cells were also loaded into the xCELLigence device

following manufacturer's instructions to measure cell proliferation[17]. Cellular circumference, area and diameter were measured using ImagePro Plus image analysis software.

Tube formation assay and wound healing

ECs were seeded in an ibiTreat µ-Slide Angiogenesis (Ibidi) following manufacturer's instructions. 3D microvascular networks (VN) were obtained by a microfluidic approach[18]. Wound closure was expressed as % of wound healed divided by area and width of original wound.

Subcutaneous sponge implantation assay for *in vivo* vascularization

Male non-obese diabetic (NOD) severe immunodeficiency genetic disorder (SCID)-IL-2 gammaRnull mice aged 10–12 weeks were bred and maintained in the animal facilities of the University of Barcelona. All procedures were conducted following the European Directive 2010/63/UE and Spanish RD 53/2013 regulations related to the Guide for the Care and Use of Laboratory Animals. The study protocol was approved by the Animal Experimentation Ethics Committee of the University of Barcelona (DAAM 10028). Anesthetic comprised Ketamina (100mg/ml) and Medetomidina (1mg/ml), given intraperitoneally at a dose of 7.5ul/10 gbw and 10ul/10 gbw. Reversal of anesthesia was induced, after at least 20 minutes of unconsciousness, using Atipamezole (5mg/ml) in water for injection. This was given subcutaneously at a dose of 2ul/10 gbw. Meloxicam was given subcutaneously after surgery (2mg/ml) at 10ul/10 gbw. Mice were anesthetized and a sterilized sponge cylinder (0.5 cm^3) (Caligen Foam) was implanted subcutaneously on each flank. Each animal had a control vehicle-impregnated sponge implanted on one flank and cell-impregnated sponge on the other flank. Mice were culled and sponges excised 21 days following implantation. Sponges were fixed and stained for identification of blood vessels[19].

Electron microscopy

CTEPH-EC or HPAE were fixed with 2.5% (w/v) glutaraldehyde in 0.1 M cacodylate buffer. Cell pellets were stored and analysed by the technologic centre of University of Barcelona.

High resolution respirometry

CTEPH-EC or HPAE were resuspended in MiR05 medium and introduced into Oxygraph-2k (Oroboros Instruments). Endogenous cell respiration and complex I-IV analysis using specific substrates and inhibitors were performed (detailed in **Supplementary Table 3**).

Mitochondrial morphology and content

Mitochondrial morphology and content was determined using confocal microscopy[20] and MitoTracker green following manufacturer´s instructions.

Detection of oxidative stress

Cellular and mitochondrial oxidation was measured using cell-permeant CellROX™ and MitoSOX (ThermoFisher Scientific). Total oxidized proteins were measured with the Oxyblot Protein Oxidation Kit (Merck Millipore) following manufacturer's instructions**.**

Statistical analysis

Statistical analyses were performed using GraphPad Prism 7(Graph Pad Software). Data are shown as mean ± standard deviation. Independent samples were analysed using the unpaired Student's t-test (Mann-Whitney U test) to compare differences between two independent groups. More than two groups were compared using One-way ANOVA with Tukey´s post-hoc test or non-parametric analysis of variance Kruskal-Wallis test with a Dunn´s post-hoc multiple comparison test. The Spearman rank correlation coefficient was used as a hypothesis test to study the dependence between two random variables. Statistical significance was assumed if a null hypothesis could be rejected at $P \leq 0.05$ (for a confidence interval of a =95%).

Ethical statement

The study was conducted in accordance with the Declaration of Helsinki, was approved by the Committee on Human Research of our institution and all subjects gave written informed consent.

RESULTS

Histological assessment of resected specimens at pulmonary endarterectomy

PEA resected specimens from the vessel wall accessible to surgery (3-5cm) appear yellowish in colour, occasionally with a red non-resolved thrombus in the proximal surface (**Supplementary Figure 1**). Haematoxylin/eosin (H/E) and orcein staining are shown in **Figure 1A-B**. **Figure 1C** shows an intima layer, expressing endothelial markers (CD31 and vWF), a small compact alpha smooth muscle actin (α-SMA) positive media layer and an enlarged α-SMA negative remodelled intima (neointima) occupying on average 89.2±3.9% of the total width of the resected specimen (**Supplementary Table 4**). **Figure 1D** illustrates the presence of mature microvessels within the neointima positive for endothelial markers surrounded by a layer of α-SMA positive cells. The average number of microvessels/mm^2 per specimen was 47.5±14.2 (**Supplementary Table 4**). The H/E staining of the non-resolved thrombus shows a fibrous structure with no cells (DAPI negative) (**Figure 1E-F**). Flow cytometry analysis of disaggregated PEA resected specimens showed a cellular average of 11.1% CD45$^+$, 11.8% α-SMA$^+$, 6.7% CD34$^+$, 4.9% CD144$^+$, 10.9% CD31$^+$among others (n=20) (**Supplementary Table 4**).

Isolation and characterization of ECs from PEA material

ECs were obtained from freshly resected specimens obtained at PEA (46% efficiency). Colonies emerged after 7-20 days in culture and continued to proliferate to form a confluent monolayer (**Figure 2A**). CTEPH-EC had cobblestone morphology, typical of ECs (**Figure 2A**). CTEPH-EC strongly expressed endothelial surface antigens comparable to HPAE (>75% for all). CTEPH-EC were negative for muscular markers calponin and α-SMA (<0.1%) (**Figure 2B**). This phenotype was maintained throughout cell culture. CTEPH-EC stained positive for CD31 UEA-1, VE-CAD, endothelial nitric oxide synthase (eNOS) and showed positive staining for vWF with localized cytoplasmic granular organelles (Weibel-Palade bodies) while staining negative for muscular markers SMA and calponin (**Figure 2C**). In addition, CTEPH-EC were confirmed to be ECs at mRNA and protein level (**Figure 3A-B**). CTEPH-EC showed an increased mRNA levels of VE-cadherin, CD31, ANG1, vWF, ICAM-1, CD44 and VCAM-1 compared to HPAE. CTEPH-EC mRNA levels were negative for muscular markers and showed a significant reduction in eNOS and caveolin-1 mRNA levels compared to HPAE (**Figure 3A-B**). The levels of VEGF-A and ANG2 were not significantly different between CTEPH-EC and HPAE (**Figure 3A**). Additionally, whereas

HOXD3, HOXD8 and HOXD9 were highly expressed in HMVEC-L, they were virtually non-expressed in both HPAE and CTEPH-EC (**Figure 3D**).

CTEPH-EC showed a hyperproliferative phenotype

Proliferative capacity was assessed by quantifying the fold cell expansion/day. Growth of CTEPH-EC was consistently enhanced and showed a greater area under the curve compared to HPAE (9.66±2.54 *vs* 4.4±0.2 respectively) (**Figure 4A**). CTEPH-EC grew for >10 passages *versus* HPAE that lost growth potential around passage 10 (**Figure 4A**). CTEPH-EC showed a significantly longer viability at late passages (**Figure 4B**). CTEPH-EC had an increased number of Ki-67^{+} cells (**Figure 4C**), a significantly higher clonogenic potential (**Figure 4D)**, and greater cell proliferation compared to HPAE at late passages (**Figure 4E**).

CTEPH-EC showed resistance to apoptosis

Compared to HPAE, CTEPH-EC showed a significant reduction of caspase-3, -8, -9 expression levels (**Figure 5A**). mRNA expression levels of BCL2, p53 and p21 remained unchanged (**Supplementary Figure 2**). Cellular circumference, area and diameter of CTEPH-EC at late passage were reduced compared to HPAE, consistent with a more immature and proliferative cellular phenotype (**Figure 5B**).

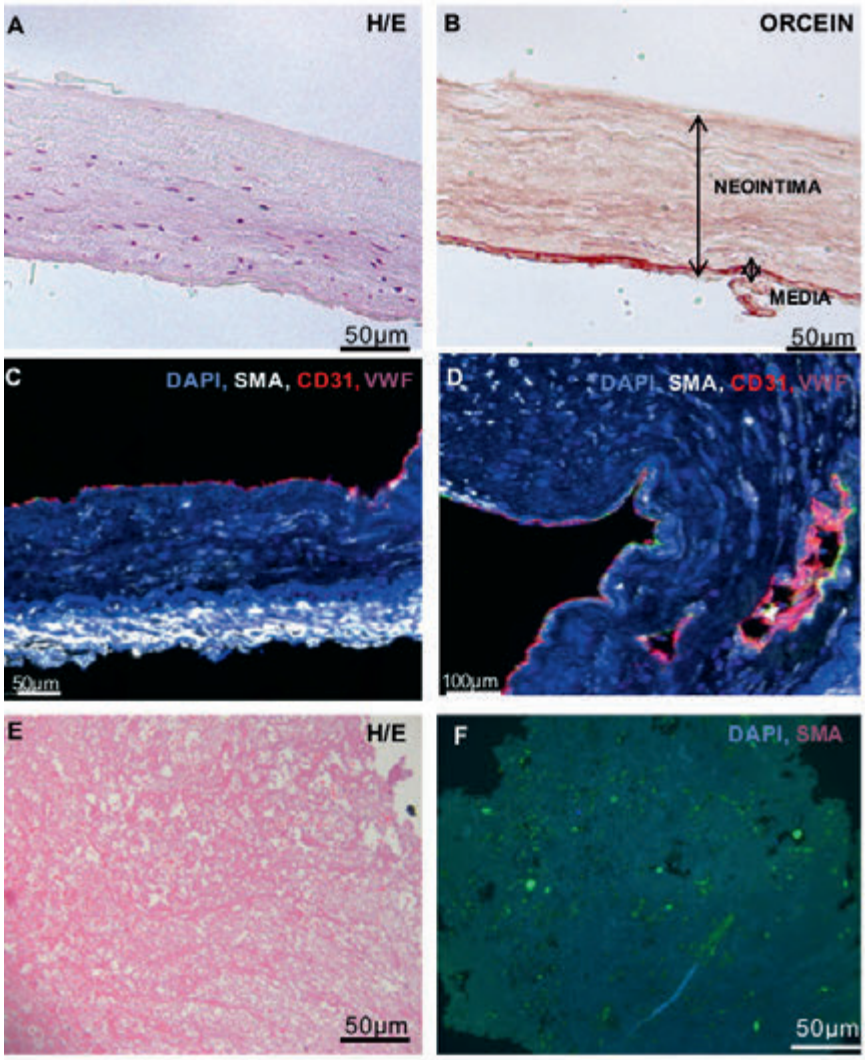

Figure 1. PEA samples. A-B) Specimens collected during PEA from CTEPH patients were stained for hematoxylin and eosin, and stained for elastin by orcein stain. **C)** PEA samples presented an intact endothelium as shown by endothelial markers CD31 and vWF, an enlarged α-SMA negative neointima and an α-SMA positive organized media. **D)** PEA samples showed the presence of mature microvessels. **E-F)** Coagulant material showed a fibrous structure lacking cells.

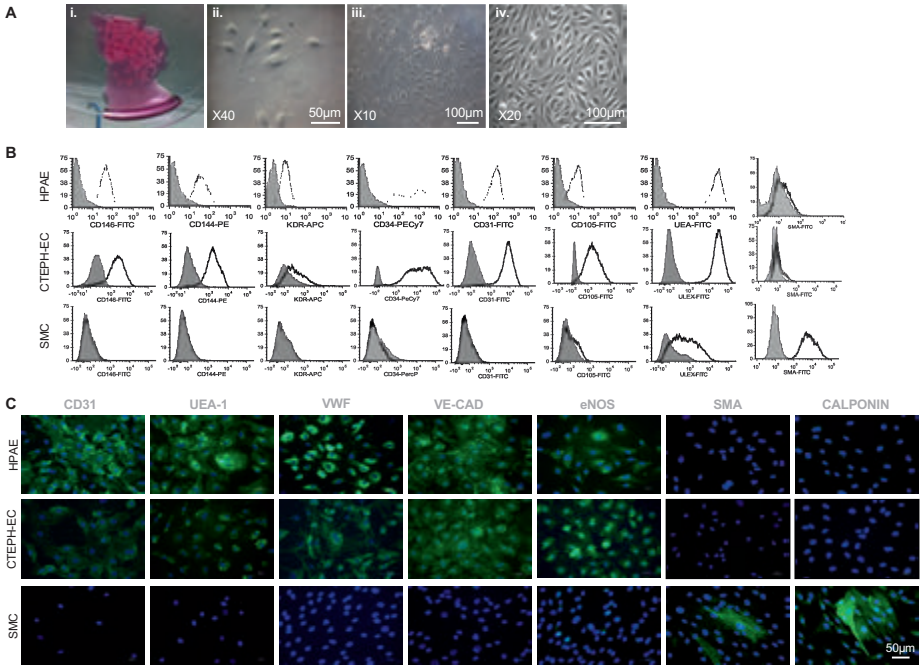

Figure 2. CTEPH-EC characterisation. A) Isolation of CTEPH-EC from PEA specimen, i) minced PEA material, ii) endothelial cell colony at day 10, iii) at day 14, iv) a confluent monolayer of CTEPH-EC. **B)** Representative flow cytometry histograms of HPAE, CTEPH-EC and smooth muscle cells (SMC) labelled with antibodies against endothelial surface makers (LTR: CD146, CD144, KDR, CD34, CD31, CD105, UEA/ULEX) and muscular markers (α-SMA) (n=10). **C)** HPAE, CTEPH-EC, and SMC were immune-labelled with antibodies against endothelial makers (CD31, UEA-1, vWF, VE-CAD, eNOS) and muscular markers (α-SMA and calponin) (n=10). Nuclei were counterstained using DAPI (blue). Scale bar=50µm.

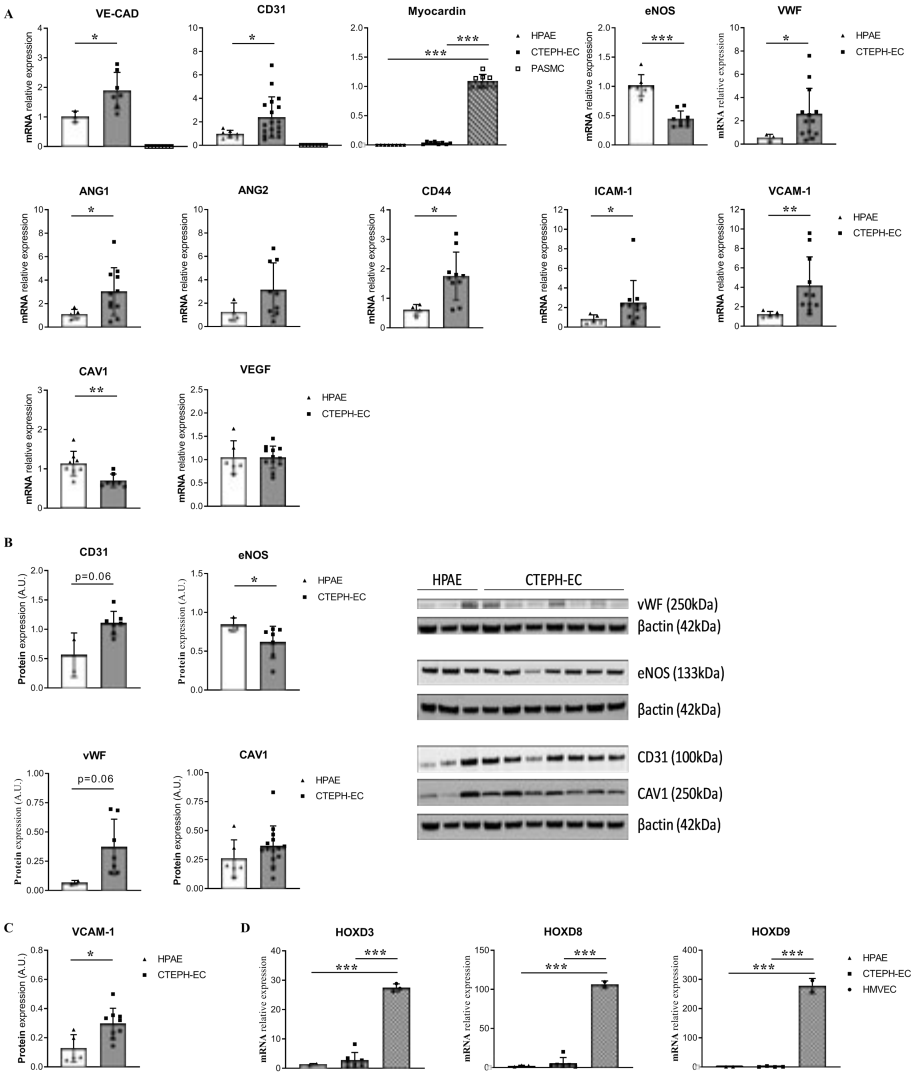

Figure 3. Expression of cell-specific markers in CTEPH-EC. A) CTEPH-EC presented endothelial markers at mRNA level and were negative for muscular markers (myocardin). p<0.05*, p<0.1**, p<0.001***, Mann–Whitney *U* test, values expressed as mean ± SD. **B**) CD31, eNOS, vWF and Cav-1 protein levels in CTEPH-EC compared to HPAE. p<0.05*, Mann–Whitney *U* test, values expressed as mean ± SD. **C)** Protein levels of VCAM-1 in CTEPH-EC compared to HPAE. p<0.05*, Mann–Whitney *U* test, values expressed as mean ± SD. **D)** CTEPH-EC and HPAE expression levels of HOXD3, -8 and -9 compared to HMVEC-L. p<0.001***, One-way Anova, values expressed as mean ± SD.

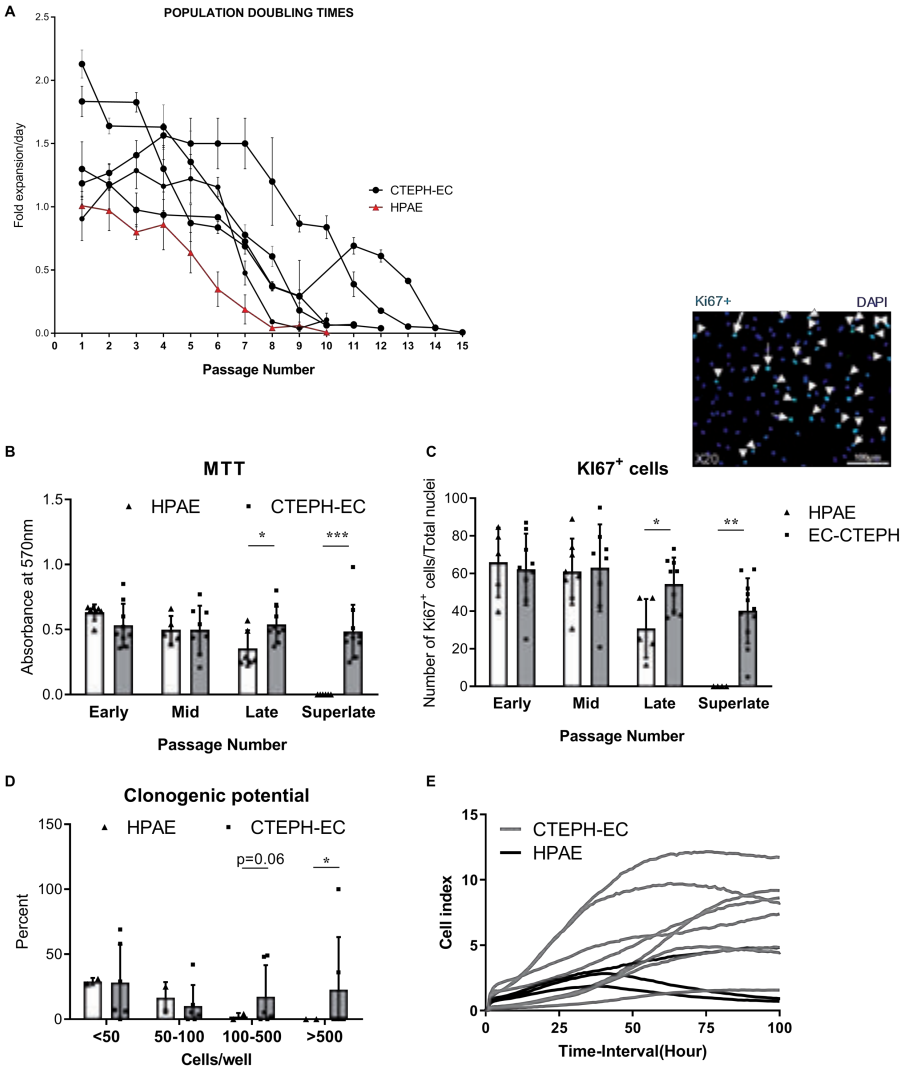

Figure 4. Proliferation of CTEPH-EC. A) Proliferative capacity of CTEPH-EC and HPAE as population doubling time. Each line represents an individual donor/ patient. **B)** Viability of CTEPH-EC compared to HPAE evaluated by MTT assay. p<0.05*, p<0.001***, Mann–Whitney *U* test, values expressed as mean ± SD. **C)** Clonogenic potential of CTEPH-EC and HPAE expressed as percentage, p<0.05*, Mann–Whitney *U* test, values expressed as mean ± SD. **D)** Quantification and stain of proliferative marker Ki-67 in CTEPH-EC and HPAE at different passages. p<0.05*, p<0.01**, Mann–Whitney *U* test, values expressed as mean ± SD. **E)** Cellular adhesion of CTEPH-EC compared to HPAE. Each line represents an individual donor/ patient.

CTEPH-EC showed reduced angiogenic capacity

Both CTEPH-EC and HPAE formed tube-like structures on a Matrigel® in 2D and on a fibrin hydrogel membrane matrix in 3D (**Figure 6A-B**, and Supplementary video 1-2). Quantification of cell-cell connections and tube length showed reduced angiogenic potential of CTEPH-EC compared to HPAE (**Figure 6A-B**). CTEPH-EC also displayed reduced recovery capacity in wound healing assay (**Figure 6C**). *In vivo*, using an established mouse model of angiogenesis, we examined the spontaneous vascularization of subcutaneously implanted sponges embedded with CTEPH-EC and HPAE. Quantification of number of vessels showed a significant reduction in growth of new vessels in CTEPH-EC loaded sponges compared to HPAE (**Figure 6D**). There was no significant difference in spontaneous vascularization between control sponges (Matrigel only) and sponges with CTEPH-EC (5.8±2.3 vs 6.48±3.1 respectively). The expression levels of Notch related genes/proteins such as DLL4 and JAG1 which differentiate tip (migrating) from stalk (proliferating) ECs did not differ between CTEPH-EC and control cells (**Supplementary Figure 3**).

Mitochondrial abnormalities in CTEPH-EC

Electron microscopy of cultured CTEPH-EC showed an irregular mitochondrial structure (inner membranes and cristae) compared to HPAE (**Figure 7A**). The number of mitochondria per cell area and mitochondria circularity did not differ between CTEPH-EC and HPAE (**Supplementary Figure 4A-B**). Aspect ratio (AR) or mitochondrial elongation parameters were also comparable between the two groups (**Supplementary Figure 4C**). Additionally, mitochondrial content assessed by mt12SrRNA gene/nRNAseP nuclear gene ratio did not differ between the two groups (**Supplementary Figure 4D**).

Oxygen consumption, measured by high resolution respirometry, was increased in CTEPH-EC compared to HPAE. Both endogenous cell respiration (basal), as well as maximum respiratory capacity were increased (**Figure 7B**). To explore whether increased basal and maximum respiration were related to a specific complex of the mitochondrial respiratory chain (MRC), we further explored each MRC complex through stimulation or inhibition with specific substrates. All oxidative activities including glutamate and pyruvate oxidation through complex I, succinate oxidation through complex II, glycerol-3-phosphate oxidation through complex III, ascorbate and 2,3,5,6-tetramethyl-p-phenylenediamine (TMPD) oxidation of complex IV were increased in CTEPH-EC compared to control cells, reaching statistical

significance for complex I and II (**Figure 7B**). Finally, significant increase in proton leakage was observed in CTEPH-EC suggesting uncoupling leakage as the main causative factor for the oxidative alterations mentioned above (**Figure 7B**).

CTEPH mitochondrial fusion/fission

Mitochondrial dynamics in HPAE and CTEPH-EC were analysed by studying both fusion and fission processes. All fusion genes studied (MFN1, MFN2 and OPA1) showed a significant downregulation in CTEPH-EC compared to HPAE (**Figure 7C**). Fission related gene DRP1 remained unchanged (**Figure 7C**). These results were also found at protein level (**Figure 7D**).

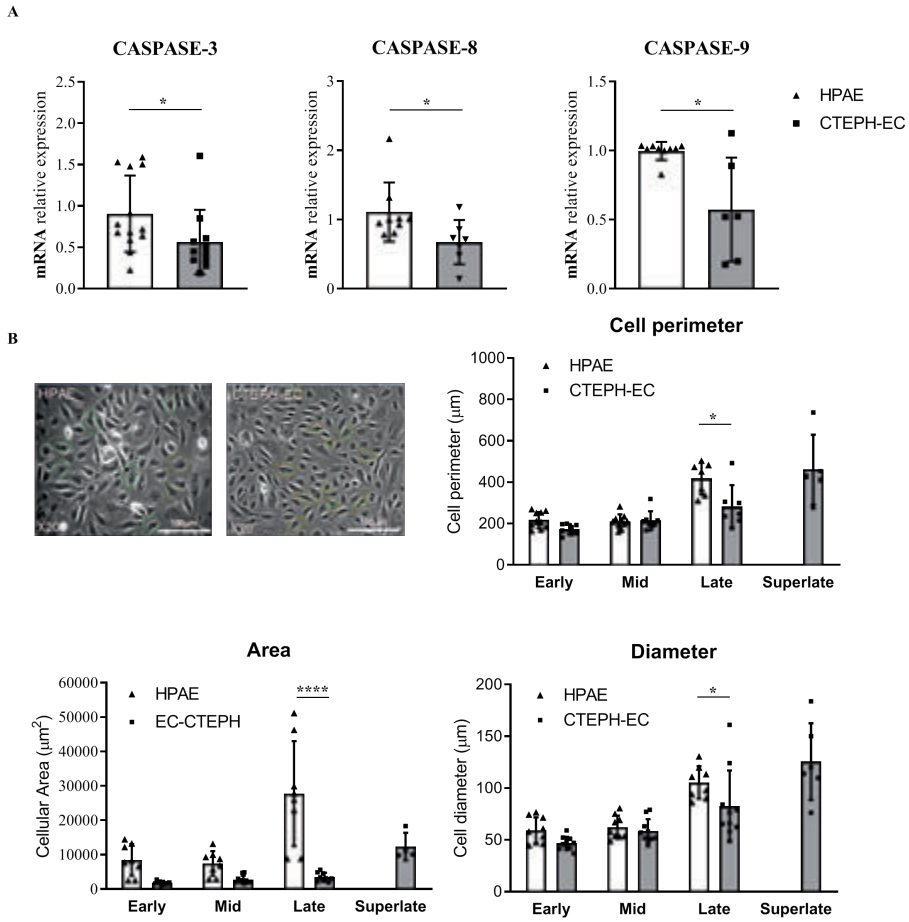

Figure 5. Apoptosis in CTEPH-EC. A) mRNA expression profile of apoptotic markers caspase-3, -8 and -9 in CTEPH-EC compared to HPAE. p<0.05*, Mann–Whitney *U* test, values expressed as mean ± SD. **B)** Cellular perimeter, area, and diameter of CTEPH-EC and HPAE measured at different passages. p<0.05*, p<0.0001****, Mann–Whitney *U* test, values expressed as mean ± SD.

CTEPH-EC showed high levels of oxidative stress

Confocal microscopy demonstrated a significant increase in the amount of mitochondrial superoxide in CTEPH-EC compared to HPAE (**Figure 8A**). Direct measurement of total ROS levels showed no difference between the two cell lines (**Figure 8A**). Oxyblot assay for immunodetection of carbonyl groups showed a significant upregulation of oxidized proteins in CTEPH-EC compared to HPAE (**Figure 8B**). Increased levels of oxidized proteins were also seen in serum of CTEPH patients compared to healthy controls (**Figure 8B**). Superoxide dismutase 2 (SOD2) expression at both mRNA and protein level was significantly reduced in CTEPH-EC compared to HPAE (**Figure 8C**). Superoxide dismutase 1 (SOD1) expression levels showed no significant difference between the two groups (**Figure 8C**). Additionally, levels of 8-hydroxyguanosine (8-OHdG), a biomarker of DNA damage were abundant in PEA samples (**Figure 8D**).

CTEPH-EC correlation with clinical data

Patient characteristics are summarized in **Table 1**. CTEPH patients studied had a mean age of 62.5±6.5 years old and gender matched. Most of the patients were in functional class (FC) III (71%), 21% in FC II and 7% in FC I (**Table 1**). Significant dysfunctional characteristics found in CTEPH-EC were related with clinical risk parameters. Significantly, higher expression levels of VCAM-1 in CTEPH-EC were associated with higher PVR (**Figure 9A**). Lower CTEPH-EC eNOS mRNA levels were observed in patients classified in worst FC (**Figure 9B**).

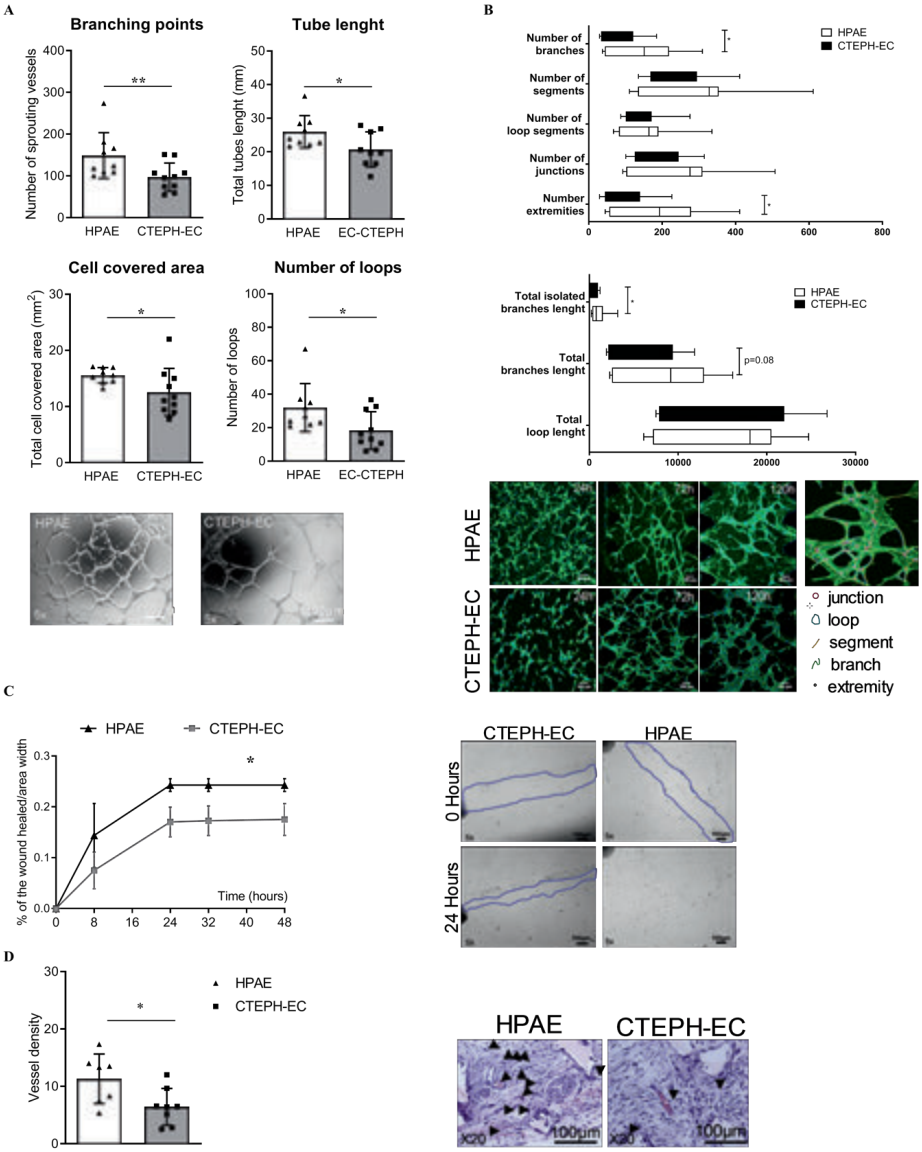

Figure 6. Functional characterisation of CTEPH-EC. A) *In vitro* angiogenic potential of quantified by the number of branching points, tube length, cell covered area and number of loops. Pictures of spontaneous tube formation in HPAE and CTEPH-EC, p<0.05*, p<0.01**. **B)** Quantification of cell connections and geometrical features measured at 24, 72 and 120 hrs and representative pictures of HPAE and CTEPH-EC. p<0.05*. Scale bar=100µm **C)** Migration of CTEPH-EC and HPAE in the wound healing assay. Wound closure is measured at 8, 24, 32 and 48 hrs and plotted as the percentage of closure over the average area of width. Pictures of wound closure at 0 hours and 24 hrs in HPAE and CTEPH-EC, p<0.01**. **D)** *In vivo* angiogenic potential of CTEPH-EC- and HPAE-embedded sponge pellets quantified by the number of vessels in the sponge sections. Picture of vessels in sponges embedded with HPAE and CTEPH-EC. p<0.02*. Test used: Mann–Whitney *U* test (all). All values expressed as mean ± SD.

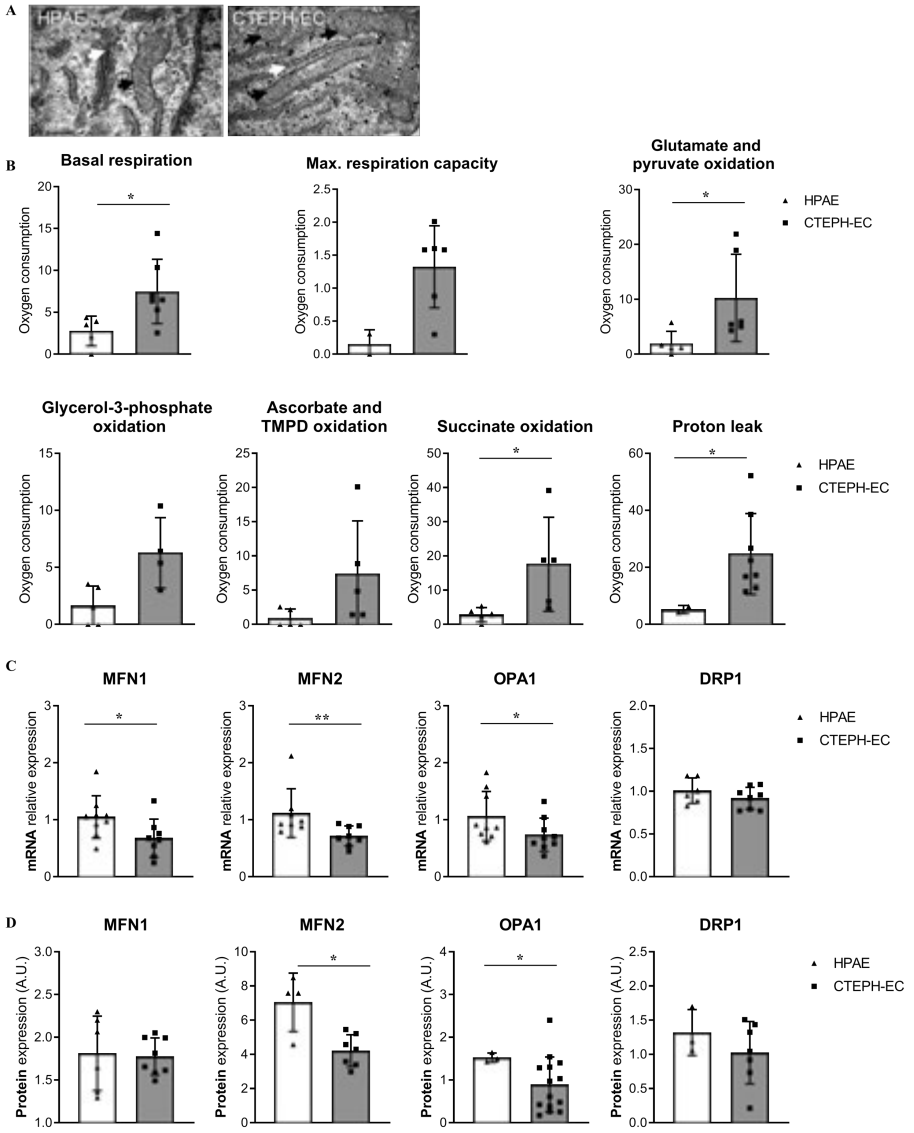

Figure 7. Mitochondria in CTEPH-EC. **A)** Visualization of the mitochondrial structure of cultured CTEPH-EC and HPAE by electron microscopy. **B)** Oxygen consumption was measured in CTEPH-EC and HPAE by high-resolution respirometry. Contribution of the individual complexes of the mitochondrial respiratory chain to total cellular respiration was determined by the use of substrates or inhibitors of specific mitochondrial respiratory chain complexes. At last, proton leak was measured in CTEPH-EC and HPAE. p<0.05*, Mann–Whitney *U* test, values expressed as mean ± SD. **C-D)** mRNA levels and protein expression of fusion (MFN1, MFN2, and OPA1) and fission (DRP1) related modulators in CTEPH-EC and HPAE. CTEPH-EC, p<0.05*, p<0.01**, Mann–Whitney *U* test, values expressed as mean ± SD.

