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Pulmonary Vascular Endothelium: Orchestra Conductor in Respiratory Diseases
Highlights from Basic Research to Therapy

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Paris, January 15th 2018

To the Associate Editor of the *European Respiratory Journal*,
Dear Prof. Paul Hassoun,

Thank you very much for offering to submit a revised version of our manuscript entitled "***Pulmonary Vascular Endothelium: Orchestra Conductor in Respiratory Diseases - Highlights from Basic Research to Therapy***" as a review article by Alice Huertas and Christophe Guignabert, who have contributed equally and are both first authors, on behalf of the ERS Research Seminar Faculty.

We have addressed all of the reviewer's comments and we strongly believe that the revised paper has been strengthened.

Please find enclosed a point-by-point answer to the comments of the reviewer.

We would be grateful if you would be willing to take into consideration our revised manuscript.

Yours sincerely,

Alice Huertas and Christophe Guignabert





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Pulmonary Vascular Endothelium: Orchestra Conductor in Respiratory Diseases

Highlights from Basic Research to Therapy

Alice HUERTAS and Christophe GUIGNABERT on behalf of the ERS Research Seminar Faculty*

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Abstract

The European Respiratory Society (ERS) Research Seminar entitled ''*Pulmonary Vascular Endothelium: Orchestra Conductor in Respiratory Diseases - Highlights from Basic Research to Therapy*'' brought together international experts of dysfunctional pulmonary endothelium, from basic science to translational medicine, to discuss several important aspects in acute and chronic lung diseases. This review will briefly sum up the different topics of discussion of this meeting held in Paris (France) on October 27th and 28th, 2016. It is important to consider that this paper does not address all aspects of endothelial dysfunction but focuses on specific themes: (1) the complex role of the pulmonary endothelium in orchestrating the host response in both health and disease (acute lung injury, chronic obstructive pulmonary disease and high altitude pulmonary edema, pulmonary hypertension); (2) the potential value of dysfunctional pulmonary endothelium as a target for innovative therapies. The main conclusions and perspectives are hereafter reported.

Key words: *Lung; Pulmonary endothelium; Endothelial dysfunction; Respiratory diseases; Innovative therapeutics.*

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1. Introduction

The lung is supplied by two distinct circulatory systems, the systemic bronchial circulation and the pulmonary circulation. From the functional point of view, the pulmonary circulation is unique among vascular beds in the human body, characterized by a high flow, low-resistance, low-pressure system. Although all of the cardiac output is pumped through the pulmonary circulation, the measured values of mean pulmonary arterial pressure (mPAP) in pulmonary arteries for a resting, healthy adult human is about 14±3 mm of mercury (Hg), when systemic arterial blood pressure is about 100±20 mm Hg. This unique feature of the pulmonary circulation is mainly explained by three~~wo~~ critical characteristics of the pulmonary vasculature: **(1)** a low impedance to blood flow; **(2)** a high compliance of pulmonary precapillary arterioles characterized by a thin vessel wall; **(3~~2~~)** a high capacity to recruit available vessels that help accommodate an increase in flow. Indeed, this circulatory system can accommodate flow rates ranging from approximately 6 L/min under resting conditions to 25 L/min in strenuous exercise, with minimal increases in pulmonary pressures[1].

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Because of their strategic localization, at the interface between bloodstream and lung tissue, the pulmonary endothelial cells (ECs) play key roles not only in optimizing gas exchanges and in controlling barrier integrity and function, but also in regulating pulmonary vascular tone (i.e. through nitric oxide (NO), prostacyclin (PGI_2), endothelin (ET) and serotonin (5-HT) pathways)[2]. Moreover, pulmonary endothelium functions as an active and dynamic receptor-effector tissue and responds to the different chemical, physical, or mechanical stimuli by secreting the correct substance(s) by which it may maintain the vasomotor balance and vascular-tissue homeostasis. Indeed, pulmonary ECs are highly metabolically active, sensing and responding to signals from extracellular environments. All these interactions with adjacent cells and circulating cells and/or mediators aim to maintain a thrombosis-free surface, to control inflammatory cell adhesion and trafficking, to assure a normal angiogenesis and the integrity of the vascular wall[3]. As a vital part of the respiratory system, alterations of pulmonary endothelium play a central role in the pathogenesis of several chronic and acute lung diseases, common or rare. The main characteristics of pulmonary endothelial alterations or dysfunction are mainly reflected by increased permeability that can lead to vascular leakage and edema formation; altered balance between vasoconstriction and vasodilation; acquisition of a pro-inflammatory phenotype with increased expression of adhesion molecules for inflammatory cell recruitment, increased activation of pro-inflammatory transcription factors, release of inflammatory mediators and

oxidative stress; pro-thrombotic phenotype; pro-proliferative and anti-apoptotic phenotype; miscommunication with adjacent vascular cell wall.

Although considerable progress has been made in the understanding of pulmonary EC dysfunction, the triggers, the mechanisms and the consequences of dysfunctional endothelium in lung disease, acute and chronic, are still not completely understood. A better knowledge of these key aspects would help in finding new disease biomarkers and/or novel therapeutical targets to preserve homeostasis in response to injury and disease(s).

2. The importance of genetic and environmental factors

Most, if not all, acute and chronic lung diseases result from interaction between multiple environmental exposures and many genetic risk factors, an aspect that has been critically evaluated in a recent review from this European Lung White book[4]. The role of vasculature in the development, lifelong repair and maintenance of the lung tissue homeostasis is of paramount importance. Indeed, many diseases involving the lung, such as emphysema, chronic obstructive pulmonary disease (COPD)[5, 6] and pulmonary hypertension (PH)[7], display major EC abnormalities. A better understanding of how pulmonary ECs contribute to normal lung development and how these mechanisms are deregulated during chronic disease is of crucial importance for the development of new therapeutic strategies for debilitating lung conditions. However, ~~inter~~individual differences in response to the same stimulus in subjects are well documented, such as environmental hypoxia at high altitude. In addition, it is well established that the pulmonary vasopressor response varies greatly between humans and animals, and even between different species, supporting the importance of unknown genetic influences.

Beyond the genetic background and specific gene mutations, other factors are known to determine structural and functional modifications of the pulmonary vasculature, such as exogenous exposures to drugs and toxins, hormones, and aging, with decreased repair capacity[8-10] and impaired productions of different vasoreactive mediators[11].

Another potential agent capable of causing endothelial impairment is represented by cigarette smoke products, that play a major role in COPD since “healthy” smokers (without COPD) already show significant abnormalities in pulmonary arteries that include among others: vessel remodeling, inflammatory cell infiltrate, endothelial dysfunction, reduced

expression of endothelial NO synthase (eNOS), increased expression of growth factors and a similar gene expression profile to that observed in COPD patients[12, 13]. In experimental models of COPD, it has been also demonstrated that chronic exposure to cigarette smoke induces endothelial dysfunction in pulmonary arteries [14], along with changes in the expression of eNOS[15] and soluble guanylate cyclase (sGC)[16]. Interestingly, vascular changes antecede the development of pulmonary emphysema[17]. Altogether, these findings strongly support relations between smoking habits and pulmonary vascular damage in COPD.

