CHAPTER 1
Introduction & Background
THE ENDOTHELIUM: INTEGRATOR OF FORCES

Structure and function of the endothelium

The endothelium is a highly dynamic organ forming the luminal lining of the heart, blood vessels, lymph vessels and the serous cavities of the body (Figure 1). It is highly metabolically active and possesses secretory, synthetic, and immunologic functions. Furthermore, it is central in the control of fluid homeostasis, vascular tone, coagulation, trafficking of circulating cells and nutrients, vasculogenesis and angiogenesis (Furchgott 1983; Furchgott & Zawadzki 1980; Palmer et al. 1987; Pries & Kuebler 2006). The endothelium is composed of a single layer of endothelial cells (EC), which form characteristic, homotypic cell-cell junctions with neighboring cells on their lateral side and contact their growth-substrate through focal adhesions on their basolateral side. Although a subject of ongoing debate, the endothelium in an adult human is estimated to home approximately $10^{12}$ EC in relation to a total number of $3.72 \times 10^{13}$ cells in the entire human body, weighs about 1 kg, and covers a surface area of circa 1000 $m^2$ (Bianconi et al. 2013; Jaffe 1987; Pries & Kuebler 2006; Wolinsky 1980).

Figure 1: Scanning electron micrograph of the smooth, tight endothelial cell monolayer covering the luminal surface of healthy blood vessels. Reprinted from (Baluk et al. 2005) with permission from Elsevier.

Regulation of the vascular endothelial barrier and vascular permeability

The central function of the vascular endothelium is to maintain fluid homeostasis and deliver nutrients to the interstitium by providing a dynamic, semi-permeable, size-selective barrier between blood and underlying tissue (Sukriti et al. 2014). The capacity of the endothelium to actively control fluid, solute and macro-molecule exchange is
defined as vascular permeability (Amado-Azevedo et al. 2014). Vascular permeability is
separated into two sub-types: basal and inducible permeability (Figure 2) (Aird 2007a).
Basal permeability describes the passive, continuous flux of material between blood
and underlying tissue. Inducible permeability is mediated by agonists such as histamine,
serotonin, vascular endothelial growth factor (VEGF), tumor necrosis factor (TNF),
thrombin, and several cytokines. The EC responses to these mediators are highly ligand
specific (Beckers et al. 2010).

Figure 2: Live-cell DIC imaging of human umbilical vein endothelial cells (HUVEC) in
an in-vitro culture system. A) Cells were maintained under basal, fully supplemented culture
conditions. Arrow heads indicate the appearance of spontaneously forming inter-endothelial
minute gaps. B) HUVEC monolayer after activation with thrombin. Notable are the contracted EC
phenotype and the increase in gap size (arrow heads).

Fluid, solutes, macro-molecules and circulating cells can pass the endothelium though
two main routes, dependent on activation status of the endothelium and molecule
size (Mehta & Malik 2006). Molecules between 3 to 11.5 nm in diameter move across
the EC barrier via the vesicle-mediated trans-cellular transport route, whereas smaller
molecules <3 nm, such as water and ions, travel along the para-cellular route (Amado-
Azevedo et al. 2014; Vandenbroucke et al. 2008). Para-cellular permeability is mainly
determined by the adhesive properties versus junctional tension of the proteins that
make-up the inter-endothelial cell-cell junctions (IEJ) (Dudek & Garcia 2001; Martinelli
et al. 2013; Oldenburg & Rooij 2014; Vandenbroucke et al. 2008). The IEJ comprise
specialized junctional structures named tight (TJ) and adherens junctions (AJ). In
addition, EC express other specific adhesion proteins that form inter-cellular contacts
and participate in signaling processes, such as the mechansensor platelet endothelial
cell adhesion molecule (PECAM-1) (Dejana 2004; Privratsky & Newman 2014). In blood
vessels, however, AJ represent the majority of junctional complexes (Vandenbroucke et al.
2008). The IEJ are actively regulated, continuously fine-tuned and can be disassembled
and assembled to either increase or decrease para-cellular permeability, respectively.