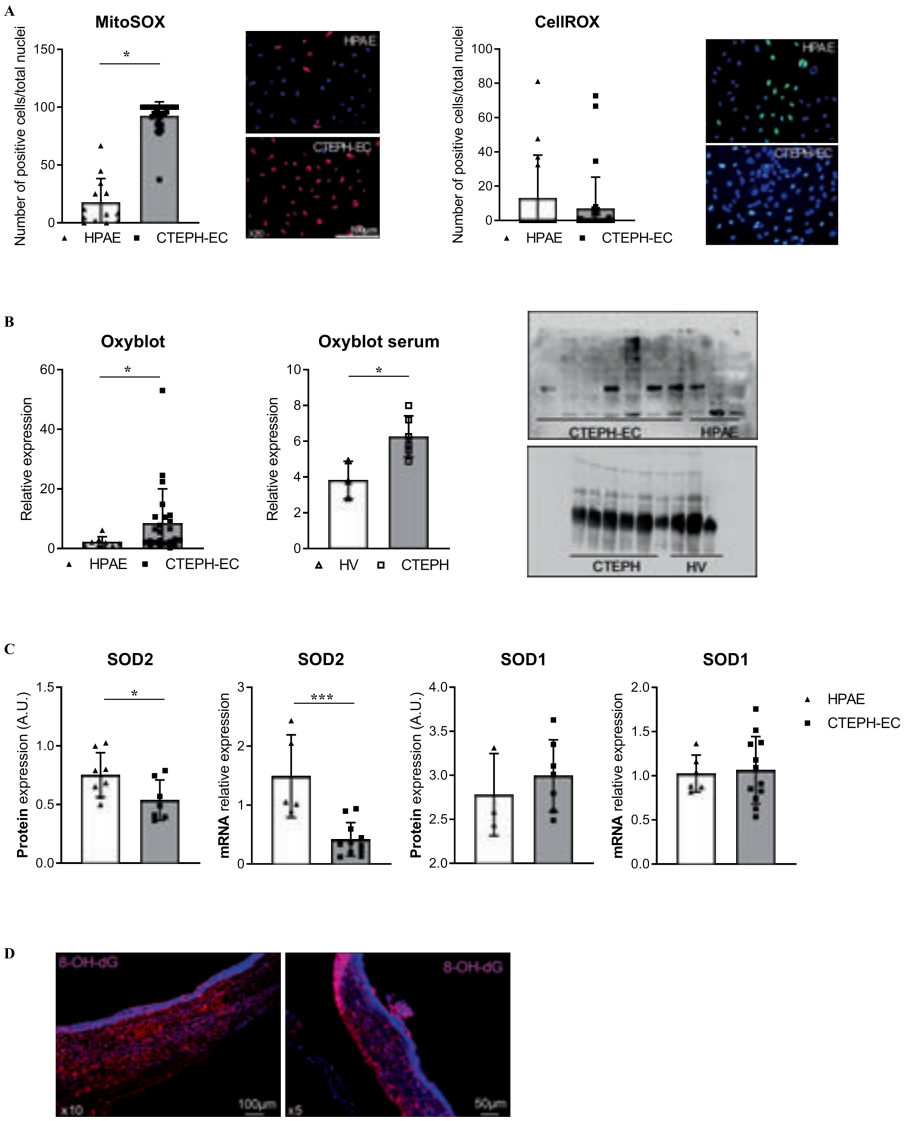

Figure 8. Oxidative stress in CTEPH-EC. A) ROS levels in CTEPH-EC and HPAE were visualized by fluorogenic dye MitoSOX (red) and CellROX (green). p<0.003**, Mann–Whitney *U* test, values expressed as mean ± SD. **B)** Oxidation status of proteins in CTEPH-EC, HPAE, patient serum and serum from healthy volunteers. p<0.05*, Mann–Whitney *U* test, values expressed as mean ± SD. **C)** mRNA and protein levels of SOD1 and SOD2 in CTEPH-EC and HPAE. p<0.05*, p<0.001***, Mann–Whitney *U* test, values expressed as mean ± SD. **D)** Staining of DNA damage induced by oxidative stress in PEA specimen by the use of 8-OHdG. Scale bar=50μm.

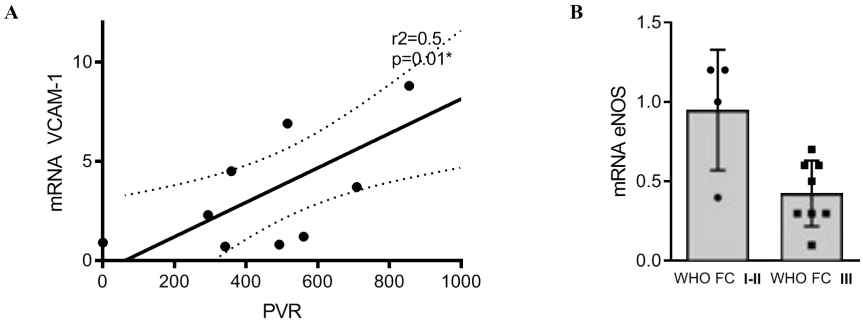

Figure 9. Correlation with clinical risk. A) Relationship between VCAM-1 mRNA levels (CTEPH-EC, n=10) and pulmonary vascular resistance (PVR). Spearman rank correlation test. **B)** Relationship between eNOS mRNA levels (CTEPH-EC, n=12) and World Health Organization functional class (WHO FC). p=0.04, Mann–Whitney *U* test, values expressed as mean ± SD

Table 1. Clinical characteristics, lung function and laboratory measurements

Characteristics	Data
Age, years	62.5±6.5
Male sex, n (%)	8 (53.3%)
BMI, kg/m^2	27.2±3.7
mPAP, mmHg	38.3±6.8
PVR, din·s·cm^{-5}	576.1±217.5
PAOP, mmHg	9.3±4.3
BNP, pg/ml	137.2 ± 280.7
CI, L/min/m^2	2.2 ± 0.4
RAP, mmHg	6.7 ± 4.3
SvO_2, %	6.2 ± 6.0
WHO FC, n (%)	
I	1 (7.1%)
II	3 (21.4%)
III	10 (71.4%)
6MWT, m	434.0 ± 81.7
Survival, years	3.0 ± 3.0

Definition of abbreviations: Body mass index (BMI), Mean pulmonary arterial pressure (mPAP), pulmonary vascular resistance (PVR), Pulmonary artery occluded pressure (PAOP), Brain natriuretic peptide (BNP), Cardiac Index (CI), Right Atrial Pressure (RAP), Pulmonary arterial oxygen saturation ($SV0_2$), World Health Organization functional class (WHO-FC) and 6-minute walk distance (6MWD, in meters). CTEPH (n=14), values expressed as mean ± SD

DISCUSSION

In this study, we isolated ECs from material collected at PEA in patients with CTEPH and, consequently, validated them as an attractive *in vitro* model of endothelial pathology in CTEPH.

Isolation and characterization of CTEPH-EC. CTEPH-EC showed typical cobblestone morphology and stained positive for a wide range of endothelial markers and negative for muscular or hematopoietic markers. Homeobox-containing genes (HOX) are well conserved among mammalian species and good predictors of EC identity[20]. Toshner *et al* identified a differential expression of certain HOX genes between lung microvascular ECs and pulmonary artery macrovascular ECs[20]. Our results indicate that CTEPH-EC are positive for the macrovascular genic pattern excluding the possibility of a peripheral circulation origin.

It has been shown, that ECs derived from patients with PAH (PAH-EC) have an hyperproliferative and an apoptosis-resistant phenotype contributing to the progression of the disease[10,11,21]. In this study, we showed that CTEPH-EC grew at a consistently faster rate and presented higher number of Ki-67^{+} cells when compared to healthy HPAE. This result is in line with a recent publication by Naito *et al*[22] in which they showed greater proliferative potential of CTEPH-EC. In this study, we expand this observation by demonstrating that CTEPH-EC had a significantly higher clonogenic potential and could survive for many more cell culture passages without losing their proliferative potential and phenotype. As reported for PAH-EC, CTEPH-EC also presented a significant decrease in apoptotic caspase related genes compared to HPAE cells. However, the expression of survival factors such as BCL2 or tumorigenic-associated genes p53 and p21 did not differ between patient and control cells. Additionally, CTEPH-EC maintained a smaller cell size consistent with a less differentiated phenotype. Altogether, these results strongly support that CTEPH-EC present a more viable, proliferative phenotype compared to pulmonary ECs from healthy subjects.

CTEPH-EC overexpression of adhesion molecules. CTEPH-EC showed a significant increase in expression of adhesion molecules compared to HPAE cells. Several studies have shown the role of CD31 in modulating apoptosis and cell growth under stress conditions[23]. Cheung *et al*[24] showed that in response to apoptotic stimuli, CD31 engages a pro-survival pathway that in some cancers influences tumour immuno-resistance. Tsuneki *et al*[25] showed that CD44 could regulate endothelial proliferation and apoptosis by modulating the expression of CD31 and

VE-cadherin. Our results show an increased expression of several adhesion molecules in CTEPH-EC and suggest the possibility that this increase could be related to pro-survival characteristics in CTEPH-EC. Indeed, Ataam *et al* has recently suggested the important role of ICAM-1 overexpression in regulating abnormal EC growth via phosphorylation of several downstream effectors such as SRC, p38, ERK[26]. Targeting adhesion molecules as early indicators of endothelial dysfunction could represent a novel and attractive strategy to prevent and treat patients with CTEPH.

CTEPH-EC function. Functionally, CTEPH-EC had a reduced capacity to form tube-like structures and reduced migration in response to wounding. In agreement with this, studies reported an impairment of angiogenesis in CTEPH patients. Alias *et al*[27] showed a downregulation of mRNA levels of VEGFR2 in PEA specimens. Also, Zabini *et al*[28] reported that homogenized PEA specimens contained several cytokines that inhibit angiogenesis. We comprehensively assessed angiogenesis, demonstrating that the formation of tubular structures by CTEPH-EC is functionally impaired both *in vitro*, in 2D and 3D cultures, and *in vivo*, using an established mouse model of angiogenesis. CD31 overexpression has also been shown to inhibit migration of ECs through a PECAM-1/γ-catenin/desmoplakin/vimentin mechanism[23]. Overexpression of adhesion molecules in CTEPH-EC could significantly affect their angiogenic potential, migration and contribute to the thrombogenic response and vessel wall remodelling.

Mitochondrial dysfunction in CTEPH-EC. Endothelial metabolism is closely linked to EC function [29]. Accordingly, a better understanding of the metabolic changes in CTEPH-EC is a crucial step in resolving CTEPH pathogenesis. It has been shown that PAH-EC have an altered mitochondrial-metabolic phenotype that includes a metabolic shift towards glycolysis and an increased mitochondrial fragmentation fusion/fission imbalance[30]. In PAH, recent studies claim that compounds that stimulate glucose oxidation and TCA cycle could potentially be re-purposed for treating PAH[31]. The question is whether such molecules could also be applied to CTEPH patients. Our results show that fusion regulatory dynamin-related GTPases MFN1, MFN2 and OPA1 were downregulated in CTEPH-EC compared to control cells. However, unlike in PAH-EC, fission regulatory protein DRP1 was not differentially expressed between CTEPH-EC and control cells. Downregulation of MFN2 in CTEPH-EC indicates mitochondrial unbalance and damage. In a mouse model of PAH, overexpression of MFN2 showed significant reversion

of the disease and improved hemodynamics[32]. Augmenting fusion proteins such as MFN2 could have a therapeutic benefit in CTEPH.

On the other hand, our electron microscopy results showed abnormal mitochondria with irregular inner membrane and cristae in CTEPH-EC. Cristae shape determines the assembly and stability of respiratory chain super-complexes, impacting mitochondrial respiratory efficiency[33]. In our study, CTEPH-EC displayed increased mitochondrial respiration activity in all the respiratory chain complexes. Increased oxygen consumption could be due to a rise of mitochondrial mass as a compensatory mechanism or due to proton leakage. As we did not find an increase in the number of mitochondria, we imply the observed uncoupling leakage as the main causative factor for the oxidative alterations in CTEPH-EC. In agreement with these results we confirmed the presence of higher levels of mitochondrial reactive oxygen species (mROS) production in CTEPH-EC. Taken together, our results show that CTEPH-EC mitochondria are dysfunctional with a fusion/fission imbalance, an increased mitochondrial respiration and uncoupling leakage. Such mitochondrial dysfunction is significantly different from the imbalance previously reported in PAH-EC[30]. Further work is required to unravel the insights of such metabolic dysfunction and its implications in the pathophysiology of CTEPH.

Oxidative stress. Higher levels of protein carbonyl groups, an indicator for the oxidative status of proteins, were observed in CTEPH-EC in comparison to control ECs. Levels of 8-OHdG, a biomarker of DNA damage induced by oxidative stress, were also abundant in PEA samples. Oxidative stress is caused by elevated ROS production or reduced cellular detoxification by antioxidants[34]. Superoxide dismutase mitochondrial antioxidant enzyme (SOD2) is the first line of defence against superoxide accumulation and cell death[34,35]. In PAH levels of SOD2 are downregulated causing ionic dysregulation and downstream pulmonary vasoconstriction[36]. Sato *et al*[37] were the first to publish on SOD2 deficiency leading to spontaneous development of PH. Our results show that SOD2 mRNA and protein levels are also significantly downregulated in CTEPH-EC. Archer *et al*[38] have shown that administration of a SOD-mimetic metalloporphyrin was able to partially reverse PAH *in vivo*. Epigenetic attenuation of SOD2 could be one of the key reasons behind CTEPH-EC dysfunctionalities. Additionally, ROS production is closely linked to NO availability [39]. Decreased eNOS activity has also been associated with mitochondrial impairment and dysregulated angiogenesis in an animal model of PH[40]. In agreement with these findings, our study showed that increased levels of mROS in

CTEPH-EC were accompanied by a significant reduction in eNOS and caveolin-1 expression. Understanding the underlying signalling in oxidative/nitrative stress-induced pathways in CTEPH, merits attention as potential novel therapeutic approaches for the prevention and treatment of CTEPH[41].

Correlation with clinical risk. Significantly, eNOS and VCAM-1 mRNA levels were related with clinical risk parameters. Higher expression levels of VCAM-1 were observed in patients with higher levels of PVR and lower CTEPH-EC eNOS mRNA levels were associated with patients classified in worst WHO-FC. eNOS and VCAM-1 patient levels could become valuable predictive and diagnostic tools in CTEPH.

CONCLUSION

Our study forms the largest comprehensive investigation of isolated CTEPH-EC. Our results show that CTEPH-EC isolated from the vessel wall accessible to surgery have an abnormal phenotype and function. CTEPH-EC present hyperproliferation, reduced angiogenesis, increased adhesion molecule expression, mitochondrial dysfunction, eNOS uncoupling and unbalanced oxidative stress/antioxidant production. Significantly, some of these dysfunctional factors were associated with parameters for disease severity such as PVR and WHO-FC. Overall, isolation of CTEPH-EC represents an attractive *in vitro* model to study the mechanisms behind endothelial dysfunction in CTEPH. In this study, we have identified several novel molecular pathways likely to influence thrombus stabilization, vessel wall remodelling and development of CTEPH. Modulation of oxidative/nitrative stress, antioxidant production, mitochondrial homeostasis and adhesion molecules deserves further study to underpin disruptive signalling molecules as potential new targets and biomarkers in CTEPH.

LIMITATIONS

This study has limitations. The use of primary cell cultures involves involuntary selection of cells with the highest growth potential. To minimize this limitation, all CTEPH-EC were isolated strictly following the same protocol and all experiments (unless otherwise stated) were carried out in early passage cells. In this study, we confirmed that CTEPH-EC derived from material collected at PEA are dysfunctional which may contribute to lack of thrombus resolution and enhance distal vascular disease. However, to date, it is not possible to conclude that functional differences found in CTEPH-EC are a primary cause or a secondary

consequence to the hemodynamic perturbations caused by the pulmonary vascular obstruction. Further studies would need to be designed to answer this specific subject.

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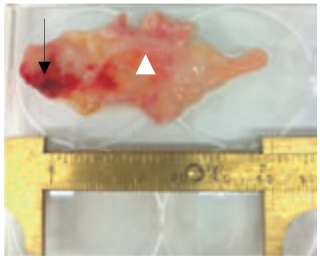
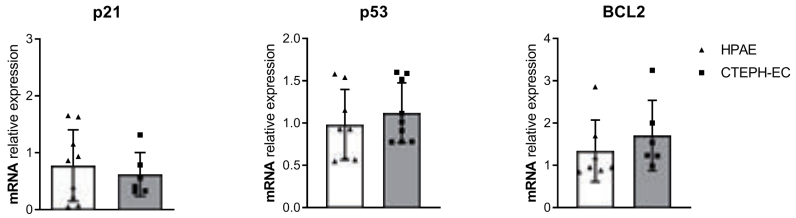
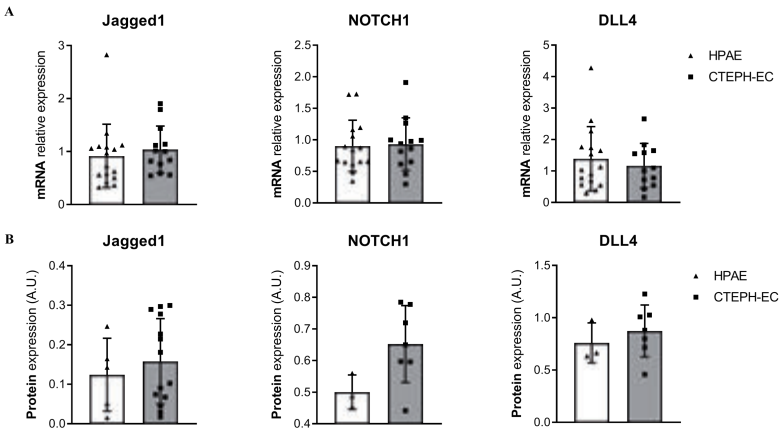
SUPPLEMENTAL FIGURES



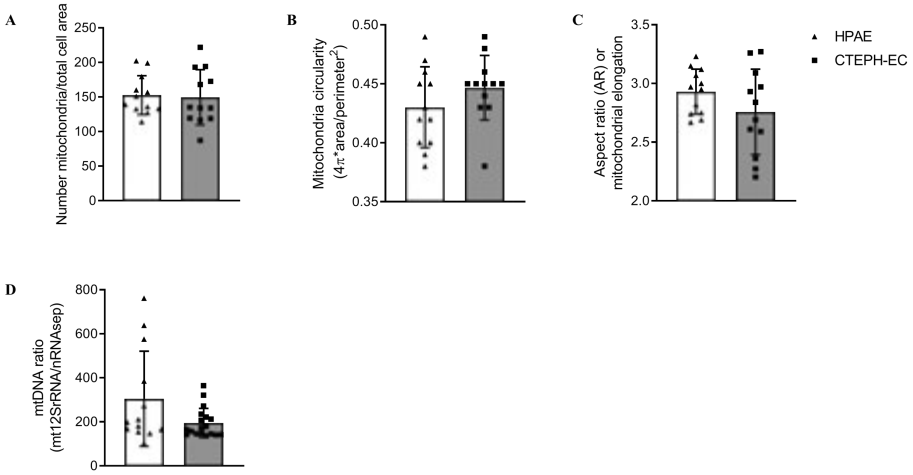
Supplementary Figure 1. Specimens collected during pulmonary endarterectomy (PEA) from a patient with CTEPH. The presence of a thrombus is indicated by the black arrow and the tissue used for isolation of pulmonary artery ECs (cell culture) is indicated by the white arrow head.



Supplementary Figure 2. **Apoptosis in CTEPH-EC.** mRNA expression profile of apoptotic related markers p21, p53 and BCL2 in CTEPH-EC and HPAE. p>0.05, Mann–Whitney *U* test, values expressed as mean ± SD.



Supplementary Figure 3. Notch signaling pathway in CTEPH-EC. A-B) mRNA and protein expression of Notch1 and its ligands DLL4 and Jagged1 in CTEPH-EC and HPAE. CTEPH-EC. p>0.05, Mann–Whitney *U* test, values expressed as mean ± SD.



Supplementary Figure 4. Mitochondria in CTEPH-EC. A-C) The number of mitochondria over total cell area, mitochondrial circularity, and mitochondrial elongation in CTEPH-EC and HPAE. p>0.05, Mann–Whitney *U* test, values expressed as mean ± SD. **D)** mt12SrRNA gene/nRNAseP nuclear gene ratio in CTEPH-EC and HPAE. p>0.05, Mann–Whitney *U* test, values expressed as mean ± SD

SUPPLEMENTAL TABLES

Supplementary Table 1. Antibodies

Flow cytometry antibodies			
Name	**Fluorochrome**	**Company**	**cat number**
CD144	PE	BD Pharmingen	560410
CD34	PECy7	eBioscience	25-0349-42
CD146	FITC	BD Pharmingen	560846
CD62	APC	BD Pharmingen	551144
KDR, VEGFR2	PerCP/Cy5.5	affymetrix (eBioscience)	45-0459
CD45	FITC	BD Pharmingen	345808
CD133	PE	BD Pharmingen	555473
CD42b	alexa fluor	Biolegend	135117
C-Kit, CD117	brilliant Violet 421TM	Biolegend	313215
CD56	FITC	Abcam	ab8211
SMA	FITC	BD Pharmingen	555445
CD31	FITC	Biolegend	323203
CD105	FITC	Sigma	L9006
UEA-1	FITC	BD Pharmingen	561712

Immunofluorescence			
Name	**Host**	**Company**	**cat number**
a-SMA	mouse	Dako	M0851
vwf	rabbit	abcam	ab6994
CD31	mouse	Dako	M0823
8-hydroxiguanosine (8-OH-dG)	rabbit	Bioss antibodies	BS-1278R
UEA-1 Ulex	Ulex europaeus	Sigma	L9006
vwf	rabbit	abcam	ab6994
ve-cad (CD144)	mouse	BD Pharmingen	555661
eNOS	mouse	abcam	ab76198
Calponin	mouse	Dako	M3556
Ki-67	mouse	Leika	ack02
Western Blot			
Name	**Host**	**Company**	**cat number**
CD31	mouse	Dako	M0823
VWF	rabbit	abcam	ab6994
CAV1	mouse	santa cruz biotechnology	sc-53564
eNOS	mouse	abcam	ab76198
MFN1	mouse	santa cruz biotechnology	sc-166644
MFN2	mouse	abcam	ab56889
OPA1	mouse	bd bioscience	bd612606
DRP1	mouse	santa cruz biotechnology	sc271583
SOD1	rabbit	SAB Signalway Antibody	32058
SOD2	rabbit	SAB Signalway Antibody	32265
Jagged1	rabbit	abcam	ab7771
NOTCH1	mouse	santa cruz biotechnology	sc-373891
Dll4	rabbit	abcam	ab176876

Supplementary Table 2. Primer sequences

Primer Name	Forward primer (5'-3')	Reverse primer (5'-3')
VE-CAD	GATGCAGACGACCCCACTGT	CCACGATCTCATACCTGGCC
CD31	AAAGTCGGACAGTGGGACGT	GGCTGGGAGAGCATTTCACA
ANG1	AATATGCCAGAACCCAAAAAG	CAATATTCACCGGAGGGATTT
Myocardin	ACAGCGCGGTTTTTTCCA	CACCGAGGAACACGGAGC
vWF	CCTTGAATCCCAGTGACCCTGA	GGTTCCGAGATGTCCTCCACAT
eNOS	GGCCCGGATCCAGTGGG	GTGGTTGCAGATGTAGGTGAACA
CAV1	CATCCCGATGGCACTCATCTG	TGCACTGAATCTCAATCAGGAAG
VEGF	GCCTTGCTGCTCTACCTCCAC	ATGATTCTGCCCTCCTCCTTCT
ANG2	TTCCTCCTGCCAGAGATGGA	TGCACAGCATTGGACACGTA
CD44	TCCAACACCTCCCAGTATGACA	GGCAGGTCTGTGACTGATGTACA
ICAM-1	CAGAGGTTGAACCCCACAGT	CCTCTGGCTTCGTCAGAATC
VCAM-1	GCAAAGGGAGCACTGGGTTGACT	GCCACATTGGGAAAGTTGCACAGG
HOXD3	CGTAAGGATTGCATCGGACT	TCCTAAGCTCGGCTGGATAA

HOXD8	TAAACCAGCTTGCTGTGTGC	GTGAGGCTATCGCTTTCCTG
HOXD9	CCTGCTCCATTGGTTCCTTA	TCAGAAACATGGGGGACATT
Cas3	AGGACTCAAATTCTGTTGCCACC	TGGAACAAATGGACCTGTTGACC
Cas8	GATTTGCTGATTACCTACCTAAACACT	TCTGAAATCTGATAGAGCATGACC
Cas9	ACACCCAGTGACATCTTTGTGT	GTCTCAACGTACCAGGAGCC
p21	CTGGAGACTCTCAGGGTCGAA	GGCGGATTAGGGCTTCCTC
p53	GAGCTGAATGAGGCCTTGGA	CTGAGTCAGGCCCTTCTGTCTT
BCL2	GGGAGGATTGTGGCCTTCTT	CAGGTACTCAGTCATCCACA
MFN1	TCTGGGCCTGATGAGGGTAA	TTCCTCCCAGGAGCTCCTAC
MFN2	CACAAGGTGAGTGAGCGTCT	TCCATGTACTCGGGCTCTGA
OPA1	TGCCTGACATTGTGTGGGAAA	TTCCGGAGAACCTGAGGTAA
DRP1	CACCCGGAGACCTTCTCATTC	CCCCATTCTTCTGCTTCCAC
SOD1	GGTGGGCCAAAGGATGAAGAG	CCACAAGCCAAACGACTTCC
SOD2	GCCCTGGAACCTCACATCAA	TCAGGTTGTTCACGTAGGCC

Supplementary Table 3. Complex I, II, III and IV specific substrates and inhibitors.

Complex	Substance	Inhibitor/Substrate
I	20µl of glutamate (0.5M)	substrate
I	8µl of malate (0.5M)	substrate
I	40µl of ADP (500mM)	substrate
I	40µl of pyruvate (250mM)	substrate
I	1µl of rotenone (0.5mM)	inhibitor
II	20µl of succinate (1M)	substrate
II	10µl of malonate (10mM)	inhibitor
III	43µl of glyceraldehyde 3-phosphate (G3P) (0.5M)	substrate
III	25µl of antimycin (0.2mM)	inhibitor
III	43µl of ascorbate (5mM)	substrate
IV	43µl of N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD) (1mM)	substrate
IV	17µl of potassium cyanide (80mM)	inhibitor

Supplementary Table 4. Flow cytometry PEA tissue

Percentage of expression	Mean ± SD
Endothelial markers	
CD34+	6.7±4.2
CD144+	4.9±2.3
CD31+	10.9±7.7
CD146+	7.6±8.1
KDR+	1.6±2.2
Endothelial and Mesenchymal markers	
CD105+	25.76±6.1
CD56+	2.5±3.2
Tie2+	24.0±5.2
Muscular marker	
α-SMA+	11.8±5.2
Progenitor markers	
CD133+	1.2±1
Leukocyte marker	
CD45+	11.1±8.2
CD14+	0,05±0,21
CD16+	0,04±0,15
Platelet marker	
CD62+	1.2±1.3
CD42b+	1.8±2.1
Morphometric measurements	
Neointima / % of total thickness	89.17±3.94
Neointima / % of total area	87.17±3.21
Number of microvessels (mm^2/tissue)	47.5±14.2

A collagen digested PEA material was analyzed by flow cytometry. Percentage of expression is given as mean ± SD. PEA material presented an enlarged neointima and the presence of microvascular vessels.

DETAILED MATERIALS AND METHODS

Subjects

Fourteen subjects with CTEPH, aged between 55-75 years (53.3% male) who underwent PEA at the Hospital Clinic of Barcelona, Spain were enrolled in the study. Patient characteristics are shown in Table 1. CTEPH was diagnosed according to current guidelines[13]. The study was conducted in accordance with the Declaration of Helsinki, approved by the institutional Committee on Human Research and all subjects gave written informed consent.

Morphometric and histological assessments

A piece of PEA resected material from the vessel wall accessible to surgery was fixed in 4% paraformaldehyde (PFA) and paraffin-embedded. Sections (5µm) were stained by hematoxylin and eosin (H/E; nuclei and cytoplasm) and orcein (elastin fibers). Staining of elastin was performed using orcein stain. The thickness of neointima (remodeled intima) was measured as the distance from lumen to the media layer. Microvessel density within the neointima was assessed as the number of blood vessels per mm^2/tissue. Distribution of endothelial markers (vWF and CD31), vascular smooth muscle cell markers (α-SMA) and 8-Hydroxyguanosine (8-OH-dG) was analyzed by immunohistochemistry. Immunohisto-fluorescence staining was performed as previously described[14]. Briefly, paraffin sections were deparaffinized, rehydrated. and boiled for 40 min in Vector® Antigen Unmasking Solution (Vector) using a pressure cooker. After blocking with 1% BSA in 0.1% Tween-PBS, sections were incubated overnight at 4°C with primary antibodies directed against alpha smooth muscle actin (SMA; 1:5000; Sigma), Von Willebrand factor (1:1000, Abcam), CD31 (1:1000; Santa Cruz) and 8-OHdG (1:150; Bioss antibodies). All sections were mounted with ProLong® Gold antifade reagent (Invitrogen) containing DAPI. In parallel, another piece of PEA resected material from the vessel wall accessible to surgery was enzymatically digested using collagenase type I and IV and incubated 60 min a 37°C. The digested sample was directly stained and analyzed by flow cytometry for phenotypic expression of surface markers using pre-conjugated anti-human monoclonal antibodies (mAbs), as previously described[15]. The antibodies used are listed in Table E1.

Primary cell cultures

Isolated ECs (CTEPH-EC) were obtained from fresh PEA resected specimens by mincing it into 1-2 mm pieces and culturing in 0.2% gelatin-coated plates in EBM-2 EC growth medium

(Lonza), supplemented with 10% fetal bovine serum (FBS) and EGM-2 SingleQuots (Lonza). Cell colonies appeared after 7-20 days in culture. Cells were used between passages 1 and 15 (proliferation, viability and senescence assays) and between passages 4 and 7 (for all other assays). Passages <4 are referred to as early, passages between 4 and 7 are referred to as mid, passages between 7 and 10 are referred to as late and passages >10 are referred to as super-late. All cellular experiments were performed at a cell confluency of 80-90% unless otherwise stated. Human pulmonary artery SMCs, human lung microvascular ECs (HMVEC-L) and human pulmonary artery ECs (HPAE) were purchased from Lonza and used as controls. Control lines were used at passages one to eight and maintained in a humidified atmosphere at 37ºC in 5% CO_2.

Cell characterization

Cells were directly analyzed by flow cytometry for phenotypic expression of surface markers using pre-conjugated mAbs, as previously described[15]. The anti-human mAbs used included endothelial markers (CD144, KDR, CD34, CD31, CD105, UEA-1 and CD146), hematopoietic lineage markers (CD45, CD14, CD16, CD56, CD62, CD133 and c-Kit), vascular smooth muscle marker α-SMA and annexin V apoptotic marker. Immunofluorescence analysis for cell phenotype was performed as previously described[19] using antibodies against endothelial markers (CD31, UEA-1, vWF, VE-CAD and eNOS) and muscular markers (α-SMA and calponin). The antibodies used are listed in Supplementary Table 1. An antibody against Ki-67 (Novocastra) was used to measure cell proliferation.

RNA Isolation and quantitative Real Time PCR

Total RNA was extracted from 80-90% confluent cultures using 1ml of TRIsure reagent (Bioline) according to the manufacturer instructions. Following reverse transcription (high capacity cDNA RT kit, Applied Biosystems), quantitative real-time PCR experiments were performed in the presence of fluorescent dye (power SYBR Green, Applied Biosystems) with a ViiA 7 Real-Time PCR System (Applied Biosystems). cDNA copy numbers were normalized against genomic DNA level of endogenous β-actin and analyzed by the 2-ΔΔCt method. All primers were delivered by IDT and primer sequences are listed in Supplementary Table 2.

Western blotting

Protein was isolated from cells at 80-90% confluency. Protein was isolated using RIPA lysis and extraction buffer (Pierce) supplemented with Halt protease/phosphatase inhibitor

cocktail (ThermoFisher Scientific). Protein concentrations were determined using BCA protein assay kit (Pierce) following manufacturer's instructions. Samples were prepared to load 15-25 µg of protein into wells of commercial NuPAGE™ 4-12% Bis-Tris Gels (Thermo Fisher) alongside Kaleidoscope™ Precision Plus Protein™ Standard (BioRad). As loading buffer, NuPAGE® LDS Sample Buffer 4X (Thermo Fisher) was used at 1X; and the NuPAGE® MES SDS Running Buffer 20X (Life Technologies) diluted to 1X was used as running buffer. Samples were heated at 70ºC for 10 min before loading into the gel for an electrophoresis duration of about 50 min at 200V followed by transfer onto nitrocellulose membrane using the iBlot® Gel Transfer Stacks Nitrocellulose, Regular Kit (Thermo Fisher) and iBlot™ Gel Transfer Device, (Invitrogen), following manufacturer's guidelines. After the transfer process, the membrane was blocked for 1 hour in 1X blocking solution using Casein Blocking Solution 10X (Sigma). Membranes were incubated overnight at 4ºC under rotation in 0.5X Casein Blocking Solution with primary antibodies following the manufacturer recommendations. Antibodies used are listed in Supplementary Table 1. The intensity of the individual bands was quantified using Image Lab (Bio-Rad). All results are shown as relative expression to β-actin protein levels.

Cell growth kinetics

CTEPH-EC and HPAE were plated in triplicate at a concentration of $3x10^4$ cells/ml. At 80% confluence cells were dissociated from the plate by trypsinization and counted. Cells were re-plated in triplicate at the same concentration and passaged until no growth was observed. Proliferative capacity was assessed by quantifying the fold cell expansion/day as number of final cells divided by the number of seeded cells/days of culture.

Cell viability

The viability potential of cells through different passages, was determined using Vybrant® MTT Cell Proliferation Assay Kit, (Thermo Fisher). Cells at different passages were plated at a density of $2x10^4$ cells per well on a 96-well microtiter plate in EGM-2 medium in a final volume of 100µl. Two wells of 100µl of EGM-2 medium without cells were used as blanks. WST-1/ECS solution was added at 10µl per well, incubated for 4 hours at 37ºC and quantified using multiwall spectrophotometer to measure absorbance of the dye solution at 570 nm.