3. Dysfunction of endothelial barrier properties

The vascular endothelium forming the innermost lining of all pulmonary blood vessels is the major barrier that protects air spaces against vascular fluid entry. The integrity of this endothelial barrier permeability is critical for the maintenance of homeostasis for all tissues and organs of the body, and changes in its permeability are a hallmark of acute and chronic lung disease.

The vascular endothelium forms a continuous uninterrupted layer of ECs, held together by complex junctional structures, namely adherens junctions, tight junctions and gap junctions, known as gate keepers for trans-endothelial movements of fluid, protein and cells. It is well established that endothelial barrier permeability under basal conditions and in response to different stimuli or certain agents varies considerably among the different vascular beds[18]. Loss of endothelial barrier causes pulmonary edema, the characteristic feature of Acute Lung Injury (ALI) and High Altitude Pulmonary Edema (HAPE). The pathogenesis of both conditions is still unclear and no drugs are approved for these life-threatening conditions so far.

ALI can progress to the Acute Respiratory Distress Syndrome (ARDS) in which multi-organ failure accounts for high mortality and morbidity. ARDS can be precipitated by either direct (i.e. pneumonia, aspiration of gastric contents) or indirect (i.e. sepsis, multiple trauma) insults to the lung. Sepsis is the most common cause of ARDS in humans and it is ~~s~~well-established also that a pulmonary cause carries with it the highest mortality compared with other etiologies of ARDS. The incidence of ARDS varies widely, from 15 to 70 cases per 100,000 persons per year, representing approximately 5% of hospitalized, mechanically ventilated patients[19].

Being at the interface between the vascular and lung airspaces, the pulmonary endothelium is positioned to play a critical role in establishing immune responses that lead to ALI. The mechanistic understanding underlying these processes remains inadequate and is considered here in light of new findings. The lung's innate immune response involves recruitment of immune cells to pulmonary vessels across which they migrate to the air space. Injury to the endothelial barrier occurs through the paracrine effect of mediators such as arachidonate[20], adenosine triphosphate (ATP)[21] and peroxide[22] released from adjoining epithelial cells, mechanical stresses such as vascular stretch resulting from increased vascular pressure[23], peroxide release from hypoxic red cells[24] and pro-coagulant proteins deposed by platelets[25].

A surprising, yet robust role for endothelial mitochondria in the lung's immune response is becoming increasingly evident: although mitochondria are generally considered to be major sites of cellular ATP generation, little is known about the functional significance of lung endothelial mitochondria in the context of the normal or the diseased lung. The old impression derived from cultured cells that ECs lack mitochondria has been dispelled by more recent real-time fluorescence imaging (RFI) of live lungs studies that provide definitive evidence for the presence of endothelial mitochondria in lung[26]. As a result of enhanced cytosolic Ca^{2+} oscillations, either spontaneous[27] or induced by conditions associated with ALI[26, 28], oscillations of mitochondrial Ca^{2+} increase causing mitochondrial hydrogen peroxide (H_2O_2) production and therefore nuclear factor-κB (NF-κB) pathway activation[26, 28]. The resulting gene transcription leads to endothelial expression of P-selectin and leukocyte adhesion receptors, E-selectin, the defining marker of proinflammatory activation in the lung vasculature. In the pulmonary endothelium, the mitochondrial density tends to be somewhat higher at capillary branch points than in the septal capillaries[23]. Hence, inflammation initiates at capillary branch points, which are also major sites of microvascular filtration – a process that generates the interstitial fluid required to drive lymph flow. Thus, lung endothelial mitochondria activate innate immune mechanisms that in turn induce physiological responses to facilitate lymphatic delivery of antigens to lymph nodes, promoting adaptive immunity **(Figure 1)**.

A protective role for endothelial mitochondria has also emerged through RFI studies addressing expression of the endothelial proinflammatory receptor, TNFR1 (tumor necrosis factor (TNF)α receptor type-1) that is expressed as a trans-membrane protein on the luminal membrane[28]. The mitochondrial H_2O_2 release, resulting from TNFα-induced Ca^{2+}

oscillations, activates the metalloproteinase TACE (TNFα–converting enzyme) that is also expressed on the luminal endothelial membrane, causing shedding of TNFR1 ectodomains, limiting the proinflammatory effects of TNFα.

The new understanding that mitochondria are immune sensors may have implications beyond the pathology of ALI, to inflammatory lung diseases in general[29]. Further research is required to understand disease processes that damage lung mitochondria, particularly in the pulmonary endothelium, and to develop therapeutic strategies for improving mitochondrial function to cure lung diseases.

Loss of endothelial barrier properties in the pulmonary circulation can also cause a high-permeability form of pulmonary edema, defined as HAPE, that can occur in previously healthy, fit, but not acclimatized individuals who~~that~~ ascend rapidly to altitudes above 3,000 – 4,000 m[30, 31]. It is a multifactorial disease, involving both environmental (altitude, rate of ascent) and genetic risk factors[32-34]. In addition, oxygen deprivation causes vasoconstriction of the small pulmonary arteries, when systemic arteries dilate under hypoxic conditions. This phenomenon, known as hypoxic pulmonary vasoconstriction (HPV) is more pronounced as the vessel diameter decreases and represents an important physiological mechanism by which pulmonary arteries constrict in hypoxic lung areas to redirect blood flow to areas with greater oxygen supply. ~~However, i~~ It is now well recognised that chronic exposure to hypoxia (i.e., COPD or living at high altitude) causes ~~ing~~ continuous alveolar hypoxia ~~results~~ resulting in sustained HPV, thought to play a crucial role by exposing the pulmonary capillaries to a high pressure, damaging their walls and leading to pulmonary vascular remodeling, ~~and~~ possibly to PH~~, or to a potentially lethal edem~~ ~~a~~[35, 36].~~:~~

~~, in which hypoxic pulmonary vasoconstriction (HPV) is thought to play a crucial role by exposing the pulmonary capillaries to a high pressure, damaging their walls and leading to a potentially lethal edema[35, 36].~~

Abnormally high PAP in hypoxia has been shown to be ~~is~~ a hallmark of HAPE. ~~and~~ HAPE susceptibility can be revealed at low altitude during a brief hypoxic challenge in people with a history of HAPE[37-39]. Right heart catheterization performed at high altitude ~~revealed~~ showed that all individuals developing pulmonary edema had capillary pressures above a leakage threshold of 19 mmHg, indicating that abnormally high PAP in HAPE translates to the capillary bed[40-42]. Areas with less HPV have a high perfusion causing flow-mediated increased capillary pressure and leakage, while areas with more vigorous

vasoconstriction have lower perfusion and are thus protected from edema formation. This hypothesis is supported by perfusion scan results in HAPE showing that pulmonary edema occurs in areas of high blood flow[31], the observation that edema has a patchy distribution in HAPE[43] and by analysis of lung perfusion in hypoxia by magnetic resonance imaging[44, 45]. In addition, broncho-alveolar lavage (BAL) performed at high altitude in individuals at the outset of HAPE reported an absence of inflammatory markers in the BAL fluid[46]. Inflammatory cells and cytokines were found in BAL fluid in more advanced cases[47] or in patients hospitalized with HAPE in Japan[48]. HAPE is a pressure induced non inflammatory edema that may induce a secondary inflammatory response[30, 49].