Cadherins, most importantly vascular endothelial (VE) cadherin, are specifically
associated with endothelial AJ (Bravi et al. 2014). These trans-membrane proteins form
extra-cellular homotypic interactions with each other and their cytosolic domain is bound to the actin-cytoskeleton via special linker-proteins, the catenins (Sukriti et al. 2014). By this functional association with all kinds of membrane proteins, actin filaments are involved in controlling the dynamics of cell shape, cell polarity, cell–substrate adhesion, cell–cell adhesion, and cell migration in both physiological and pathological conditions (Schnittler 2014). Thereby, cytoskeletal organization, its intra-cellular orientation, and localization are central for its function (Figure 3A and B). As such junction-associated intermittend lamellipodia (JAIL) are particularly found in sub-confluent cells at sites of VE-cadherin free large inter-cellular gaps (Schnittler et al. 2014). These actin-rich, locally restricted membrane protrusions are essential for cell-junction formation and for the dynamic maintenance of the junctional integrity by initiating and stabilizing VE-cadherin mediated cell adhesions. Junction associated circumferential actin bundles that are supposed to stabilize VE-cadherin mediated cell adhesions, and thereby facilitate barrier function and integrity are alternatively found under non-pathological, unstimulated conditions in mature EC barriers at sites where a continuous junctional organization of VE-cadherin is already established (Schnittler et al. 2014). On the contrary, in migrating EC, under pathological conditions, or upon activation by vasoactive agents, such as thrombin, VEGF, or inflammatory molecules, EC can retract their cell borders based on formation of actin stress fibers that are spanning from one side of the cell to the other. The initiation of actin-myosin based cytoskeletal contraction leads to the opening and disassembly of IEJ and formation of inter-cellular gaps (Vandenbroucke et al. 2008). This transient, precisely regulated opening of the endothelial barrier is necessary for proper homeostasis, as well as the formation and remodeling of blood vessels (Amado-Azevedo et al. 2014), whereas chronic or non-reversible loss of junctional integrity and uncontrolled fluid exchange, dubbed hyper-permeability (also known as vascular leakage), is associated with many clinical syndromes including edema, ALI, and ARDS (Goldenberg et al. 2011; Lampugnani 2012; Matthay et al. 2012).

The formation of stress fibers and actin-myosin controlled cytoskeletal contraction is the result of actin remodeling, increased myosin light chain (MLC) phosphorylation, hampered MLC de-phosphorylation and the formation of focal adhesions (van Hinsbergh & van Nieuw Amerongen 2002). These mechanisms are to a predominant part regulated by calcium/calmodulin signaling and the activation of small guanosine triphosphatases (GTPases), members of the Ras superfamily that act as a binary molecular switches (Sukriti et al. 2014; Wennerberg et al. 2005). Within this delicate balance the Rho GTPase RhoA and its downstream kinases ROCK1/2 were historically seen as mediators of hyper-permeability through their effect on actin cytoskeletal organization (stress fiber formation) as well as their function as initiators of cell contraction and retraction of cell boarders (van Nieuw Amerongen et al. 2000). This notion started to shift when reports, from our own group and others, indicated a role of RhoA in barrier maintenance (van Nieuw Amerongen et al. 2007). Subsequent studies identified active RhoA in the leading edge of migrating cells (Kardash et al. 2010; Kurokawa 2005) pointing out the functional importance of spatial regulation of RhoA activity in dependence of
specific cues in the control of its biological effect (Pertz et al. 2006; Timpson et al. 2011). Regarding this new dual role of RhoA (Figure 3C) a key question to be investigated was, whether a spatial association exists between local RhoA activation and IEJ disassembly or whether RhoA acts indirectly at sites remote from newly formed inter-endothelial gaps (Szulce et al. 2013).
Mechanobiology of the endothelium

Function and structure of the vascular EC is not only controlled though agonists and receptor-ligand interactions, but EC also sense and adapt to physical and mechanical forces in their three-dimensional environment. Forces are vectors with magnitude and direction that act on objects with mass (Lessey et al. 2012). They influence a diversity of biological processes, such as organ and tissue development, arterial versus venous phenotype as well as basic cellular functions, like migration, proliferation, apoptosis, gene regulation and protein synthesis (Eyckmans et al. 2011). The direction and magnitude of the applied forces is temporarily highly variable and dependent on the vascular bed (Lessey et al. 2012). EC possess intrinsic capabilities to sense, translate, transmit and dynamically adapt their morphology, structure and function to physical and mechanical stimulation (Davies 1995). The cellular mechanosensing is based on force induced conformational changes of mechanosensitive proteins that result in altered affinities to binding partners, thereby activating signaling pathways that convert physical stresses (force per unit area) into biochemical signals (Amado-Azevedo et al. 2014; Hahn & Schwartz 2009; Jaalouk & Lammerding 2009). This mechanism is named mechanotransduction. The EC mechanotransduction loops are essential for the maintenance of tissue homeostasis and several cellular structures have been shown to play an integrative role coupling cell function to structure and the extracellular environment, including IEJ, focal adhesions, the cytoskeleton, cell surface receptors and mechanosensitive ion channels (DuFort et al. 2011; Jaalouk & Lammerding 2009).