Single Cell clonogenic assay

Single CTEPH-EC and HPAE were plated in a 96-well plate and cultured as previously described[16] for 14 days changing media every 4 days. The number of cells per well was

counted by visual inspection and classified into four different categories: 2–50 cells/well, 50–500 cells/well, 100–500 cells/well, >500 cells/well.

Cell growth and proliferation assay using xCELLigence

Experiments were carried out using the xCELLigence RTCA DP instrument (Roche Diagnostics) in a humidified incubator at 37ºC and 5% CO_2. 100 mL of cell-free growth medium (10% FBS) was added to the wells and the background impedance for each well was measured. Cells were seeded in parallel into 0.2 % gelatin coated wells at 5,000 cells/well in 150ul medium/well. After leaving the plates at RT for 30 min to allow cell attachment, in accordance with the manufacturer's guidelines, they were loaded into the RTCA DP device in the incubator. Impedance value of each well was monitored by the xCELLigence system and expressed as a Cell Index value (CI). The CI represents the measure of cellular adhesion across each individual well. In the absence of living cells the CI values will be close to zero. After cellular attachment onto the electrode, the measured signal correlates linearly with cell number throughout the experiment[17]. Cells were incubated for 5 days in EGM-2 growth medium (10% FBS) and CI was monitored every 5-15 min.

Cell Morphology

Cellular circumference, area and diameter were measured using ImagePro Plus image analysis software in triplicates fields of 5-10 cells/picture (20x magnification).

Tube formation assay

10µL of Matrigel (BD Biosciences) was added to each well of an ibiTreat µ-Slide Angiogenesis, (Ibidi) and allowed to polymerize for a minimum of 30 min at 37°C. EC lines were resuspended in EC medium and seeded in each well at a concentration of 1×10^4 cells/well in a 50µl total volume. Cells were monitored to determine the formation of tube-like structures and pictures (5x) were taken at baseline and at 16h. HPAE were used as a positive control–forming capillary-like structures. Number of branching points, tube lengths, cell covered area and number of loops were quantified in triplicate for CTEPH-EC and HPAE in 5 random fields.

3D microvascular networks (VN) were obtained by a microfluidic approach[18]. Microfluidic chips were fabricated in house using standard soft-lithography techniques, from a SU-8 master with micro-features using polydimethylsiloxane (PDMS)[18]. The master design included three channels for injection of a mixture of ECM-like fibrin hydrogel and cells, flanked by four channels injected with culture media. All channels in the chip were 100µm thick, allowing for

3D culture. CTEPH-EC and HPAE were injected at a seeding density of 6-9×106 cells/ml and suspended in fibrin in one of the three gel channels. Human lung fibroblasts (HLF, Lonza) were suspended in fibrin and injected in the remaining two channels. Vertical micro-pillars separated by 100µm populate the boundaries of each gel channel with the corresponding media. This configuration allows surface tension effects during the filling of cell-laden hydrogels and paracrine interactions between endothelial cells and flanking HLF[18]. In such a culture system, endothelial cells self-assemble into VN through a vasculogenesis process. Thus, endothelial cells form vacuoles and establish connections as early as few hours after the seeding. Further maturation of microvascular structures with tubulogenesis and lumen formation usually requires more than 48 hours of cultures. These structures are stable up to one week[18]. For visualization of these structures, cells were fixed with 4% PFA and stained using standard immunofluorescent protocols[18]. Acquisition and visualization are done by confocal microscopy. The analyses were performed on 3D microvascular networks after 24 hours of *in vitro* culture, when network connections are fully established. Quantifications were obtained using a freely available imaging analysis tool angiogenesis analyzer, ImageJ applied on 2D maximum projected confocal stacks of fluorescent signal from phalloidin staining. These values were normalized taking into account the image size.

Wound healing assay

Cell migration was evaluated using a scratch wound assay. Twenty thousand sub-confluent EC-CTEPH and HPAE were seeded in 24-well plates and starved prior to scratching the cell monolayer with a p200 pipette tip to generate a wound. Non-adherent cells were removed by washing and normal growth medium was added for 48h. Pictures were taken at baseline and 8h, 24h, 32h and 48h. Wound closure was expressed as percentage of regrowth divided by area and width of original wound. The healing area was analyzed with Image-Pro Plus software.

Subcutaneous Sponge Implantation Assay for *in vivo* Vascularization

Male non-obese diabetic (NOD) severe immunodeficiency genetic disorder (SCID)-IL-2 gammaRnull mice aged 10–12 weeks were bred and maintained in the animal facilities of the University of Barcelona. All procedures were conducted following the European Directive 2010/63/UE and Spanish RD 53/2013 regulations related to the Guide for the Care and Use of Laboratory Animals. The study protocol was approved by the Animal Experimentation Ethics

Committee of the University of Barcelona (DAAM 10028).

Anesthetic comprised Ketamine (100mg/ml) and Medetomidine (1mg/ml), given intra-peritoneally at a single dose of 7.5ul/10 gbw and 10ul/10 gbw. Reversal of anesthesia was induced, after at least 20 minutes of unconsciousness, using Atipamezole (5mg/ml) in water for injection. This was given subcutaneously at a single dose of 2ul/10 gbw. Meloxicam was given subcutaneously after surgery (2mg/ml) at 10ul/10 gbw. Mice were anesthetized and a sterilized sponge cylinder (0.5 cm^3) (Caligen Foam) was implanted subcutaneously on each flank. Each animal had a control vehicle-impregnated sponge implanted on one flank and cell-impregnated sponge on the other flank. Each animal had a control vehicle-impregnated sponge (growth-factor-reduced [GFR]-Matrigel alone) implanted on one flank and cell-impregnated sponge (GFR-Matrigel plus CTEPH-EC or HPAE) on the other flank. Sponges were impregnated with $1x10^5$ cells cells/mL of CTEPH-EC or HPAE in complete EGM-2 medium and mixed with 250µL of GFR-M.

Mice were humanely euthanized 21 days following implantation with overdose of anesthesia by intraperitoneal single dose (100 mg/Kg. Stock solution 200 mg/ml) of sodium Pentobarbital. Confirmation of death was carried out by cervical dislocation. Sponges were fixed in 4% PFA before embedding in paraffin wax. Sections (5µm) were stained with H/E for identification of blood vessels, as described[19]. Vessel density within sponges was determined using the mean of triplicate vessel counts on each of two sections per sponge.

Electron microscopy

CTEPH-EC or HPAE were washed twice with PBS and fixed with 2.5% (w/v) glutaraldehyde in 0.1 M cacodylate buffer (Electron Microscopy Sciences) for 10 min at RT. Cells were recovered by scraping and centrifuged at 1200rpm 4°C for 4 min. Cell pellets were stored at 4°C and analyzed by the scientific and technologic center of University of Barcelona.

High resolution respirometry (OROBOROS)

Oxygraph-2k (Oroboros Instruments) was used to study cellular respiratory metabolism. This system is composed of two chambers for cell loading and two electropoles for sensing the consumption of oxygen in each chamber. DataLab software was used to calculate results based on the number of cells introduced and on the protein concentration. Calibration prior to each experiment was required following the manufacturer's instructions. $1x10^6$ CTEPH-EC or HPAE per ml were resuspended in 100µl of MiR05 medium and introduced into one of the

chambers at a final volume of 2ml. Two different assays were run in parallel testing the respiratory flux control of both HPAE and CTEPH-EC simultaneously. i) Respiratory capacity assessment: First initial monitoring of endogenous cell respiration (routine) was measured. Cells were then subjected to different exogenous compounds -0.6µl of oligomycin (0.25mM) (inhibitor of complex V) as an indicator of proton leakage, increasing concentrations of CCCP (1mM) until respiration no longer increased, indicative of maximal respiratory capacity, 0.25µl of antimycin (0,2mM) (inhibitor of complex III) was added to end the assay by completely inhibiting respiration. ii) Complex I, II, III and IV were also analyzed using specific substrates and inhibitors allowing the different complexes to be analyzed separately (see Table E4 for details). All data was recorded using DataLab software v5.1.1.9 (Oroboros Instruments). Results were expressed as median and as 25% and 75% percentile, statistical analysis was performed with GraphPad Prism 7 software.

Mitochondrial morphology and content

Immunocytochemistry was performed as previously described using confocal microscopy[42] . One cell from three different fields for each cell line was randomly selected and analyzed with ImageJ software to quantify the following parameters of mitochondrial dynamics: i) Mitochondrial content: Total number of mitochondria/total cell area; ii) Circularity (Circ): 4π.area/perimeter^2; circular mitochondria have fewer interaction sites with other mitochondria, thus, Circ=1 refers to poor mitochondrial dynamics of isolated mitochondria[42] iii) Aspect ratio (AR) or mitochondrial elongation: major/minor axis, AR = 1 indicates a perfect circle; AR increases as mitochondria elongate and become more elliptical, considered a beneficial sign of mitochondrial dynamics.

Mitochondrial content was also determined using mitotracker green (MTG) following manufacturer´s instructions. Briefly, a total of 1 ml of complete culture media containing roughly $2x10^5$ cells was prepared for different reaction procedures and incubated: (i) in the absence of any dye as control for autofluorescence, (ii) with 200nM MTG fluorophore (Molecular Probes) for 30 min. Cytometric analyses were performed using a FACScalibur cytometer (Becton Dickinson). Results were expressed as median or percentage of cells with specific fluorescence.

Detection of oxidative stress

Cellular oxidation in HPAE and CTEPH-EC was measured using cell-permeant CellROX™ Deep Green reagent (ThermoFisher Scientific) following manufacturer's instructions. 250ul/well of 5 µM CellROX® was added to cells seeded in triplicate at 80% confluence in µ-Slide 8 Well (Ibidi) and incubated for 30 min at 37°C. Cells were washed three times with HBSS/Ca/Mg buffer and fixed with 3.7% formaldehyde for 15 min before analysis using fluorescence microscopy 485/520nm. Nuclei were stained with blue-fluorescent Hoechst 33342. MitoSOX, mitochondrial Superoxide Indicator (ThermoFisher Scientific) was used to detect generation of the mitochondrial superoxide anion following manufacturer's instructions. 5mM MitoSOX™ reagent stock solution was diluted in HBSS/Ca/Mg to make a 5µM MitoSOX™ working solution. 250ul/well of 5 µM MitoSOX™ was added in triplicate in a 80% confluent µ-Slide 8 Well (Ibidi). Cells were incubated for 10 min at 37˚C, washed three times with HBSS/Ca/Mg buffer and analyzed using fluorescence microscopy 640/665 nm. Nuclei were stained with blue-fluorescent Hoechst 33342.

Oxyblot

Total oxidized protein content was measured with the Oxyblot Protein Oxidation Kit (Merck Millipore) following manufacturer's instructions**.** Briefly, 20µg of protein samples were mixed with an equal volume of 12% SDS and then incubated with an equal volume of 1X dinitrophenylhydrazine (DNPH) derivation solution at RT for 15 min before addition of neutralization solution rto terminate the reaction. . The DNPH-tagged proteins were then used for SDS-PAGE/Western blot and loaded directly onto a PVDF membrane. An anti-DNP antibody was used for detection of the DNPH-tagged proteins. The blots were developed using the SuperSignal West Dura Kit (ThermoFisher). The intensity of bands was quantified using Imagequant LAS 4000 Software and analyzed by Image J software.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 7(Graph Pad Software). Data are shown as mean ± standard deviation. Independent samples were analyzed using the unpaired Student's t-test (Mann-Whitney U test) to compare differences between two independent groups. More than two groups were compared using One-way ANOVA with Tukey´s post-hoc test or non-parametric analysis of variance Kruskal-Wallis test with a Dunn´s post-hoc multiple comparison test. The Spearman rank correlation coefficient was used as a hypothesis

test to study the dependence between two random variables. Statistical significance was assumed if a null hypothesis could be rejected at P≤ 0.05 (for a confidence interval of a = 95%).

CHAPTER 4

METABOLIC ALTERATIONS IN CARDIOPULMONARY VASCULAR DYSFUNCTION

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ABSTRACT

Cardiovascular diseases (CVD) are the leading cause of death worldwide. CVD comprise a range of diseases affecting the functionality of the heart and blood vessels, including acute myocardial infarction (AMI) and pulmonary hypertension (PH). Despite their different causative mechanisms, both AMI and PH involve narrowed or blocked blood vessels, hypoxia, and tissue infarction. The endothelium plays a pivotal role in the development of CVD. Disruption of the normal homeostasis of endothelia, alterations in the blood vessel structure, and abnormal functionality are essential factors in the onset and progression of both AMI and PH. An emerging theory proposes that pathological blood vessel responses and endothelial dysfunction develop as a result of an abnormal endothelial metabolism. It has been suggested that, in CVD, endothelial cell metabolism switches to higher glycolysis, rather than oxidative phosphorylation, as the main source of ATP, a process designated as the Warburg effect. The evidence of these alterations suggests that understanding endothelial metabolism and mitochondrial function may be central to unveiling fundamental mechanisms underlying cardiovascular pathogenesis and to identifying novel critical metabolic biomarkers and therapeutic targets. Here, we review the role of the endothelium in the regulation of vascular homeostasis and we detail key aspects of endothelial cell metabolism. We also describe recent findings concerning metabolic endothelial cell alterations in acute myocardial infarction and pulmonary hypertension, their relationship with disease pathogenesis and we discuss the future potential of pharmacological modulation of cellular metabolism in the treatment of cardiopulmonary vascular dysfunction. Although targeting endothelial cell metabolism is still in its infancy, it is a promising strategy to restore normal endothelial functions and thus forestall or revert the development of CVD in personalized multi-hit interventions at the metabolic level.

Keywords: Pulmonary hypertension – acute myocardial infarction – endothelial dysfunction – cellular metabolism – glycolysis – metabolic targets – systems biology

IMPORTANCE OF CARDIOPULMONARY DISEASES

Cardiovascular diseases (CVD) are leading cause of death in the world. In 2015, The World Health Organization (WHO) estimated that 17.7 million people died from CVD, representing 31% of all global deaths[1].Death from CVD is associated with increasing age, with 1.4 million deaths in individuals under 75 and 700,000 in individuals under 65 [2].

CVD encompass a range of diseases affecting the functions of the heart and blood vessels, driven by diverse underlying mechanisms. In this review, we will focus on acute myocardial infarction (AMI), a type of coronary artery disease, and pulmonary arterial (PAH) and chronic thromboembolic pulmonary hypertension (CTEPH). Both types of CVD share a diseased endothelium conducive to atherosclerosis and endothelial hyperproliferation, respectively, with consequent narrowing of arteries, compromised blood flow and reduced oxygen and nutrient supply to the vascular cells, eventually leading to cardiac hypertrophy and myocardial infarction.

Atherosclerosis

Atherosclerosis, or the formation of atherosclerotic plaques, underlies one of the major causes of morbidity and mortality in developed and developing nations[1]. The World Health Organization attributes an estimated 16.7 million deaths to atherosclerotic cardiovascular disease[3,4]. Atherosclerotic plaque formation and rupture is one of most common pathogenic mechanisms of coronary artery disease, stroke and peripheral artery disease[5]. Atherogenesis is the result of complex sequences of events associated with processes such as endothelial dysfunction, neovascularization, vascular proliferation, apoptosis, matrix degradation, inflammation, oxidative stress and thrombosis[6]. All these processes affect the inner lining of the artery. Eventually, arteries become thicker by virtue of an accumulation of calcium, fat deposits and inflammatory cells, leading to the formation of the atherosclerotic plaque[7]. Although the pathophysiological mechanisms of atherosclerosis are yet to be fully unveiled, increasing evidences point to a critical role played by endothelial and metabolic dysregulation involving downregulation of oxidative phosphorylation and fatty acid metabolism. A complete understanding of endothelial metabolic reprogramming underlying atherosclerosis requires further investigation and could open new avenues in the prevention and treatment of this disease[8].

Current therapies in atherosclerosis and AMI

The four major risk factors for developing atherosclerosis are hypercholesterolemia, diabetes, hypertension and cigarette smoking[9]. Recent studies have shown that an adequate control of lipoprotein levels reduces the risk of atherosclerosis events. As such, a modification in daily diet, an increase in physical activity and cessation of smoking constitute the cornerstones of any intervention aimed at the prevention and/or treatment of atherosclerosis. While the role of the other parameters is still not clear, TG (triglycerides), LDL-C (low density lipoprotein cholesterol) and HDL-C (high density lipoprotein cholesterol) remain very strong predictors of premature atherosclerosis[10]. Hypercholesterolemia alters vascular permeability, allowing the leaking of LDL cholesterol and deposition on the arterial walls. There, LDL is subject to modifications that include oxidation, enzymatic processing and aggregation, that render the lipoprotein particles proinflammatory and induce an immune response. As part of this response, monocytes are recruited to the sub-endothelial space where they differentiate into macrophages. Macrophages may also derive from pluripotent cells associated with blood vessels. Regardless of their origin, macrophages in atherosclerotic lesions actively participate in lipoprotein ingestion and accumulation giving rise to foam cells filled with cholesterol-rich lipid droplets. These processes lead to vascular modifications visible as fatty streaks, intimal thickening and ruptured plaques, causing acute coronary disease[9]. Vulnerable plaques contain monocytes, macrophages and T cells, which accounts for their instability.

The critical role played by LDL-C in atherosclerosis has prompted the development of rational strategies to counter the pathogenic effects of hypercholesterolemia. The use of statins (inhibitors of HMG-CoA reductase, the rate-controlling enzyme of the mevalonate and cholesterol synthesis pathways) as a pharmacological approach to lower cholesterol levels is one of the most widely used therapies in the treatment of atherosclerosis and acute coronary syndromes, as these drugs show consistent clinical event reductions with a very good safety profile[11]. Other clinical studies reveal that the use of ezetimibe (an inhibitor of intestinal cholesterol absorption) considerably reduces LDL-C blood levels when combined with statins[10]. An alternative approach is the administration of fibrates, a particular class of agonists of the peroxisome proliferator-activated receptor α (PPAR-α) a regulator of lipoprotein metabolism, showing a good effect in lowering TG levels as mono-therapy, even though the results are not as promising as for the statins[11].

Recently, a large multi-scale and multiethnic study was undertaken to better understand the role of genetic and environmental factors in CVD and to identify genetic variants associated to blood lipids levels[12]. By using a genome wide association study (GWAS) on Million Veterans Program (MVP), this work depicted some novel lipid associated coding variants with CVD risk or incidence. Individuals with mutations in ANGPTL4 (Angiopoietin-like4) presented a lower risk to develop diabetes mellitus (type2), those with a loss of function in PCSK9 (Proprotein convertase subtilisin/kexin type 9) showed a reduced risk of abdominal aortic aneurysm, and those treated with inhibitors of PDE3B (Phosphodiesterase 3B), presented reduced levels of triglycerides in blood[13-17]. These data highlight the complexity in humans to control blood lipid composition and the potential of a genetic approach to develop novel therapeutic agents for the prevention of cardiovascular disease.

When the combination of healthier diet, lifestyle and pharmacological treatments fail to improve the pathological and clinical conditions in atherosclerotic patients with an associated coronary disease, surgical intervention is considered the best option. Coronary artery thrombosis with complete occlusion typically leads to ST-segment elevation myocardial infarction (STEMI). Partial occlusion, or occlusion in the presence of collateral circulation, results in non-STEMI or unstable angina, an acute coronary syndrome without ST-segment elevation. Once a definitive or likely diagnosis of an acute coronary syndrome without ST-segment elevation has been made, the patient is triaged to either an invasive strategy or an ischemia-guided strategy. An invasive strategy leads to improved outcomes and is favored for the majority of patients[18].

In patients presenting with an unstable condition or with STEMI, urgent Percutaneous Coronary Intervention (PCI) is performed. An ischemia-guided strategy is chosen for patients at low risk of recurrent ischemia, especially for women. Although PCI is currently the intervention of choice for most of these patients, individual coronary anatomy and clinical features may dictate the use of a different approach, such as coronary artery bypass grafting (CABG), a surgery that reinstates cardiac blood flow[19]. In some cases, current guidelines also recommend an antiplatelet therapy combined with non-vitamin K antagonist oral anticoagulant (NOACs) therapy[20,21]. Indeed, the rates of major complications of acute myocardial infarction have declined dramatically with early reperfusion (PCI) associated with antiplatelet therapy[22]. Nevertheless, complications are still a leading cause of death and

deserve careful consideration. Several new therapeutic approaches, such as reducing inflammation, mitigating reperfusion injury or inducing myocardial regeneration, are under active investigation although, except for angiotensin converting enzyme (ACE) inhibition, have so far not proved beneficial in the acute care setting. Acute myocardial infarction continues to have a major impact on global health and its management remains a crucial challenge for scientific advancement in medicine[23].

Pulmonary hypertension

Pulmonary hypertension (PH) is a hemodynamic disease state involving multiple clinical conditions including pulmonary arterial hypertension (PAH) and chronic thromboembolic pulmonary hypertension (CTEPH)[24]. They are defined by hemodynamic parameters characterized by a mean pulmonary artery pressure ≥ 25 mmHg at rest, measured during right heart catheterization[24]. The pathophysiological consequence is a gradual obstruction of the arterial lumen leading to the development of increased resistance of the pulmonary vasculature and, ultimately, right ventricular failure[25,26]. PAH is characterized by disease-specific lesions mainly involving the smaller pulmonary arteries (< 500µm in diameter). These lesions feature thickening of both the external and medial layers and changes in the endothelial monolayer accompanied by an inflammatory infiltration and formation of complex thrombotic lesions[25]. Unlike PAH, where obstruction caused by remodeling likely occurs in the more distal pulmonary arteries, CTEPH is largely associated with prominent obstructions in the main pulmonary arteries caused by unresolved thrombi affecting the medial and intimal layers of the arteries[26,27]. Subsequently, distal pulmonary arteriopathy and microvascular disease can be triggered, indistinguishable from classic PAH[26,28]. PAH and CTEPH are both rare and progressive vascular diseases with poor prognosis if early diagnosis is not performed. Diagnosis is complicated by the lack of biomarkers and patient-specific symptoms[29]. Whereas PAH has an estimated prevalence of 15 to 50 per million population[29], the prevalence of CTEPH is not easy to estimate, due to a long asymptomatic period between the initiating event (pulmonary embolism; PE) and the overt symptomatic disease. With this caution, the prevalence of CTEPH is estimated at between 0.1% and 9.1% after diagnosis of PE[27,28]. Current therapies for PAH mainly focus on targeting endothelial dysfunction, whereas pulmonary endarterectomy (PEA) is the treatment of choice for CTEPH, with a possible curative outcome[30,31]. To date, additional research is needed to learn more about the onset

and development of PH. This review will touch the recent findings concerning the endothelium dysfunction and metabolic alterations in PAH and CTEPH, its likely relationship with the disease pathogenesis, and whether pharmacological modifications on cellular metabolism might be a potential future treatment for PH.

Current therapies in PH

PAH is the most studied condition of all PH clinical groups. Three key vasomotor pathways are the targets of the main approved PAH therapies: 1) prostacyclin, 2) endothelin-1 and 3) nitric oxide-cyclic guanosine monophosphate (cGMP). Interventions on all three pathways aim at restoring the imbalance of endothelial vasodilator and vasoconstrictors mediators. However, because they have little impact on vascular remodeling and coagulation homeostasis, they are not curative for PAH[29].

No targeted therapy is currently available for CTEPH patients and, as mentioned above, pulmonary endarterectomy (PEA) is the treatment of choice in operable patients. Features for determining patient operability are the accessibility of the thromboembolic material and patient comorbidities influencing the peri- and post-operative risk. Effective PEA results in improvement of clinical symptoms, normalization of hemodynamics and increased survival[30]. For patients who suffer from inoperable CTEPH or persistent/recurrent CTEPH, Riociguat was recently approved as the only pharmaceutical targeted treatment of CTEPH based on the findings from the CHEST study[32] and the PATENT-1 study[33]. Riociguat is a member of the family of soluble guanylate cyclase stimulators (sGC). It has a two-faceted mode of action, on the one hand by increasing the sensitivity of sGC to endogenous NO, and on the other hand by stimulating sGC activity independently of NO, resulting in restoration of the NO-sGC-cGMP (cyclic guanosine monophosphate) pathway, accompanied with vasodilation and anti-fibrotic, anti-proliferative and anti-inflammatory effects[32]. The drug significantly improves the patients' exercise capacity and pulmonary vascular resistance and possibly constitutes a first targeted therapy for CTEPH[34].

The limited benefits of existing therapies for PAH and the high risk associated with PEA for CTEPH patients have fostered recent studies that explore new therapeutic avenues. An emerging topic suggests the occurrence of potentially actionable metabolic abnormalities in pulmonary hypertension. We will now focus on recent observations on the role of EC

dysfunction and metabolism in the pathophysiology of PAH and CTEPH and the therapeutic opportunities they may provide.

ENDOTHELIAL CELLULAR ENERGY METABOLISM

The endothelium is a dynamic organ consisting of a single layer of endothelial cells (ECs) lining the entire vascular system. Independently of their anatomic location (artery, arteriole, capillary, venule, vein) all endothelial share the common function of maintenance of vessel homeostasis[35,36]. The control of vessel functions involves regulation of the blood flow, vascular tone, physical barrier, blood coagulation and the inflammatory response. A balanced production of various hormones, neurotransmitters and vasoactive factors is crucial for maintaining a homeostatic vessel function[36]. An important vasoactive factor is eNOS-derived nitric oxide (NO), that promotes vasodilatation and inhibits important events that contribute to the development of vascular remodeling diseases, such as platelet aggregation, adhesion of leukocytes and oxidative stress[37]. NO produced by the endothelium also plays an important role in mitochondrial respiration to maintain the oxygen gradient in oxygen limiting situations[38]. When the NO precursor arginine and the eNOS cofactor tetrahydrobiopterin (BH_4) are not available, eNOS fails to produce NO and may promote the formation of reactive oxygen species (ROS), causing endothelial dysfunction and leading to atherosclerosis and PH pathogenesis[37].

The importance of maintaining physiological and homeostatic EC functions is underlined by the development of major diseases like cardiovascular disease, diabetes and cancer, consequent to endothelial dysfunction[39]. The endothelium can be disrupted by EC damage or apoptosis which leads to a re-endothelialization response and in some cases to the selection of ECs with an altered phenotype[31,35]. Environmental stresses, such as oxidative stress and metabolic disturbances, are important sources of endothelial dysfunction, injury and death[35].

Glycolysis

When exposed to hypoxic conditions, mitochondrial alterations or growth factors, ECs can rapidly shift from a quiescent cellular mode to an angiogenic state accompanied with changes in their cell metabolism. Under basal conditions, ECs rely mostly (> 80%) on glycolysis for generating their cellular energy and thus leaving the circulating oxygen available for underlying oxygen-requiring tissues[38-40]. In pathological conditions with sustained suppressed

oxidative phosphorylation (OXPHOS), glycolytic flux is increased regardless of the oxygen supply as long as glucose is available (Warburg effect) promoting the development of highly proliferative vascular disorders[38,41]. This predominantly reliance on glycolysis over the use of more efficient mitochondrial oxidation is supported by the presence of fewer mitochondria when compared to cell types relying on mitochondrial respiration. Regardless the presence of sufficient oxygen to maintain mitochondrial respiration, glycolytic flux quickly converts pyruvate to lactate, followed by an increase in glucose uptake[41]. Sustained upregulation of glycolysis and suppression of OXPHOS is often accompanied with a normoxic activation of HIF1α, leading to activation/upregulation of several glycolytic enzymes and further suppressing mitochondrial respiration[38].

The glycolytic enzyme phosphofructokinase-2/fructose-2,6-biphosphatase 3 (PFKFB3) is important for maintaining glycolysis and it has been shown that PFKFB3 inactivation reduces EC proliferation[40]. PFKFB3 catalyzes the synthesis of fructose-2,6-biphosphate (Fru-2,6-P_2), an allosteric activator of phosphofructokinase 1 (PFK-1) and a potent stimulator of glycolysis. Partial reduction of the glycolytic flux by PFKFB3 inhibition was shown to be sufficient to impair EC proliferation without induction of cell death as caused by 2DG (2-deoxy-D-glucose)[42]. Another important glycolytic enzyme is the mitochondrial "gate-keeper" enzyme pyruvate dehydrogenase (PDH) that acts as a promoter of the entry of pyruvate in mitochondria[43]. PDH can be phosphorylated and inhibited through the activity of pyruvate dehydrogenase kinases (PDKs), resulting in a reduced mitochondrial contribution to energy production and concomitant promotion of aerobic glycolysis[44].

PDKs are induced by HIF1α and PDHs are additionally inhibited by altered mitochondrial Ca^{2+} signaling[38]. Upon PDH inhibition, pyruvate, rather than entering the mitochondrial respiratory chain, is converted to lactate by the enzyme lactate dehydrogenase (LDH) allowing the transformation of NADH to NAD^+, which is crucial for maintaining further glycolysis. Lactate is released to the extracellular microenvironment via monocarboxylate transporter 4 (MCT4). It can also enter the cells through monocarboxylate transporter 1 (MCT1) and used to feed the TCA cycle under low oxygen conditions, promoting the so-called "reverse Warburg effect", a process that illustrates the symbiotic relationship between lactate-producing and lactate-consuming normal and pathological cells[45], an important adaptive mechanism to continuously changing micro-environmental conditions[45,46].Moreover, the increase in lactate production

generates an extracellular acidification of the microenvironment which promotes the activity of certain metalloproteases that disrupt the extracellular matrix, which also leads to an infiltration of the vascular wall with inflammatory cells and other cell types[47].

Glycolysis is also connected to the pentose phosphate pathway (PPP), a metabolic pathway that generates NADPH and ribose-5-phosphate, essential for the biosynthesis of lipids and nucleotides. PPP plays a pivotal role in the production of NADPH moieties which provide the reducing equivalents necessary for the synthesis of fatty acids and for the scavenging of ROS to promote cell survival[48]. The altered metabolic profile of mitochondrial suppression along with increased glycolytic rates causing a dysfunctional vasculature is similar to that of other rapidly proliferating healthy and malignant cell types, in which a shift occurs from mitochondrial respiration to lactate-producing aerobic glycolysis in order to sustain their rapid growth and to block apoptosis[41,49,50].

Mitochondrial respiration

Mitochondria are considered the quintessential cellular engine since its primary function is energy production in the form of adenosine triphosphate (ATP). ATP is essential to sustain cellular bioenergetic demands, glucose being the principal carbon substrate needed to generate ATP. OXPHOS is the mitochondrial metabolic pathway that enables cells to synthesize ATP from oxidation of nutrients. For each glucose molecule that enters the glycolytic pathway, 2 pyruvate molecules are produced with a net energy of 2 ATP molecules. Subsequent pyruvate uptake into mitochondria results in 36 ATP molecules with minimal production of lactate under physiological conditions[41,50].This process is oxygen-dependent and, considering its high efficiency, it is the predominant source of energy in mammalian cells[41]. Individual cell growth is controlled by environmental nutrient availability; therefore, cells only take up nutrients for cell division when stimulated by growth factors to avoid abnormal proliferation. In addition to ATP, active cell division requires nucleotides, amino acids, and lipids. To maintain cell growth and concomitant increase in biomass, part of the glucose must be redirected to the generation of critical macromolecular precursors such as acetyl-CoA, glycolytic intermediates and ribose for the biosynthesis of, respectively, fatty acids, nonessential amino acids and nucleotides[50]. In order to promote this flow of carbon substrates towards biomass accumulation, rapidly growing cells are endowed with mechanisms that favor glycolysis over mitochondrial oxidation.

Although the major function of mitochondria is ATP production, these organelles are also important regulators of cell survival, ion homeostasis (H^+, Ca^{2+}) and cellular redox status[51]. Tight regulation of the mitochondrial ion status is of great importance in tissues with limited oxygen consumption, like the vasculature and the lung, which facilitate the diffusion of oxygen to more oxygen-requiring tissues. Disturbances in mitochondrial ion status have direct and indirect consequences on cell function, growth and survival[38]. Altered mitochondrial morphology and function prompted by factors such as NO status have been associated with vascular endothelial dysfunction and to diverse pathological conditions, including cardiovascular disorders, muscular degeneration and cancer[38,51]. Mitochondrial metabolism contributes actively to the production of reactive oxygen species (ROS). Mitochondria regulate redox signaling to and from mitochondria[51] and initiate cellular apoptosis[52]. Oxidative stress is considered a major contributor to the destruction of well-balanced homeostatic mechanisms, causing cell injury either through direct oxidation of cellular proteins, lipids, and DNA or via cell death signaling pathways[4,53].

The sensitivity of cells to glycolytic and OXPHOS inhibitors (such as2DG, and Oligomycin, respectively) can be used to help unveil the cell dependency on a specific energy-generating pathway[54]. Such studies have shown that, in spite of the importance of the glycolytic pathway, especially under hypoxic conditions, the majority of cells use a combination of OXPHOS and glycolysis as a strategy for energy production, pointing out the importance of metabolic plasticity for cell survival under shifting environments and the complexity of metabolic adaptations in disease[55].

ROS-Oxidative stress

During OXPHOS, electrons from NADH and $FADH_2$ molecules (electron donors) are transferred to electron acceptors, such as oxygen. These redox reactions are carried out by protein complexes located in the mitochondrial inner membrane, and release energy which is used to form ATP[56]. Small amounts of these electrons form prematurely mitochondria-derived reactive oxygen species (mROS), such as superoxide (O_2^-). ROS are oxygen-containing chemically reactive species which play important signaling roles to sustain fundamental cellular functions under various physiological conditions[57]. High levels of oxygen and increased mitochondrial activity leads to excessive ROS production overcoming the buffer capacity of usable antioxidant systems results in oxidative stress causing increased cell death and

endothelial dysfunction[4,38,58,59]. Mitochondria-based manganese superoxide dismutase (MnSOD, or SOD2) has an immediately anti-oxidative effect through the conversion of superoxide to the more stable and diffusible H_2O_2. After leaving mitochondria, physiological levels of hydrogen peroxide (H_2O_2) function as an important signaling system, acting on several redox sensitive targets in the cytoplasm (HIF1α) and the cell membrane (K+ channels) and also with some proliferation-related enzymes[38,60]. Several other enzyme systems can produce ROS in the vascular wall, notably NADPH oxidase, xanthine oxidase, and eNOS, a dysfunctional endothelial NO synthase in which oxygen reduction is uncoupled from NO synthesis[57,58,61,62]. These oxidases are multisubunit enzyme complexes that produce superoxide from molecular oxygen and NADPH as electron donor[63].

Imbalances in the cellular oxidative status play significant roles in the pathophysiology of vascular diseases[64]. Some cells might resort to a metabolic switch to glycolysis as a mechanism to reduce production of ROS and thus to protect themselves from mitochondrial-mediated apoptosis[44].The crucial role of oxidative stress in CVDs makes it an attractive target for therapy. However, recent studies in patients with cardiovascular symptoms showed that the use of supplementary antioxidants such as vitamins E and C had little therapeutic effect. This was mainly due to the limited specificity for ROS producing factors and to the requirement of high antioxidant doses which could worsen vascular function[65,66]. Whilst, novel therapeutic strategies propose to target more precise ROS producing sites such as the mitochondria, it is not an easy task as those are important dose-dependent signaling molecules which could have a detrimental effect on key body functions. Therefore, more detailed studies are needed to elucidate the potential therapeutic effects of antioxidant treatments[65].