A recent meta-analysis of high-altitude studies in which PAP had been measured by echocardiography in people living at altitudes between 3,600 and 4,350 m has concluded that altitude-induced-PH appears to be rare[50]. ~~Mean systolic PAP at high altitude was 25.3 mmHg, modestly elevated compared to that seen in PH clinics.~~ Mean systolic PAP at high altitude was 25.3 mmHg, modestly elevated compared to that seen in healthy individuals from the general population living below 1,200 m. Nonetheless, there is a wide distribution and some people are ~~more prone than~~protected compared to others to PH at altitude[51]. Others have reported that the magnitude of HPV in humans can vary almost five-fold between individuals[52, 53]. This has a genetic basis and understanding the genetic mechanisms involved offers important insights into the molecular regulators of pulmonary vascular tone and structure which can be exploited as drug targets. Studies examining extreme phenotypes in high altitude populations and rat strains have begun to support that approach and open up new areas of research.

HAPE-susceptible individuals display endothelial dysfunction in forearm blood flow studies in hypoxia but not in normoxia, reduced exhaled NO at high altitude[54, 55], decreased pulmonary NO-bioavailability[31, 46] and an increase in production of reactive oxygen species (ROS) across the lung, in particular at the pulmonary artery smooth muscle cell (PA-SMC) level in hypoxia[56]. In addition, plasma levels of ET-1 are increased in parallel with increased PAP in HAPE-susceptible individuals at high altitude[57]. Besides these endothelium-dependent mechanisms accounting for increased HPV in HAPE-susceptible individuals, additional factors must be considered such as a low hypoxic ventilatory response[58] and a smaller capillary bed as suggested by lower lung volumes[59, 60], which can both contribute to an increase of PAP in hypoxia.

In conclusion, there is a need to better understand the mechanisms underlying the differences in endothelial permeability of various vascular beds, the different signaling pathways that modulate interendothelial junctions and how tissue-fluid homeostasis is controlled under normal conditions and pathological processes. Indeed, the restoration of the endothelial barrier is critical for tissue homeostasis and also for recovery following exposure to acute inflammatory events or injury. But our understanding of how this process occurs at the molecular level still remains obscure. More effort should be geared toward developing drugs that facilitate the formation of interendothelial junctions or that enhance the functions of key mediators of barrier integrity.

4. Altered balance between vasoconstriction and vasodilation

As already underlined, the pulmonary circulation has unique hemodynamic features, characterized by a more relaxed vasomotor tone in the normal physiological state, in contrast to the systemic circulation. ~~In addition, oxygen deprivation causes vasoconstriction of the small pulmonary arteries, when systemic arteries dilate under hypoxic conditions. This phenomenon, known as HPV is more pronounced as the vessel diameter decreases and represents an important physiological mechanism by which pulmonary arteries constrict in hypoxic lung areas to redirect blood flow to areas with greater oxygen supply.~~ In the fetus where gas exchange occurs in the placenta, HPV acts to maintain a high pulmonary vascular resistance to divert blood flow away from the lungs through the *ductus arteriosus*. Different ion channels, including potassium (K^+) channels, are expressed and distributed in several types of pulmonary vascular cells including ~~pulmonary artery smooth muscle cells (~~PA-SMCs~~)~~, and contribute to this large functional diversity in the hypoxic response. ~~However, it is now well recognised that chronic exposure to hypoxia (i.e., COPD or living at high altitude) causing continuous alveolar hypoxia results in sustained HPV, leading to pulmonary vascular remodeling, and possibly to PH.~~

Pulmonary ECs synthesize a variety of paracrine and endocrine factors that are capable of controlling vascular tone, either by favoring relaxation (i.e., PGI_2 and NO), or by causing contraction (i.e., ET-1 and 5-HT) under basal conditions and in response to different stimuli or certain agents[61]. There is evidence to suggest that background release of NO contributes to the normally low pulmonary vascular tone in normoxia[61]. Although there are theoretical grounds to hypothesize that hypoxia reduces the synthesis of NO, lack of the latter does not seem to account for the acute HPV. Instead, there is evidence to suggest that NO activity is

increased in order to modulate the pulmonary vasopressor response to acute alveolar hypoxia. The impaired NO production, whilst reducing the ability of the pulmonary vasculature to relax, also favors the occurrence of excessive pulmonary vasoconstriction[5]. Lack of NO synthesis might also permit mitogenesis and proliferation of various cell types within the vascular wall[62]. It is also well established that the RhoA/Rho-associated kinase (ROCK) pathway also regulates pulmonary vascular tone through both NO-dependent and -independent mechanisms[63]. Hypoxia-induced activation of ROCKs increases Ca^{2+} sensitivity of the contractile myofilaments in PA-SMCs, thereby increasing vascular tone and causing sustained HPV and increased pulmonary vascular resistance[63-65].

In the adult, HPV has at least two phases: the initial constrictor response starts within seconds and reaches a maximum within minutes, followed after 30-120 minutes by a sustained phase[52, 64, 66]. These phases are regulated, at least in part, by different signaling pathways. The second phase of HPV is influenced by EC function through changes in the release of vasoactive mediators, such as ET-1, PGI_2 and NO. *In vivo*, neurohumoral mediators, red blood cells and lung innervation may also influence the response. The mechanisms underlying the sensing of low oxygen levels by cells have been the subject of discussion. Both mitochondria and nicotinamide adenine dinucleotide (phosphate) (NAD[P]H) oxidases have been suggested as oxygen sensors. A change in the levels of ROS is thought to be important but there is lack of agreement about whether the signal is an increase or decrease in ROS. Hypoxia-inducible factor (HIF)-1 and HIF-2 that transactivate a number of common as well as distinct downstream target genes are clearly candidates for explaining variability in the pulmonary vascular response to chronic hypoxia[67]. In addition to differing in terms of downstream targets, their α subunit expression levels are also known to vary in cells and tissues. Recently, a comparison of two rat strains, F344 and WKY, that differ in their response to chronic hypoxia has highlighted the importance of zinc in several cell types, and in particular in PA-SMCs, in regulating pulmonary vascular homeostasis[68]. A mutation in the *Solute carrier family 39 member 12* (*Slc39A12*,) gene, which encodes the zinc transporter ZIP12, predicts a truncated and inactive protein in the F344 rat strain. Introduction of a similar mutation in the WKY rat strain reduced responsiveness of the WKY strain to hypoxia. Upregulation of ZIP12 expression in the lungs of humans living at high altitude (and patients with idiopathic pulmonary arterial hypertension (PAH)) has now stimulated a search for small molecule inhibitors of ZIP12, and interest in the role of intracellular labile zinc in pulmonary vascular disease.