Generally, two forms of physical forces are being distinguished that functionally cooperate (Figure 4). Endogenous forces, which are generated within or exerted by EC, and exogenous forces that are externally applied and affect the endothelium directly or indirectly through its environment (Jaalouk & Lammerding 2009; Lessey et al. 2012). Endogenous forces were briefly discussed previously, for the reason that the balance of tethering and contractile forces within EC is critical in the regulation of vascular permeability (Eyckmans et al. 2011). Tethering or adhesive forces, are exerted through cell-cell and cell-matrix adhesions, whereas contractile forces are generated and transduced by the cytoskeleton. These competing endogenous forces are intimately linked, determine cell shape, transduce signals within EC, act on neighboring cells, and exhibit traction forces onto their growth substrate (Dudek & Garcia 2001; Krishnan et al. 2011). Here, especially Rho GTPases, as major regulators of cytoskeletal tension, are central for the EC force-feedback (Jaalouk & Lammerding 2009).

Furthermore, through their location within the vasculature, EC are continuously exposed to the content of the vascular lumen, wherefore blood flow, blood pressure, the pumping heart, as well as vascular tone, and the lung respiratory activity induce exogenous forces onto the EC monolayer (Gulino-Debrac 2013). These include forces that are exerted from or onto the vessel wall, such as compression, torsion and cyclic stretch, as well as forces acting on the EC surface, like fluid flow induced shear stress (Kwak et al. 2014). These externally applied forces are typically referred to as ‘classical’ biomechanical forces and their involvement in health and disease is well recognized.
(Chiu & Chien 2011). As such, it is known from atherosclerosis research that EC can sense and adapt to different rates of shear stress and even differentiate between laminar and disturbed blood flow profiles. A large body of evidence proofs that disturbed flow patterns, such as found in curvatures, branches and bifurcations of large arteries, induces pro-inflammatory and pro-apoptotic signaling proning or even inducing endothelial dysfunction (athero-prone). Laminar shear stress, on the contrary, protects cells from apoptosis, locks them in a quiescent state, increases wound healing capabilities and decreases pro-inflammatory signaling (athero-protective) (Abe & Berk 2014; Conway & Schwartz 2013; Davies 1995).

Generally, the EC responses to biomechanical stimuli encompass often transient changes in signaling as well as the remodeling of EC morphology, topology and structure to minimize extra-cellular drag forces, intra-cellular stresses, and prevent cell injury (Chien 2007). Cytoskeletal remodeling is a major process during the adaptive response of the EC (Ngu et al. 2008; Wille et al. 2004).

### Biomechanical forces generated and exerted by endothelial cells (endogenous) - Contraction

**Actin-myosin contraction**

Myosin dependent EC contraction generates forces in between cell-cell junctions and onto their growth substrate. In a healthy, non-activated EC monolayer these forces are counterbalanced by the adhesive properties of the cell junctions.

### Externally applied biomechanical forces (exogenous) - Deformation

**Shear Stress**

The frictional force of the flowing blood generates shear stress, a force per area, parallel to the EC. When elevated, this increased drag-force causes the EC to flatten, elongate, and re-orientate in flow direction.

**Compression**

Caused by increased load and muscle tension, EC respond by elongation without predominant orientation.

**Stretch**

Can be either induced by the muscular vessel layer, adjacent cells or blood pulses. EC respond by reorientation away from the direction of applied force so as to be perpendicular. The cell response is dependent on time and magnitude of applied strain.

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**Figure 4: Endogenous and ‘classical’ exogenous biomechanical forces influencing EC morphology, structure and function.** Adapted with permission from (Lessey et al. 2012). Copyright (2012) American Chemical Society.

In addition to the previously described biomechanical forces, EC are exposed to other physical forces in everyday situations. These are less recognized and insufficiently studied. When in a car, plane, elevator or while conducting sports, EC are exposed to brief alterations in gravitational forces (g-forces, a type of acceleration). Before the first
space flights g-forces were believed to have little to no effects on eukaryotic cells, as the at time known cytosolic and nuclear components were either too small to be affected or expected to be incapable to move and sediment (Pollard 1965; Todd 1977). Nowadays, g-forces have been proven to profoundly affect the cardiovascular system and cellular functions similarly to age related diseases, when applied over long durations (Biolo et al. 2003; Vorselen et al. 2014). Extensive periods of weightlessness, during space flights have, shown detrimental effects on the human cardiovascular system defined as ‘cardiovascular deconditioning’ (Antonutto & di Prampero, PE 2003). Yet, no drugs are available, to prevent the vascular impairments under real weightlessness, for the reason that the underlying causes remain elusive and gravisensors are poorly defined (Zhang et al. 2014). Hence, it was of importance to elucidate, if EC are capable to sense fast, temporary changes in g-forces and whether EC gravisensing shares concurrent mechanisms with mechanosensing or represents a fully independent process (Szulcek et al. 2015; Versari et al. 2013).