THE IMPORTANCE OF THE ENDOTHELIUM IN DISEASE

Endothelial dysfunction in acute myocardial infarction

Acute myocardial infarction (AMI) is the development of myocardial necrosis caused by an unstable ischemic state. The disorder is diagnosed and assessed based on clinical evaluation, electrocardiogram (ECG), biochemical testing, invasive and noninvasive imaging, and pathological evaluation. The usual triggering event of acute myocardial infarction is the rupture or erosion of a vulnerable, lipid-laden, atherosclerotic coronary plaque. This event results in the exposure of circulating blood to highly thrombogenic core and matrix materials

in the plaque[67]. In response to various stimuli, the normal endothelium endures phenotypic changes and variations, collectively known as endothelial dysfunction, characterized by a loss of the majority of the homeostatic mechanisms present in normal healthy endothelial cells. Usually, this dysfunction is associated with upregulation of adhesion molecules, enhanced production of ROS, synthesis of pro-inflammatory factors and loss of vascular tone (**Figure 1**) [68]. Recent studies suggest that this dysfunction contributes to the progression of the atherosclerotic plaque[69]. The pioneer role played by endothelial dysfunction in vascular pathology is supported by observations that individuals without any clinical sign of atherosclerosis but with high cardiovascular risk already present an endothelial dysfunction indicated by a diminished response to some vasodilators, such as acetylcholine[69]. These findings suggest that endothelial dysfunction may precede and constitute a link between different vascular diseases and represents a good predictor of future cardiovascular events, including atherosclerotic diseases and AMI[68]. Endothelial dysfunction is considered a systemic vascular process that not only leads to plaque formation, but also determines the clinical course of atherosclerosis progression and associated coronary syndromes. Because several metabolic pathway abnormalities, such as the deregulation of the nitric oxide production and the excessive generation of ROS (**Table 1**), are associated with atherosclerosis, the identification of key metabolic mechanisms underlying such alterations should provide fresh opportunities for the development of new strategies for the treatment of endothelial cell dysfunction in atherosclerosis and related vascular pathologies[70].

Metabolic alterations and requirements in acute myocardial infarction

Atherosclerosis is characterized by the presence of an uncoupled and reduced eNOS, causing an imbalance between the production of NO, an anti-atherogenic molecule, and superoxide, a pro-atherogenic factor, thereby losing the atheroprotective function of eNOS[71]. As such, two general processes are largely responsible for the angiogenic growth observed in early atherogenesis: inflammation and oxidative stress. These metabolic alterations also affect, to a variable degree, vascular remodeling and coagulation homeostasis[29]. Beyond eNOS, a critical role in atherogenesis is also played by NADPH oxidase (NOX) enzymes, a large family of enzymes that are pivotal in the generation of ROS in the vasculature. NOX-4 is universally expressed in vascular smooth muscle cells (VSMCs), the primary components of the vascular wall and crucial determinants of vascular homeostasis and disease[72]. Its expression and

activation during the angiogenic process promotes a chain of events leading to vascular inflammation, cellular dysfunction and atherosclerosis.

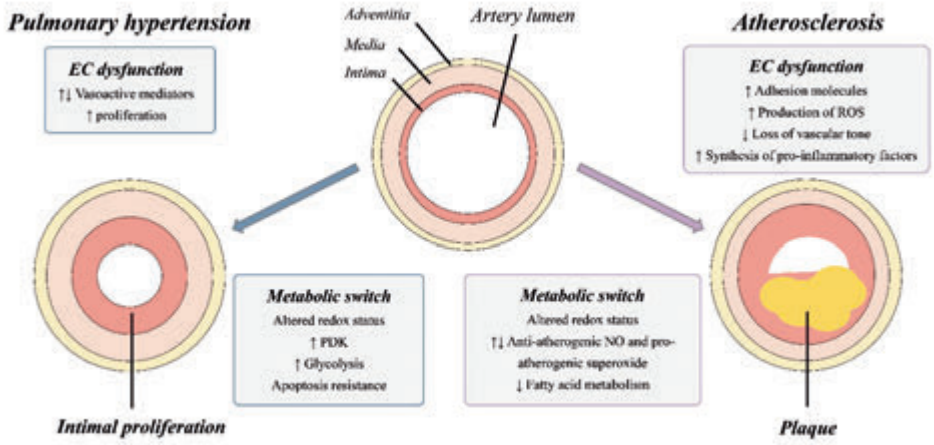

Figure 1. Graphical representation of vascular changes occurring in PH and atherosclerosis. Narrowing of the artery lumen is caused by intimal proliferation (PH) and by plaque formation (atherosclerosis) and is a result of disease specific EC dysfunction and cellular metabolic switches. EC, endothelial cell; PDK, pyruvate dehydrogenase kinases; ROS, reactive oxygen species; NO, nitric oxide; PH, in pulmonary hypertension.

Additional metabolic mechanisms may contribute to the generation of a pro-inflammatory environment leading to atherogenesis. For example, the high glycolytic mode encountered in endothelial dysfunction implies a relative reduction in available ATP as compared to cells with OXPHOS-predominant metabolism. This results in diminished intracellular adenosine levels which drives hydrolysis of S-adenosylhomocysteine (SAH) to adenosine and L-homocysteine (Hcy). Reduced SAH levels foster histone H3 lysine 4 hypomethylation and overexpression of a pro-inflammatory gene repertoire[73]. A recent study[74] identified three metabolic biomarkers, arginine and two lysophosphatidylcholines (LPC 17:0 and LPC 18:2) associated with incident myocardial infarction (MI). This study also focus on the association between these metabolites and the high-sensitivity C reactive protein (hsCRP), which is a measure for inflammation[75]. The three biomarkers correlated with each other and with the hsCRP levels, suggesting that inflammation can represent a pathway through which these biomarkers are associated with MI[76]. As a consequence of these processes, increased rates of apoptosis are evident in more advanced atherosclerotic plaques. This is a key point in the progression of atherosclerosis and consists of a programmed cell death, morphologically expressed as cellular contraction,

condensation of chromatin and disruption of the cellular membrane. All cells existing in the atherosclerotic plaque, including lymphocytes, endothelial cells, smooth muscle cells and macrophages, can undergo cellular apoptosis[77].

Future treatment options in AMI

Currently, the use of beta blockers and angiotensin-converting enzyme (ACE)-inhibitors are under study for the treatment of AMI, as well as improvements in antithrombotic therapies. Despite the advances of the last decades, a lot is unknown about the biological processes involved in cardiac development and repair for this reason there is a strong need to find new successful therapeutic targets to struggle this disease[78].

Metabolomic approaches can identify potential biomarkers with predictive value for CVD[79] and correlate metabolic profiles with risk of death or incident MI[80]. Several studies focus on the limitations of current vascular biomarkers, like hsCRPs (high sensitive C reactive proteins) and encourage the discovery and validation of novel biomarkers using emerging omics technologies. HDL cholesterol is considered strictly linked to cardiovascular diseases: low HDL cholesterol is associated to a very high cardiovascular risk while high HDL cholesterol seems to be linked to cardiovascular protection[81]. It has been described, that the key role of HDL is to promote the efflux of the cholesterol and to invert its transport from the periphery to the liver, which seems to be correlated to a low incidence of cardiovascular events, suggesting the potential use of HDL as a new biomarker for coronary heart disease[79].

The detection of plasma-derived markers which include microparticles (MPs), microvesicles and exosomes in human plasma, has triggered increasing interest for their potential as biomarkers. In particular, levels of MPs expressing CD31 or CD144 seem inversely correlated to the endothelium associated vasodilation highlighting that MPs' levels might be good indicators of vascular lesions and acute endothelial dysfunction[82]. Moreover, the complex composition of these microparticles which comprise proteins, lipids and nucleic acids represent an interesting onset for omics based analysis[83]. Recent studies have also unveiled that micro-RNAs (miRNAs) not only present relevant intracellular functions, but also show potential value as cardiovascular disease biomarkers. miRNAs also circulate within microvesicles and may contribute to forecast heart failure, early atherosclerosis and plaque vulnerability by targeting vascular and cardiac cells[84].

Metabolomic studies using general population-based cohorts have recently been performed using LC-MS/MS-based lipidomics and NMR-based approaches to identify species associated with incident CVD with a potential link to systemic inflammation and in particular, pro-inflammatory lipid metabolites have acquired great interest in the cardiovascular disease frame[79]. Molecular lipid profiling by mass spectrometry and nuclear magnetic resonance spectroscopy, as proteomic identification and quantification of small metabolites, can improve the individual cardiovascular risk prediction[85]. The identification of potential metabolic targets for novel therapeutic approaches for acute myocardial infarction is therefore an interesting approach that should be investigated in earnest in the near future.

Endothelial dysfunction in pulmonary hypertension

The pulmonary vascular barrier consists of three cellular layers: an external layer, the adventitia containing fibroblasts, a medial layer mainly consisting of smooth muscle cells (SMC) lined by an elastic membrane and an internal intima composed of a single layer of endothelial cells in direct contact with the blood circulation[86]. Upon abnormal activation, the normally quiescent endothelium loses its homeostatic function, leading to a disorganization of the three-layer structure of the vascular wall as a key element in the development of pathological lesions[87,88]. Several endothelium activation stimuli such as ROS, shear stress and inflammation are known to stimulate the endothelium, causing changes in its proliferation status and production of vasoactive mediators and growth factors. In PH, it has been shown that there is an imbalance between vasodilators, such as nitric oxide (NO) and prostacyclin (PG), and vasoconstrictors, such as endothelin-1 (ET-1) and thromboxane, resulting in the disruption of basal pulmonary vascular tone, vascular injury repair and growth[87]. In addition to changes in vasoactive mediators, it is hypothesized that initial endothelial damage induces a widespread endothelial cell death cascade leading to an apoptosis-resistant population, from which rapidly proliferating cells regenerate the vascular lining (**Figure 1**) [89]. Additionally, endothelium injury also causes exposure of the medial and adventitial layer to growth factors, inducing the proliferation of fibroblasts and smooth muscle cells[87]. Furthermore, disease-related alterations in the function and expression of specific ion channels in pulmonary artery smooth muscle cells (PASMC) and endothelial cells contribute to increased vascular tone, proliferation and decreased apoptosis in PAH. Well studied as important contributors to continuous vasoconstriction and remodeling of pulmonary arteries are dysfunctional K^+

channels and altered levels of cytosolic Ca^{2+} [90]. Regulatory dysfunctions in fibroblasts are an additional factor leading to impaired vascular function and remodeling. Studies in PAH have shown a rise in the deposition of extracellular matrix (ECM) proteins in the adventitia facilitating the migration of fibroblasts to the medial and endothelium layers[91]. The overall result is the emergence of an aberrant apoptosis-resistant, highly proliferative pulmonary vascular endothelium together with disruptions in both fibroblasts and PASMC layers.

Previous research has suggested that PAH ECs derived from obstructive plexiform lesions are likely associated with alterations of cellular functions involved in apoptotic and proliferative processes[92]. PAH ECs display faster growth rates as compared to non-diseased pulmonary ECs, as shown by enhanced cellular survival signals and increased cell division. This increased ability to maintain cell viability is accompanied with an upregulation of pro-survival factors and continuous activation of signal transducers and activators of transcription (STATs), such as STAT3, with a known involvement in cell growth regulation[89].

In CTEPH, several studies have highlighted the existence of an altered cellular phenotype in cells derived from large arteries. Pulmonary arterial ECs (PAECs) derived from CTEPH patients showed enhanced mitogenic activity *in vitro*[93]. This was concomitant with the presence of small-vessel abnormalities such as thickening of the medial layer and increased proliferative characteristics of cells lining the internal intimal layer, and formation of obstructive plexiform lesions comparable with features seen in PAH, which suggests the possibility that both diseases might develop from a common endothelial dysfunction that contribute to vascular remodeling[94]. A deeper understanding of the cellular processes behind these endothelial abnormalities might therefore shed light on the precise mechanisms that underlie the pathological changes occurring in the vascular wall of PAH and CTEPH.

Metabolic alterations and requirements in pulmonary hypertension

To better understand pulmonary vascular remodeling processes, we will take a closer look at the metabolic alterations and requirements of ECs in PH. As described above, endothelial cells are highly dynamic and rely mostly on glycolysis for their energy production and, when stimulated, they further boost the glycolytic rate to support their higher growth rates.

Both *in vitro* and *in vivo* studies have described an increase in glycolytic rate and lactate release in PAECs derived from PAH patients, as compared to non-diseased PAECs[88,95]. These

findings suggest that glucose metabolism is the primary energy source in PAEC. Additionally, PAH PAEC showed decreased oxygen consumption and maintained similar ATP levels under normoxia and hypoxia, compared to control PAECs[95]. Despite a significant scientific effort in the past years, we are still not able to fully understand regulatory mechanisms that promote the switch from oxidative glucose metabolism to glycolysis. A possible explanation is an impaired mitochondrial function, including pathological activation of pyruvate dehydrogenase kinase (PDK) activity and MnSOD deficiency[96,97]. It has been shown that PDK are highly expressed in PAH, which may imply a stronger inhibition of PDH and thus a proneness towards aerobic glycolysis[43,44]. Reduced levels of MnSOD in PAH disturb the cellular redox status leading to an accumulation of superoxide anion O_2^- and a reduced production of signaling moleculeH_2O_2 followed by normoxic activation of the redox-sensitive HIF1α. This pseudohypoxic state, decreased MnSOD and increased PDK in the presence of normal PO_2, further favors glycolysis and causes a cascade of downstream pathways promoting proliferation and inhibiting apoptosis through increased cytosolic Ca^{2+} and K^+ concentrations, respectively, both induced by a downregulation of $K_{v1,5}$ channel[96].

Recent metabolic studies focus on the less invasive technique, metabolomics of biofluids in PH. Despite contrasting reports using this approach regarding PAH[98,100], it is a promising technique in search of disease specific biomarkers. Metabolic profiling in PH has complement findings from *in vitro* and *in vivo* studies regarding increased glycolysis and has additionally found an increase in PPP, decrease in fatty acid oxidation (FAO) and impaired TCA[98,99]. All these observations point to similarities in metabolic profiles between diseased endothelia in PAH and rapidly growing cells, thus suggesting the existence of a Warburg effect in PAH PAECs together with the presence of mitochondrial abnormalities as summarized in **Table 1**. It will be interesting to determine whether CTEPH ECs also present similar pathophysiological metabolic processes.

Table 1. Summary main metabolic perturbations in AMI and PH

	AMI	PH
Nitric Oxide	↓ NO [37,71]	↓ NO [87]
Proliferation	↓ proliferation [72]	↑ proliferation [89,92]
Metabolism	↓ Glycolysis [71]	↓ OXPHOS ↑ Glycolysis [88,95,98,99]
Antioxidants and ROS	↑ mROS [37,71]	↓ MnSOD (↑O_2^-/↓H_2O_2) [96]

Abbreviations. AMI: acute myocardial infarction; PH: pulmonary hypertension; NO: nitric oxide; mROS: mitochondrial ROS; MnSOD: manganese-dependent superoxide dismutase

Future treatment options in PAH and CTEPH

The above described metabolic transformations in PAH ECs (enhanced glycolytic flows and diminished oxidative metabolism) bear similarities to the metabolic profiles of hyperproliferative ECs. On that basis, pharmacological blockade of PFKFB3, which restraints angiogenesis[42], offers a window of opportunity to rein in the hyperproliferative state in PAH ECs by reducing their glycolytic rate[39]. *In vitro* studies indicated that a dose-dependent inhibition of PFKFB3 by small compounds such as 3PO (3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one) successfully reduced glycolysis partially (35%-40%) although sufficiently to impair EC proliferation[42]. The enhanced expression of the PDK enzymes in PAH and CTEPH ECs offers an additionally opportunity for new targeted therapies. Preclinical intervention models of pulmonary hypertension have been studied to evaluate the efficacy of PDK inhibition in limiting and/or overturning pulmonary vascular changes[101]. The small molecule dichloroacetate (DCA), a pyruvate analogue, has a relatively high specific binding to PDK[102]. In rat models of monocrotaline-induced PAH (MCT-PAH), DCA was shown to prevent and reverse pulmonary vascular remodeling. Further, DCA induced apoptosis in the MCT-PAH pulmonary arteries (PAs) and suppressed cell growth rates measured by bromodeoxyuridine (BrdU) uptake in the medial layer of remodeled MCT-PAH PAs with little impact on normal pulmonary vascular cells in rats and humans, making it a promising small molecule for targeted restoration of normal metabolic dysfunctions in PAH ECs[44,102,103]. A recently published work demonstrated in a first-in-human clinical trial that PDK inhibition has a positive effect on the hemodynamics of PAH patients, further supporting the potential of DCA as a pharmacological agent in this disease[104]. Growing evidence of the existence of metabolic remodeling beyond the glycolytic pathway leads to a growing list of possible metabolic targets. One of these targets is the methylation of SOD2 (MnSOD) which suppresses expression of this redox enzyme. 5-AZA (5-aza-2´-deoxycytidine) inhibits methylation of SOD2 followed by restoration of the mitochondrial respiration[101]. Nevertheless, future metabolic studies in larger PAH patient groups and CTEPH patients will be a necessary, as additional steps in unraveling further pathophysiological mechanisms. However, one needs to bear in mind the challenges of metabolic therapies linked to interpatient variations and target selectivity but also due to the complexity of cell metabolism itself. Together with the search for new pharmacological interventions, a broad metabolomic screening is indispensable in the search for biomarkers in

PAH and CTEPH that may help early diagnosis and uncover metabolic-mediated remodeling in PH.

CONCLUSIONS AND PROSPECTS

There is increasing evidence that endothelial dysfunction in cardiopulmonary vascular disorders is associated with disease-specific metabolic alterations in endothelial cells. Such reorganization of metabolic networks is a double-edged sword as, on the one hand, it can function as a defense mechanism against disease-associated external insults, such as changes in glucose or oxygen availability, while, on the other hand, it can contribute to the generation of toxic end-products, anomalous accumulation of metabolic intermediates and alterations in energetic and redox metabolism that compromise physiological endothelial functions. This review points out current metabolic changes that have a great impact on the onset and progression of both atherosclerosis and PH. Endothelial cells' ability to easily switch between glycolysis, OXPHOS, PPP and FAO makes the cellular metabolic switch a complex and challenging target in the search for future pharmacological interventions.

Targeting endothelial cell metabolism is a promising strategy to restore normal endothelial function and it has been reported that moderate inhibition of PFKFB3 can block pathological angiogenesis and normalize EC dysfunction, whereas strong inhibition can result in vessel disintegration[105]. This strategy could be useful to restore endothelial cell function in pulmonary arterial hypertension (PAH), and possible also in CTEPH, both characterized by abnormal growth and enhanced glycolysis of pulmonary artery endothelial cells[106]. The main metabolic perturbations in pathological ECs in atherosclerosis, acute myocardial infarction and PH impact distinct pathways that lead to an imbalance in NO metabolism and ROS production. These diverse metabolic alterations in different EC dysfunctional pathologies highlight the need to apply metabolic network modelling approaches to identify key players that may be specific of endothelial metabolic dysfunctions and to rationally design interventions to target pathological EC metabolism for therapeutic benefit. Although the key players in glycolysis and energetic metabolism as well as in NO and ROS balance have been described, and communalities among different cell types and tissues have been reported, we still have only partial knowledge on the weight of each enzyme on the metabolic network fluxes in different cell types or in pathological versus healthy states. Techniques and algorithms developed in the past few years permute an accurate modelling of metabolic

network fluxes that, in conjunction with "omics" approaches, lay the foundations for unbiased and large-scale identification of targets with the greatest potential for specifically modulating metabolic pathway flux in disease conditions.

For medium-scale metabolic network models, among the main approaches used to model metabolic pathways are those based on the use of stable-isotope tracers (such as ^{13}C labelled metabolites). Computational tools to reconstruct metabolic flux maps from the quantification of the incorporation of ^{13}C-atoms into metabolites have been developed in recent years[107-109].

Models considering the kinetic properties of each enzyme in the metabolic network, in combination with methodologies such as Metabolic Control Analysis (MCA), a methodology to quantitatively evaluate the relative contribution and importance of each metabolic step in controlling overall metabolic flux distribution, have also been successfully used to predict putative drug targets[110]. The control that each individual enzyme has on the flux through a metabolic pathway is quantified using MCA methodology in terms of the so-called "flux control coefficients". MCA can be used also to compare metabolic flux distribution in healthy versus pathological condition. These comparisons will permit to identify the steps with the greatest potential for target specific metabolic adaptations accompanying pathological states and restore the flux distribution in health condition.

For large-scale models, constraint-based genome-scale metabolic models (GSMMs) have been developed and used in the last years to successfully predict putative therapeutic targets in different types of cancer[111-114]. In brief, GSMMs mainly use transcriptomics and other "omics" data to constraint metabolic flux maps as well as flux balance analysis (FBA) methods to optimize for a cellular objective function. The main challenge in this approach is to appropriately define the objective function. To identify drug targets that impact abnormal endothelial cell growth, the most used objective function has been the maximization of biomass production. Using this approach, putative drug targets have been identified relevant to cancer through a systematic search for essential genes and combinations of target genes interacting in a synthetic lethal fashion able to impair biomass production.

As a word of caution, although topology, stoichiometry and chemical reaction properties are the major constraints on metabolic network flux, the consequences of inhibiting an enzyme activity on the overall network flux redistribution will depend on the relative concentrations of the different enzymes in the network[115-117]. Relative levels of enzyme concentrations in

human metabolic networks are different not only in different tissues and in health versus disease conditions but also between individuals which can result in different patient responses to identical metabolic drug interventions. Endothelial cell metabolic alterations associated with cardiopulmonary vascular disorders have been mainly studied using immortalized cell models and there is a shortage of studies characterizing ECs from patient tissues, a necessary next step forward in the field.

The therapeutic possibilities of targeting EC metabolism to improve cardiopulmonary vascular dysfunction are still understudied. However, we predict that deeper characterization of metabolic reprogramming in patient-derived cells and systemic approaches that integrate "omics" data into comprehensive metabolic network flux models will soon permit to identify putative key players in endothelial dysfunction associated with cardiovascular disorders and to design personalized multi-hit interventions at the metabolic level to restore physiological endothelial functions.

Finally, growing interest has focused on the modulation of gut microbiota as a therapeutic strategy in cardiovascular diseases. Microbiota stability is essential in human physiology, as it is involved in the regulation of many host functions such as blood pressure control, glucose tolerance, insulin sensitivity and body weight control among others. Since hypertension and metabolic disturbances are well-known risk factors of CVD development, it is inevitable to touch in this review the possible role of gut microbiota and its impact on the cardiovascular system[118,119]. In humans, the bacterial proportion of the gut microbiota consists of mainly Firmicutes and Bacteroidetes phyla and its ratio is an important indicator of microbiota stability[119]. Since gut microbes primarily use ingested nutrients as fuel, it is not surprising that changes in dietary patterns alters the gut composition and its functions[120]. One way of interaction between the gut microbiota and the host is through production of metabolites that are biologically active or further metabolized by the host[119]. A meaningful example is trimethylamine (TMA), a metabolite produced by Firmicutes phyla that promotes foam cell formation through its hepatic oxidized form TMA-N-oxide (TMAO)[118]. Increased levels of this atherogenic microbial metabolite have been associated with increased risks of cardiovascular events[118,119]. Individual differences in the composition of the gut bacteria, combined to the plasticity of the microbiota, indicate that a gut microbiota-targeted strategy could be a promising approach for the prevention and the treatment of several metabolic diseases.

Despite, to date, little evidence have provided direct evidence of mechanistic or causal roles of gut microbiota in human cardiovascular disease suggesting that the relationship between human and gut microbiota must be further investigated[120,121].

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CHAPTER 5

DECREASED GLYCOLYSIS AS METABOLIC FINGERPRINT OF ENDOTHELIAL CELLS IN CHRONIC THROMBOEMBOLIC PULMONARY HYPERTENSION

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ABSTRACT

Chronic thromboembolic pulmonary hypertension (CTEPH) is caused by non-resolved thrombi obliterating pulmonary arteries and peripheral vasculopathy in non-occluded arteries. We hypothesized that metabolic dysregulation of endothelial cells (EC) contributes to cellular changes that promote vascular narrowing in CTEPH. The study aimed to characterize the metabolic fingerprint of EC-CTEPH. We used EC isolated from specimens removed at pulmonary endarterectomy. Human pulmonary artery endothelial (HPAE) cells from healthy donors were used as control cells. Expression levels of metabolic enzymes were studied at mRNA and protein levels by RT-PCR and western blot, respectively. Compared to HPAE, EC-CTEPH showed lower mRNA and protein levels of hexokinase-2 and lactate dehydrogenase-A, which represent the first and last steps of the glycolytic process. mRNA levels of the glycolytic regulators 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-3 and phosphofructo-kinase-1 were reduced in EC-CTEPH. Transcript levels of pyruvate dehydrogenase kinase-1, inhibitor of pyruvate dehydrogenase, and glutamate dehydrogenase-1, involved in glutamine metabolism, were also downregulated in EC-CTEPH. Fatty acid oxidation and the pentose phosphate pathway did not differ between EC-CTEPH and HPAE. Our results show reduced glycolytic metabolism in pulmonary artery EC in patients with CTEPH. This reduced glycolytic activity could contribute to endothelial dysfunction and vascular remodeling in CTEPH.

Keywords: Endothelial cell – cell metabolism – glycolysis – pulmonary hypertension – chronic thromboembolic pulmonary hypertension

INTRODUCTION

Chronic thromboembolic pulmonary hypertension (CTEPH) is a severe complication of pulmonary embolism (PE) that may lead to right heart failure and death [1]. Chronic obstruction of pulmonary arteries with unresolved thrombi is the initial trigger of CTEPH. Unresolved thrombi disturb the normal laminar blood flow of pulmonary arterial tree by limiting blood flow in affected arteries and, as a consequence, diverting it to nonoccluded arteries [1]. These hemodynamic changes disturb pulmonary endothelial cells (ECs) by altering shear stress, triggering inflammation and promoting a local imbalance of vasoactive mediators (nitric oxide (NO), prostacyclin (PGI_2) and endothelin-1 (ET1), among others), which leads to endothelial dysfunction [2,3]. Endothelial dysfunction is known to play a leading role in vascular remodeling via secretory imbalance of vasoactive factors and (pulmonary) vascular constriction among others [3,4]. Endothelial dysfunction induced vascular remodeling together with proximal chronic obstruction of the pulmonary vessels are thought to play a pivotal role in the development of CTEPH [1,2,5-7]. EC metabolism is currently regarded as an important co-determinant for endothelial (dys)function and vascular remodeling. Dysregulated is involved in EC proliferation, migration and energy production [8-12]. In addition, knock-down and inhibitory studies in animals and humans, respectively, showed the role of metabolic remodeling in pulmonary hypertension (PH) [13-15]. In this context, studying EC metabolism gains relevance to better understand EC behavior in CTEPH disease pathogenesis.

The gold standard treatment for CTEPH is the surgical removal of the thrombotic material along with the adhered and enlarged arterial intima by pulmonary endarterectomy (PEA) [5]. Approximately 40% of patients with CTEPH are not eligible for PEA due to thrombotic lesions not accessible to surgery or concomitant comorbidities. In addition, in about 35% of patients, pulmonary hypertension persists after surgery [16]. A deeper understanding of the molecular mechanisms leading to CTEPH is crucial to minimize persistence of PH after PEA and to find novel therapies for patients that are non-candidates to surgery.

Cellular metabolism involves glucose, fatty acids (FAs) and glutamine as main substrates for energy (ATP) and biomass production [17]. Glucose oxidation through glycolysis is the primary source of energy in endothelial cells and is linked via pyruvate dehydrogenase (PDH) to mitochondrial respiration, named oxidative phosphorylation (OXPHOS) (**Figure 1**). The pentose phosphate pathway (PPP) parallels the glycolysis pathway and generates reductive

power and macromolecules for endothelial cell viability and migration (**Figure 1**). Glutamine and FAs, through fatty acid oxidation (FAO), feed metabolic side branches of the tricarboxylic acid (TCA) cycle with additional metabolites for maintaining cellular energy and biomass production (**Figure 1**) [11,17,18].

Based on the relationship between EC metabolism and vascular function, we hypothesize that ECs-CTEPH present metabolic alterations which might explain EC dysfunction and subsequently vascular changes occurring in the pulmonary vessels. In this study, we used EC harvested from specimens extracted in PEA to study cellular metabolic changes in CTEPH.

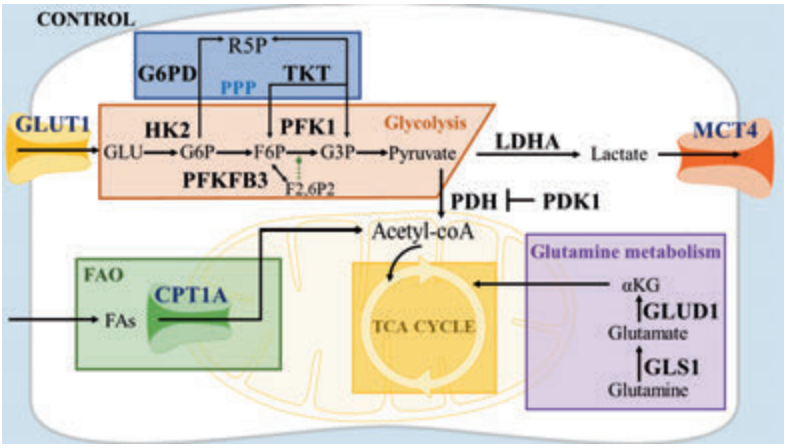

Figure 1. Metabolic pathways in endothelial cells. ECs use glucose, fatty acids (FAs) and glutamine as main source of energy. Glucose is converted into lactate in a multistep process during glycolysis. Glycolytic intermediate, pyruvate can also be shunted into the TCA cycle for oxidative phosphorylation. Pyruvate entry is dependent on activity of pyruvate dehydrogenase (PDH) and its inhibitor pyruvate dehydrogenase kinase-1 (PDK1). Glycolytic intermediate glucose-6-phosphate (G6P) can be shuttled into the pentose phosphate pathway (PPP) where it is converted into ribose-5-phosphate (R5P). The non-oxidative branch of the PPP generates R5P out of glycolytic intermediates, catalyzed by transketolase (TKT), this reaction is bidirectional. Both FAs and glutamine serve as precursors for the generation of TCA cycle intermediates via fatty acid oxidation (FAO) and glutamine metabolism, respectively.

MATERIAL AND METHODS

Study population

Endovascular material from proximal pulmonary arteries obtained in surgical PEA in 9 CTEPH patients was used as a source of pulmonary ECs. The study was approved by the institutional Ethics Committee of the Hospital Clínic of Barcelona and informed consent was obtained from all patients. All patients were diagnosed according to the 2015 ESC/ERS Guidelines, had an average age of 54 ± 6 years and a 3:6 female-male ratio. The full table can be found at the end of the document.

EC-CTEPH cell isolation and cell culture

Endarterectomy specimens collected from patients was finely dissected immediately after PEA. The tissue section used to isolate cells was free of thrombotic material and comprised intimal and superficial medial vessel layers. Endothelial cells were isolated (referred to as EC-CTEPH) as previously described [19]. Human pulmonary artery endothelial cells (HPAE) (Lonza) were used as controls. EC-CTEPH and HPAE cells were maintained in endothelium cell growth medium-2 (EGM-2) BulletKit (Lonza) supplemented with 10% FBS (GE Healthcare) on plates pre-coated with gelatin (0.2%) at 37°C, 5% CO_2. Primary cultures between passage 4 and 8 were used in experiments. The endothelial nature of isolated cells was confirmed by immunocytochemistry and flow cytometry with a panel of endothelial and smooth muscle cell-specific markers, including endothelial nitric oxide synthase (eNOS) and alpha-smooth muscle actin (α-SMA) as previously shown [20].

Expression profile mRNA

Total RNA was extracted from cultured HPAE and EC-CTEPH when 80%-90% confluent using TRIsure reagent (Bioline). Following reverse transcription (high capacity cDNA RT kit Applied Biosystems), quantitative real-time PCR experiments were performed in the presence of SYBR Green (Applied biosystems) with a ViiA 7 Real-Time PCR System (Applied Biosystems). cDNA copy numbers were normalized against endogenous control β-actin. All experiments were performed in triplicate and are expressed as $2^{-\Delta\Delta Ct}$. All primers were delivered by IDT and primer sequences can be consulted in supplementary data files.

Western blotting

Protein was isolated from HPAE and EC-CTEPH at 80%-90% confluency. To isolate protein, cells were treated with cold RIPA lysis and extraction buffer (Pierce) supplemented with Halt

protease and phosphatase inhibitor cocktail (ThermoFisher scientific). After centrifugation, lysate supernatant was collected and stored at -80°C. Protein concentrations were determined using BCA protein assay kit (Pierce). Equal amounts of protein lysates were separated by NuPAGE™ 4-12% Bis-Tris Protein Gels (Invitrogen) and transferred onto nitrocellulose membranes using iBlot Transfer Device (Invitrogen). After transfer, membranes were blocked in Casein Blocking buffer (Sigma-Aldrich). The membranes were incubated with primary antibodies overnight at 4°C with the following antibodies: GLUT1 (sc-377228, 1:500), HK2 (sc-374091, 1:800), LDHA (sc-137243, 1:800), PFKFB3 (sab-1402305, 1:1000) and G6PD (ab-993, 1:10000). After washing with TBS-Tween, the blots were incubated with secondary horseradish peroxidase-conjugated antibodies. Signal was detected using WesternBright Quantum substrate. The intensity of the individual bands in the blots was quantified using Image Lab (Bio-rad). All experiments were performed in triplicate and are expressed as relative expression normalized by β-actin (NB600-503) protein levels.

Statistical analysis

To evaluate the significance of differences between patient derived samples and non-diseased controls, Mann-Whitney test was used. Results are expressed as mean ± SEM. Differences were considered significant when $p < 0.05$ (*), $0.001 < p < 0.01$ (**), $p < 0.001$ (***). Statistical tests were undertaken using GraphPad software.

RESULTS

Glycolytic flux in EC-CTEPH

We first explored the mRNA expression of glycolytic related enzymes in EC-CTEPH compared to control human pulmonary artery endothelial cells (HPAE). Compared with control cells, EC-CTEPH showed 40% and 22% lower levels of hexokinase-2 (HK2) (p=0.0007) and phosphofructokinase-1 (PFK1) (p=0.006), respectively, which represent the first and second rate-limiting enzyme of the glycolytic pathway. mRNA levels of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-3 (PFKFB3), master regulating factor of the downstream glycolytic pathway were 42% lower in EC-CTEPH compared to control cells (p=0.002). Lactate-dehydrogenase-subunit-A (LDHA), the final glycolytic enzyme, showed 37% lower levels in EC-CTEPH compared to HPAE (p=0.004) (**Figure 2 A-D**). Overall, these results show a significant reduction in the expression levels of all glycolytic enzymes studied in EC-CTEPH.

The expression levels of glycolytic transporters of the first and final steps of the glycolysis pathway, such as glucose transporter 1 (GLUT1) and lactate monocarboxylate transporter 1 and 4 (MCT1 and MCT4, respectively), did not differ between EC-CTEPH and HPAE (**Figure 2E-F**).

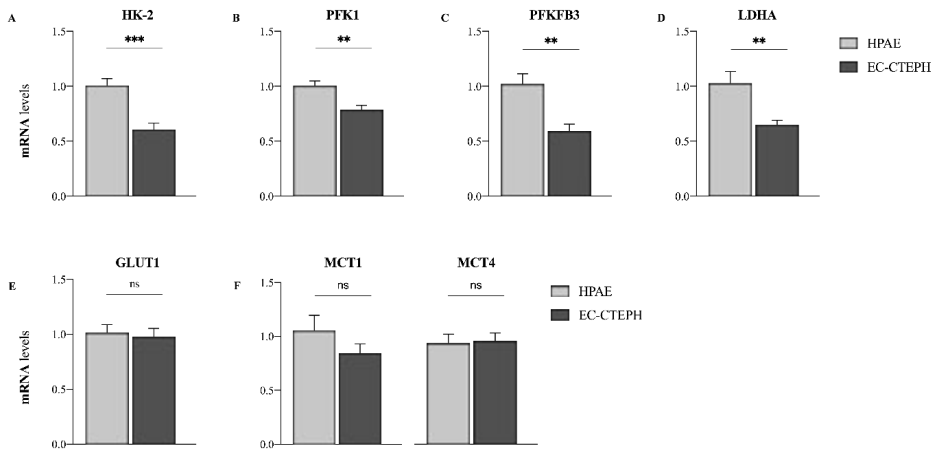

Figure 2. Glycolytic flux in EC-CTEPH. mRNA expression of glycolytic enzymes in EC-CTEPH compared to control cells. **(A-D)** HK2, PFK1, PFKFB3 and LDHA expression was reduced in EC-CTEPH compared to HPAE (p=0.0007; p=0.006; p=0.002; p=0.004). **(E-F)** GLUT1 expression was similar in EC-CTEPH and HPAE (p=0.6) and also lactate transporters MCT1 and MCT4 were similar in EC-CTEPH and HPAE (p=0.1 and p=0.9, respectively). Results are expressed as mean ± SEM. N=9 (EC-CTEPH), N=3 (HPAE)

To confirm these findings at the protein level, we performed western blot analyses . Protein levels of HK2 and LDHA showed 58% and 60% lower levels, respectively, in EC-CTEPH (p=0.0063 and p<0.0001 respectively) (**Figure 3A**). PFKFB3 protein levels were numerically lower in EC-CTEPH but not statistically significant (p=0.2) (**Figure 3A**). GLUT1 protein levels were similar in EC-CTEPH compared to control cells, (p=0.4) (**Figure 3B**).