5. From a dynamic to an unadapted pulmonary vascular remodeling

The pulmonary circulation responds to alveolar hypoxia by increasing pulmonary vascular resistance and HPV is responsible for the initial rise in pulmonary pressures[4, 52, 53]. With continued exposure to hypoxia, other mechanisms which drive structural changes in resistance vessels contribute to the elevated pressures, thus explaining why after two or three weeks of hypoxia there is little response to rebreathing 100% oxygen. However, pulmonary pressures decrease progressively with time on re-exposure to a normal oxygen environment, suggesting that the pulmonary vascular remodeling-induced by chronic hypoxia is reversible. Histological examination of lungs from mammals, including rat, cow and humans, exposed chronically to hypoxia demonstrate structural remodelling of pulmonary arterioles[69]. All layers of the vascular wall, including fibroblasts, are involved in the remodeling, but the hallmark of the vascular response to chronic hypoxia is increased muscularisation of distal vessels with extension of muscle into previously non muscularised arterioles **(Figure 2)**. The relative contributions of HPV and vascular remodeling to chronic hypoxia-induced PH are the subject of debate[70]. Histological studies in the rat contest the extent to which remodeling narrows the vascular lumen and so provides an obstruction. A recent consideration in this debate is vascular stiffness. The changes in vascular structure lead to an increase in stiffness which alters the transmission of pulse waves along affected vessels and their reflection from branch points. The right heart then senses an increased pressure load from the combination of advancing and reflected waves. The role of haemodynamic forces and shear stress in initiating and sustaining pulmonary vascular remodeling in hypoxia has been highlighted recently by studies in the rat. Pulmonary artery banding reduces haemodynamic shear stress and not only prevented the development of vascular remodeling but also reversed occlusive lesions and perivascular inflammation in the Sugen-hypoxia model[71]. It is easy to speculate that haemodynamic stress and occlusive vascular lesions could form a vicious cycle. If so, these observations suggest that mechanisms to reduce shear stress, for example with vasodilator therapies, should not be ignored in the search for treatments for PH.

PH, defined as a mean PAP $\geq$ 25 mmHg at rest as assessed by right heart catheterization, can present in a number of clinical scenarios, including several with a high mortality rate [72, 73]: pulmonary arterial hypertension (PAH or group 1), PH due to lung diseases and/or hypoxia such as COPD and emphysema (group 3), chronic thromboembolic pulmonary hypertension (CTEPH or group 4) and PH with unclear multifactorial

mechanisms, such as hematological disorders (group 5)[74]. In all these PH subgroups, pulmonary endothelial dysfunction contributes to pulmonary vascular remodeling [75, 76]. In particular, major functional alterations in the pulmonary vascular endothelium in PAH have been demonstrated, including among others: 1) a transition from a quiescent state (without adhesion capacity) to an activated state with adhesive capacity[77]; 2) an aberrant pro-proliferative and apoptosis resistant phenotype[78]); 3) a pro-inflammatory phenotype characterized by an excessive release of various key cytokines and chemokines: interleukin (IL)-1α, IL-6, IL-8, IL-12, CCL2/monocyte chemotactic protein (MCP)-1[77]; 4) an excessive production and secretion of various key growth factors including fibroblast growth factor-2[78, 79], angiotensin-II (Ang II)[80], and leptin[81-83]. Additional insights into the altered pulmonary EC phenotype and the endothelial communication with both resident vascular cells (PA-SMCs, myofibroblasts) and immune cells are a prerequisite for a better understanding of PAH pathogenesis that could lead to novel therapeutical strategies.

More recently, an endothelial communication was reported with pericytes, a key progenitor cell-type that are found around pre-capillary arteries, capillaries, and post-capillary venules and ~~that~~ occupy a strategic position at the interface between circulating blood and interstitial space, and at close proximity to ECs and PA-SMCs. Ricard and coauthors ~~We~~ have demonstrated an excessive pericyte coverage in distal pulmonary arteries in human PAH (a 2- to 3-fold increase), a phenomenon that contributes to pulmonary vascular remodeling as a source of smooth muscle-like cells[84]. Pericytes are central regulators of vascular development, stabilization, maturation, and remodeling modulating: (i) EC growth, proliferation, differentiation and migration; (ii) PA-SMC contraction and capillary blood flow; (iii) immune cell functions; (iv) and their possible differentiation in SMC-like cells and dendritic cells, acting as progenitor cells.

Deciphering the intracellular pathways that modulate pulmonary vascular remodeling has paved the way for precise molecular targeting strategies with new treatments for PH ~~Deciphering the intracellular pathways modulating the actin-myosin interaction mechanisms have paved the ways for precise molecular targeting strategies with new treatments for PH~~ [85]. For example, one can now choose between specific and nonspecific ET-1 receptors, or stimulation of synthesis or inhibition of degradation of the key intracellular, second messenger cyclic guanosine monophosphate (cGMP) to alter pulmonary vascular tone in diseased states. Even though the prognosis remains poor, these additional pathways may represent~~ent~~ innovative tools for the treatment of PH in the future.

In PH due to lung disease, such as COPD, pulmonary vascular impairment is highly prevalent: about 40% of patients with COPD GOLD 4 and 27% of GOLD 3 stages display an increased mPAP[86]. Among patients w~~h~~ithout ~~o do not present~~ PH at rest, a significant proportion of them develop it during exercise[87, 88]. These findings concur with the frequent observation of histological changes in small pulmonary arteries, mainly consisting of intimal hyperplasia, in patients with end-stage disease who underwent lung transplantation[5], but even in patients with mild-to-moderate disease[6, 89]. In addition to vascular remodeling, dysregulated vascular tone is a major contributor to pulmonary hypertension in COPD: the expression of eNOS[90], sGC[16] and PGI_2 synthase[36] are reduced in the pulmonary arteries of COPD patients, thus decreasing their vasodilator and antiproliferative capacity **(Figure 3)**.

With this background it is interesting to note that some patients with extensive emphysema develop severe PH[91], suggesting a link between the emphysematous destruction of lung parenchyma and the severity of pulmonary vascular impairment. In fact, in explanted lungs of COPD patients who underwent lung transplant, the severity of emphysema was significantly higher in those patients who had PH[92]. Accordingly, the combination of vascular remodeling, endothelial dysfunction and parenchymal derangement might explain the development of severe PH in a subset of patients with COPD[93].