Heterogeneity of the vascular endothelium

The ‘typical’ EC is thin and elongated with a length of roughly 50-70 μm, width of 10-30 μm and height of up to 10 μm around the nucleus that thins out to 0.1 μm towards the periphery (Féléto 2011; Florey 1966). EC express numerous membrane bound receptors for proteins, metabolites, lipid-transporting particles, hormones and interact with blood, ECM and neighboring cells through mediator release and specialized junctions (Ribatti et al. 2002). However, EC are not intrinsically identical. Structural and functional characteristics of individual EC differ in time and space along the vascular tree, by organ and even within the same organ, which is important for site-specific vascular homeostasis (Huxley 2013; Schnittler 2014). As such EC differ in their morphology as well as expression pattern of molecular markers, protein secretion, release of mediators, antigen presentation and stress response depending on organ, vascular bed, and embryonic origin (Aird 2007a; Molema 2010; Pusztaszeri et al. 2006). These differences are partly genetically imprinted and partly driven by prevailing biochemical and biomechanical environmental conditions (Pries & Kuebler 2006). Therefore, it is essential to realize that EC heterogeneity is a hallmark of EC function, wherefore its loss is indicative for disease or pathological changes (Aird 2005).

In the vascular system three classes of blood vessels are generally discriminated on the basis of their diameter, cross-sectional area and composition: arteries, veins and capillaries (Figure 5). Arteries and veins are made of three layers containing different cell types and possess an external diameter of 0.02-10 mm and 0.03-12.5 mm, respectively. A single EC monolayer (Tunica intima) forms the inner layer of arteries and veins that is surrounded by a smooth muscle cell (SMC) layer (Tunica media) followed by a layer of connective tissue and fibroblasts (Tunica adventitia). Arteries and veins are termed conduit vessels, which exemplifies their main function to transport blood throughout the body. Capillaries, on the contrary, are the major exchange vessels of the vascular system with an enormous cross-sectional area of >4000 m² and a diameter of 5-8 μm.
Capillaries are made of only a single layer of EC that is in direct contact with the ECM (Aird 2005). EC thickness in these vessels varies from 0.1 µm in capillaries and veins to 1 µm in the aorta (Aird 2007a; Pries & Kuebler 2006).

**Figure 5:** A) Schematic overview of the different types of blood vessels, their structure and organization. Reprinted with permission by Kelvinsong [CC BY-SA 3.0 (http://creativecommons.org/licenses/by-sa/3.0)] from Wikimedia Commons. B) Direct ex-vivo immunostaining and side-by-side comparison emphasizes morphological heterogeneity and differences in junctional organization between the different vessel classes. Reprint from (Dejana & Orsenigo 2013) with permission from the Journal of Cell Science.

These morphological differences are closely linked to the hemodynamic condition within the three vessel classes. Arteries are a high pressure, high flow system (which varies eight orders of magnitude throughout the body), veins are exposed to low pressure and moderate flow, whereas capillary EC experience low pressure and flow.
Hence, EC in straight segments of the aorta are reported to be long and narrow (55x10 µm) with their longitudinal axes oriented in the direction of blood flow, EC of the pulmonary artery are broader and shorter (30x14 µm) forming a rectangular shape, EC in terminal arterioles are generally elongated, reaching a width-to-length ratio of 1:6.8, whereas capillary (1:4.7) and venular EC (1:2.4) are rounder (Aird 2007a, Kibria et al. 1980, 1980; Pries & Kuebler 2006). On the contrary, EC of veins are larger and more rounded than arterial cells, wherefore one can conclude, the higher the intra-vascular pressure, the smaller and more polarized the EC (Kibria et al. 1980). Of importance is thereby, that although heterogeneity is partly genetically imprinted, EC maintain their ability to adapt to changes in their environment. As such, it was shown that EC of vein grafts transplanted into the arterial system undergo morphological and cytoskeletal changes characteristic for the arterial endothelium (Pries & Kuebler 2006, 14; Yoshida & Sugimoto 1996).

The pulmonary capillary endothelium
In the human lung EC occupy a surface area of approximately 130 m², more than one tenth of the total EC surface area in the human body (Figure 6). This enormous interface is needed to allow the pulmonary endothelium to filter the entire cardiac output, before it enters the systemic circulation (Pinsky et al. 2006). The pulmonary microcirculation is special in that it is a low pressure, high volume system and facilitates blood gas exchange with the ambient air (Aird 2007b; Pries & Kuebler 2006). It follows that, more than any other vessel type, lung capillary EC are uniquely adapted to their function and environment.

