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

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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.

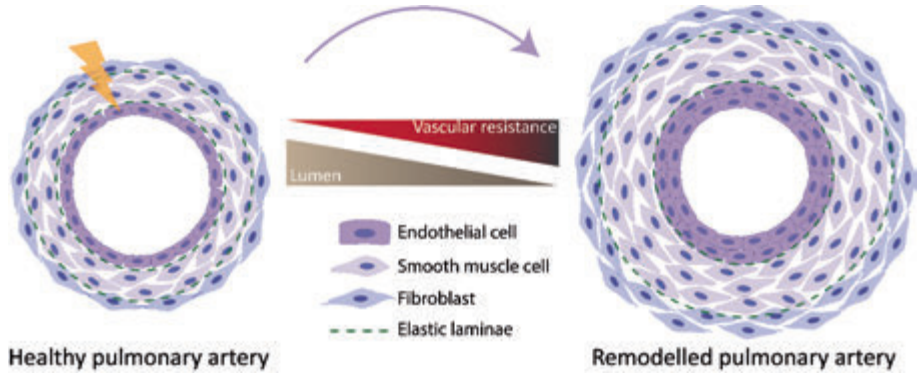

Figure 1. Pulmonary vascular remodeling. Vascular remodeling is a hallmark of PH that involves structural changes in all three layers of the vessel wall that impair blood flow. On the left a healthy pulmonary artery and on the right a remodeled artery. The decrease in lumen size because of remodeling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

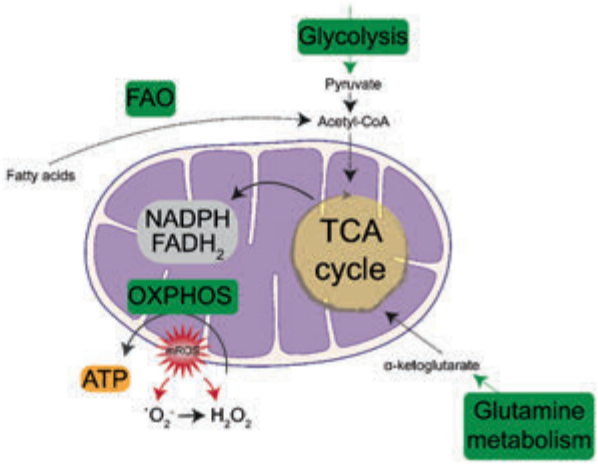

Figure 2. Aerobic cell respiration. Cells rely on glycolysis coupled with mitochondrial OXPHOS for the production of ATP. Once Pyruvate is converted into acetyl-coA it is metabolized in the TCA cycle with the production of NADPH and $FADH_2$. These molecules are essential to complete cell respiration 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.

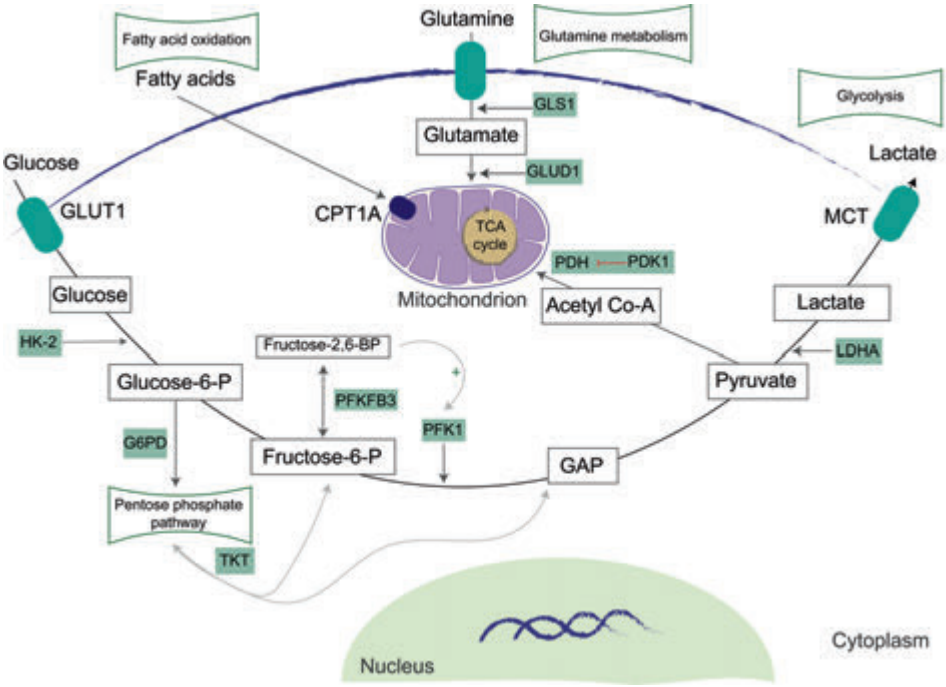

Figure 3. Endothelial cell metabolism. Cellular metabolism consists of aerobic glycolysis linked with oxidative phosphorylation in the mitochondria. In normal conditions, glucose is converted into pyruvate which is shuttled into the mitochondria where it participates in the TCA cycle. The TCA cycle is 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.

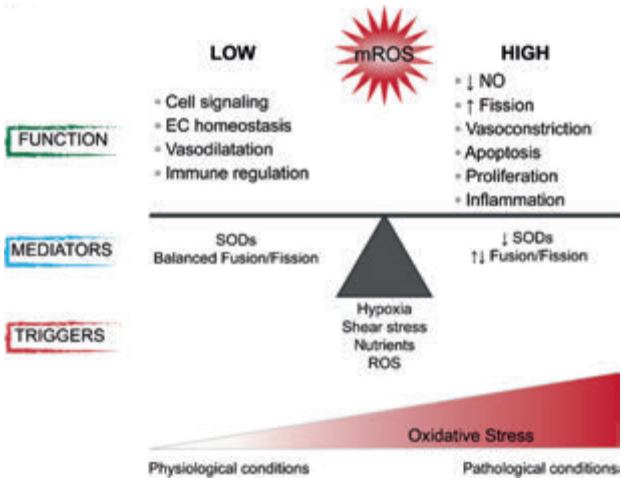

Figure 4. Mitochondria and ROS production. Mitochondrial reactive oxygen species (mROS) are natural byproducts of mitochondrial respiration. Low concentrations of mROS have several physiological functions, but excessive mROS on the other hand have several pathological functions that mediate EC dysfunction. 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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