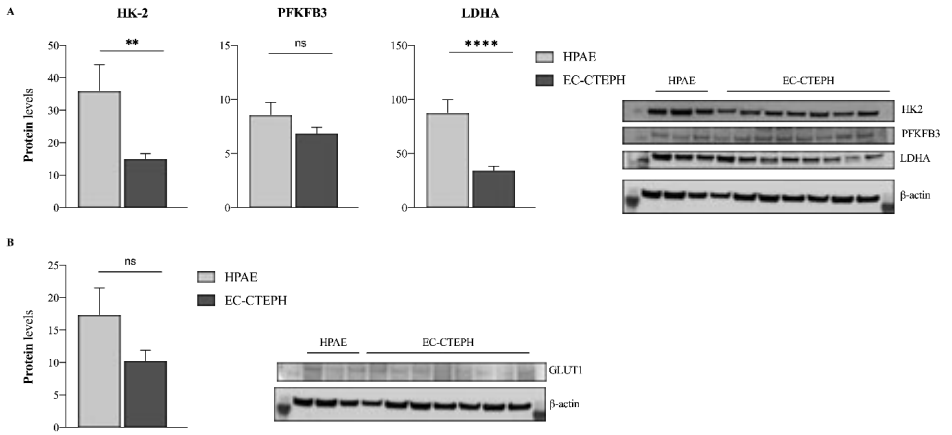

Figure 3. Glycolytic flux in EC-CTEPH. Protein levels of glycolytic enzymes in EC-CTEPH compared to control cells. **(A)** HK2 protein levels were reduced in EC-CTEPH compared to HPAE (p=0.006). LDHA protein levels were reduced in EC-CTEPH compared to HPAE (p<0.0001). PFKFB3 showed a reduction at protein levels in EC-CTEPH compared to HPAE but did not reach significance (p=0.2). **(B)** GLUT1 protein levels were similar in EC-CTEPH and HPAE (p=0.4). Corresponding blots of proteins studied in EC-CTEPH compared against endogenous control β-actin are shown. Results are expressed as mean ± SEM. N=9 (EC-CTEPH), N=3 (HPAE)

Other metabolic pathways in EC-CTEPH

We additionally investigated the involvement of other metabolic pathways in CTEPH development by analyzing mRNA expression of enzymes involved in mitochondrial OXPHOS, PPP, FAO and glutamine metabolism. Compared to control cells , EC-CTEPH had were 34% lower levels of the PDH inhibitor pyruvate-dehydrogenase-kinase (PDK1) (p=0.02) (**Figure 4A).** mRNA levels of its target, pyruvate dehydrogenase subunit E1 alpha 1 (PDHA1) and promotor of pyruvate entry into the mitochondria,, did not differ between EC-CTEPH and control cells (p=0.2) (**Figure 4A**).

mRNA levels of TCA cycle associated enzymes isocitrate dehydrogenase 1 (IDH1) did not differ in EC-CTEPH compared to HPAE (p=0.4) (Figure 4B). Although, subunit alpha of IDH isoform 3 (IDH3A) showed a trend towards 14% lower levels in EC-CTEPH compared to controls (p=0.09).

No difference was found in mRNA expression levels of subunit gamma of isoform 3 (IDH3G) between EC-CTEPH and HPAE (p=0.9) (**Figure 4B**). Additionally, subunit B of the succinate dehydrogenase (SDH) complex (SDHB) showed 12% lower levels in EC-CTEPH compared to HPAE (p=0.05). Both subunits A and C of the SDH complex (SDHA and SDHC, respectively) were similar in EC-CTEPH and HPAE (p=0.2 and p=0.6, respectively) (**Figure 4C**).

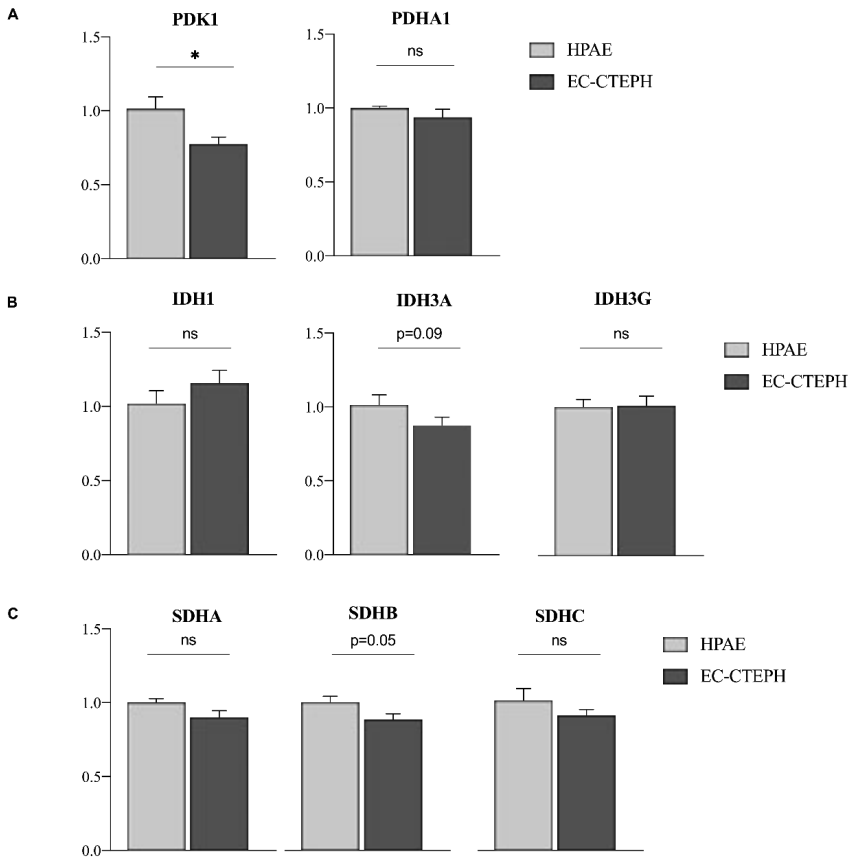

Figure 4. Oxidative phosphorylation in EC-CTEPH. (**A)** mRNA levels of PDH inhibitor PFK1 were reduced in EC-CTEPH compared to HPAE (p=0.02), whereas PDHA1 itself did not show a difference between EC-CTEPH and HPAEC (p=0.2). **(B)** Gene expression of both isoforms 1 and 3 from isocitrate dehydrogenase (IDH) were not found different in EC-CTEPH compared to HPAE with p=0.4 for IDH1, p=0.09 for IDH3A and p=0.9 for IDH3G. **(C)** Only subunit B of the succinate dehydrogenase (SDHB) complex showed reduced gene expression in EC-CTEPH compared to HPAE (p=0.05). Subunit A and C were similar in EC-CTEPH and HPAE (p=0.2 and p=0.6, respectively) Results are expressed as mean ± SEM. N=9 (EC-CTEPH), N=3 (HPAE)

The PPP showed 22% lower transcript levels in transketolase (TKT), regulator of the non-oxidative branch, in EC-CTEPH compared to HPAE (p=0.05) (**Figure 5A**). Glucose-6-phosphate-dehydrogenase (G6PD) expression, rate-limiting enzyme of the oxidative branch, was similar in the two cell lines at both mRNA (p=0.2) and protein level (p=0.3) (**Figure 5A** and **Supplement 1**).

The mitochondrial FA transporter carnitine palmitoyl-transferase-1 isoform A (CPT1A) was not differently expressed in EC-CTEPH (p=0.1) (**Figure 5B**). Additionally, glutamate-dehydrogenase-1 (GLUD1) (p=0.05) but not glutaminase (GLS1) (p=0.6) showed 24% lower mRNA levels in EC-CTEPH compared to HPAE (**Figure 5C**). An overview of results found in EC-CTEPH are summarized in **Figure 6.**

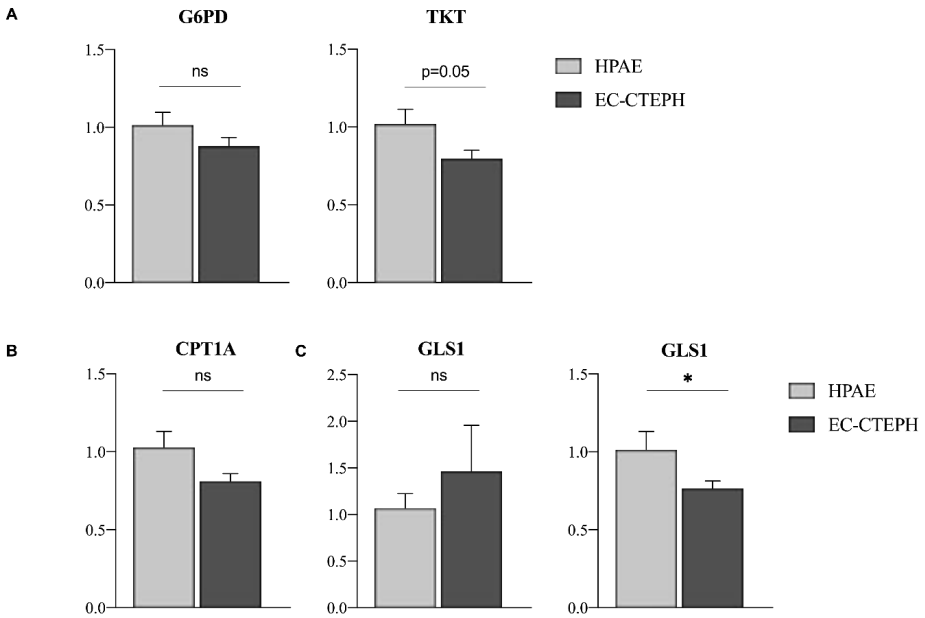

Figure 5. Additional metabolic pathways in EC-CTEPH. (A) mRNA levels of G6PD from the oxidative branch of the PPP was not different in EC-CTEPH compared to HPAE (p=0.2). mRNA levels of TKT from the non-oxidative branch of the PPP were lower in EC-CTEPH compared to HPAE (p=0.05) **(B)**. Gene expression of fatty acid transporter CPT1A was similar in EC-CTEPH and HPAE (p=0.1) (C). Gene expression of GLS1 was not different in EC-CTEPH and HPAE (p=0.6), whereas GLUD1 was lower in EC-CTEPH compared to HPAE (p=0.05). Results are expressed as mean ± SEM. N=9 (EC-CTEPH), N=3 (HPAE)

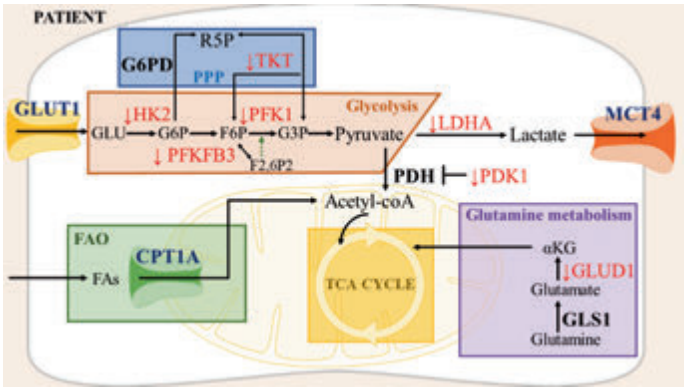

Figure 6. Metabolic pathways in endothelial cells from CTEPH patients. The key and rate-limiting enzymes of the glycolytic flux are downregulated in EC-CTEPH. Also the enzymes TKT and GLUD1 from the PPP and glutamine metabolism, respectively, are downregulated in EC-CTEPH. Enzymes from the metabolic pathways FAO and oxidative phosphorylation were not found differently expressed in EC-CTEPH compared to HPAE.

DISCUSSION

In this study, we aimed to examine EC metabolism to address a potential relationship between EC metabolism, EC dysfunction and subsequent vascular remodeling occurring in pulmonary arteries surrounding the thrombi in CTEPH. an interesting metabolic fingerprint in human EC-CTEPH isolated from PEA specimens. In contrast to the increased glycolytic profile observed in PAH [11,21], we observed a decreased glycolytic profile in EC-CTEPH based on the reduction in mRNA levels of main glycolytic enzymes HK2, PFKFB3, PFK1 and LDHA and a similar reduction in the protein levels of HK2 and LDHA. In addition, this work showed both a downregulation of PDH inhibitor PDK1 and the glutamine converting enzyme GLUD1 in EC-CTEPH. PPP and FAO associated key metabolic enzymes were not found different in EC-CTEPH compared to control cells. These data indicate a clear altered metabolic profile in EC-CTEPH compared to healthy endothelial cells.

ECs-CTEPH showed an interesting metabolic fingerprint: downregulation of controlling glycolytic enzymes PFK1 and PFKFB3 and a downregulation of PDH inhibitor PDK1. The role of metabolic alterations in the development of cardiovascular diseases such as pulmonary arterial hypertension (PAH) points out the importance of a better understanding of the EC metabolism in CTEPH [13-15,22-24]. Activating events such as shear stress, growth factors and inflammation promote endothelial dysfunction, which causes the endothelium to switch to an angiogenic state promoting vascular remodeling [3,18]. In such conditions, ECs reprogram their

metabolism towards increased glycolysis for fast production of ATP whereby upregulating key glycolytic enzymes such as GLUT1, HK2, PFK1, and PFKFB3 and suppressing OXPHOS through upregulation of PDK1 [17,18]. PDK1 shifts pyruvate away from mitochondria towards fermentation into lactate by LDHA. Increasing lactate levels are subsequently exported outside the cell by MCT4 where it acts as a pro-angiogenic factor among others to promote a sustained activated state of the endothelium [17,25,26]. In our study, the downregulation of the controlling glycolytic enzymes PFK1 and PFKFB3 and a reduction of PDH inhibitor PDK1 suggest a decreased metabolic flux towards lactate. This decreased metabolic flux is further supported by a downregulation of LDHA and no upregulation of lactate transporters MCT1 and MCT4 in ECs-CTEPH. Although EC dysfunction and EC metabolism are well studied in vascular diseases such as PAH [13,15,23,24], underlying mechanisms regarding vascular remodeling are still not completely understood, pointing out the complexity of processes involved in vascular remodeling [21,27]. As endothelial metabolism is closely linked to EC function [8-12], decreased glycolysis in EC-CTEPH might be a trigger in the development of endothelial dysfunction which impairs angiogenic capacities of the endothelium.

EC-CTEPH are isolated from larger vessels surrounding the thromboembolic lesions and might be involved in the lack of thrombus recanalization and resolution. Nonresolution of thrombi might result from underlying mechanisms such as inflammation, fibrinogen abnormalities, platelet dysfunction and impaired angiogenesis [1,28] In this study, patient derived EC-CTEPH showed a downregulation of the glycolytic flux and glutamine metabolism which might result in impaired angiogenic and/or vascular repair capacity of EC-CTEPH. PFKFB3-driven glycolysis is the key driver of the angiogenic process but also glutamine metabolism and FAO are indispensable for vessel sprouting [9,10,17,29]. Animal studies showed the importance of angiogenesis in recanalization of the thrombus [1,30]. In addition, absence of infiltrating capillaries in human CTEPH thrombi support the idea of impaired angiogenesis and its contribution to thrombus persistence followed by occlusive vascular remodeling after PE [30]. We believe that a decrease in glycolysis and impaired glutamine metabolism in EC-CTEPH, isolated from vessels surrounding the thromboembolic lesions, could provide insights into the lack of thrombus recanalization and resolution occurring in CTEPH.

Our results also showed a reduction in GLUD1 expression in EC-CTEPH, indicating that less glutamine enters the TCA cycle. Glutamine is an important metabolic substrate in activated

ECs to replenish the TCA cycle with carbons and nitrogen since the majority (>90%) of glucose-derived carbons exit the cell in the form of lactate during the glycolytic process [8,11]. As our data indicates a reduced metabolic flux towards lactate and because of that an increased amount of the total glucose-derived-pyruvate enters the TCA cycle, this results in the fact that less glutamine will be oxidized through the TCA cycle.

FAO and CPT1A are indispensable for TCA cycle replenishment during elevated cell proliferation [10]. A reduced metabolic flux towards lactate in EC-CTEPH is also here suggestive for a decreased need of FAO in EC-CTEPH which can explain no difference in CPT1A expression in EC-CTEPH compared to HPAE.

EC-CTEPH showed no change in the PPP as G6PD was not found significantly different in EC-CTEPH compared to HPAE. The PPP is a side pathway paralleling glycolysis through which NAPDH and/or ribose-5-phosphate (R5P) is generated [17,18]. R5P is mainly produced via oxidation of glycolytic intermediate glucose-6-phosphate (G6P) by G6PD in the oxidative branch of the PPP, generating at the same time reductive power in the form of NAPDH. TKT, an enzyme of the non-oxidative branch, on the other hand, recycles excess of pentose phosphates back to glycolytic intermediates fructose-6-phosphate (F6P) and glyceraldehyde-3-phosphate (G3P). Low expression of TKT indicates a low glycolytic flux [17,18,31]. In EC-CTEPH all main glycolytic enzymes were downregulated together with a downregulation of PDK1. A low flux through glycolysis together with a relative higher amount of pyruvate shunted into TCA cycle could be a reason for a low flow of metabolites into the PPP.

It has been previously shown that isolated pulmonary arterial endothelial cells (PAECs) from patients with pulmonary arterial hypertension (PAH) show metabolic reprogramming towards glycolysis together with a suppression of glucose oxidation in the mitochondria, an increase in fatty acid and glutamine metabolism while suppressing glucose oxidation in the mitochondria [32-34]. Despite histological resemblances between CTEPH and PAH [35,36], our study showed a decrease in glycolysis and no additional upregulation of fatty acid and glutamine metabolism in patient derived EC-CTEPH compared to non-diseased pulmonary endothelial cells, pointing to altered molecular mechanisms in EC-CTEPH. However, it cannot be excluded that anatomic specific hemodynamic features on pulmonary endothelial cells can also contribute to the difference in results obtained between cells isolated from CTEPH patients compared to PAH patients. PAH is a vascular disease at the level of the microvascular

endothelium compared to CTEPH with thrombotic lesions and remodeling mainly in surgically accessible large (~3cm) and medium sized arties (≥ 2mm) [22,37,38]. A different location in the pulmonary vasculature tree exposes ECs to different blood pressures and blood flow which has an influence on the endothelium and cellular behavior of ECs studied [39]. So endothelial cells derived from PEA specimen are from a different anatomic position in the pulmonary vasculature tree compared to PAECs that are isolated from PAH recipient lungs [3,32,33]. A better understanding of metabolic changes in EC-CTEPH is a crucial step in improving our understanding of CTEPH pathogenesis. Taken together, our data indicate an altered metabolic profile in human EC isolated from CTEPH PEA specimens, compared to healthy ECs, that might diminish EC angiogenic capacities and might play a role in thrombus nonresolution, an important disease trigger in CTEPH development.

CONCLUSION

Human EC isolated from CTEPH pulmonary endarterectomy specimens present an altered metabolic fingerprint. EC-CTEPH showed a reduced glycolytic metabolism compared to healthy EC. In addition, PDK1 and glutamine metabolism through the TCA cycle were downregulated. No difference has been found in FAO or PPP pathway. The altered glycolytic activity of EC-CTEPH could provide insights into the lack of thrombus recanalization and resolution occurring in CTEPH.

LIMITATIONS

This study has some limitations. We used commercially available HPAE as control cells which might be not as optimal as EC isolated from healthy transplant lung via the same procedures we used, but these lung samples were not available to us. To minimize this limitation, all cells were extensively characterized in the laboratory. Nevertheless, future studies should verify our findings in control cells from healthy transplant lungs. The number of cells needed to perform the experiments also did not allow us to use control ECs freshly isolated from patients. To minimize differences induced by the culture process, all experiments were carried out with HPAE and EC-CTEPH at the same passage (four to eight). In the current brief report we have not been able to describe findings between EC function and alterations in metabolism. The lack of protein measurements of PDK1 could be considered as a limitation of this study. Despite extensive attempts to detect PDK1 at the protein level using several

antibodies, we were not successful to perform adequate protein levels detection and WB analysis. Studies characterizing cell function of EC-CTEPH regarding angiogenesis, migration and proliferation that might help to resolve molecular insights in CTEPH disease development, are under way. For some parts of our study larger sample sizes would be ideal but this a challenge as the number of surgeries/year are limited in our institution and endothelial cells are not always successfully derived.

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CHAPTER 6

COMPARISON OF METABOLIC PROFILE IN ENDOTHELIAL CELLS OF CHRONIC THROMBOEMBOLIC PULMONARY HYPERTENSION AND PULMONARY ARTERIAL HYPERTENSION

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ABSTRACT

Chronic thromboembolic pulmonary hypertension (CTEPH) and pulmonary arterial hypertension (PAH) are two forms of pulmonary hypertension (PH) characterized by an obstructive vasculopathy that can be fatal when left untreated. Endothelial dysfunction along with metabolic changes towards increased glycolysis are thought to be important in PAH disease initiation and progression. Much less is known of the existence of such abnormalities in endothelial cells (ECs) from CTEPH patients. This study provides a systematic metabolic comparison of ECs derived from CTEPH and PAH patients.

Metabolic gene expression (glucose transporter 1 (GLUT1), hexokinase 2 (HK2), 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-3 (PFKFB3), pyruvate dehydrogenase kinase 1 (PDK1), lactate dehydrogenase (LDHA), pyruvate dehydrogenase (PDH), glutamate dehydrogenase 1 (GLUD1), glucose-6-phosphate-dehydrogenase (G6PD)) and HOXD gene expression was studied by the use of qPCR in cultured ECs from CTEPH and PAH patients. Western blot analyses were done for HK2, LDHA, PDH and G6PD. Basal viability and residual viability of CTEPH-EC and PAH-EC after incubation with metabolic inhibitors was measured by MTT. Additionally, migration capacity was assessed by use of a wound healing assay. Human pulmonary arterial endothelial cells (HPAEC) and human lung microvascular endothelial cells (HMVEC-L) were used as healthy control cells.

CTEPH-EC showed significant lower mRNA levels of GLUT1, HK2, LDHA and GLUD1 compared to PAH-EC. At the protein level phosphorylated PDH was found lower expressed in CTEPH-EC compared to PAH-EC. The pentose phosphate pathway was not found different between CTEPH-EC and PAH-EC. PAH-EC, CTEPH-EC and HPAEC presented similar HOXD gene expression. Viability after incubation with metabolic inhibitors was not differently affected between PAH-EC, CTEPH-EC and HPAEC. At last, CTEPH-EC and PAH-EC showed similar migration capacity and viability.

CTEPH-EC and PAH-EC show differences in glycolysis and glutamine metabolism. They have similar migration capacity and similar viability when treated with metabolic inhibitors. More studies are needed to better understand the importance of reduced glycolysis and glutamine metabolism in CTEPH-EC, and whether such differences may lead to the development of novel therapeutic approaches to treat CTEPH.

Keywords: Endothelial cell – cell metabolism – glycolysis – OXPHOS – chronic thromboembolic pulmonary hypertension – pulmonary arterial hypertension

INTRODUCTION

Pulmonary hypertension (PH) is defined as a mean pulmonary arterial pressure (mPAP) >20 mmHg[1]. Based on similar pathogenesis, clinical and hemodynamics characteristics PH is categorized into 5 clinical groups: pulmonary arterial hypertension (PAH); PH due to left heart disease; PH associated with lung disease and/or hypoxia; Chronic thromboembolic pulmonary hypertension (CTEPH) and PH with unclear or multifactorial mechanisms[2]. Although the most common cause of PH is left heart disease, PAH and CTEPH have received over the years the largest interest of researchers [2,3]. PAH and CTEPH result from remodeling of pulmonary blood vessels that causes increase in pulmonary vascular resistance, subsequent right heart failure and, ultimately death. This vascular remodeling is characterized by thickening of the endothelial and ⁄or smooth muscle layer of muscular vessels and by presence of vaso-occlusive lesions[4-6]. To date, exact molecular mechanisms and regulatory pathways behind vascular remodeling in PAH and CTEPH are not fully resolved. Overlap in the presence of pulmonary vascular remodeling between PAH and CTEPH suggest possible similar cellular and molecular disease mechanisms that might help to better understand CTEPH pathology.

The pulmonary vascular endothelium, at the interface between the blood and lung tissue, plays key roles in maintaining vessel homeostasis[7]. Under normal conditions, the endothelium maintains a quiescent state with at basal level a predominant use of glycolysis rather than oxidative phosphorylation in the mitochondria. However, upon stimulation by growth factors such as vascular endothelial growth factor (VEGF) or endothelial injury, the endothelium switches to an activated state which is associated with changes in cellular metabolism that are thought to contribute to EC dysfunction and pulmonary vascular remodeling[8,9]. It is known that, under sustained pathological conditions, EC metabolic alterations promote vascular diseases by mean of excessive cellular proliferation, increased angiogenesis and a pro-survival cellular phenotype[8,10]. Endothelial cells (ECs) originating from vascular lesions in patients with PAH are found to have a hyperproliferative and apoptosis-resistant phenotype that is supported by a metabolic switch towards glycolysis, changes in oxidative phosphorylation and increased glutaminolysis[11-13]. Increased expression of pyruvate dehydrogenase kinase (PDK) in PAH is thought to be responsible for the increased reliance on glycolysis[14,15]. Overall, those studies have been performed with PAH-EC isolated from distal small arterioles but not much is known about the involvement of macrovascular pulmonary arterial ECs (PAECs) in PAH.

Expression patterns of homeobox (HOX) gene clusters have been found to be associated with the positional fate of ECs. In mammals HOX genes appear in 4 clusters, the HOXA, HOXB, HOXC and HOXD and especially the HOXD expression pattern has shown to cluster based on the type of blood vessels[16,17]. To date, it is unknown whether macrovascular PAECs from CTEPH patients share the same metabolic characteristics as PAECs from PAH or whether metabolic modulation might be beneficial in patients with CTEPH.

ECs rely on glucose, fatty acids and amino acids as their main sources of energy and biosynthetic precursors[10]. During glycolysis, glucose is metabolized into lactate by rate-limiting glycolytic enzymes hexokinase-2 (HK2), phosphofructokinase-1 (PFK1) and lactate dehydrogenase A (LDHA). The glycolytic process is controlled by the glycolytic regulator 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-3 (PFKFB3) but also by the enzyme activity of the pyruvate dehydrogenase (PDH) complex. PDH is phosphorylated and inhibited by pyruvate dehydrogenase kinase (PDK) which blocks pyruvate entry into the mitochondria and promote pyruvate fermentation into lactate by LDHA instead [8,10,18]. The glycolytic pathway is paralleled by the pentose phosphate pathway (PPP) that uses glycolytic intermediates, mediated through the expression of glucose-6-phosphate-dehydrogenase (G6PD), but also shunts intermediates back into the glycolysis pathway when needed. The PPP is an additional pathway for nucleotide production and redox homeostasis[8]. Despite the fact that ECs mainly rely on glycolysis for ATP production, mitochondria remain fully functional and rather function as suppliers for cellular building blocks through metabolism of amino acids such as glutamine and glutamate that serve as biosynthetic precursors for the production of nucleotides and macromolecules through glutaminolysis[19].

In this study, we used isolated ECs from vascular tissue collected at pulmonary endarterectomy (PEA) and after lung transplantation from CTEPH and PAH patients, respectively. We perform a systemic metabolic comparison of PAECs from patients with CTEPH and PAH. In addition, we asses migration, basal viability and residual viability upon inhibition by metabolic regulators of glycolysis and glutamine metabolism.

MATERIAL AND METHODS

Study population and samples collected

This study included ECs from 12 CTEPH patients who underwent pulmonary endarterectomy at the Hospital Clinic of Barcelona, Spain. This study further included 6 patients with end-stage PAH who underwent lung transplantation or lung autopsy at the Amsterdam VU University Medical Center, The Netherlands. The study was approved by the institutional Ethics Committee of the Hospital Clínic of Barcelona and the IRB of the Amsterdam VU University Medical Center, the Netherlands. Informed consent was signed by all patients. All patients were diagnosed according to the 2015 ESC/ERS Guidelines[20].

Pulmonary endothelial cell isolation and culture

ECs isolated from endarterectomy specimens from CTEPH patients, referred to as CTEPH-EC, were isolated and cultured as previously described[21]. EC phenotype was characterized by staining the cells with antibodies against a FACS panel of endothelial and smooth muscle cell-specific markers, including endothelial nitric oxide synthase (eNOS) and alpha-smooth muscle actin (α-SMA). ECs from 2 heritable and 4 idiopathic PAH patients (referred to as PAH-EC) were isolated from lung tissues and artery rings of end-stage PAH patients obtained from lung transplantations or from autopsies and cultured as previously described[22,23]. ECs were purified by magnetic affinity cell sorting (MACS, Miltenyi Biotec) based on CD144 antibody labeling and purity was ensured by regular FACS testing. In short, these ECs were plated onto 0.2% gelatin-coated wells and grown in endothelium cell growth medium-2 (EGM-2) supplemented with EGM™-2 SingleQuots (Lonza, USA) and 10% fetal bovine serum (FBS) (GE Healthcare, USA). Patients characteristics are presented in **Supplementary Table 1**. Human pulmonary artery endothelial cells (HPAEC) (Lonza, CC-2530) were used as control cells. Human lung microvascular endothelial cells (HMVEC-L) (Lonza, CC-2527) were grown in EBM™-2 Basal medium supplemented with EGM™-2 MV microvascular endothelial cell growth medium singleQuots™ supplements (Lonza).

Gene expression analyses

The mRNA levels of metabolic genes were measured by qPCR (N=10 CTEPH-EC, N=6 PAH-EC and N=3 or 6 HPAEC). ECs were seeded at a density of 6×10^4 per 40 mm cell culture dish (pre-coated with 0.2% gelatin). Total RNA was extracted using TRIsure™ (Bioline, Germany) and concentrations were determined by spectrophotometry. Reverse transcription was

performed using reactive mix high-Capacity cDNA RT kit (Applied Biosystems, USA). For qRT-PCR, SYBR Green I (ThermoFisher Scientific, USA) was used and specific primers were used on the ViiA7 Real-Time PCR system (Applied Biosystems, USA). Relative quantification was calculated by normalizing the Ct (threshold cycle) of the gene of interest to the Ct of an endogenous control (β-actin) in the same sample, using the comparative ΔΔCt method. All primers were designed with Primer 3Plus and delivered by Integrated DNA Technologies. Primer sequences can be found in **supplementary Table 2.**

Protein expression analyses

ECs from 6 CTEPH donors, 5 PAH donors and 3 HPAEC were seeded into T75 flaks pre-coated with 0.2% gelatin, at a density of 4 x 10^5 cells per flask, and allowed to adhere and grow in complete endothelial medium till confluency was reached. Next, cells were washed twice with ice-cold PBS and treated with Pierce® RIPA buffer (ThermoFisher Scientific, USA) supplemented with a Halt protease/phosphatase inhibitor cocktail (ThermoFisher Scientific, USA). After incubation on ice, the cell lysate was obtained by centrifugation. Protein concentrations in the lysates were determined using the Pierce™ BCA protein assay kit (ThermoFisher Scientific, USA). Then, 15-25µg of total protein was loaded onto 4-12% Bis-Tris Gels (ThermoFisher Scientific, USA). The proteins were transferred to nitrocellulose membranes and blocked for 1 hour at room temperature with Casein Blocking Buffer (Sigma-Aldrich, USA). The membranes were incubated with specific primary antibodies (HK-2, LDHA, G6PD, PDHA1, phospho-PDHA1, PDK1, OXPHOS cocktail and vinculin) overnight at 4°C. Horseradish peroxidase-conjugated anti-IgG was used as the secondary antibody. Immunoreactive bands were detected by WesternBright™ Quantum™ substrate (Advansta, USA). Images were analyzed with ImageJ software. The antibodies list can be found in **supplementary Table 3.**

Functional analyses

Wound healing assay

ECs from 5 CTEPH donors, 4 PAH donors and 3 HPAEC were seeded into 24-wells pre-coated with 0.2% gelatin, at a density of 5 x 10^4 cells per well and allowed to adhere and grow in 10% FBS EGM-2 medium at 37℃ till confluency was reached. The cells were then starved for 2 hours in 2% FBS EGM-2 medium at 37℃ prior to wound healing assay. The wound healing assays were performed with the 2% FBS EGM-2 starving medium containing hydroxyurea at

a final concentration of 2mM to exclude the effect of cell proliferation. Microscopic screenshots of each well (Zeiss Axiovert 200 Microscope) were taken at 0, 5, 10, and 24-hour time points since the wound was made.

MTT

ECs from 3-6 CTEPH donors, 3-5 PAH donors and 3 HPAEC were seeded into 96-wells pre-coated with 0.2% gelatin, at a density of 5 x 10^3 cells per well and allowed to adhere and grow in 10% FBS EGM-2 medium till 80% confluency was reached.. For residual viability, cells were starved with EGM-2 medium containing only 0.5% FBS overnight (about 15 hours). Inhibition treatments were performed in starving medium (0.5% FBS EGM-2) and incubated at 37℃ for 24 hours. 3PO (15 and 30 µM), BPTES (2 and 4 µM), UK5099 (2 and 4 µM), Etomoxir (2 and 4 µM), and DCA (40 and 80mM) were the inhibitors and concentrations of choices to evaluate the effects on cell viability by blocking different biological pathways. The Vybrant® MTT Cell Proliferation Assay Kit (ThermoFisher Scientific, USA) was used for assessing basal and residual viability. For residual viability, medium was initially exchanged for 0.5% FBS EGM-2 phenol-red-free medium containing or without inhibitors depending on the experimental conditions. Basal viability was measured in 10% FBS EGM-2 phenol-red-free medium. 20mM MTT solutions were added and the plates were then incubated at 37℃ for 4 hours. SDS 0.01M HCl solution was then loaded followed by further 4-hour incubation at 37℃. The absorbance was recorded at 570nm on Synergy™ HTX Multi-Mode Microplate Reader.

Statistical analysis

Results are described as means ± standard deviation and were compared using Unpaired t-test, one-way ANOVA or two-way ANOVA. Statistical analyses were performed using GraphPad Software. P-values <0.05 were considered statistically significant.