Pulmonary endothelium represents an interesting therapeutic target in COPD. Studies conducted in experimental models show that targeting the NO-cGMP signaling pathway with sGC stimulators[16] or phosphodiesterase-5 (PDE5) inhibitors[94, 95] prevents the development of PH, pulmonary vascular remodeling and right ventricle hypertrophy. Interestingly, sGC stimulators and PDE5 inhibitors also attenuated the development of pulmonary emphysema[16, 95], an effect that was associated with a reduction in the number of neutrophils and alveolar macrophages in lung tissue, as well as a reduction in leukocyte adhesion[16]. Taken together these findings suggest that therapeutic interventions in the NO-cGMP signaling pathway exert a dual effect: on one hand, relaxation and eventually attenuation of cell proliferation through protein kinase G-dependent mechanisms in smooth muscle cells; on the other, reduction of leukocyte adhesion in ECs, presumably by downregulating P-selectin[96].

These results have been obtained using a preventive experimental design but it remains to be shown to what extent the therapeutic intervention exerts the same effects when lung damage is already established, using a “therapeutic” design. In fact, treatment with PDE5

inhibitors or sGC stimulators in COPD patients with PH has been ineffective in improving exercise tolerance[86]. However, exercise tolerance might not be the most adequate surrogate of the effects of treatment on vascular endothelium. Further experimental and clinical studies addressing the effects of therapeutic interventions on the disturbed endothelium are warranted to elucidate the significance of this approach in COPD.

In PH due to hematological disorders, such sickle cell disease (SCD)[97-101], the mechanistic linkage between hemolytic anemia and vasculopathy has been the subject of extensive study in pre-clinical animal models, in vascular studies in patients, and in large human cohort studies[99, 102, 103]. Intravascular hemolysis releases cell-free hemoglobin (Hb) in the plasma, which can scavenge NO and generate ROS, impairing redox balance and leading to proliferative systemic and pulmonary vasculopathy[103, 104]. It has been recently appreciated that products released from ~~the~~ red cell during hemolysis can be considered danger associated molecular pattern molecules or erythrocyte "DAMPs" (eDAMPs)[105]. These studies in SCD support a more general pathological role for intravascular hemolysis and cell-free Hb in various human diseases and in transfusion medicine[106].

6. Biological factors to assess pulmonary endothelial dysfunction

In light of the critical role played by the pulmonary endothelium in acute and chronic lung diseases, identification of biological factors to assess its (dys)function is needed in the field, but represents a real challenge for clinicians and researchers. Indeed, a broad appreciation of the numerous functions of the endothelium request the assessment of a large panel of molecules of endothelial origin in circulating blood (Table 1), and a comprehensive prior assessment and validation in large and well-characterized cohorts of patients before routine clinical use. These EC products could include, among others: measures of NO biology, circulating miRNAs, cytokines, chemokines, circulating adhesion molecules, growth factors, regulators of thrombosis, as well as markers of endothelial damage (i.e. detachment of mature ECs, derived microparticles (EMPs)) and repair (i.e., number and functional charasteristics of circulating endothelial progenitor cells).

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Besides changes in vasoactive mediators, COPD patients present structural damage in pulmonary endothelium. Areas of cell denudation and detachment between endothelial cells have been shown in pulmonary arteries of COPD[107]. The presence of endothelial damage in COPD has also been demonstrated by increased numbers of EMPs[108, 109]. Circulating

EMPs may originate from endothelial cell apoptosis or activation, which can be identified by the expression of the CD31 and CD62E markers, respectively. The number of circulating EMPs is inversely related to the FEV1[109]. Interestingly, whereas $CD31^+$ EMPs (apoptotic) are already significantly elevated in patients with mild COPD, $CD62E^+$ EMPs (activated) are increased only in patients with severe disease. The number of circulating EMPs further increases during exacerbation episodes[110]. The number of circulating EMPs correlates with the severity of emphysema[109] pointing to a connection between parenchymal destruction and endothelial damage. Furthermore, in COPD, circulating EMPs are also correlated with vascular function of systemic arteries. An increased number of circulating EMPs is associated with impaired endothelial function and increased stiffness in systemic arteries[111]. COPD patients also show reduced numbers of bone marrow-derived circulating progenitor cells (EPCs)[112-115], which play a key role in the repair process of damaged endothelium and are closely related to endothelial function[116]. Studies carried out during the last years have made a great effort in trying to identify, define and characterize cell populations with phenotypic, biochemical, molecular and functional properties of EPCs[117, 118]. EPCs have been suggested as a precious source for generating functionally competent endothelial cells (ECs), candidate for various clinical applications. However, the paucity of these progenitor cells and the technical difficulties for their *in vitro* growth represent a main limitation to their use. Interestingly, recent studies have led to the identification in hematopoietic tissues (blood, bone marrow and cord blood) of very rare EPCs not originating from the bone marrow, called endothelial colony-forming cells (ECFCs) or late outgrowth ECs, ~~and~~ capable of generating a large progeny of phenotypically and functionally competent mature ECs *in vitro* and of sustaining *in vivo* angiogenetic processes[119, 120]. These EPCs are also present at the level of the vessel wall, particularly in the vascular endothelial intima[117, 118].

This methodology can be easily extended to the study of circulating EPCs and can facilitate and improve the functional characterization of EPCs in various pathologic conditions, particularly in various lung diseases.

7. Conclusion

In conclusion, it is now well established that the pulmonary vascular endothelium is involved in most if not all acute and chronic lung disease, either as a primary determinant of these disease processes or as a victim of collateral damage **(Figure 4)**. Although considerable

progress has been made in the understanding of pulmonary endothelial cell (dys)function, the triggers, the mechanisms and the consequences of dysfunctional endothelium in lung disease, both acute and chronic, are still not completely understood. In addition, further efforts are also needed to elucidate the remarkable heterogeneity of the pulmonary endothelium in space and time, in structure and function, as well as in health and disease. An understanding of the molecular basis for these key aspects would help finding new disease biomarkers and/or novel therapeutical targets to preserve homeostasis in response to injury and disease and to reduce the "bench-to-bedside" gap in endothelial biomedicine.