![Figure 6: Scanning electron micrograph illustrating the architecture of the normal arterial and venous pulmonary system, which is connected through a densely packed capillary meshwork.](image-url) Reprint from (Gebb & Stevens 2004) with permission from Elsevier.
Capillaries are the major exchange vessels in the circulation with a diameter of less than 10 µm (Aird 2007b). They are made up from flat EC, which are to a variable extent surrounded by pericytes and are in direct contact with ECM proteins (Armulik et al. 2011). The pulmonary capillary walls are extremely thin and fragile (West 2013). Only 0.2 µm of tissue separates the capillary endothelium from the alveolar space to minimize diffusional path length and pulmonary blood flow (PBF) is slow to optimize the time of perfusion to guarantee proper gas exchange (Aird 2007b; West 2013). Pulmonary capillary EC have a high number of caveolae, express higher levels of ACE, ICAM-1 and CD166 (ALCAM), but low levels of E-selectin and VCAM-1 (Molema 2010). Their permeability is lower than of other vascular beds, which is probably due to increased storage of cAMP, and capillary EC express more VE-cadherin and less eNOS compared to pulmonary arteries (Pries & Kuebler 2006). In addition to the structural and secretory differences, lung capillary EC respond totally different to agonist induced activation, than the endothelium of the intermediate to large arteries. As such, they demonstrate reduced basal and inflammation mediated permeability to solutes and calcium (Aird 2007b; Pries & Kuebler 2006). Additionally, lung capillary EC express a more pro-inflammatory endothelial phenotype that may be regarded as a beneficial defense mechanism, because the lung is continuously challenged by inhaled toxins and pathogens (van ‘t Wout, Emily F A et al. 2014, 1).

Through their location, function and environmental pre-conditioning, pulmonary capillary EC are the first line of defense and also the first target for diseases. Consequently, impaired lung capillary EC function is described under conditions of hypoxemia (impaired gas exchange), pulmonary edema (excessive fluid accumulation in lung cavities), sepsis induced ALI (increased vascular permeability and inflammation), and pulmonary hypertension (increased pulmonary artery pressure) (Aird 2007b; Pinsky et al. 2006).

**PULMONARY ARTERIAL HYPERTENSION: THE ENDOTHELIUM IN FOCUS**

**Pulmonary Arterial Hypertension at a glance**

WHO Group I Pulmonary Arterial Hypertension (PAH) comprises a severe and deadly group of lung diseases defined by a mean pulmonary artery pressure (mPAP) that equals or exceeds 25 mmHg at rest with normal left ventricular filling pressures and normal pulmonary wedge pressure (PWP, ≤15 mmHg) in the absence of other disorders, such as left heart disease, chronic thromboembolic disease, other lung diseases or hypoxemia (Humbert et al. 2010; Simonneau et al. 2013). PAH defines a highly heterogeneous patient population and can be idiopathic (iPAH), heritable (hPAH), drug or toxin induced, or associated with other diseases (see Table 1).

The vasculopathy of PAH is characterized by proliferative and occlusive remodeling of the small sized distal and medial capillaries and pre-capillary pulmonary arteries (Hatano & Strasser 1975), as well as a reduction in vessel number (Rabinovitch 2012). Progressive intimal hyperplasia and neo-intima formation put a special focus on EC alterations that precede and trigger the occlusive changes and the formation of generic
plexiform lesions (Figure 7) (Cool et al. 1999; Rabinovitch 2012). The drastic remodeling progressively decreases the cross-sectional area of the vascular lumen causing the rise in pulmonary vascular resistance (PVR, >3 Wood units) and mPAP, as well as a reduction in vascular perfusion leading to initial compensatory right ventricular hypertrophy, followed by decreased cardiac output, and eventually right heart failure (Sutendra & Michelakis 2013; Voelkel et al. 2012). Lung transplantation is often the only treatment option for end-stage disease, but 5-year post-transplant survival is only about 50% (Fadel et al. 2010).

Although survival, exercise capacity and quality of life has improved compared to prediction models derived from the early NIH registry in the late 1980s (Rich 1987), prognosis is still detrimental. Patients continue to be diagnosed late, because of their unspecific syndromes and if remained untreated PAH leads to death within 1 to 3 years. However, even with treatment 5-year survival rates after diagnosis are 57% in the US (Benza et al. 2012), 67%, in France (Humbert et al. 2010) and 69% in Switzerland (Mueller-Mottet et al. 2015). Outcome among PAH sub-populations varies substantially favoring females, despite a female predominance, and younger patients before their fifties (Benza et al. 2012, 452; Ling et al. 2012; McGoon et al. 2013). A report of the American College of Cardiology and the American Heart Association estimated overall PAH prevalence to be between 15 to 50 per million dependent on the PAH etiology (McLaughlin et al. 2009; Sutendra & Michelakis 2013). The French registry provides similar prevalence numbers and an incidence of 2.4 PAH cases per million of adults per year (Humbert et al. 2006). For the three sub-groups of idiopathic, heritable, and drug-induces PAH numbers are even smaller, wherefore PAH is referred to as a seldom lung disease. Even though PAH is rare, it has a high impact on society and kills patients at a productive and reproductive age and at a tremendous financial cost. The annual costs for single therapy range from 10,000 to 100,000 dollar and reach 216,953 dollar for combination therapy for a single patient with advanced PAH (McLaughlin et al. 2009; Sutendra & Michelakis 2013).