RESULTS

HOXD expression

Based on previous studies, this study particularly focused on the HOXD expression as it has been shown that the expression patterns of the HOXD gene family, and especially HOXD3, -8 and -9, allow to distinguish between the microvascular and macrovascular origin of ECs.[17] Compared to HMVEC-L, CTEPH-EC and PAH-EC showed 1.9 fold (p=0.0005) and 1.7 fold (p=0.002) lower HOXD3 expression levels, respectively, whereas HOXD8 expression levels were 1.9 fold (p<0.0001) lower in both CTEPH-EC and PAH-EC. At last, HOXD9 showed 2 fold (p=0.003) lower expression levels in both CTEPH-EC and PAH-EC compared to HMVEC-L. Expression levels of all three HOXD genes were not found differently expressed between CTEPH-EC and PAH-EC. Control HPAEC showed similar expression profiles as CTEPH-EC and PAH-EC for all HOXD genes studied (**Figure 1A**).All ECs studied present a typical cobblestone morphology (**Figure 1B**)

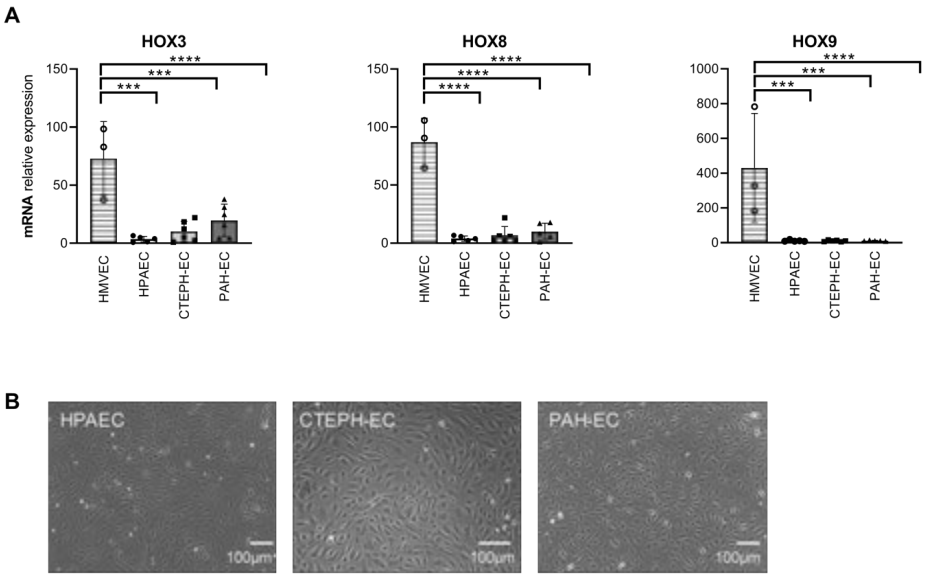

Figure 1. HOXD gene expression. A) mRNA expression levels of HOXD3, HOXD8 and HOXD9 were found significantly increased in HMVEC-L compared to HPAEC, CTEPH-EC and PAH-EC. No differences in gene expression was found between HPAEC, CTEPH-EC and PAH-EC. HMVEC-L, n=3; HPAEC, n=5; CTEPH-EC, n=6; PAH-EC, n=3; One-way ANOVA, p<0.001= ***; p<0.0001 = ****, data is expressed as mean ± SD. **B)** Pictures of cultured HPAEC, CTEPH-EC and PAH-EC. All ECs present a cobblestone morphology, typical of ECs. Scale bar =100µm

Metabolic gene expression

mRNA levels of GLUT1 in CTEPH-EC were 1.7 fold (p=0.006) lower compared to PAH-EC and 1.8 fold (p=0.01) higher in PAH-EC compared to HPAEC. mRNA levels of glycolytic rate-limiting enzyme HK-2 were 2.2 fold lower in CTEPH-EC as compared to PAH-EC (p=0.0003) and 1.5 fold higher in PAH-ECs compared to HPAEC (p=0.02). Glycolytic master regulator PFKFB3 did not show a difference at mRNA level between the three subject groups. Final glycolytic enzyme LDHA showed 1.8 fold lower mRNA levels in CTEPH-EC compared to PAH-EC (p=0.002) and 1.5 fold higher levels in PAH-EC compared to HPAEC (p=0.04) (**Figure 2A**). Mitochondrial gate-keeper enzyme pyruvate dehydrogenase (PDH) subunit E1 alpha (PDHA1) showed 1.3 fold (p=0.04) lower mRNA levels in CTEPH-EC compared to PAH-EC and 1.4 fold (p=0.02) higher levels in PAH-EC compared to HPAECs. mRNA levels of PDK1 were not found differently expressed between all three groups (**Figure 2B**). Also, mRNA levels of PPP associated G6PD were similar in the three studied groups (**Figure 2C**). Glutamate converting enzyme glutamate-dehydrogenase-1 (GLUD1) showed 1.5 fold (p=0.0001) lower mRNA levels in CTEPH-EC compared to PAH-EC. GLUD1 mRNA levels in PAH-EC were 1.2 fold (p=0.06) higher compared to HPAEC. In addition, GLUD1 mRNA levels in CTEPH-EC were 1.2 fold (p=0.06) lower compared to HPAEC. mRNA levels of glutamine converting enzyme, glutaminase 1 (GLS1), were similar in all three PAH-EC, CTEPH-EC and HPAEC (**Figure 2D**).

Metabolic protein expression

Protein levels of glycolytic rate-limiting enzymes HK-2 and LDHA were found similarly expressed between all three study groups, only for HK-2 mRNA expression levels seemed to show a tendency towards upregulation in CTEPH-EC compared to PAH-EC (**Figure 3A**). Protein levels of G6PD were also similar between CTEPH-EC, PAH-EC and HPAEC (**Figure 3B**). Protein levels of phosphorylated PDHA1 (inactive form) showed a tendency towards 1.4 fold (p=0.09) lower levels in CTEPH-EC compared to PAH-EC. No differences were found in phosphorylated PDHA1 protein levels between CTEPH-EC and HPAEC, and between PAH-EC and HPAEC. Protein levels of the non-phosphorylated form of PDHA1 (active form) showed a tendency towards lower protein levels in CTEPH-EC as compared to PAH-EC (p=0.08). The same tendency of lower levels was observed in HPAEC compared to PAH-EC (p=0.08) (**Figure 3C**). No difference was observed in protein levels of PDK1 between the three groups (**Figure 3C**). Protein levels for oxidative phosphorylation associated proteins from complex-I, complex-II

and complex-IV showed respectively 1.7 fold (p=0.06), 1.7 (p=0.04) and 1.8 (p=0.005) lower levels in CTEPH-EC compared to PAH-EC. The same 3 complexes showed respectively 3.9 fold (p=0.005), 2.6 fold (p=0.01) and 3.9 fold (p=0.0004) higher protein levels in PAH-EC compared to HPAEC. Protein levels of complexes-I, -II and -III were similar between CTEPH-EC and HPAEC. Complex-IV and -V showed similar protein levels between all three groups (**Figure 4**).

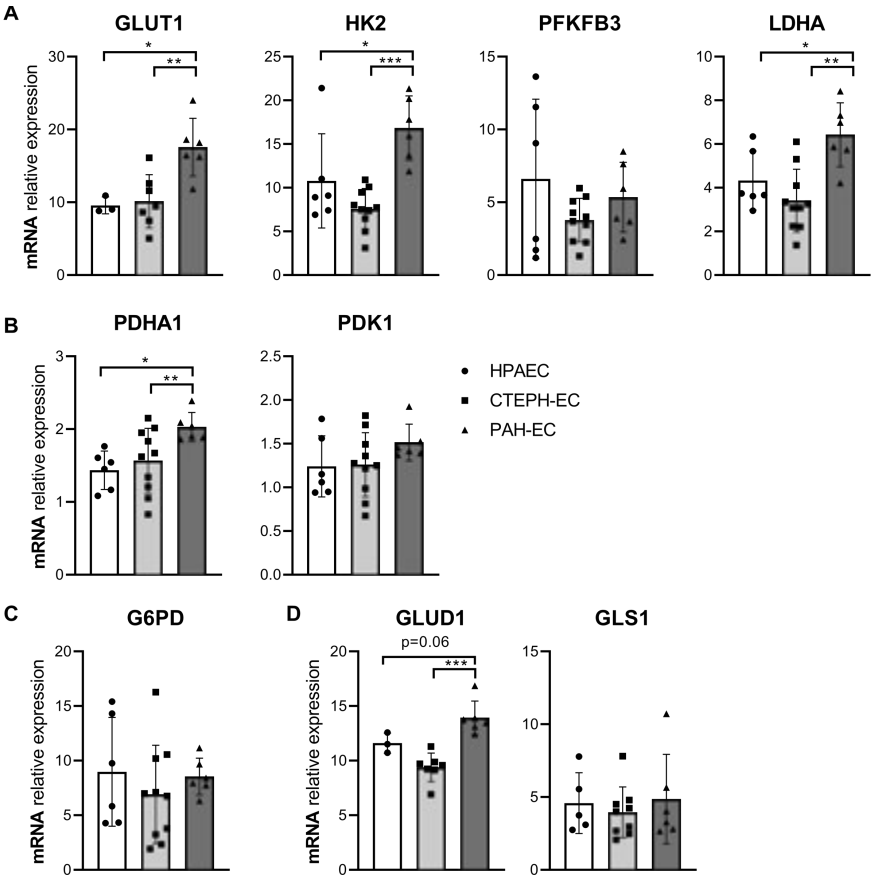

Figure 2. Gene expression of key metabolic enzymes. A) mRNA expression levels of GLUT1 were found significantly downregulated in CTEPH-EC compared to PAH-EC and, in PAH-EC compared to HPAEC. Similar mRNA levels were observed between CTEPH-EC and HPAEC. HK2 mRNA levels were found significantly downregulated in CTEPH-EC compared to PAH-EC and in PAH-EC compared to HPAEC. No difference in PFKFB3 mRNA expression was observed between all three groups. mRNA expression levels of LDHA were significantly lower in CTEPH-EC compared to PAH-EC and in PAH-EC compared to HPAEC. **B)** mRNA levels of PDHA1 were only found significantly different between CTEPH-EC and PAH-EC. mRNA levels of PDK1 were similar between all three groups. **C)** G6PD mRNA levels were found similar between all three groups. **C)** mRNA levels of GLUD1 were significantly decreased in CTEPH-EC compared to PAH-EC and a tendency to lower levels in PAH-EC compared to HPAEC was observed. mRNA levels of metabolic enzyme GLS1 was found similar between all three groups studied. CTEPH-EC, n>7; PAH-EC, n=6; HPAEC, n>3; One-way ANOVA, data is expressed as mean ± SD, p<0.05= *; p<0.01= **; p<0.001= ***.

Residual viability

Residual viability after incubation with metabolic inhibitors was assessed in all three groups. Incubation with glycolytic inhibitor 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) showed a dose-dependent reduction in viability in all three groups (p=0.0001). However, no difference in residual viability was observed between CTEPH-EC, PAH-EC and HPAEC after incubation with 3PO. Also, for glutaminase inhibitor bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)-ethyl-sulfide (BPTES) and mitochondrial pyruvate carrier blocker 2-Cyano-3-(1-phenyl-1H-indol-3-yl)-2-propenoic-acid (UK-5099) a dose-dependent reduction in residual viability was observed in all three groups (p=0.02 and p<0.0001, respectively) but no difference was found in viability between CTEPH-EC, PAH-EC and HPAEC after incubation with both inhibitors. PDK inhibitor dichloroacetate (DCA) showed a dose-dependent reduction in residual viability in all three groups (p=0.0001) but in between groups no difference in response was observed for CTEPH-EC, PAH-EC and HPAEC (**Figure 5**).

Basal viability and migration

Because of clear metabolic differences observed between CTEPH-EC and PAH-EC, basal viability and migration were assessed to investigate a relationship between the observed changes in cell metabolism and cellular functions. Basal viability was found to show a trend towards a 1.2 fold (p=0.09) decrease in CTEPH-EC compared to PAH-EC. Basal viability was similar between CTEPH-EC and HPAEC, and between PAH-EC and HPAEC (**Figure 6A**). Both CTEPH-EC and PAH-EC showed reduced wound closing speed compared to HPAEC but did not reach the statistical level (p=0.09 and p=0.07, respectively). No difference in closing speed was observed between CTEPH-EC and PAH-EC (**Figure 6B**). The results of this study are summarized in **Table 2.**

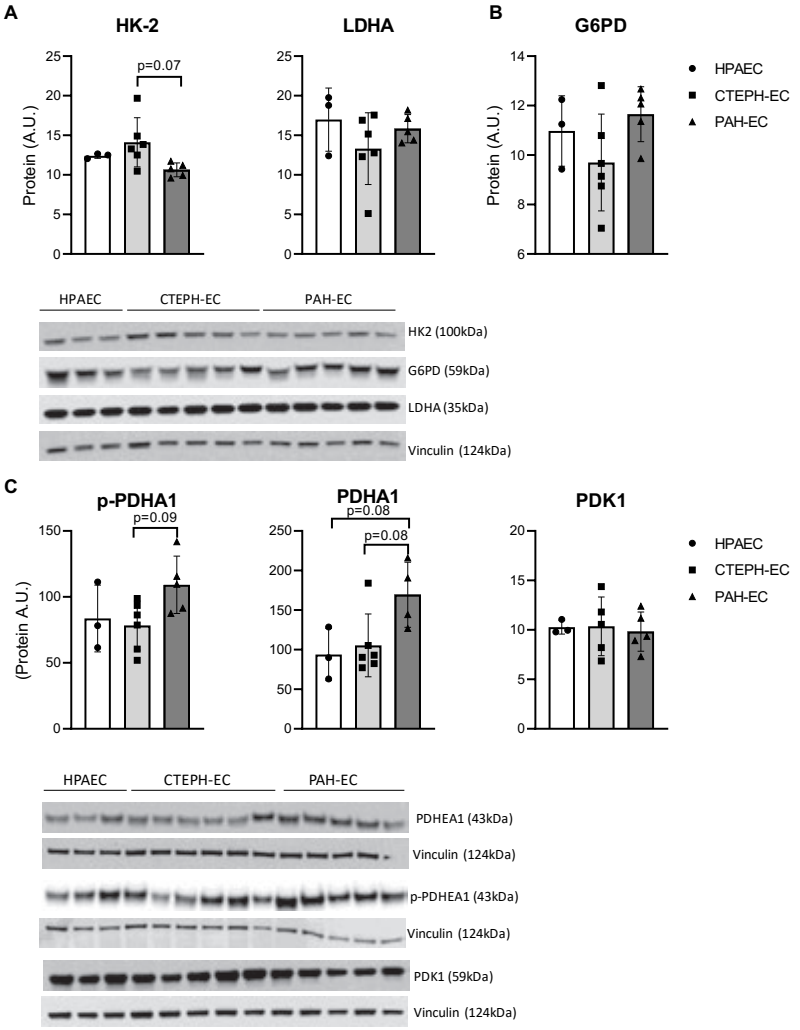

Figure 3. Protein expression of key metabolic enzymes. **A)** Protein levels of HK2 showed a tendency towards upregulation in CTEPH-EC compared to PAH-EC. **B)** Protein levels of PPP key enzyme G6PD were similar in all three groups studied **C)** Protein levels of the phosphorylated E1 alpha PDH subunit (active PDH) showed a tendency towards a decrease in CTEPH-EC compared to PAH-EC. Protein levels of the active form of PDHA1 (non-phosphorylated subunit) also showed a tendency towards a decrease in CTEPH-EC compared to PAH-EC. Protein levels of PDHA1 inhibitor, PDK1, were found similar in all three groups. CTEPH-EC, n=5-6; PAH-EC, n=5; HPAEC, n=3; One-way ANOVA, data is expressed as mean ± SD.

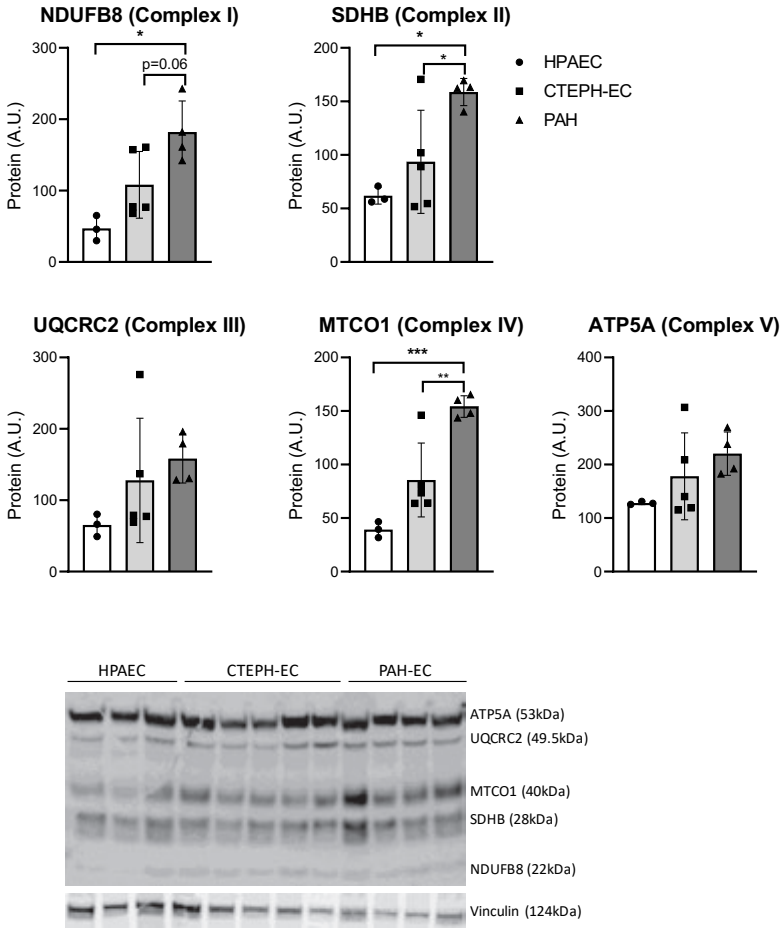

Figure 4. Oxidative associated proteins. Protein levels of complexes-I, -II and -IV were found significantly downregulated in CTEPH-EC compared to PAH-EC. All three complexes are also significantly different between PAH-EC and HPAEC. Similar levels were observed for complexes-III and -V between all three groups studied. CTEPH-EC, n=5; PAH-EC, n=4; HPAEC, n=3; One-way ANOVA, data is expressed as mean ± SD, p<0.05= *; p<0.01= **; p<0.001= ***.

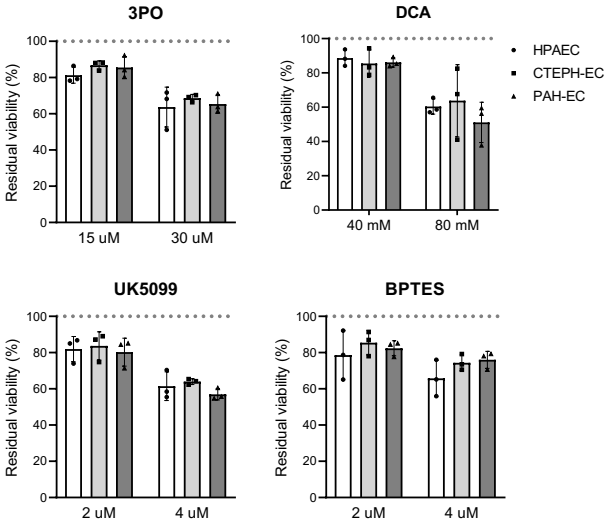

Figure 5. Residual viability upon metabolic inhibition. The effect of metabolic inhibition was evaluated by use of MTT. 3PO (glycolytic inhibitor), BPTES (glutaminase inhibitor), UK5099 (mitochondrial pyruvate carrier blocker) and DCA (PDK1 inhibitor). A dose dependent effect on the viability of all three groups studied is found. No difference in residual viability was observed between CTEPH-EC, PAH-EC and HPAEC in response to metabolic inhibitors. CTEPH-EC, n=3; PAH-EC, n=3; HPAEC, n=3; Two-way ANOVA, data is expressed as mean ± SD.

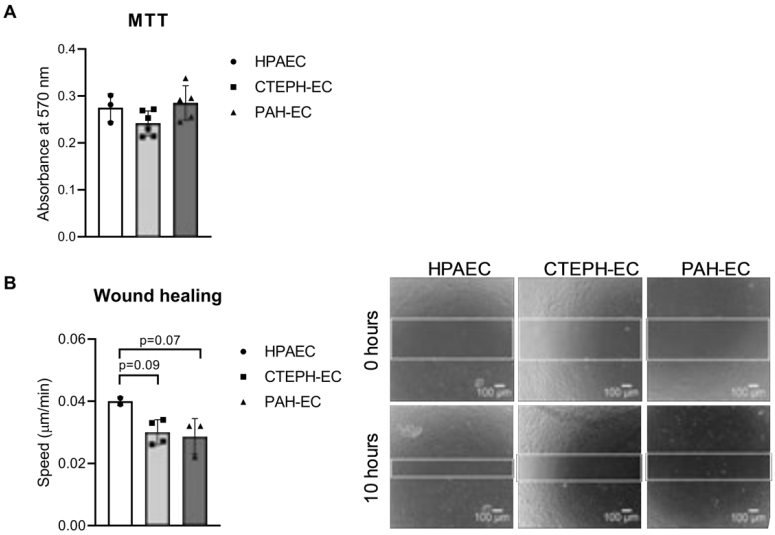

Figure 6. Basal viability and wound healing. A) CTEPH-EC showed a trend towards less viability compared to PAH-EC. **B)** Migration capacity was assessed by use of the wound healing assay and expressed as speed of closure over time (µm/min). Both CTEPH-EC and PAH-EC showed a trend towards a reduction in speed of closure compared to HPAEC. No difference in wound closure was found between CTEPH-EC and PAH-EC. CTEPH-EC, n>6; PAH-EC, n>2; HPAEC, n>2; One-way ANOVA, data is expressed as mean ± SD.

Table 1. Comparison of CTEPH-EC and PAH-EC

	CTEPH-EC
HOXD (mRNA)	=
Glycolysis (mRNA)	↓
p-PDHA1 (inactive) (protein)	↓ [a]
Glutamine metabolism (mRNA)	↓
Viability (metabolic inhibition)	=
Basal viability	↓ [a]
Migration	=

Summary of the most relevant findings of CTEPH-EC in comparison with PAH-EC.
[a] Tendency was observed

DISCUSSION

This study showed a pronounced difference in gene expression of glycolytic and glutamine-related enzymes between CTEPH-EC and PAH-EC. The decrease in glycolytic enzymes in CTEPH-EC was further accompanied by a decrease in PDHA1 mRNA levels. Furthermore, reduced phosphorylated PDHA1 protein levels in CTEPH-EC compared to PAH-EC were observed. In addition, protein levels of oxidative phosphorylation complexed I, II and IV were reduced in CTEPH-EC compared to PAH-EC. No differences were seen in PPP related enzymes both at mRNA and protein level. All ECs showed a response to metabolic inhibitors despite a distinct metabolic profile between CTEPH-EC and PAH-EC. No difference in migration capacity could be observed between CTEPH-EC and PAH-EC. Finally, a trend towards lower viability was observed in CTEPH-EC compared to PAH-EC.

In this study CTEPH-EC and PAH-EC presented a similar HOXD gene expression pattern. Toshner *et al.* described that, taking into account the HOXD expression patterns, ECs could be clustered based on the type of blood vessel that they were derived from. Their study showed that high expression of HOXD3, HOXD8 and HOXD9 was associated with ECs that are microvascular in origin[17]. The expression profiles of all 3 HOXD genes, both in CTEPH-EC and PAH-EC, were significantly lower than those in HMVEC-L. Based on this data it can be concluded that CTEPH-EC and PAH-EC origin from the same macrovascular lineage.

It is known that, in PAH, vascular ECs have adopted several metabolic changes that are associated with vascular hyperproliferation and resistance to apoptosis. PAH-EC are found to increase glycolysis in order to assure proliferation[12,26]. Not much is known about metabolic alterations in CTEPH-EC, but, based on few recent *in vitro* and *in vivo* studies from our group, CTEPH-EC seem to be associated with impairments in their metabolism that point towards lower glycolysis and glutamine metabolism in CTEPH-EC compared to healthy ECs[27,28]. In accordance with those observations, our study showed the downregulation of glycolytic genes GLUT1, HK2 and LDHA in CTEPH-EC compared to PAH-EC, accompanied by a tendency towards higher viability of PAH-EC. Glycolytic regulator PFKFB3 was not found differently expressed between all ECs types studied. An explanation could be that PFKFB3 activity is dependent on post-translational modifications [29,30]. The current study indicates an unexpected difference in the reliance on glycolysis between CTEPH-EC and PAH-EC that could possibly explain the difference in viability between CTEPH-EC and PAH-EC. Another important key feature of metabolic adaptations in ECs from PAH patients is increased inhibition of PDHA1 by PDK1. PDK1 phosphorylates PDHA1, which blocks mitochondrial oxidative phosphorylation and further promotes glycolysis [31,32]. The present study showed increased gene expression of PDHA1 in PAH-EC compared to CTEPH-EC but not difference in gene expression of PDK1. More importantly, protein levels of phosphorylated (inactive) PDHA1 were higher expressed in PAH-EC compared to CTEPH-EC. No differences were found at the protein level for PDK1. Although this study could not show an increased expression of PDK1 in PAH-EC, the increase in phosphorylated PDHA1 is in line with the increase use of glycolysis by PAH-EC. Next to increased levels of phosphorylated (inactive) PDHA1, protein levels of active PDHA1 seemed also higher in PAH-EC compared to CTEPH-EC, and, even though it seems unexpected, higher levels of oxidative phosphorylation associated proteins in PAH-EC is indicative for functional mitochondrial respiration and could explain the increase in active PDHA1. Besides glycolysis, glutamine metabolism is thought to be involved in PAH pathology and has shown to be altered in CTEPH-EC[27,33]. Glutamine metabolism is essential in EC proliferation and is driven by the expression of GLS1 and GLUD1[19]. The current study showed increased gene expression of GLUD1 but not GLS1 in PAH-EC compared to CTEPH-EC. This observation implies a role of glutamine metabolism in PAH-EC but, also, further confirms a difference in metabolism between PAH-EC and CTEPH-EC that needs deeper attention. At last, the oxidative arm of the PPP, important for maintaining cell viability under high rates of

proliferation[34], was not found different between CTEPH-EC and PAH-EC, neither between PAH-EC and HPAEC. Despite similar vasculopathy between both diseases, a different metabolic profile at the level of glycolysis, oxidative phosphorylation and glutamine metabolism is present in CTEPH-EC compared to PAH-EC and suggests differences in molecular mechanisms and regulatory pathways that could be important in disease pathology.

Based on the different EC metabolic profile between CTEPH and PAH, ECs were treated with metabolic inhibitors to see whether differences in metabolism could be translated into differences in viability upon inhibition. All ECs studied showed a dose-dependent reduction in viability after incubation with metabolic inhibitors 3PO, DCA, BPTES and UK5099 but, no significant differences in response could be found between PAH-EC, CTEPH-EC and HPAEC. The lack of significant response to glycolytic inhibitor 3PO and DCA, and oxidative phosphorylation inhibitor UK5099 in PAH-EC compared to CTEPH-EC is unexpected. Not only because this study showed an increase in glycolysis in PAH-EC, but, also, because previous studies have shown beneficial effects of DCA in both human PAH and experimental PAH[14,15]. Both studies have reported mainly effect of DCA on pulmonary artery smooth muscles, and together with the existence of non-responders due to genetic alterations, this could be an explanation for similar responses between PAH-EC and CTEPH-EC. Nonetheless, regardless their metabolism, ECs of all three groups did show a reduction in residual viability after incubation with metabolic inhibitors which could imply lack of selective inhibition of diseased ECs. Moreover, based on the results of this study, blocking glycolysis may not be beneficial in CTEPH-EC and could even further compromise CTEPH-EC viability. Overall, those observations show a clear response to several metabolic inhibitors in all three groups studied but no differences in response to those inhibitors between the three groups.

Besides proliferation and viability also migration is controlled by metabolic pathways such as glycolysis, glutamine metabolism and PPP [35,36]. Interestingly, both CTEPH-EC and PAH-EC showed a reduction in migration speed compared to control HPAEC, but no difference was observed between CTEPH-EC and PAH-EC despite the significant differences in cell metabolism observed. These results of reduced migration are in line with previous studies in ECs from CTEPH and PAH patients[21,37], and indicate that both CTEPH-EC and PAH-EC present inherent endothelial dysfunctionalities compared to healthy ECs. A possible explanation for a similar reduction in migration could be the existence of alterations in other pathways that, in

parallel to cell metabolism, control migratory behavior such as VEGF and Notch signaling pathways [38].

This study compared CTEPH-EC and PAH-EC that present a similar HOXD gene profile. CTEPH-EC and PAH-EC show differences in glycolysis and glutamine metabolism that could support higher viability of PAH-EC. Nevertheless, no difference in response to metabolic inhibitors is observed between CTEPH-EC and PAH-EC. Moreover, reduced migration is observed in both CTEPH-EC and PAH-EC compared to control ECs despite differences in cell metabolism. More studies are needed to better understand the importance of reduced glycolysis and glutamine metabolism in CTEPH-EC, and whether such differences may lead to the development of novel therapeutic approaches to treat CTEPH.

LIMITATIONS

This study has several limitations. Patients with non-operable disease or increased risk factors (e.g. high BMI or PVR > 900 dyn·s·m^{-5}) for surgery are not included in this study, which can cause a certain selection bias of CTEPH patients with a more moderate-to-severe disease state compared to the PAH population group. However, CTEPH patients still show hemodynamic severity (mean mPAP >40 mmHg, low cardiac index and PVR > 500 dyn·s·m^{-5}). The PAH patients cohort is comprised of 6 individuals, therefore these results should be verified with a bigger PAH group. Also, some functional data is lacking in this group (we could only trace one 6-minute-walking-distance and four evaluations of NYHA functional class at baseline, however, the values we found for NYHA FC are similar to those found in previous literature[39]). Differences in disease severity could potentially contribute to the differences in metabolism and viability between endothelial cells isolated from PAH and CTEPH patients.

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SUPPLEMENTAL

Clinical data

Clinical data is summarized in **supplementary table 1**. ECs were isolated from a total of 18 PH patients, 12 patients with CTEPH and 6 with PAH. Gender was equally disturbed in the CTEPH patient group but in the PAH patient group women accounted for 83% of the patient population. CTEPH patients were significantly older than PAH patients (58.02±7.62 vs 37.17±10.72, p=0.0007). Body mass index (BMI) was equally distributed in both groups. mPAP was severely elevated in both groups. mPAP was significantly higher in PAH patients than in CTEPH patients (68.17±24.21 vs 40.67±8.86, p=0.01), as well as pulmonary vascular resistance (PVR) (1070.00±464.78 vs 607.00±246.46, p=0.05). Mean cardiac index (CI) was reduced in CTEPH but not in PAH patients, however there was no statistical difference between the groups. Pulmonary artery occlusion pressure (PAOP) was normal but not different between both groups. Right arterial pressure was elevated but not different between both groups. Both groups showed a diminished mixed oxygen blood saturation (SvO2). Both groups showed increased brain natriuretic peptide (BNP), with PAH patients presenting significantly higher levels than CTEPH patients (6021.00±4939.87 vs 166.02±300.68, p=0.006), reflecting their worse hemodynamic condition. All PAH patients were in NYHA class III/IV, whereas CTEPH patients were in NYHA class I/II (25%) and class III/IV (75%). Some patients received PH-targeted therapy either as treatment of the disease (PAH), or as a bridge to the intervention (hemodynamic values are those of the baseline, before drug treatment) (CTEPH). In PAH patients combination therapy prevailed with endothelin antagonists (ERAs) plus phosphodiesterase type 5 inhibitors (PDE5i) and/or plus prostanoids, whereas in CTEPH patients received either monotherapy (ERAs/PDE5i/Riociguat) (33%), combination therapy (ERA plus PDE5i or Riociguat (33%)) or no therapy (25%) (data of one patients was not available).

Supplementary Table 1. Clinical features and hemodynamic parameters

	CTEPH (n=12)	PAH (n=6)
Female/male	6/6	5/1
Age years	58.02 ± 7.62	37.17 ± 10.72 *
BMI kg·m^{-2}	26.69 ± 4.09	27.83 ± 16.53
mPAP mmHg	40.67 ± 8.86	68.17 ± 24.21 *
PVR dyn·s·m^{-5}	607.00 ± 246.46	1070.00 ± 464.78 $^{p=0.05}$
PAOP mmHg	9.75 ± 4.41	12.33 ± 6.66
Cardiac index L·min^{-1}·m^{-2}	2.38 ± 0.49	2.56 ± 0.82
Right atrial pressure mmHg	7.08 ± 5.23	13.83 ± 7.36
SvO2 %	61.82 ± 7.61	52.00 ± 4.97 $^{p=0.07}$
BNP pg·mL^{-1}	166.02 ± 300.68	6021.00 ± 4939.87 *
WHO FC [b]		
I	0	0
II	3	0
III	9	3
IV	0	1

Data are presented as n or mean ± SD. CTEPH: chronic thromboembolic pulmonary hypertension; PAH: pulmonary arterial hypertension; BMI: body mass index; mPAP: mean pulmonary artery pressure; PVR: pulmonary vascular resistance; PAOP: pulmonary artery occlusion pressure; SvO2: mixed venous oxygen blood saturation; BNP: brain natriuretic peptide; WHO FC: world health organization functional class. [b] Data from 2 PAH patients are not available. * p< 0.05, unpaired t-test, data expressed as mean ± SD.

PAH patients were significantly younger and mostly female which is in line with the observed female predominance of PAH[24] and the earlier onset of disease[25]. The older age of CTEPH patients can be explained by the fact that CTEPH results from a pulmonary embolism after which it can take years before patients show clinical signs of CTEPH. Furthermore, older patients might have more comorbidities and treatments, which could increase the risk of pulmonary embolism. The significant increase in hemodynamic parameters in PAH patients compared to CTEPH patients could be explained by the more severe disease state of PAH patients included in this study. In addition, this study only used ECs from CTEPH patients that

underwent pulmonary endarterectomy, and patients with persistently elevated levels of PVR are not eligible for surgery.

Material and methods

Supplementary Table 2. Primers sequences

Gene name	Forward (5'-3')	Reverse (5'-3')
G6PD	CCAAGCCCATCCCCTATATTT	CCACTTGTAGGTGCCCTCAT
GLS1	GCTGTGCTCCATTGAAGTGACT	TTGGGCAGAAACCACCATTAG
GLUD1	GGGATTCTAACTACCACTTGCTCA	AACTCTGCCGTGGGTACAAT
GLUT1	GGTTGTGCCATACTCATGACC	CAGATAGGACATCCAGGGTAGC
HK2	TCCCCTGCCACCAGACTA	TGGACTTGAATCCCTTGGTC
LDHA	GCAGATTTGGCAGAGAGTATAATG	GACATCATCCTTTATTCCGTAAAGA
PDHA1	CCTGACTTTATATGGCGATGG	CTGCCATGTTGTAAGCTTCG
PDK1	GGTTACGGGACAGATGCAGT	CGTGGTTGGTGTTGTAATGC
PFKFB3	CCTACCTGAAATGCCCTCTTC	GTCCCTTCTTTGCATCCTCTG
HOXD3	CGTAAGGATTGCATCGGACT	TCCTAAGCTCGGCTGGATAA
HOXD8	TAAACCAGCTTGCTGTGTGC	GTGAGGCTATCGCTTTCCTG
HOXD9	CCTGCTCCATTGGTTCCTTA	TCAGAAACATGGGGGACATT

Supplementary Table 3. Antibody list

Name	Dilution	Company	Code	Characteristics	Host	Reactivity	kDa
HK2	1/800	santa cruz	sc-374091	monoclonal	mouse	human	100
LDHA	1/800	santa cruz	sc-137243	monoclonal	mouse	human	35
G6PD	1/10000	abcam	ab993	polyclonal	rabbit	human	59
PDH-E1α	1/1000	santa cruz	sc-377092	monoclonal	mouse	human	43
p-PDH-E1α	1 µg/mL	abcam	ab92696	polyclonal	rabbit	human	43
Total OXPHOS	1/250	abcam	ab110413	monoclonal	mouse	human	/[a]
PDK1	1/2000	abcam	ab207450	monoclonal	rabbit	human	49
β-actin	1/1000	novus biologicals	NB600-503	polyclonal	rabbit	human	37
Vinculin	1/10000	abcam	ab129002	monoclonal	rabbit	human	124

[a] This kit contains 5 antibodies. The kDa for each antibody is indicated in figure 4

CHAPTER 7

THE INFLAMMATORY PROFILE OF CTEPH DERIVED ENDOTHELIAL CELLS IS A POSSIBLE DRIVER OF DISEASE PROGRESSION

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Submitted

ABSTRACT

Chronic thromboembolic pulmonary hypertension (CTEPH) is a form of pulmonary hypertension characterized by the presence of fibrotic intraluminal thrombi causing obliteration of the pulmonary arteries. Although both endothelial cell (EC) dysfunction and inflammation are linked to CTEPH pathogenesis, regulation of the inflammatory response of ECs in CTEPH is not fully understood. Therefore, in the present study we investigate the role of NF-κB proinflammatory signaling pathway in ECs in CTEPH.