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Table 1 - A partial list of pulmonary endothelium-derived factors

Vasoconstrictors:	Serotonin (5-HT) Endothelin (ET)-1, ET-2, and ET-3 Angiotensin (Ang-II) Thromboxane A2 Oxidative stress
Vasodilators:	Nitric oxide (NO) Prostacyclin (PGI_2) Apelin Adrenomedullin
Cell accumulation:	Fibroblast growth factor (FGF)-2 Vascular endothelial growth factor (VEGF) Platelet derived growth factor (PDGF) Tumor growth factor (TGF)

	Epidermal growth factor (EGF) Nerve growth factor (NGF)
Inflammation:	Interleukin (IL)-1, IL-6, IL-8… Tumor necrosis factor (TNF) Interferon Leptin (Ob) Leukotrienes
Other proteins:	Plasminogen activator inhibitor (PAI)-1 Soluble E-selectin Soluble intercellular adhesion molecule (ICAM)-1 Soluble vascular cell adhesion protein 1 (VCAM)-1 Matrix metalloproteinases (MMP)-2, MMP-9, MMP-10… Tissue plasminogen activator (tPA) Platelet-activating factor Thrombomodulin

Figure legends

Figure 1: Inflammation and the pulmonary microcirculation

Inflammation-mediated events occurring in pulmonary arterioles, capillaries and venules contributes to impaired capillary perfusion. The presented sample was taken from the lungs of a patient with chronic thromboembolic pulmonary hypertension. Note the moderate to severe microvascular inflammation (in the absence of any major vascular wall remodeling)

Figure 2: Vascular response to chronic hypoxia

Previously non muscularized arterioles (A) show "muscularization" with recruitment of smooth muscle cells and myofibroblasts (B). Note the mild inflammatory infiltrate (predominating on the right side of the vessel) which accompanies the remodeling process

Figure 3: Pulmonary artery in a patient with chronic obstructive pulmonary disease

Histological changes of a small pulmonary artery in a patient with chronic obstructive pulmonary disease (COPD). The vessel (here at a dichotomous branching point) presents with important intimal fibrosis. Note the mild perivascular infiltrate (predominating beneath the artery).The surrounding pulmonary parenchyma displays emphysematous remodeling

Figure 4:

Schematic overview of pulmonary endothelial dysfunction main characteristics

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Pulmonary Vascular Endothelium: Orchestra Conductor in Respiratory Diseases

Highlights from Basic Research to Therapy

Alice HUERTAS and Christophe GUIGNABERT on behalf of the ERS Research Seminar Faculty*

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Abstract

The European Respiratory Society (ERS) Research Seminar entitled ''*Pulmonary Vascular Endothelium: Orchestra Conductor in Respiratory Diseases - Highlights from Basic Research to Therapy*'' brought together international experts of dysfunctional pulmonary endothelium, from basic science to translational medicine, to discuss several important aspects in acute and chronic lung diseases. This review will briefly sum up the different topics of discussion of this meeting held in Paris (France) on October 27th and 28th, 2016. It is important to consider that this paper does not address all aspects of endothelial dysfunction but focuses on specific themes: (1) the complex role of the pulmonary endothelium in orchestrating the host response in both health and disease (acute lung injury, chronic obstructive pulmonary disease and high altitude pulmonary edema, pulmonary hypertension); (2) the potential value of dysfunctional pulmonary endothelium as a target for innovative therapies. The main conclusions and perspectives are hereafter reported.

Key words: *Lung; Pulmonary endothelium; Endothelial dysfunction; Respiratory diseases; Innovative therapeutics.*

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1. Introduction

The lung is supplied by two distinct circulatory systems, the systemic bronchial circulation and the pulmonary circulation. From the functional point of view, the pulmonary circulation is unique among vascular beds in the human body, characterized by a high flow, low-resistance, low-pressure system. Although all of the cardiac output is pumped through the pulmonary circulation, the measured values of mean pulmonary arterial pressure (mPAP) in pulmonary arteries for a resting, healthy adult human is about 14±3 mm of mercury (Hg), when systemic arterial blood pressure is about 100±20 mm Hg. This unique feature of the pulmonary circulation is mainly explained by three~~wo~~ critical characteristics of the pulmonary vasculature: **(1)** a low impedance to blood flow; **(2)** a high compliance of pulmonary precapillary arterioles characterized by a thin vessel wall; **(3~~2~~)** a high capacity to recruit available vessels that help accommodate an increase in flow. Indeed, this circulatory system can accommodate flow rates ranging from approximately 6 L/min under resting conditions to 25 L/min in strenuous exercise, with minimal increases in pulmonary pressures[1].

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Because of their strategic localization, at the interface between bloodstream and lung tissue, the pulmonary endothelial cells (ECs) play key roles not only in optimizing gas exchanges and in controlling barrier integrity and function, but also in regulating pulmonary vascular tone (i.e. through nitric oxide (NO), prostacyclin (PGI_2), endothelin (ET) and serotonin (5-HT) pathways)[2]. Moreover, pulmonary endothelium functions as an active and dynamic receptor-effector tissue and responds to the different chemical, physical, or mechanical stimuli by secreting the correct substance(s) by which it may maintain the vasomotor balance and vascular-tissue homeostasis. Indeed, pulmonary ECs are highly metabolically active, sensing and responding to signals from extracellular environments. All these interactions with adjacent cells and circulating cells and/or mediators aim to maintain a thrombosis-free surface, to control inflammatory cell adhesion and trafficking, to assure a normal angiogenesis and the integrity of the vascular wall[3]. As a vital part of the respiratory system, alterations of pulmonary endothelium play a central role in the pathogenesis of several chronic and acute lung diseases, common or rare. The main characteristics of pulmonary endothelial alterations or dysfunction are mainly reflected by increased permeability that can lead to vascular leakage and edema formation; altered balance between vasoconstriction and vasodilation; acquisition of a pro-inflammatory phenotype with increased expression of adhesion molecules for inflammatory cell recruitment, increased activation of pro-inflammatory transcription factors, release of inflammatory mediators and

oxidative stress; pro-thrombotic phenotype; pro-proliferative and anti-apoptotic phenotype; miscommunication with adjacent vascular cell wall.

Although considerable progress has been made in the understanding of pulmonary EC dysfunction, the triggers, the mechanisms and the consequences of dysfunctional endothelium in lung disease, acute and chronic, are still not completely understood. A better knowledge of these key aspects would help in finding new disease biomarkers and/or novel therapeutical targets to preserve homeostasis in response to injury and disease(s).

2. The importance of genetic and environmental factors

Most, if not all, acute and chronic lung diseases result from interaction between multiple environmental exposures and many genetic risk factors, an aspect that has been critically evaluated in a recent review from this European Lung White book[4]. The role of vasculature in the development, lifelong repair and maintenance of the lung tissue homeostasis is of paramount importance. Indeed, many diseases involving the lung, such as emphysema, chronic obstructive pulmonary disease (COPD)[5, 6] and pulmonary hypertension (PH)[7], display major EC abnormalities. A better understanding of how pulmonary ECs contribute to normal lung development and how these mechanisms are deregulated during chronic disease is of crucial importance for the development of new therapeutic strategies for debilitating lung conditions. However, ~~inter~~individual differences in response to the same stimulus in subjects are well documented, such as environmental hypoxia at high altitude. In addition, it is well established that the pulmonary vasopressor response varies greatly between humans and animals, and even between different species, supporting the importance of unknown genetic influences.