Currently, PAH patients receive drugs sequentially, with physicians adding additional treatment after reassessment of the patient response (Zamanian et al. 2014). Cases

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Table 1: Simplified clinical classification for Pulmonary Hypertension in accordance to the 5th WHO World Symposium held 2013 in Nice, France (Simonneau et al. 2013).

<table>
<thead>
<tr>
<th>Group 1 - Pulmonary Arterial Hypertension (PAH)</th>
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<tr>
<td>Idiopathic PAH (iPAH)</td>
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<td>Heritable PAH (hPAH)</td>
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<td>Drug or toxin induces PAH</td>
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<td>Associated PAH (assoc. PAH)</td>
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<td>Group 2 - Pulmonary Hypertension (PH) due to left heart disease</td>
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<td>Group 3 - PH due to lung disease and/or hypoxia</td>
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<td>Group 4 - Chronic Thromboembolic Pulmonary Hypertension (CTEPH)</td>
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<td>Group 5 - PH with unclear multifactorial mechanisms</td>
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Figure 7: The vasculopathy of PAH is characterized by muscularization of distal non-muscularized small arteries and reduction in their number, thickening of the vascular wall of larger arteries by medial hypertrophy and neo-intima formation, as well as the development of generic plexiform lesions. A) Schematic representation of the lung vascular abnormalities and the involved cell types associated with PAH. Republished with permission of the Journal of Clinical Investigation, from (Rabinovitch 2012); permission conveyed through Copyright Clearance Center, Inc. B) Immunohistochemically stained human lung samples from a healthy control and a PAH patient exemplify the different types of vascular alterations found in PAH. Images were kindly provided by Nina Rol (VU University Medical Center, Amsterdam, The Netherlands).

with severe disease typically receive double or mostly triple therapy. In addition, upfront combination therapy is under investigation (Galiè et al. 2015). Therapy involves mainly three classes of drugs, endothelia receptor antagonist (ERA), phosphodiesterase type V inhibitor
(PDE5-I) and prostacyclin synthetics/analogs (PGI₂), which all target the endothelium to release pulmonary vasoconstriction (Ling et al. 2012, 794; Mueller-Mottet et al. 2015, 131). New anti-remodeling drugs are being tested with the anti-angiogenic tyrosine kinase inhibitors (TKI), known from cancer treatment, leading the way. Clinical trials, however, highlight particular concerns pertaining the use of TKIs, as they can have serious side-effects including cardiotoxicity, vascular harmfulness, risk of subdural hematoma, and, to some extent, even promote the development of PH (Godinas et al. 2013; Hoeper et al. 2013). Riociguat, a soluble guanylate cyclase stimulator represents another new drug that acts like PDE5-I on cGMP. Riociguat has shown to be beneficial in a phase 3 clinical trial (Ghofrani et al. 2013, 330), but results are being discussed critically as the primary outcome 6-minute walk distance (6-MWD) has limited predictive value for survival and long-term data are missing (Farber et al. 2015, 362). Despite limited variety and success of current treatment, only 2.5% of recent clinical trials test new therapies aiming to reverse the vascular pathology. The rest focuses on drugs from the three already approved drug classes or drugs which are used for other indications (Sutendra & Michelakis 2013).

In summary, PAH therapies are not disease or organ specific as they were designed to treat systemic vascular diseases and cannot reverse the cardiac or vascular changes (Sutendra & Michelakis 2013). In consequence, PAH remains a progressive and fatal disease. Hence, the only option for recent treatment guidelines is to recommend more active treatment strategies including systematic and quick referral to expert centers, timely therapies in symptomatic PAH patients and earlier use of combination therapy (Humbert et al. 2010; Zamanian et al. 2014). The central problem, however, remains that therapies specifically targeting the pathology of the lung microvasculature are missing.

**Endothelial mechanopathobiology in Pulmonary Arterial Hypertension**

The phenotype of the vascular endothelium constantly varies between a quiescent and an activated state. Thereby, activation defines the initiation of specific, pre-defined cellular processes and is not necessarily linked to disease (Aird 2005, 2008). If this balance is for any reason shifted towards one side of the spectrum, one generally refers to endothelial dysfunction, defined as non-adaptive changes in EC structure and function that represent a net liability to the host (Aird 2008). EC dysfunction can be provoked by extrinsic acute or chronic pathophysiological stimuli and/or intrinsic EC defects.