To study if NF-κB is activated, pulmonary endarterectomy (PEA) specimens from CTEPH patients and ECs isolated from PEA specimens were stained for phospho-NF-κB-P65. Especially the vessels within the thrombus and CTEPH-ECs are positive for phospho-NF-κB-P65. Moreover, in CTEPH-ECs, IL-8, IL-1β, MCP-1, CCL5, and VCAM-1 mRNA levels were upregulated compared to controls. To assess the involvement of NF-κB signaling in the inflammatory activation, CTEPH-ECs were incubated with the NF-κB inhibitor Bay 11-7085. The increase of pro-inflammatory cytokines was abolished when cells were incubated with the NF-κB inhibitor .

In summary we show that CTEPH-ECs have a pro-inflammatory status and that blocking NF-κB signaling reduced the production of inflammatory factors in CTEPH-ECs. Therefore our results show that the increased pro-inflammatory status of CTEPH-ECs is, at least partially, regulated through activation of NF-κB signaling.

Keywords: Chronic thromboembolic pulmonary hypertension – inflammation – nuclear factor-κB signaling – endothelial dysfunction

INTRODUCTION

Chronic thromboembolic pulmonary hypertension (CTEPH) is a severe cause of pulmonary hypertension (PH) resulting from unresolved pulmonary emboli (PE) that obstruct main pulmonary arteries. In addition, vascular remodeling of muscular pulmonary arteries similar to the arteriopathy observed in pulmonary arterial hypertension (PAH) is found in CTEPH [1-3]. CTEPH patients, without medical intervention, have poor prognosis with a 5-year survival rate between 10-30%, depending on the mean pulmonary artery pressure [4]. Pulmonary endarterectomy (PEA), to remove fibrotic organized clots from pulmonary arteries, is the gold standard treatment for eligible patients with CTEPH and significantly improves patients' survival and hemodynamics [5]. The invasiveness of PEA surgery together with insufficient effects of additional treatment options for inoperable patients and for patients with recurrent/persistent PH (up to 35%) after PEA point out the importance of resolving new, potential curative, targets in CTEPH pathogenesis [4].

Only 75% of patients with CTEPH have history of symptomatic acute PE [6]. To date, the molecular and cellular mechanisms behind the lack of thrombus resolution and vascular remodeling that result in CTEPH remain unclear. The frequently observed remodeling in non-occluded arteries and small pulmonary arteries similar to that observed in PAH supports the presence of endothelial dysfunction in CTEPH pathogenesis [2]. Studies have reported the involvement of endothelial cells (ECs) in the process of thrombi organization and remodeling of surrounding pulmonary arteries through impaired angiogenesis, changes in EC function and increased production of inflammatory cytokines and adhesion molecules [7-12]. The pulmonary endothelium is an important interface between the circulating blood and underlying tissues. Through the production and release of cytokines, chemokines and adhesion molecules it controls inflammatory cell adhesion and trafficking [13]. Disturbances in endothelial inflammatory processes are central in cardiovascular disease development and progression including PH. Studies, both in PAH lungs and animal models of PAH, showed crucial involvement of inflammation in pulmonary vascular remodeling [14-19]. However, the regulation of inflammation in CTEPH-EC is largely unknown.

The expression and production of inflammatory mediators in the endothelium is controlled by nuclear factor (NF)-κB, a central regulator of inflammation [20,21]. Activated NF-κB translocates into the nucleus to promote expression of target genes such as tumor necrosis

factor alpha (TNFα), interleukin-1-beta (IL-1β), IL-8, monocyte chemoattractant protein 1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) among others [20]. Several of these endothelial-derived inflammatory factors have shown to influence cell survival, proliferation and migration of cells within the vascular wall and thereby contributing to vascular remodeling [10,21-23]. Therefore, blocking nuclear translocation of NF-κB using inhibitory small molecules like Bay 11-7085 [24], may be a method to study the involvement of the NF-κB pathway in the regulation of inflammatory pathways in CTEPH-ECs.

Here, we hypothesized that ECs from CTEPH patients exhibit a pro-inflammatory status through sustained activation of the NF-κB pathway which contributes to EC dysfunction and abnormalities involved in CTEPH pathophysiology.

MATEREIAL AND METHODS

Study population and samples collected

This study included CTEPH-ECs derived from 8 different endarterectomy specimens from patients with CTEPH who underwent pulmonary endarterectomy at the Hospital Clinic of Barcelona, Spain. The study was approved by the institutional Ethics Committee of the Hospital Clínic of Barcelona and informed consent was obtained from all patients. All patients were diagnosed according to the 2015 ESC/ERS Guidelines [25].

Pulmonary endothelial cell isolation and culture

ECs isolated from endarterectomy specimens, referred to as CTEPH-ECs, were cultured as previously described [26]. In short, ECs were plated onto 0.1% gelatin-coated wells and grown in endothelium cell medium (ScienceCell Research Laboratories) supplemented with endothelial cell growth supplement, 5% fetal bovine serum (FBS) and Penicillin/Streptomycin solution (ScienceCell Research Laboratories). The cell phenotype was characterized by staining the cells with antibodies against a panel of endothelial and smooth muscle cell-specific markers, including endothelial nitric oxide synthase (eNOS) and alpha smooth muscle Actin (αSMA) [26]. Patients characteristics are presented in supplementary Table 1. Three different batches of human pulmonary artery endothelial cells (HPAEC) (Lonza, CC-2530) were used as control cells.

Gene expression analysis

The levels of IL-8, MCP-1, C-C motif chemokine ligand 5 (CCL5), IL-1β, ICAM-1 and VCAM-1 were measured from CTEPH-EC cultured in low serum conditions (endothelial cell medium with 0.1% FBS) by real-time quantitative PCR (n=8 per group). Bay 11-7085 (Cayman Chemicals, 14795, USA), a potent NF-κB inhibitor, was applied at 1μM final concentration. Treatments were performed in endothelial cell medium with 0.1% FBS and stimuli were provided for 6 hours. Total RNA was extracted using the ReliaPrep™ RNA Cell Miniprep system (Promega) and concentrations were determined by spectrophotometry. Reverse transcription was performed using a RevertAid First strand cDNA synthesis kit (ThermoFisher Scientific). For qRT-PCR, QuantiTect® SYBR® Green PCR kit (Qiagen) and specific primers were used on the ViiA7 Real-Time PCR system (Applied Biosystems). Relative quantification was calculated by normalizing the Ct (threshold cycle) of the gene of interest to the Ct of an endogenous control (TBP and ARP) in the same sample, using the comparative ΔΔCt method. All primers were produced by Invitrogen and primer sequences can be found in supplementary Table 2.

Immunostaining

Paraffin-embedded sections (5μm) were incubated at 4˚C overnight with antibodies against phospho-NF-κB P65 (rabbit anti-phospho-p65, 1:100; Signalway antibody, 11260), platelet endothelial cell adhesion molecule (goat anti-CD31, 1:1000; R&D Systems AF3628). Sections were then incubated with anti-rabbit Alexa Fluor 555 (Invitrogen, A31572) or anti-goat Alexa Fluor 647 (Invitrogen, A21447) secondary antibodies for 2 hours. Nuclei were counterstained using Hoechst 34580 (1:800; Sigma-Aldrich, 63493). Sections were imaged using slidescanner 3DHistech Pannoramic 250.

CTEPH-EC and HPAEC were seeded at 1 x 10^5 cells/mL in 24-well plates pre-coated with 1% gelatin and covered with glass cover slides and allowed to grow for 48 hours. Cells were washed with cold PBS and fixated with 4% paraformaldehyde. Next, cells were permeabilized with PBS/0.25% Triton and blocked with PBS/0.1% Triton/10% FBS. Next, slides were incubated at 4˚C overnight with antibodies against phospho-P65 (rabbit anti-phospho-P65 1:100; Signalway antibody, 11260) and CD31 (mouse anti-CD31 1:250; Dako, M0823). The slides were then incubated with anti-rabbit Alexa Fluor 488 (Invitrogen, A21206) or anti-mouse Alexa Fluor 647 (Invitrogen, A21447) secondary antibodies for 1 hour. Nuclei were

counterstained with DAPI (ProLong™ Gold Antifade Mountant with DAPI, Invitrogen, P36931). Slides were imaged using a Leica DM6B microscope and the mean fluorescence intensity was quantified using ImageJ software.

Statistical analysis

Results are described as mean ± standard deviation and were compared using unpaired t-test (immunofluorescence analysis) or unpaired t-test with Welch's correction (gene expression analysis). Statistical analyses were performed using GraphPad Software. P-values <0.05 were considered statistically significant.

RESULTS

Endothelial localization of phospho-P65 in CTEPH specimen

Pulmonary endarterectomy specimens were stained for the presence of phospho-NF-κB P65 (pP65) and PECAM/CD31. Thrombus vessels present in the tissue stained positive for PECAM/CD31. Interestingly, in these areas pP65 showed to colocalize with PECAM/CD31, indicating the presence of pP65 ECs lining vessels within the thrombus. Cells positive for pP65, but negative for PECAM/CD31, were also observed throughout the tissue. These cells are most likely infiltrating inflammatory cells (**Figure 1A-B**).

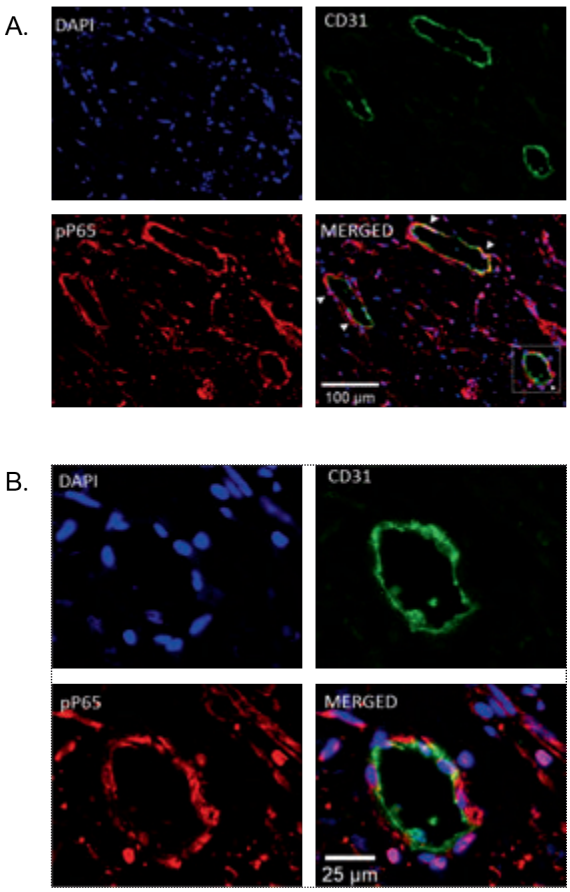

Figure 1. PEA immunofluorescence. (A) Localization of phospho-NF-κB P65 (pP65) in vessels in endarterectomy specimens from patients with CTEPH, using double labeling with PECAM (green) and pP65 (red). **(B)** pP65 immunoreactivity was observed in endothelial cells from vessels within the thrombus (magenta, indicated by the white arrows). Nuclei were counterstained with DAPI (blue). Scale bar 100µm (panel A) and 25µm (panel B).

Fluorescence staining of phospho-P65 in cultured CTEPH-ECs

NF-κB activation in CTEPH-ECs was studied by monitoring nuclear translocation of the p65 subunit by immunofluorescence. Cultured CTEPH-ECs, isolated from four different pulmonary endarterectomy specimens, and three different control HPAEC were stained for PECAM/CD31 and pP65. Both CTEPH-ECs and HPAECs were positive for endothelial marker PECAM/CD31 (**Figure 2A**). The number of cells showing nuclear anti-p65 was measured from random selected areas of the coverslip. CTEPH-ECs and HPAECs showed positive fluorescence signal for pP65 which was mainly found within DAPI positive nuclei (**Figure 2A**). The amount of nuclear translocation of the pP65 subunit was determined by quantifying the intensity of the fluorescence signal inside the nuclei, and CTEPH-ECs showed a trend towards a 2.4 fold higher (p=0.06) nuclear signal intensity compared to control cells (**Figure 2B**).

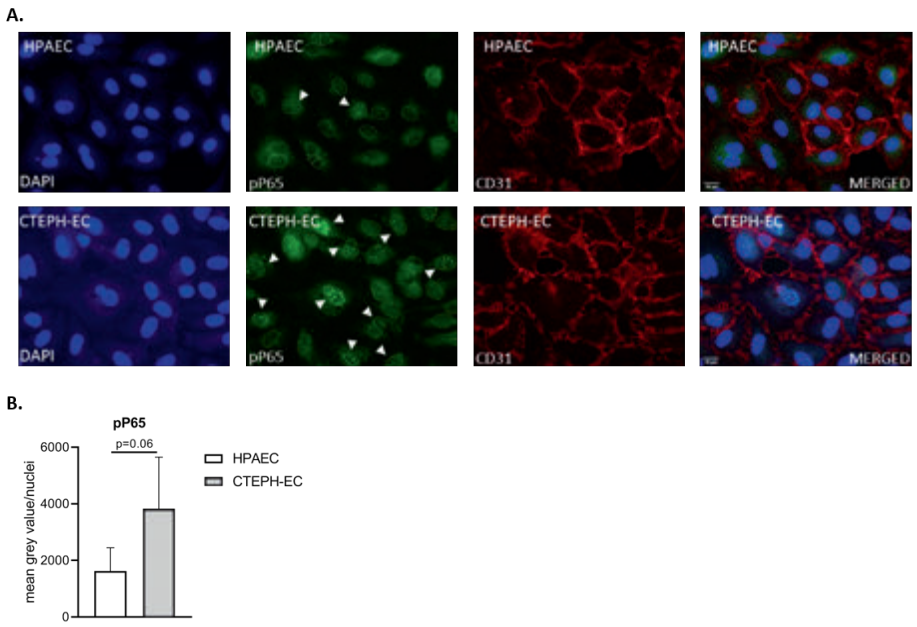

Figure 2. Fluorescence staining of phospho-P65 in cultured ECs. (A) HPAECs (top) and CTEPH-ECs (bottom) were stained for endothelial marker PECAM/CD31 (red) and phospho-NF-κB P65 (pP65; green). Both ECs showed presence of PECAM/CD31 and nuclear pP65 staining (indicated by the white arrows). Nuclei were counterstained with DAPI, scale bar 10μm. **(B)** Presence of nuclear PP65 was quantified in both CTEPH-ECs and HPAECs. CTEPH-ECs showed 2.4 fold higher presence of nuclear PP65 compared to control cells (p=0.06) (unpaired t-test); CTEPH-ECs, n=4; HPAECs, n=3 Data is expressed as mean ± SD.

Expression of pro-inflammatory cytokines in CTEPH-ECs

To investigate the role of increased NF-κB activation in CTEPH-ECs, cytokines downstream the NF-κB signaling pathway were measured. mRNA expression levels of IL-8 and MCP-1 showed a 5.5 fold (p=0.009) and a 2.5 fold (p=0.05) increase, respectively, compared to control cells. CCL5 showed a 7 fold increase (p=0.03) in mRNA expression levels compared to control cells. ICAM-1 showed a trend towards increased levels in CTEPH-ECs but did not reach significance (p=0.07). IL-1β showed a 6 fold increase (p=0.02) in mRNA levels compared to HPAECs. At last, mRNA levels of VCAM-1 showed a 3 fold (p=0.03) increase in CTEPH-ECs compared to control cells (**Figure 3**).

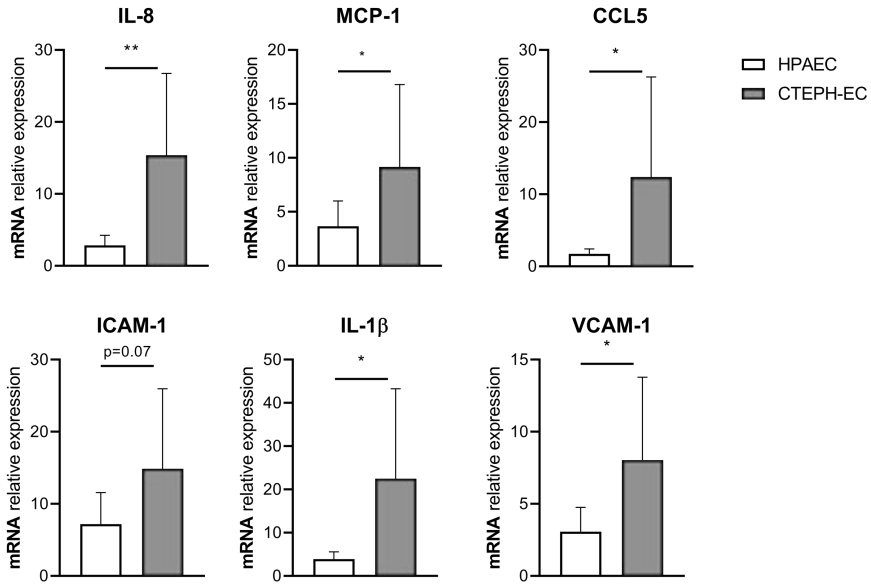

Figure 3. Inflammatory cytokines in cultured CTEPH-ECs. mRNA expression levels of IL-8, IL-1β, MCP-1, CCL5, VCAM-1 and ICAM-1 were found increased in CTEPH-ECs compared to HPAECs. CTEPH-EC, n=8; HPAEC, n=3; unpaired t-test with Welch's correction , p<0.05 = *, data is expressed as mean ± SD.

Effect of NF-κB inhibition in CTEPH-ECs

To examine if a reduction in NF-κB activity leads to reduced expression of inflammatory cytokines, cultured CTEPH-ECs were incubated with 1μM Bay 11-7085, a NF-κB inhibitor, which at the same time demonstrates the role of NF-κB signaling on the expression of pro-inflammatory cytokines. VCAM-1 showed a 1.6 fold reduction (p=0.02) in mRNA levels in CTEPH-ECs compared to control condition without inhibitor. ICAM-1 and MCP-1 showed a tendency towards reduction after incubation with Bay 11-7085 but did not reach significance (p=0.09 and p=0.08, respectively). mRNA levels of IL-8 were found not changed in CTEPH-ECs after incubation with Bay 11-7085 compared to the control condition (**Figure 4**).

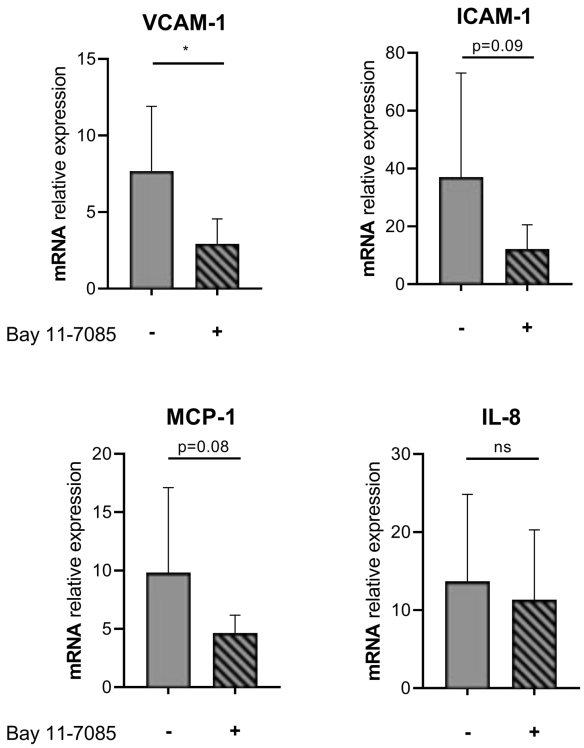

Figure 4. Effect of NF-κB inhibition on CTEPH-ECs. Cultured CTEPH-ECs were incubated with NF-κB inhibitor Bay 11-7085 at a final concentration of 1μM. VCAM-1 showed a significant reduction in mRNA levels compared to control condition without Bay. mRNA levels of ICAM-1 and MCP-1 showed a trend towards reduction after Bay 11-7085 incubation. IL-8 mRNA expression levels were found similar between CTEPH-ECs incubated with Bay 11-7085 and without. CTEPH-ECs, n=5; unpaired t-test, $p<0.05$ = *, data is expressed as mean ± SD.

DISCUSSION

In this study we showed the presence of pP65 positive thrombus vessels in CTEPH PEA specimens. Furthermore, we showed increased nuclear localization of pP65 in cultured CTEPH-ECs indicating the presence of more active NF-κB signaling in CTEPH-EC compared to control HPAECs. Increased expression of IL-8, IL-1β, CCL5, MCP-1 and VCAM-1, all inflammatory factors downstream of NF-κB signaling pathway, in CTEPH-ECs compared to controls confirmed this observation. Furthermore, CTEPH-ECs incubated with the NF-κB inhibitor Bay 11-7085 showed a decrease in the expression of VCAM-1, ICAM-1 and MCP-1.

Our observation that cytokines are elevated in CTEPH-ECs is in line with a comprehensive analysis by Zabini *et al.* where they found that the expression of inflammatory cytokines, including IL-6, IL-8 and MCP-1, was significantly higher in serum and PEA tissue of CTEPH patients compared to healthy controls and healthy lung tissue, respectively [27]. Despite the clear evidence of an inflammatory component in CTEPH, the regulation of the inflammatory response of ECs in CTEPH is less studied and only few studies have been performed to better understand the regulatory pathways of inflammation in CTEPH pathogenesis. Ataam *et al*. recently reported that increased ICAM-1 contributes to EC dysfunction in CTEPH [11]. In the present study we show for the first time that cultured CTEPH-ECs have increased nuclear phospho-p65 compared to control cells. NF-κB activation involves besides phosphorylation and degradation of inhibitory proteins IκBs, also phosphorylation of the P65 subunit. This phosphorylation is an important event to enhance the transcriptional capacity of DNA bound NF-κB and activates the transcription of key targets VCAM-1, ICAM-1 and MCP-1 in ECs [28-30]. Therefore, our results indicate that the observed increase in phosphorylation of the P65 subunit is key in the elevated expression of inflammatory cytokines in CTEPH-ECs. Several inflammatory-related diseases such as cancer, atherosclerosis, restenosis and asthma have been associated with increased activation of NF-κB and expression of its downstream mediators [31-35]. Based on the increase in NF-κB activation observed, we hypothesized that inhibition of NF-κB signaling could reverse the pro-inflammatory profile in CTEPH-ECs. In the current study we found that inhibition of NF-κB signaling, by blocking the phosphorylation of NF-κB inhibitor IκB-α with inhibitory small molecule Bay 11-7085, results in reduced expression of inflammatory cytokines VCAM-1, ICAM-1 and MCP-1 in CTEPH-ECs. Cancers such as multiple myeloma, where NF-κB signaling plays a significant role in the pathogenesis,

have been successfully treated with drugs that have NF-κB as their primary or secondary target [36]. These findings confirm that the increased inflammatory cytokines in CTEPH-ECs are, at least partially, regulated through NF-κB signaling.

Based on the results obtained in this study we can conclude that CTEPH-ECs have a pro-inflammatory status as shown by the increased production of inflammatory cytokines IL-8, MCP-1, IL-1β, CCL5, ICAM-1 and VCAM-1. More importantly, we showed that the increased inflammatory cytokines observed in CTEPH-ECs are, at least partially, regulated through NF-κB signaling and that blocking NF-κB activation might be an important target in CTEPH to prevent disease progression or recurrent PH.

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Supplementary Table 1. Clinical features and hemodynamic parameters

	CTEPH (n=8)
Female/male	5/3
Age years	63.15 ± 10.88
BMI kg·m^{-2}	25.97 ± 4.35
mPAP mmHg	42.13 ± 9.52
PVR dyn·s·m^{-5}	706.75 ± 230.07
PAOP mmHg	10.25 ± 3.77
Cardiac index L·min^{-1}·m^{-2}	2.23 ± 0.61
Right atrial pressure mmHg	9.38 ± 4.63
SvO2 %	59.50 ± 7.86
6MWD m	398.13 ± 102.02
BNP pg·mL^{-1}	209.54 ± 360.12
WHO FC	
I	0
II	2
III	6

Data are presented as n or mean ± SD. CTEPH: chronic thromboembolic pulmonary hypertension; BMI: body mass index; mPAP: mean pulmonary artery pressure; PVR: pulmonary vascular resistance; PAOP: pulmonary artery occlusion pressure; SvO2: mixed venous oxygen blood saturation; 6MWD: 6-minute walking distance; BNP: brain natriuretic peptide; WHO FC: world health organization functional class

Supplementary Table 2. Primer sequences

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
IL8	CTGGCCGTGGCTCTCTTG	CTTGGCAAAACTGCACCTTCA
MCP1	CTGTGCCTGCTGCTCATAG	AGCTTCTTTGGGACACTTGC
CCL5	GCATCTGCCTCCCCATATTC	AGTGGGCGGGCAATGTAG
IL-1β	CGAATCTCCGACCACCACTAC	TCCATGGCCACAACAACTGA
ICAM	CTGCAGACAGTGACCATC	GTCCAGTTTCCCGGACAA
VCAM	CAGGCTGGAAGAAGCAGA	GGCCTTTCGGATGGTATAGG
ARP	CACCATTGAAATCCTGAGTGATGT	TGACCAGCCGAAAGGAGAAG
TBP	TGGAAAAGTTGTATTAACAGGTGCT	GCAAGGGTACATGAGAGCCA

CHAPTER 8

SUMMARY AND FUTURE PERSPECTIVES

GENERAL SUMMARY

Rationale

Pulmonary hypertension (PH) is a condition of increased blood pressure within the arteries of the lung (mPAP > 20mmHg) which affects approximately 1% of the global population [1,2]. CTEPH, group 4 PH, is characterized by unresolved pulmonary emboli and pulmonary vascular remodeling of both occluded and non-occluded vessels. CTEPH patients, if left untreated, have poor prognosis with a 5-year survival between 10-30% depending on the patient's mPAP [3]. The gold standard treatment for CTEPH patients is pulmonary endarterectomy, the removal of occluding thromboembolic material from the pulmonary arteries, with a possible curative outcome [4]. Although positive outcome associated with PEA surgery, 40% of CTEPH patients are not operable and up to one-third has persistent or recurrent PH pointing out the need for better understanding of CTEPH pathogenesis [5,6]. To date, the molecular and cellular mechanisms behind the lack of thrombus resolution and vascular remodeling that result in CTEPH remain unclear. One hypothesis is the existence of primary arteriopathy with endothelial dysfunction as a driving factor.

This thesis

The general aim of this thesis was to improve the understanding of CTEPH pathophysiology by focusing on patient endothelial cell (EC) behaviour and function. For this purpose, we isolated ECs from vascular material collected at pulmonary endarterectomy from patients with CTEPH (referred to as CTEPH-EC) and validated them as an *in vitro* model for studying endothelial pathology in CTEPH. Currently, studies assessing EC abnormalities and molecular mechanisms behind pulmonary vascular remodelling are scarce in CTEPH. Therefore, investigating the underlying mechanisms of pulmonary embolism nonresolution and subsequent vascular remodelling are essential to create a better understanding of CTEPH pathology but also might help to find novel targets for CTEPH treatment. We found several molecular abnormalities in CTEPH-EC that can give rise to a better understanding of disease development and that offer new therapeutic targets.

In **Chapter 2** we addressed the latest advances on the role of endothelial dysfunction in all forms of PH. Current therapies mainly focus on three key vasomotor pathways: 1) prostacyclin; 2) endothelin-1; 3) nitric oxide-cyclic guanosine monophosphate, to control

symptoms by targeting pulmonary dilatation but hardly focus on the origin of the disease: endothelial dysfunction. We described a wide range of factors such as endothelial to mesenchymal transition, inflammation, thrombosis and coagulation, apoptosis, vasoactive imbalance and genetic factors that are known to contribute to endothelial dysfunction. Moreover, the fact that many PAH-based therapies are not always beneficial for patients with other forms of PH, illustrates the complexity of the phenomenon of endothelial dysfunction. We also provided an update on potential targets to restore EC function and highlighted pitfalls that need to be overcome in order to find pharmacological curative treatments for PH. Overall, a better understanding of cellular changes and molecular mechanisms involved in the process of vascular remodeling will be the stepping stone to future targeted PH therapies and earlier disease diagnosis.

Intravascular occluding material extracted at pulmonary endarterectomy offers a unique opportunity to evaluate ECs at the site of damage in the pulmonary arteries of patients with CTEPH. In **Chapter 3** we described the isolation of EC from pulmonary endarterectomy specimen from patients with CTEPH. We validated these cells as an attractive *in vitro* model for endothelial pathology by assessing endothelial and mitochondrial function. In this way we identified key targets and molecular pathways for prevention and treatment of the disease. CTEPH-EC were characterized as endothelial cells in origin by the presence of endothelial cobblestone morphology of the monolayer cultures and by the expression of endothelial markers such as eNOS, CD31 and von Willebrand Factor, both at mRNA and protein level. Furthermore, CTEPH-EC were found to be hyperproliferative and apoptosis resistant compared to healthy human pulmonary artery endothelial cells (HPAEC). Despite an increase in proliferation, CTEPH-EC showed reduced angiogenesis and migration as the number of tube-like structures formed and scratch wound closure, respectively, was lower compared to control cells. We showed that CTEPH-EC mitochondria presented an abnormal morphology with irregular inner membrane and cristae together with an imbalance in mitochondrial dynamics and increased mROS. As increased mROS indicates a dysregulation between anti-oxidant systems and ROS production, we questioned whether dismutase enzymes were differently expressed in CTEPH-EC. We found that anti-oxidant enzyme SOD2 was downregulated in CTEPH-EC compared to control cells. Next to that, we also demonstrated increased oxidative stress in pulmonary endarterectomy specimen. Taken together, we have

identified for the first time several novel molecular pathways in CTEPH-EC likely to influence thrombus resolution, vessel wall remodelling and development of CTEPH. We showed alterations in oxidative/nitrative stress, antioxidant production, mitochondrial homeostasis and adhesion molecules that could be involved in the pathophysiology of CTEPH.

In **Chapter 4** we described an emerging topic in cardiovascular disease that suggests the occurrence of potentially actionable metabolic abnormalities in pulmonary hypertension. We focused on recent observations on the role of EC dysfunction and metabolism in the pathophysiology of PAH and CTEPH, and the therapeutic opportunities they may provide. We described the unique role of EC metabolism in regard to its function to maintain a quiescent non-proliferative endothelium. Increasing evidence points to a critical role played by metabolic dysregulation involving downregulation of oxidative phosphorylation and fatty acid metabolism and an increase in glycolysis that promotes proliferation and inhibits apoptosis. Pre-clinical studies have gained insight in targeting EC metabolism to restore normal endothelial function. Nonetheless, due to the challenges of target selectivity and the complexity of cell metabolism itself, future metabolic studies in larger PAH and CTEPH patient groups will be necessary in the search for new pharmacological interventions and biomarkers.

As mentioned in chapter 4, a deeper understanding of EC metabolism in CTEPH will contribute to the improved understanding of molecular mechanisms behind CTEPH pathophysiology and as such will help to define novel therapies. Therefore, in **Chapter 5** we examined alterations in metabolism of CTEPH-EC that might be involved in EC dysfunction and subsequent vascular remodeling occurring in pulmonary arteries surrounding thrombi in CTEPH patients. We observed, in contrast to the increased glycolytic profile present in PAH-EC, a decrease in key and rate-limiting enzymes of the glycolytic pathway. In addition, we found a downregulation in pyruvate dehydrogenase kinase, the enzyme that prevents pyruvate entry into the mitochondria, together with a downregulation in glutamine metabolism. At last, pentose phosphate pathway- and fatty acid oxidation-associated key metabolic enzymes were not found different in CTEPH-EC compared to control ECs. Modulation of glycolytic regulator PFKFB3 but also glutamine metabolism has previously been shown to be involved in the angiogenic process. Therefore, we believe that a decrease in glycolysis and impaired glutamine metabolism in CTEPH-EC, isolated from vessels surrounding the thromboembolic

lesions, could provide insights into the lack of thrombus recanalization and resolution occurring in CTEPH.

Both PAH and CTEPH are characterised by pulmonary vascular remodeling so the idea of similar molecular and cellular changes in the search for a better understanding of disease pathology is very appealing. However, in chapter 5 we showed that CTEPH-EC are less glycolytic compared to control EC. This finding is very intresting because in PAH it is well known that PAH-EC are higly glycolytic. Therefore we performed in **Chapter 6** a systemic metabolic comparison of EC isolated from CTEPH patients that underwent pulmonary endarterectomy and from PAH patients that underwent lung transplantation. To better understand potential differences in metabolism, we assessed basal viability and migration, and viability upon inhibition by metabolic regulators of glycolysis and glutamine metabolism. Interestingly, we showed a pronounced difference in gene expression of glycolytic and glutamine-related enzymes. A decrease in glycolytic enzymes in CTEPH-EC was accompanied by a decrease in mRNA and protein levels of pyruvate controlling enzyme pyruvate dehydrogenase in CTEPH-EC compared to PAH-EC. No differences were observed in pentose phosphate pathway-related enzymes at both mRNA and protein level. PAH-EC seemed to have a higher basal viability compared to CTEPH-EC, which is in line with an increase in glycolysis in PAH-EC. Remarkably both CTEPH-EC and PAH-EC showed a drop in viability in response to metabolic inhibitors but no difference in viability between both groups was observed. At last, although clear metabolic differences between CTEPH-EC and PAH-EC are detectable, no difference in migration capacity could be observed between CTEPH-EC and PAH-EC. This study confirmed the reduced glycolysis in CTEPH-ECs observed in chapter 5 but more interestingly showed clear differences in glycolysis and glutamine metabolism between CTEPH-EC and PAH-EC. Such a difference in metabolism between both diseases creates better understanding of CTEPH specific disease pathophysiology and eventually could contribute to development of disease specific therapies.

At last, inflammatory processes are central in cardiovascular disease development and progression, including PH. In CTEPH there is increasing evidence of inflammatory changes that promote vascular remodeling, thrombus formation and endothelial dysfunction. However, the regulation of basal inflammation in CTEPH-EC is largely unknown. Therefor we studied in **Chapter 7** the potential involvement of sustained activation of the NF-κB pathway as possible

driver behind the pro-inflammatory status in CTEPH-EC that might be play a role in CTEPH progression. We found that phospho-p65 was present in PECAM/CD31 positive vessels within pulmonary endarterectomy specimens from CTEPH patients. Furthermore we showed increased presence of nuclear phospho-p65 in cultured CTEPH-EC, indicating the presence of more active NF-κB in CTEPH-EC compared to control cells. In line with these observations, several inflammatory factors downstream NF-κB signaling, IL-8, IL-1β, MCP-1, ICAM-1 and VCAM-1, were found to be upregulated in CTEPH-EC compared to control cells. When CTEPH-EC were incubated with a NF-κB inhibitor, we showed a significant decrease in mRNA levels of MCP-1 and VCAM-1. Based on the results obtained in this study we can conclude that CTEPH-EC have a pro-inflammatory status under basal conditions as shown by the increased production of inflammatory cytokines IL-8, IL-1β, ICAM-1 and VCAM-1. More importantly, we showed that the increased basal inflammatory cytokines observed in CTEPH-EC are, at least partially, regulated through NF-κB signaling. This implies that the NF-κB pathway and inflammation could be important contributors to development and progression of CTEPH.