Beyond the genetic background and specific gene mutations, other factors are known to determine structural and functional modifications of the pulmonary vasculature, such as exogenous exposures to drugs and toxins, hormones, and aging, with decreased repair capacity[8-10] and impaired productions of different vasoreactive mediators[11].

Another potential agent capable of causing endothelial impairment is represented by cigarette smoke products, that play a major role in COPD since “healthy” smokers (without COPD) already show significant abnormalities in pulmonary arteries that include among others: vessel remodeling, inflammatory cell infiltrate, endothelial dysfunction, reduced

expression of endothelial NO synthase (eNOS), increased expression of growth factors and a similar gene expression profile to that observed in COPD patients[12, 13]. In experimental models of COPD, it has been also demonstrated that chronic exposure to cigarette smoke induces endothelial dysfunction in pulmonary arteries [14], along with changes in the expression of eNOS[15] and soluble guanylate cyclase (sGC)[16]. Interestingly, vascular changes antecede the development of pulmonary emphysema[17]. Altogether, these findings strongly support relations between smoking habits and pulmonary vascular damage in COPD.

3. Dysfunction of endothelial barrier properties

The vascular endothelium forming the innermost lining of all pulmonary blood vessels is the major barrier that protects air spaces against vascular fluid entry. The integrity of this endothelial barrier permeability is critical for the maintenance of homeostasis for all tissues and organs of the body, and changes in its permeability are a hallmark of acute and chronic lung disease.

The vascular endothelium forms a continuous uninterrupted layer of ECs, held together by complex junctional structures, namely adherens junctions, tight junctions and gap junctions, known as gate keepers for trans-endothelial movements of fluid, protein and cells. It is well established that endothelial barrier permeability under basal conditions and in response to different stimuli or certain agents varies considerably among the different vascular beds[18]. Loss of endothelial barrier causes pulmonary edema, the characteristic feature of Acute Lung Injury (ALI) and High Altitude Pulmonary Edema (HAPE). The pathogenesis of both conditions is still unclear and no drugs are approved for these life-threatening conditions so far.

ALI can progress to the Acute Respiratory Distress Syndrome (ARDS) in which multi-organ failure accounts for high mortality and morbidity. ARDS can be precipitated by either direct (i.e. pneumonia, aspiration of gastric contents) or indirect (i.e. sepsis, multiple trauma) insults to the lung. Sepsis is the most common cause of ARDS in humans and it is ~~s~~well-established also that a pulmonary cause carries with it the highest mortality compared with other etiologies of ARDS. The incidence of ARDS varies widely, from 15 to 70 cases per 100,000 persons per year, representing approximately 5% of hospitalized, mechanically ventilated patients[19].

Being at the interface between the vascular and lung airspaces, the pulmonary endothelium is positioned to play a critical role in establishing immune responses that lead to ALI. The mechanistic understanding underlying these processes remains inadequate and is considered here in light of new findings. The lung's innate immune response involves recruitment of immune cells to pulmonary vessels across which they migrate to the air space. Injury to the endothelial barrier occurs through the paracrine effect of mediators such as arachidonate[20], adenosine triphosphate (ATP)[21] and peroxide[22] released from adjoining epithelial cells, mechanical stresses such as vascular stretch resulting from increased vascular pressure[23], peroxide release from hypoxic red cells[24] and pro-coagulant proteins deposed by platelets[25].

A surprising, yet robust role for endothelial mitochondria in the lung's immune response is becoming increasingly evident: although mitochondria are generally considered to be major sites of cellular ATP generation, little is known about the functional significance of lung endothelial mitochondria in the context of the normal or the diseased lung. The old impression derived from cultured cells that ECs lack mitochondria has been dispelled by more recent real-time fluorescence imaging (RFI) of live lungs studies that provide definitive evidence for the presence of endothelial mitochondria in lung[26]. As a result of enhanced cytosolic Ca^{2+} oscillations, either spontaneous[27] or induced by conditions associated with ALI[26, 28], oscillations of mitochondrial Ca^{2+} increase causing mitochondrial hydrogen peroxide (H_2O_2) production and therefore nuclear factor-κB (NF-κB) pathway activation[26, 28]. The resulting gene transcription leads to endothelial expression of P-selectin and leukocyte adhesion receptors, E-selectin, the defining marker of proinflammatory activation in the lung vasculature. In the pulmonary endothelium, the mitochondrial density tends to be somewhat higher at capillary branch points than in the septal capillaries[23]. Hence, inflammation initiates at capillary branch points, which are also major sites of microvascular filtration – a process that generates the interstitial fluid required to drive lymph flow. Thus, lung endothelial mitochondria activate innate immune mechanisms that in turn induce physiological responses to facilitate lymphatic delivery of antigens to lymph nodes, promoting adaptive immunity **(Figure 1)**.

A protective role for endothelial mitochondria has also emerged through RFI studies addressing expression of the endothelial proinflammatory receptor, TNFR1 (tumor necrosis factor (TNF)α receptor type-1) that is expressed as a trans-membrane protein on the luminal membrane[28]. The mitochondrial H_2O_2 release, resulting from TNFα-induced Ca^{2+}

oscillations, activates the metalloproteinase TACE (TNFα–converting enzyme) that is also expressed on the luminal endothelial membrane, causing shedding of TNFR1 ectodomains, limiting the proinflammatory effects of TNFα.

The new understanding that mitochondria are immune sensors may have implications beyond the pathology of ALI, to inflammatory lung diseases in general[29]. Further research is required to understand disease processes that damage lung mitochondria, particularly in the pulmonary endothelium, and to develop therapeutic strategies for improving mitochondrial function to cure lung diseases.

Loss of endothelial barrier properties in the pulmonary circulation can also cause a high-permeability form of pulmonary edema, defined as HAPE, that can occur in previously healthy, fit, but not acclimatized individuals who~~that~~ ascend rapidly to altitudes above 3,000 – 4,000 m[30, 31]. It is a multifactorial disease, involving both environmental (altitude, rate of ascent) and genetic risk factors[32-34]. In addition, oxygen deprivation causes vasoconstriction of the small pulmonary arteries, when systemic arteries dilate under hypoxic conditions. This phenomenon, known as hypoxic pulmonary vasoconstriction (HPV) is more pronounced as the vessel diameter decreases and represents an important physiological mechanism by which pulmonary arteries constrict in hypoxic lung areas to redirect blood flow to areas with greater oxygen supply. ~~However, i~~ It is now well recognised that chronic exposure to hypoxia (i.e., COPD or living at high altitude) causes ~~ing~~ continuous alveolar hypoxia ~~results~~ resulting in sustained HPV, thought to play a crucial role by exposing the pulmonary capillaries to a high pressure, damaging their walls and leading to pulmonary vascular remodeling, ~~and~~ possibly to PH~~, or to a potentially lethal edem~~ ~~a~~[35, 36].~~:~~