Chronic increased PBF represents such an extrinsic stimulus, which was early on associated with the typical occlusive vascular remodeling in PAH (Figure 8) (Heath & Whitaker 1957). Mostly because the lesions were found in patients with a hyperdynamic circulation, i.e. congenital heart diseases with nonrestrictive, post-tricuspid shunts (Krishnan & Rosenzweig 2013), and developed specifically at sites of vascular bifurcations and branches (Cool et al. 1999), which are characterized by abnormal blood flow patterns. Consequently, increased PBF was suggested as an etiological factor. Mathematical models supported this hypothesis showing that pulmonary vascular remodeling and the increase in fluid flow induced shear stress are interdependent.
(Postles et al. 2014). However, increased PBF alone, such as in the flow only animal models of chronic hypoxic vasoconstriction and pulmonary shunt, could only partly resemble the PAH vasculopathy (de Raaf et al. 2014; Dickinson et al. 2013; Kulik 2012). Clinical and experimental observations indicated that the increase in PBF needs to synergize with acute EC injury or an increased pulmonary artery pressure to initiate the occlusive alterations (Kulik 2012; Okada et al. 1997; Taraseviciene-Stewart et al. 2001). It follows, that increased PBF is more likely to be either a pre-disposition that increases susceptibility of EC towards injury or a maintenance factor sustaining the progressive remodeling. The latter is supported by reports showing reversal of occlusive remodeling after single lung transplantation (Deb et al. 2006; Levy et al. 2001).

Figure 8: The vascular remodeling and loss of vessels causes altered lung vascular perfusion and an abnormal pulmonary blood flow (velocity and flow pattern). A) Region-growing algorithm, based on CT pulmonary angiograms, was used to model the pulmonary artery tree of PAH patients in different disease stages. Results visualize the immense loss of potent vessels during pathogeneses. Reprint from (Moledina et al. 2011) with permission from the BMJ Publishing Group Ltd. B) Computational modeling of wall shear stresses (WSS, red = high, blue = low) in the lung vasculature of an end-stage iPAH patient indicates that the structural changes and the increase in shear stress are interrelated. WSS was highest in terminal arteries and slowly increased towards the proximal vessels as disease/remodeling progressed. Reprinted, with permission, from (Postles et al. 2014). Copyright 2014 IEEE. C) The endothelium of pre-acinar pulmonary arteries expresses a sheared, arterial-like phenotype in the chronic hypoxia rat model of pulmonary hypertension and in patients with medial hypertrophy suggestive for increased shear stresses. Arrows indicate the direction of blood flow. Scanning electron micrographs are reprints from (Rabinovitch et al. 1986).
In addition to the changes in their extracellular environment a multitude of intrinsic EC dysfunctions were reported in PAH. Remarkably, although a non-adapted EC shear-phenotype was described in the human and rat PAH lung, the causal link with intrinsic defects in EC mechanosensing has never been made (Budhiraja et al. 2004; Kibria et al. 1980; Rabinovitch et al. 1986). This is especially interesting, as any changes in cellular or extracellular structure, the mechanosensing process itself, the subsequent downstream signaling or the consequent adaptation could potentially lead to disease, which would make increased PBF an acute trigger for the vascular changes in PAH (Jaalouk & Lammerding 2009). Thus, a central question to be answered was, whether pulmonary EC derived from PAH patients are capable to adapt morphology, structure and function to supra-physiological PBF (Szulcek et al. 2016)?

Past, present and future: A short historical overview of explanatory models for Pulmonary Arterial Hypertension

Several explanatory models have been developed defining different aspects of the PAH pathology and pathogenesis (Figure 9). In the 1940s and 50s it became clear that pulmonary vasoconstriction has a central role in PAH, which was then called Primary Pulmonary Hypertension (PPH) (Fishman 2004; Wagenvoort 1972). Chronic and sustained pulmonary vasoconstriction and consequent increase in PBF were seen as the cause for the vascular remodeling and the discovery in the 1990s that the endothelial derived vasoconstrictor endothelin-1 was up-regulated and the endothelial specific vasodilator nitric oxide synthase (NOS) decreased in patient lungs, led to the ‘vasoconstrictor hypothesis’ (Sutendra & Michelakis 2013). Current drug treatments of PAH still rely on this concept aiming to reverse the imbalance between vasoconstriction and vasodilatation (Humbert Marc et al. 2004; Zamanian et al. 2014). Yet, the overall success of vasodilator treatment is modest, because it does not reverse the occlusive vascular changes, wherefore PAH cannot result from vasoconstriction alone.

In the late 1960s a PPH epidemic caused by the appetite suppressant Aminorex occurred worldwide and still continues to spark the interest of researchers for a role of sympatric nerve activation and the serotonin 5-HT-receptor in PAH (Fishman 2004; Hatano & Strasser 1975). Aminorex is related to amphetamine and has both alpha- and beta-adrenergic receptor stimulating effects (Hatano & Strasser 1975). Its actions include release of norepinephrine and an increase in circulating serotonin levels (Fishman 2004). Aminorex was introduced in November 1965 and the epidemic significantly decreased 2 years after its withdrawal in October 1968 (Fishman 2004; Montani et al. 2013). Appetite suppressant drugs were the first well established risk factors for the development of PAH and still remain a problem, since correlations between drugs and such a rare disease is challenging (Montani et al. 2013). Today, several additional drug classes and toxins have been identified to be risk factors for PAH. Nevertheless, it is widely accepted that rather two or more ‘hits’ are needed to initiate PAH, than one risk factors alone (Yuan & Rubin 2005). The ‘multiple-hit model’ entails the combination of pre-disposition and acute stimulation. Herein, genetic mutations (mainly in the TGFβ superfamily), hyper-dynamic
circulation, autoimmune diseases, and other factors might prone the pulmonary system to increased vascular injury and cause defective vascular repair, whereas acute stimuli, such as hypoxia, inflammation, viral infections or environmental factors initiate pathogenesis (Voelkel et al. 2012). However, the difficulty with this model is that the sequence of pre-disposition and injury seems interchangeable and the term injury is not defined from a molecular point of view (Botney 1999), wherefore this model is too unspecific.