In this thesis, the role of endothelial dysfunction as driver in CTEPH pathology has been investigated. Our data provide a first, but important indication of endothelial cell abnormalities in CTEPH-EC that might be amenable for the development of therapeutic strategies, as well be potential biomarkers for early detection of CTEPH. Endothelial dysfunction has long been implicated in the initiation and progression of several vascular diseases such as atherosclerosis and PAH. We have shown that patient specific derived EC are a valuable tool to study EC characteristics at the lesion side. We have found that these CTEPH-EC are hyperproliferative but less angiogenic, have increased expression of adhesion molecules, present dysfunctional mitochondria and unbalanced oxidative stress/antioxidant production. In addition, we have shown that CTEPH-EC have metabolic impairments that are associated with a reduction in glycolysis and glutamine metabolism. At last, we have also shown that CTEPH-EC are pro-inflammatory in a, at least partially, NF-κB dependent manner.

FUTURE PERSPECTIVES

The endothelial dysfunction that we demonstrated in CTEPH might be key in the non-resolution of thrombi, promote thrombi stabilisation, vessel wall remodelling and contribute to the progression towards CTEPH. We identified several abnormalities in CTEPH-EC that

could play a role in CTEPH pathophysiology. We described alterations in key processes such as angiogenesis and migration, oxidative stress, metabolism and inflammation. Each of these processes may represent targets for novel therapies or biomarkers.

CTEPH differs with other forms of PH in being potentially curable by surgery. Nevertheless, surgical invasiveness and complications, patient comorbidities, surgical inaccessible lesions and persistent or recurrent PH are important reasons for a better understanding of disease pathology but also for the development of novel therapeutic strategies. Current drug-based therapies mainly focus on symptom control by restoring pulmonary vasodilatation but they have no or limited effect on progressive remodeling. Furthermore, their use in CTEPH is mainly based on clinical and pathological similarities between PAH and CTEPH [5]. As we have shown in **Chapter 6**, there are differences in cell viability and cell metabolism between ECs from PAH and CTEPH patients. This also indicates that drugs, currently being studied in experimental PAH, might not always be beneficial in CTEPH. Hence, studies with CTEPH-EC are a powerful tool to get more insight in the pathways involved in CTEPH pathology and in the search for CTEPH-specific therapeutic interventions. The next step will be using these disease-specific CTEPH-EC in more advanced 3D cell culture systems such as flow models and organ-on-a-chip models. Such dynamic models allow the exposure of ECs to both physiological and pathological stimuli such as flow, stretch and nutrients, which mimics stimuli present *in vivo*. This will improve the mechanistic understanding of dysfunctional CTEPH-EC in thrombus non-resolution and vascular remodeling. In addition, these systems are also valuable for testing novel potential pharmacological compounds that will benefit inoperable CTEPH patients or patients with residual or recurrent pulmonary hypertension after surgery.

Angiogenesis and metabolism

Angiogenesis is a physiological process through which new blood vessels form. The angiogenic process is regulated by genetic signals such as VEGF and Notch but also majorly influenced by EC metabolism. It is known that glycolysis is key in angiogenesis and reduction in glycolysis has been shown to impair vessel sprouting [7]. Impaired angiogenesis has been proposed as potential mechanism behind the delay of thrombus resolution in CTEPH experimental models [8]. Therefore, based on the reduced angiogenesis observed in **Chapter 3** and the reduced cell

metabolism found in **Chapter 5 and 6** we believe that angiogenesis is an important target in CTEPH.

Augmenting local angiogenesis via therapeutics can be interesting to recanalize obstructive remodeled thrombi, (partially) restore the blood flow and reduce long-term complications in CTEPH such as right heart failure. Based on the data presented in this thesis, a first step towards restoring the angiogenic potential of CTEPH-EC will be the delivery of factors that promote angiogenesis. These factors can be either energy substrates such as glucose, glutamine and fatty acids or oxidative phosphorylation inhibitors, since an in vitro study in ECs has shown that blocking oxidative phosphorylation induces a switch towards increased glycolysis [9]. Oxidative phosphorylation inhibitors are already available to treat certain types of cancer but aim for cell death rather than promoting glycolysis[10]. Future studies will be needed to investigate whether these drugs in adjusted concentrations could be interesting in CTEPH to promote glycolysis and subsequent angiogenesis. Although the use of pro-angiogenic therapies in CTEPH seems very appealing, it is important to be cautious with regards to the use of pro-angiogenic therapies. Several other vascular diseases such as pulmonary arterial hypertension and cancer are associated with uncontrolled angiogenesis in parallel with an increase in cell metabolism. It will therefore be important to carefully monitor for any potential off-target effects that promote unwanted angiogenesis and remodeling in nonoccluded arteries in CTEPH patients. One possible way to assure more specific delivery in areas of occlusion by remodeled thrombi, is via local delivery of pro-angiogenic factors or genes by e.g. shear-stress-sensitive nanoparticles. The application of such site-specific shear-sensitive drug delivery systems has been shown to be effective in the local delivery of antiplatelet drugs in microfluid and mouse thrombosis models [11]. In addition, another study using a nanoparticle delivery of pro-angiogenic transcription factors *Foxf1* and *Foxm1* has shown to effectively enhance angiogenesis in a mouse model of bronchopulmonary dysplasia [12]. It will be of great interest for future studies to investigate if the use of those delivery methods will also be applicable in the therapy of CTEPH.

Oxidative stress

In **Chapter 3** we observed mitochondrial dysfunction and an increase in oxidative stress in CTEPH-EC. Factors of oxidative stress were also observed in serum and tissue samples from CTEPH patients. It is to assume that the existence of increased oxidative stress in EC is an

important contributor to the induction of endothelial dysfunction and vascular changes observed in CTEPH.

CTEPH is known to be diagnosed at advanced stage of the disease due to a long period of nonspecific symptoms after initial pulmonary embolism. Oxidative stress biomarkers in the form of serum oxidized proteins and urinary levels of oxidative markers could allow earlier disease detection. One such oxidative marker is 8-oxo-2'-deoxyguanosine (8-oxo-dG), which has been found to be detectable in the urine of prediabetes patients as an indicator for early oxidative damage/endothelial dysfunction [13]. The non-invasive character of sampling urine opens a window for measuring oxidative markers in CTEPH to evaluate the degree of cellular damage. More interestingly, sampling urine in patients after a thrombotic event over a longer period of time should be considered. Oxidative stress is involved in a wide range of vascular diseases, and the comparison of oxidative markers between patients that develop and do not develop CTEPH after pulmonary embolism could be promising to find one or more oxidative markers that are specifically related to key mechanisms involved in CTEPH.

The finding of increased oxidative stress in CTEPH holds also great therapeutic potential. Anti-oxidative therapy could focus on lowering mitochondrial ROS by the use of exogenous antioxidant systems such as vitamins [14]. However, due to limited or no therapeutic effect of such systems in cardiovascular diseases [15], its use will not be optimal in CTEPH. Specific delivery of antioxidants to the mitochondria or stimulating the expression of endogenous SOD2 is therefore more promising. In this manner off-target effects that impair the physiological functioning of ROS will be avoided. The use of mitochondria-targeted antioxidants will be of great interest as potential therapeutic in CTEPH to lower mitochondrial ROS and prevent further oxidative damage and vascular remodeling. One such antioxidant, MitoQ, is currently being investigated in a clinical trial (NCT2364648) in patients with chronic kidney disease, a disease also characterized by endothelial dysfunction. Administration of this agent in our cell culture model will help to determine if those type of agents can reverse or prevent oxidative stress in CTEPH. Restoring the healthy balance between fusion and fission to lower oxidative stress is another important target in CTEPH that could be promising to restore endothelial function. A study by Yue *et al.* has identified a small molecule S3, which potently activates mitochondrial fusion and restores mitochondrial function [16]. The use of such small molecules could be interesting in CTEPH-EC to enhance activity of fusion related

proteins MFN1/2 and OPA1 and restore EC function. In addition, overexpression of fusion related proteins also seems a promising path in restoring the fusion and fission balance in CTEPH [17,18]. Finally, mitochondrial transplantation therapy could be a way to ameliorate various points of mitochondrial dysfunction simultaneously in CTEPH. This technique has been successfully applied in a small group of patients who suffered ischemia-reperfusion injury and has shown to improve post-ischemic myocardial function[19]. In addition, studies in a mild PH rat model (chronic hypoxia) has shown that mitochondria transplanted into pulmonary arteries seemed to improve hemodynamic parameters [20]. Future studies therefore should focus on the potential of mitochondrial transplantation in CTEPH and if this could be a novel intervention to prolong the survival of CTEPH patients.

Inflammation.

In **chapter 7** we showed that the pro-inflammatory character of CTEPH-EC is, at least partially, regulated by increased NF-κB signaling. Signaling through NF-κB is also often found dysregulated in inflammatory diseases and cancer, and therefore our finding represents an important step forward in our understanding of key drivers in the pathophysiology of CTEPH [21,22]. Inflammation can cause local thrombosis, and thrombosis can amplify inflammation (thrombo-inflammation) which makes it an interesting target in CTEPH [23]. Whether NF-κB activation is a driver of chronic inflammation in CTEPH or bystander after pulmonary embolism remains to be determined. In either case, conditions of sustained inflammation are thought to be involved in thrombus nonresolution through increased collagen production and fibrosis [24]. Because of that, future studies should focus on expanding our knowledge on the causes and consequences of chronic activated NF-κB and whether blocking inflammation could also prevent recurrent thrombotic events in CTEPH.

If anti-inflammatory interventions are sufficient to treat advanced disease stage remains to be discovered but certain is that such interventions can have a positive impact on EC behavior and function which could be important to slow down disease progression in CTEPH patients. General drug-based anti-inflammatory strategies, such as systemic corticosteroids or NSAIDs, have many limitations as the side effects limit the therapeutic benefits. However, targeting specific inflammatory pathways has already demonstrated to be a promising strategy in cardiovascular diseases as the administration of a specific anti-IL-1β antibody improved the cardiovascular outcomes of patients with high systemic inflammatory

levels in the CANTOS trial [25]. In this thesis, we have showed that CTEPH-EC display higher levels of two strong pro-inflammatory mediators, IL-1β and IL-8. Hence, specific blockage of these cytokines by antibodies could be a promising therapeutic strategy in CTEPH. In addition, the involvement of increased NF-κB signaling in CTEPH-EC suggests that inhibition of the NF-κB pathway could also be a promising approach to reverse the pro-inflammatory state of the endothelium. Baicalein, a natural flavonoid, has been shown to possess anti-proliferative and anti-inflammatory properties through blocking NF-κB activation, but more interestingly, has shown to inhibit pulmonary vascular remodeling in experimental PAH models and therefore could be interesting in CTEPH [26].

In conclusion, this thesis provides several novel insights in the function and behaviour of patient-ECs that help to better understand CTEPH disease pathophysiology. We showed the existence of endothelial dysfunction, changes in cell metabolism and increased inflammation in CTEPH-EC. All factors that can significantly contribute to thrombus nonresolution and vascular remodelling that is observed in CTEPH. These key contributors provide novel leads in the search for novel biomarkers and therapeutic targets, and eventually prolong patient survival.

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APPENDIX

NEDERLANDSE SAMENVATTING

Introductie

Pulmonale hypertensie is een te hoge bloeddruk in de pulmonale arteriën die de werking van het hart nadelig beïnvloedt. Chronische trombo-embolische pulmonale hypertensie (CTEPH) is een bijzondere vorm van pulmonale hypertensie. CTEPH is een levensbedreigende aandoening die voortvloeit uit een blijvende obstructie van de pulmonale arteriën na een longembolie. Daarnaast vertonen patiënten een progressieve vasculaire remodellering van zowel de grote als de kleine bloedvaten. Naarmate de ziekte vordert en patiënten niet behandeld worden, komen tekens van rechterhartfalen meer op de voorgrond met een hoge kans op overlijden. Pulmonale trombo-endarteriëctomie (PTEA) is de voorkeursbehandeling bij CTEPH in geselecteerde patiënten. Ondanks verbetering van de symptomen, met nagenoeg een normalisatie van de hemodynamica na deze ingreep, is 40% van de patiënten inoperabel en heeft tot een derde van de patiënten aanhoudende of terugkerende pulmonale hypertensie. De pathofysiologische processen van CTEPH zijn momenteel onvolledig begrepen en blijven een belangrijke uitdaging voor wetenschappers. Een van de huidige hypotheses over de pathogenese van CTEPH stelt dat de aanwezigheid van een primaire endotheeldysfunctie in de pulmonale arteriën een drijvende factor is in het ontstaan van CTEPH.

Dit proefschrift

Het doel van dit proefschrift was om enkele mechanismen te ontrafelen achter de pathofysiologie van CTEPH, waarbij we voornamelijk focussen op de endotheelcellen van de patiënten. Om dit te kunnen onderzoeken hebben we endotheelcellen geïsoleerd uit vasculair materiaal van de patiënt, verzameld tijdens PTEA, en hierna vermeld als CTEPH-EC. Studies over de rol van endotheelcellen in CTEPH zijn schaars en net daarom is onderzoek naar veranderingen in moleculaire mechanismen belangrijk om de kennis van de pathofysiologie van CTEPH te verbeteren. Het beter begrijpen van de pathofysiologie is ook noodzakelijk voor de zoektocht naar nieuwe behandelingsstrategieën voor CTEPH-patiënten.

In **hoofdstuk 2** werd een overzicht gegeven van de laatste ontwikkelingen op het gebied van endotheeldysfunctie bij de 5 vormen van pulmonale hypertensie. De huidige therapieën in de behandeling van pulmonale hypertensie zijn voornamelijk gericht op pulmonale vasodilatatie om zo de symptomen en de klinische tekens te verbeteren, maar deze zijn helaas niet curatief. Hoewel deze therapieën de bloeddruk in de pulmonale arteriën verminderen,

hebben ze slechts een beperkt effect op de belangrijkste mechanismen van de ziekte, namelijk endotheeldysfunctie en remodellering van de vaatwand. Dit hoofdstuk beschreef verschillende factoren zoals afname in de beschikbaarheid van endogene vasodilatoren, inflammatie en genetische factoren waarvan gekend is dat ze bijdragen aan endotheeldysfunctie. Daarnaast werd in dit hoofdstuk ook dieper ingegaan op specifieke aspecten van endotheeldysfunctie die een potentieel doelwit kunnen zijn voor nieuwe behandelingsstrategieën in pulmonale hypertensie, maar werden ook valkuilen aangehaald die omzeild moeten worden om specifiek endotheeldysfunctie en de vasculaire pathologie in pulmonale hypertensie te behandelen.

Hoofdstuk 3 beschreef de isolatie en karakterisering van endotheelcellen uit vasculair materiaal, verzameld van CTEPH-patiënten tijdens PTEA. Op basis van de aanwezigheid van de typerende *cobblestone* (kasseien) morfologie van de cellen en de expressie van endotheel geassocieerde genen en enzymen, kan gesteld worden dat de geïsoleerde cellen, gebruikt in deze thesis, endotheelcellen zijn. Wanneer de functie van deze cellen werd vergeleken met deze van gezonde endotheelcellen, bleek dat CTEPH-EC sterk proliferatief zijn maar resistent voor geprogrammeerde celdood. Ondanks de toename in proliferatie, was migratie en angiogenese verminderd in CTEPH-EC. Daarnaast werden er ook verschillende afwijkingen gevonden in de functie en morfologie van de mitochondriën van de patiëntencellen. Bovendien was er een duidelijke toename merkbaar van schadelijke reactieve zuurstofverbindingen en een afname van de enzymen die verantwoordelijk zijn voor de opruiming van deze zuurstofverbindingen. Samenvattend toonde deze studie voor het eerst verschillende moleculaire en cellulaire veranderingen in CTEPH-EC die mogelijk bijdragen tot de pathologie van CTEPH.

In **hoofdstuk 4** werd ingegaan op de belangrijke rol van endotheelcelmetabolisme in het behouden van het normaal functioneren van het vaatendotheel. Metabole veranderingen zijn onder andere geassocieerd met een toename in de proliferatie van de endotheelcellen, een afname in celdood en remodellering van de vaatwand. Het verkrijgen van inzichten in de metabole veranderingen van pulmonale hypertensie is een belangrijke stap in het beter begrijpen van mechanismen die aan de basis liggen van de blijvende obstructie en remodellering van de pulmonale arteriën, twee belangrijke kenmerken van CTEPH.

Daarom beschreef **hoofdstuk 5** de aanwezigheid van metabole afwijkingen in CTEPH-EC. Dit hoofdstuk heeft aangetoond dat CTEPH-EC een afname vertonen in de expressie van enzymen die betrokken zijn in het gebruik van glucose en glutamine, twee belangrijke bronnen van cellulaire energie. Een toename in glucose en glutamine consumptie door endotheelcellen is belangrijk voor het vormen van nieuwe bloedvaatjes. Deze bevindingen suggereren dat een verminderd gebruik van glucose en glutamine door CTEPH-EC er voor zorgt dat er geen nieuwe bloedvaatjes, die nodig zijn om de trombus te re-kanaliseren en de bloedstroom te herstellen, worden gevormd.

Ondanks de gelijkenissen in remodellering van de vaatwand van groep 1 pulmonale hypertensie en groep 4 CTEPH, zagen we in hoofdstuk 5 een afname van het gebruik van glucose door CTEPH-EC. Uit voorgaande studies blijkt dat groep 1 pulmonale hypertensie gekenmerkt wordt door een verhoogd glucosegebruik. Om dit verschil in metabolisme beter te begrijpen werd in **hoofdstuk 6** een vergelijkende studie uitgevoerd met endotheelcellen van patiënten met groep 1 pulmonale hypertensie en patiënten met groep 4 CTEPH. Deze studie is uniek in het rechtstreeks vergelijken van endotheelcellen van beide groepen en bevestigt de afname in glucose gebruik door CTEPH-EC in vergelijking met endotheelcellen van groep 1. Ondanks de verschillen in celmetabolisme, zagen we geen functionele verschillen tussen de cellen van beide groepen. Deze bevinding is belangrijk voor het beter begrijpen van de pathologie van CTEPH, maar ook voor de zoektocht naar alternatieve therapeutische doelwitten die meer specifiek zijn voor CTEPH.

Ontstekingsprocessen staan centraal in het ontstaan en de progressie van hart- en vaatziekten. Ook in CTEPH wordt gedacht dat inflammatoire processen een belangrijke rol spelen in de ontwikkeling van de ziekte. Omdat de inflammatoire mechanismen in CTEPH-EC momenteel onvolledig begrepen zijn, werd in **hoofdstuk 7** de rol van NF-κB signaal transductie in CTEPH-EC als mogelijke drijfveer van een persisterend pro-inflammatoir endotheel in CTEPH bestudeerd. Deze studie heeft aangetoond dat NF-κB signaal transductie is toegenomen zowel in gekweekte CTEPH-EC als in het vasculaire materiaal dat verzameld werd tijdens PTEA. Daarnaast lieten we zien dat een verhoogde activiteit van NF-κB signalisatie in CTEPH-EC geassocieerd was met een toename in de productie van verschillende ontstekingsfactoren waarvan gekend is dat hun aanmaak rechtstreeks gestimuleerd wordt door NF-κB. Wanneer vervolgens de NF-κB signaal transductie geblokkeerd werd kon een

afname in de productie van de ontstekingsfactoren waargenomen worden. Dit suggereert dat het pro-inflammatoire karakter van de CTEPH endotheelcellen minstens ten dele wordt aangestuurd door een verhoogde NF-κB signalisatie. Deze bevinding brengt onderzoekers een stap dichter bij het ontrafelen van de mechanismen die leiden tot CTEPH en maakt het ook duidelijker welke processen interessant zijn voor de ontwikkeling van nieuwe behandelingsstrategieën in CTEPH.

Concluderend kan gezegd worden dat het onderzoek in dit proefschrift meer licht werpt op de onderliggende mechanismen die mogelijk leiden tot endotheeldysfunctie en structurele veranderingen in de pulmonale arteriën van patiënten met CTEPH. Verschillende processen en intermediaire factoren zijn onderzocht die interessante onderzoekopties en perspectieven bieden voor nieuwe medicamenteuze behandelingsstrategieën bij patiënten met CTEPH. Dit proefschrift heeft laten zien dat overmatige zuurstofverbindingen, endotheel celmetabolisme en inflammatie nuttige aangrijpingspunten kunnen zijn om endotheeldysfunctie te herstellen.

Razón fundamental

La hipertensión pulmonar (HP) es una condición patológica que se caracteriza por un aumento de la presión arterial dentro de las arterias del pulmón (PAPm> 20 mmHg) y afecta aproximadamente al 1% de la población mundial. La hipertensión pulmonar tromboembólica crónica (HPTEC), encuadrada en el grupo 4 de la clasificación de HP, se caracteriza por la falta de resolución de una o varias embolias pulmonares y por el proceso de remodelado vascular de los vasos ocluidos y no ocluidos. Los pacientes con HPTEC, si no se tratan, tienen mal pronóstico con una supervivencia a 5 años entre el 10 y el 30%, dependiendo de la PAPm del paciente. El tratamiento de referencia para pacientes con HPTEC es la endarterectomía pulmonar (PEA), eliminación quirúrgica del material tromboembólico oclusivo de las arterias pulmonares, con un posible resultado curativo. No obstante, el 40% de los pacientes con HPTEC no son operables y hasta un tercio de los que se operan tiene HP persistente o recurrente, con lo que se requiere una mejor comprensión de la patogénesis de la HPTEC para poder desarrollar nuevas estrategias terapéuticas. Hasta la fecha, los mecanismos moleculares y celulares detrás de la falta de resolución del trombo y la remodelación vascular que resultan en HPTEC siguen sin estar claros. En este sentido, una de las hipótesis en estudio se basa en la existencia de una disfunción endotelial primaria como factor determinante para el progreso de la enfermedad.

Estructura de la tesis

El objetivo general de esta tesis es profundizar en la comprensión de la fisiopatología de la HPTEC, centrándose en el comportamiento y en la función de las células endoteliales (CE) obtenidas de pacientes. Con este propósito, se aislaron CE (definidas como HPTEC-CE) a partir de material vascular recogido en PEA de pacientes con HPTEC y se validaron como modelo *in vitro* para estudiar la patología endotelial en HPTEC. Actualmente, los estudios que evalúan las anormalidades de la CE y los mecanismos moleculares detrás de la remodelación vascular pulmonar son escasos en la HPTEC. Por lo tanto, la investigación de los mecanismos subyacentes de la no resolución de la embolia pulmonar y la posterior remodelación vascular no solo es clave para crear una mejor comprensión de la patología HPTEC, sino que también podría ayudar a diseñar nuevas dianas para el tratamiento de la HPTEC. Como resultado,

describimos varias anormalidades moleculares de las HPTEC-EC que pueden dar lugar a una mejor comprensión del desarrollo de la enfermedad y que ofrecen nuevos objetivos terapéuticos.

En **el capítulo 2** se abordan los últimos avances sobre el papel de la disfunción endotelial en todas las formas de HP. Actualmente, la terapia farmacológica en la HP se centra en tres vías vasomotoras clave: prostaciclina, endotelina-1 y óxido nítrico- monofosfato de guanosina cíclica. Estas estrategias, dirigidas a aumentar la vasodilatación pulmonar, tienen como finalidad el control de los síntomas, pero no actúan sobre el origen de la enfermedad: la disfunción endotelial. En este capítulo se describen los principales factores que contribuyen a la disfunción endotelial, como la transición endotelial a mesenquimal, la inflamación, la trombosis y los desórdenes en la coagulación, la apoptosis, el desequilibrio vasoactivo y los factores genéticos. El hecho que muchas terapias para un tipo específico de HP no sean beneficiosas para pacientes con otras formas de HP, ilustra la gran variedad de factores y la complejidad de los fenómenos implicados en la disfunción endotelial. En este sentido, proporcionamos una actualización de potenciales dianas moleculares para restaurar la función endotelial y enumeramos las principales dificultades que deben superarse para encontrar tratamientos farmacológicos curativos para la PH. En general, una mejor comprensión de los cambios celulares y los mecanismos moleculares involucrados en el proceso de remodelación vascular será el trampolín para futuras terapias dirigidas contra la HP y para un diagnóstico temprano de la enfermedad.

El material oclusivo intravascular extraído en las PEA nos ofrece una oportunidad única para evaluar las CE de la zona dañada de las arterias pulmonares de pacientes con HPTEC. En el **capítulo 3** describimos los posibles usos del aislamiento de CE de especímenes PEA de pacientes con HPTEC. Concretamente, validamos el uso de HPTEC-CE como modelo *in vitro* para el estudio de la patología endotelial mediante la evaluación de la función endotelial y mitocondrial. De esta manera identificamos posibles objetivos de diferentes vías moleculares para la prevención y el tratamiento de la enfermedad. Las HPTEC-CE, aisladas y expandidas en cultivo celular, se caracterizaron como células endoteliales por la presencia de células con la típica morfología *cobblestone* en cultivos monocapa y por la expresión de marcadores endoteliales tales como eNOS, CD31 y en factor de von Willebrand, tanto a nivel de ARNm como proteína. Además, se descubrió que HPTEC-CE son hiperproliferativas y resistentes a la

apoptosis en comparación con las células endoteliales de la arteria pulmonar (HPAEC) provenientes de donantes sanos. A pesar de un aumento en la proliferación, las HPTEC-CE mostraron una capacidad angiogénica y migratoria reducida, ya que el número de estructuras tubulares formadas y la velocidad de cierre de la herida, respectivamente, fue menor en comparación con las células control. También demostramos que las HPTEC-CE poseen mitocondrias con una morfología anormal, con la membrana interna y crestas irregulares junto con un desequilibrio de las dinámicas mitocondriales y un aumento de especies reactivas de oxígeno mitocondriales (mROS). Como el aumento de mROS indica una desregulación entre los sistemas antioxidantes y la producción de ROS, investigamos si las dismutasas se expresaban de manera diferente en las HPTEC-CE y encontramos que la SOD2 (antioxidante) estaba regulada negativamente en las HPTEC-CE en comparación con las células de control. Además, también demostramos un aumento general del estrés oxidativo en la muestra de PEA. En resumen, hemos identificado por primera vez varias vías moleculares alteradas en HPTEC-CE que probablemente influyan en la resolución del trombo, la remodelación de la pared de los vasos y el desarrollo de HPTEC. Las alteraciones en el estrés oxidativo/nitrativo, producción de antioxidantes, homeostasis mitocondrial y moléculas de adhesión podrían estar involucradas en la fisiopatología de la HPTEC.

En el **capítulo 4** describimos un tema emergente en las enfermedades cardiovasculares: el potencial papel de las anormalidades metabólicas en la hipertensión pulmonar. Nos centramos en las recientes observaciones sobre el papel del metabolismo y la disfunción endotelial en la fisiopatología de la enfermedad (HPTEC) y en las oportunidades terapéuticas que se derivan. Describimos la función clave del metabolismo de las CE en el mantenimiento del estado no proliferativo e inactivo del endotelio. Una creciente evidencia apunta a un papel crítico de la desregulación metabólica, mediante regulación negativa de la fosforilación oxidativa y el metabolismo de los ácidos grasos y un aumento en la glucólisis, en la promoción de la proliferación e inhibición de la apoptosis. En estudios preclínicos se ha indagado sobre la posibilidad de modular el metabolismo de las CE para restaurar la función endotelial normal. No obstante, dada la complejidad del metabolismo celular en sí y la dificultad de intervenir sobre el metabolismo de un solo tipo celular, son necesarios estudios metabólicos en grupos más grandes de pacientes con HAP y HPTEC para progresar en la búsqueda de

nuevos intervenciones farmacológicas y biomarcadores (en pacientes HAP y HPTEC) de la remodelación metabólica en la HP.

Como se mencionó en el capítulo 4, una comprensión más profunda del metabolismo de la CE en la HPTEC contribuirá a mejorar la comprensión de los mecanismos moleculares detrás de la fisiopatología de la HPTEC y, como tal, ayudará a definir nuevas terapias. Por lo tanto, en el **capítulo 5** examinamos las alteraciones en el metabolismo de las HPTEC-CE que podrían estar involucradas en la disfunción de CE y la posterior remodelación vascular que ocurre en las arterias pulmonares que rodean a los trombos en HPTEC. Observamos, en contraste con el aumento del perfil glucolítico presente en HAP-CE, una disminución en las enzimas clave y limitantes de la vía glucolítica. Además, encontramos una regulación negativa en la piruvato deshidrogenasa quinasa, la enzima que impide la entrada de piruvato en las mitocondrias, junto con una regulación negativa en la glutaminólisis. Por último, no se observan cambios en las vías de la pentosa fosfato y la oxidación de ácido grasos, enzimas metabólicas asociadas clave, en las HPTEC-CE en comparación con las CE control. Tanto la modulación del regulador glucolítico PFKFB3 como el metabolismo de la glutamina, han demostrado estar involucrados en la angiogénesis. Consecuentemente, creemos que la disminución en la glucólisis y el metabolismo alterado de la glutamina en las HPTEC-CE pueden ser factores relevantes para explicar la falta de recanalización y resolución de trombos en HPTEC.

Tanto la HAP como la HPTEC se caracterizan por la remodelación vascular pulmonar, por lo que la idea de que las dos enfermedades comparten cambios celulares y moleculares es muy atractiva. Sin embargo, en el capítulo 5 mostramos que las HPTEC-CE son menos glucolíticas en comparación con las CE de control. Este hallazgo es muy interesante porque se ha demostrado que las HAP-CE son altamente glucolíticas. Por lo tanto, en el **capítulo 6** realizamos una comparación metabólica sistémica de ambas CE aisladas de pacientes con HPTEC que se sometieron a PEA y de pacientes con HAP que se sometieron a un trasplante de pulmón. Para entender mejor las posibles diferencias en el metabolismo, se evaluó la viabilidad de la inhibición por los reguladores metabólicos de glucólisis y glutaminolisis y también la migración. Hemos encontrado que las CE utilizadas en este estudio, tanto de pacientes con HPTEC o HAP, tienen un perfil de expresión génica de homeobox-D similares, vinculado a un origen macrovascular, pero mostraron una diferencia pronunciada en la

expresión génica de enzimas relacionadas con las vías glucolítica y de la glutamina. En las HPTEC-CE, la disminución de las enzimas glucolíticas fue acompañada de una disminución de los niveles de ARNm y proteínas de la enzima piruvato deshidrogenasa, que controla la entrada de piruvato en las mitocondrias, en comparación con HAP-CE. No se observaron diferencias en las enzimas relacionadas con la ruta de la pentosa fosfato, ni a nivel de expresión ARNm ni de proteína. A pesar de la diferencia en el perfil metabólico entre HPTEC-CE y HAP-CE, no se observaron diferencias en la viabilidad cuando las CE se incubaron con inhibidores metabólicos. Por último, aunque se pueden detectar claras diferencias metabólicas entre HPTEC-CE y HAP-CE, no se pudo observar diferencia en la capacidad de migración. Este estudio confirmó la reducción de la glucólisis en HPTEC-CE observada en el capítulo 5, pero más interesantemente mostró diferencias claras en la glucólisis y glutaminólisis entre HPTEC-CE y HAP-CE. Tal diferencia en el metabolismo entre ambas enfermedades crea una mejor comprensión de la fisiopatología específica de la enfermedad de la HPTEC y eventualmente podría contribuir al desarrollo de nuevas terapias específicas de la enfermedad.

Por último, se ha estudiado el proceso inflamatorio, que es clave en el desarrollo y progresión de las enfermedades cardiovasculares, incluida la HP. En la HPTEC hay una evidencia creciente de que las alteraciones inflamatorias pueden promover el remodelado vascular, la formación de trombos y la disfunción endotelial. Sin embargo, la regulación de la inflamación en HPTEC-CE es aún desconocida en gran medida. Por lo tanto, en el **capítulo 7** estudiamos la posible participación de la activación sostenida de la vía NF-κB y estado proinflamatorio en HPTEC-CE como posible impulsor del desarrollo de HPTEC. Descubrimos que el phospho-p65 estaba presente en los vasos intra-trombóticos del material vascular obtenido de PEA de pacientes HPTEC. Además, mostramos una mayor presencia de fosfo-p65 nuclear en HPTEC-CE cultivadas, indicando una mayor activación del complejo NF-κB en las HPTEC-CE en comparación con las células control. En línea con estas observaciones, observamos que varios factores inflamatorios de la cascada de señalización de NF-κB como la IL-8, la IL-1β, el MCP-1, el ICAM-1 y el VCAM-1, estaban regulados positivamente en las HPTEC-CE en comparación con las células de control. Cuando las HPTEC-CE se incubaron con un inhibidor de NF-κB, el compuesto Bay, mostramos una disminución significativa en los niveles de ARNm de MCP-1 y VCAM-1. En base a los resultados obtenidos en este estudio, podemos concluir que los HPTEC-

CE tienen un estado proinflamatorio, como lo demuestra el aumento de la producción de citocinas inflamatorias IL-8, IL-1β, ICAM y VCAM. Aún más importante, demostramos que el aumento de las citocinas inflamatorias observadas en HPTEC-CE está regulado, al menos parcialmente, a través de la señalización de NF-κB. Esto implica que la vía de NF-κB y la inflamación podrían ser contribuyentes importantes para el desarrollo y la progresión de la HPTEC y que su inhibición podría ser una opción terapéutica novedosa el la HPTEC.

En esta tesis se ha investigado el papel de la disfunción endotelial como conductor en la patología de la HPTEC. Nuestros datos proporcionan una primera, pero importante, descripción de anormalidades de células endoteliales HPTEC, las cuales podrían ser estudiadas para el desarrollo de nuevas estrategias terapéuticas y también ser utilizadas como biomarcadores para la detección temprana de HPTEC. La disfunción endotelial ha sido relacionada con en el inicio y la progresión de varias enfermedades vasculares como la aterosclerosis y la HAP. Hemos demostrado que las CE derivadas de pacientes con HPTEC son una herramienta valiosa para estudiar las características de CE en el sitio de la lesión. Hemos encontrado que las HPTEC-CE son hiperproliferativas y menos angiogénicas, tienen una mayor expresión de moléculas de adhesión, presentan mitocondrias disfuncionales junto con desacoplamiento de eNOS y producción de estrés oxidativo/antioxidante desequilibrado. Además, hemos demostrado que los HPTEC-CE tienen alteraciones metabólicas asociadas con una reducción en la glucólisis y glutaminólisis. Por último, también hemos demostrado que HPTEC-ECs se encuentran en un estado proinflamatorio que depende, al menos parcialmente, del complejo NF-κB.

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The author of this thesis was born on March 1st, 1992 in Antwerp, Belgium. She grew up in Schoten together with her younger brother David. She attended the Onze-Lieve-Vrouw-van-Lourdes college in Edegem. After graduating in 2010, she started her studies in Pharmaceutical Sciences at the University of Antwerp, Belgium. During her first three years she engaged with the student association UFKA where she, besides organizing group activities, also was involved in mentoring students in their first year at the university.

In her last year at the University of Antwerp (Belgium) she did a six month internship in a public pharmacy. Afterwards, she started her Master thesis in the lab of Medicinal Chemistry where she focused on the design and synthesis of inhibitors for RIP kinases. In September 2015 she received her diploma as a pharmacist. In October 2015 she moved temporarily to Bristol, United Kingdom, to study English. While in Bristol, she got accepted into the European Horizon 2020 MSCA joint doctoral project MoGlyNet. She started her PhD in Barcelona, Spain on February 1st 2016 in the lab of Prof. Dr. Joan Albert Barberà under supervision of Dr. Olga Tura-Ceide and Prof. Marta Cascante. Her main project focused on endothelial cells in CTEPH. In September 2016 she was awarded the best poster communication prize at the CIBERES 9th symposium in Madrid, Spain. Over the years she presented her work at several national and international congresses. As part of the Marie-Curie joint doctoral program she did an industrial secondment at HistoGeneX, Belgium under the supervision of Prof. Dr. Dorien Schrijvers. Later on she moved to Leiden, The Netherlands to start the last part of her PhD in the group of Prof. Paul H.A Quax where she acquired knowledge in cell and tissue staining techniques. In addition to her work at the Vascular Surgery department of Prof. Quax, she also started working closely with the research group of Prof. Marie-José Goumans where she, together with Dr. Kondababu Kurakula, could pick up her work with endothelial cells from patients with CTEPH.

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