More modern concepts of PAH account for the many parallels to other diseases, in particular cancer. The so called ‘cancer paradigm’ defines the phenotypic alterations of the lung vasculature as ‘quasi-malignant’, characterized by an acquired apoptosis-resistant, hyper-proliferative EC phenotype (Rai et al. 2008). Within this model EC are described as the key initiating cell type that drive the occlusive vascular remodeling. Unfortunately, until today the cancer paradigm is widely unproven and the question, whether molecular evidence supporting one or more hallmarks of cancer is sufficient to imply a quasi-malignant transformation or rather represents a normal or misguided repair response to sustained cellular stress, is unclear (Sutendra & Michelakis 2013). Nevertheless, although the cancer paradigm is controversially discussed, it offers a unique mechanistical look on the multifactorial and multi-cellular nature of the disease.

As a result of the limitations of previous models and the difficulties to find a singular root cause in the very heterogeneous PAH patient population, two other models became popular. The ‘inflammation’ and the ‘metabolic theory’ of PAH changed the viewing angle and attempted to define common integrators of the numerous molecular abnormalities, rather than finding the disease initiator (Huertas et al. 2014). These approaches are based
on the idea that a combination of many, seemingly unrelated abnormalities in PAH finally cause or promote systemic changes, dysfunctions and imbalances in cell metabolisms, mitochondrial function or innate, adaptive and autoimmunity (Hatano & Strasser 1975; Paulin & Michelakis 2014; Tuder et al. 2013). Of particular interest hereby is, that drug treatment would address several molecular abnormalities simultaneously, which might be beneficial especially in more advanced stages of PAH. Nonetheless, as appealing as this idea is, only a small percentage of patients respond to anti-inflammatory treatment and mitochondria targeted therapies might be detrimental for the heart (Huertas et al. 2014; Paulin & Michelakis 2014).

In conclusion, multiple models have been created and mechanisms implicated that define individual parts of the disease and helped to develop treatment and management strategies. However, time course and sequence of cellular and vascular changes are still poorly understood, wherefore a remaining challenge is to integrate the complex genetic, epigenetic, inflammatory, metabolic and multi-cell models into the classic mechanical concepts of pressure, flow, and vascular injury (Happé et al. 2016).

HYPOTHESES & AIMS

a. We hypothesized that RhoA balances barrier maintaining and disruptive endogenous forces dependent on its spatio-temporal activity (Chapter III).

   Aim 1: Visualize RhoA activity in EC under basal and thrombin activated conditions in real-time.

   Aim 2: Correlate RhoA activity with force-generating/maintaining intra-endothelial structures (e.g. IEJ or F-actin cytoskeleton).

   Aim 3: Relate RhoA activity to overall barrier integrity.

b. We theorized that rapid, short-lived changes in gravitational forces are sensed by EC and influence endothelial barrier integrity (Chapter IV).

   Aim 1: Record the effect of rapid changes in simulated hypo- and hyper-gravity on endothelial barrier function.

   Aim 2: Compare the gravity effects to responses following classical biomechanical stimulation by increased shear stress and hydrostatic pressure.

   Aim 3: Determine, whether EC gravisensing shares concurrent causes and mechanisms with EC mechanosensing, or represents a distinct process.

c. We hypothesized that intrinsic defects in PAH patient derived lung EC interfere with their ability to sense and adapt to altered hemodynamic forces (Chapter V).

   Aim 1: Test, if the pulmonary endothelium of PAH patients with different etiologies adapts form and function to supra-physiological levels of fluid shear stress (HSS).
Aim 2: Elucidate, whether HSS represents a pivotal source for EC injury (loss or activation).

Aim 3: Explore pharmacological restoration of EC shear-responses \textit{in-vitro} and possible reversal of vascular remodeling in the Sugen-Hypoxia (SuHx) animal model for PAH.

d. We postulated that high PBF velocity is necessary to induce and maintain the obliterative changes in the PAH lung vasculature (Chapter VI).

Aim 1: Review current and past paradigms of PAH.

Aim 2: Determine, if the altered PBF in the PAH lung can potentially account for the ‘quasi malignant’ EC phenotype found in patient lungs.
REFERENCES