~~, in which hypoxic pulmonary vasoconstriction (HPV) is thought to play a crucial role by exposing the pulmonary capillaries to a high pressure, damaging their walls and leading to a potentially lethal edema[35, 36].~~

Abnormally high PAP in hypoxia has been shown to be ~~is~~ a hallmark of HAPE. ~~and~~ HAPE susceptibility can be revealed at low altitude during a brief hypoxic challenge in people with a history of HAPE[37-39]. Right heart catheterization performed at high altitude ~~revealed~~ showed that all individuals developing pulmonary edema had capillary pressures above a leakage threshold of 19 mmHg, indicating that abnormally high PAP in HAPE translates to the capillary bed[40-42]. Areas with less HPV have a high perfusion causing flow-mediated increased capillary pressure and leakage, while areas with more vigorous

vasoconstriction have lower perfusion and are thus protected from edema formation. This hypothesis is supported by perfusion scan results in HAPE showing that pulmonary edema occurs in areas of high blood flow[31], the observation that edema has a patchy distribution in HAPE[43] and by analysis of lung perfusion in hypoxia by magnetic resonance imaging[44, 45]. In addition, broncho-alveolar lavage (BAL) performed at high altitude in individuals at the outset of HAPE reported an absence of inflammatory markers in the BAL fluid[46]. Inflammatory cells and cytokines were found in BAL fluid in more advanced cases[47] or in patients hospitalized with HAPE in Japan[48]. HAPE is a pressure induced non inflammatory edema that may induce a secondary inflammatory response[30, 49].

A recent meta-analysis of high-altitude studies in which PAP had been measured by echocardiography in people living at altitudes between 3,600 and 4,350 m has concluded that altitude-induced-PH appears to be rare[50]. Mean systolic PAP at high altitude was 25.3 mmHg, modestly elevated compared to that seen in healthy individuals from the general population living below 1,200 m. Nonetheless, there is a wide distribution and some people are ~~more prone than~~protected compared to others to PH at altitude[51]. Others have reported that the magnitude of HPV in humans can vary almost five-fold between individuals[52, 53]. This has a genetic basis and understanding the genetic mechanisms involved offers important insights into the molecular regulators of pulmonary vascular tone and structure which can be exploited as drug targets. Studies examining extreme phenotypes in high altitude populations and rat strains have begun to support that approach and open up new areas of research.

HAPE-susceptible individuals display endothelial dysfunction in forearm blood flow studies in hypoxia but not in normoxia, reduced exhaled NO at high altitude[54, 55], decreased pulmonary NO-bioavailability[31, 46] and an increase in production of reactive oxygen species (ROS) across the lung, in particular at the pulmonary artery smooth muscle cell (PA-SMC) level in hypoxia[56]. In addition, plasma levels of ET-1 are increased in parallel with increased PAP in HAPE-susceptible individuals at high altitude[57]. Besides these endothelium-dependent mechanisms accounting for increased HPV in HAPE-susceptible individuals, additional factors must be considered such as a low hypoxic ventilatory response[58] and a smaller capillary bed as suggested by lower lung volumes[59, 60], which can both contribute to an increase of PAP in hypoxia.

In conclusion, there is a need to better understand the mechanisms underlying the differences in endothelial permeability of various vascular beds, the different signaling pathways that modulate interendothelial junctions and how tissue-fluid homeostasis is

controlled under normal conditions and pathological processes. Indeed, the restoration of the endothelial barrier is critical for tissue homeostasis and also for recovery following exposure to acute inflammatory events or injury. But our understanding of how this process occurs at the molecular level still remains obscure. More effort should be geared toward developing drugs that facilitate the formation of interendothelial junctions or that enhance the functions of key mediators of barrier integrity.

4. Altered balance between vasoconstriction and vasodilation

As already underlined, the pulmonary circulation has unique hemodynamic features, characterized by a more relaxed vasomotor tone in the normal physiological state, in contrast to the systemic circulation. ~~In addition, oxygen deprivation causes vasoconstriction of the small pulmonary arteries, when systemic arteries dilate under hypoxic conditions. This phenomenon, known as HPV is more pronounced as the vessel diameter decreases and represents an important physiological mechanism by which pulmonary arteries constrict in hypoxic lung areas to redirect blood flow to areas with greater oxygen supply.~~ In the fetus where gas exchange occurs in the placenta, HPV acts to maintain a high pulmonary vascular resistance to divert blood flow away from the lungs through the *ductus arteriosus*. Different ion channels, including potassium (K^+) channels, are expressed and distributed in several types of pulmonary vascular cells including ~~pulmonary artery smooth muscle cells (~~PA-SMCs~~)~~, and contribute to this large functional diversity in the hypoxic response. ~~However, it is now well recognised that chronic exposure to hypoxia (i.e., COPD or living at high altitude) causing continuous alveolar hypoxia results in sustained HPV, leading to pulmonary vascular remodeling, and possibly to PH.~~

Pulmonary ECs synthesize a variety of paracrine and endocrine factors that are capable of controlling vascular tone, either by favoring relaxation (i.e., PGI_2 and NO), or by causing contraction (i.e., ET-1 and 5-HT) under basal conditions and in response to different stimuli or certain agents[61]. There is evidence to suggest that background release of NO contributes to the normally low pulmonary vascular tone in normoxia[61]. Although there are theoretical grounds to hypothesize that hypoxia reduces the synthesis of NO, lack of the latter does not seem to account for the acute HPV. Instead, there is evidence to suggest that NO activity is increased in order to modulate the pulmonary vasopressor response to acute alveolar hypoxia. The impaired NO production, whilst reducing the ability of the pulmonary vasculature to relax, also favors the occurrence of excessive pulmonary vasoconstriction[5]. Lack of NO

synthesis might also permit mitogenesis and proliferation of various cell types within the vascular wall[62]. It is also well established that the RhoA/Rho-associated kinase (ROCK) pathway also regulates pulmonary vascular tone through both NO-dependent and -independent mechanisms[63]. Hypoxia-induced activation of ROCKs increases Ca^{2+} sensitivity of the contractile myofilaments in PA-SMCs, thereby increasing vascular tone and causing sustained HPV and increased pulmonary vascular resistance[63-65].

In the adult, HPV has at least two phases: the initial constrictor response starts within seconds and reaches a maximum within minutes, followed after 30-120 minutes by a sustained phase[52, 64, 66]. These phases are regulated, at least in part, by different signaling pathways. The second phase of HPV is influenced by EC function through changes in the release of vasoactive mediators, such as ET-1, PGI_2 and NO. *In vivo*, neurohumoral mediators, red blood cells and lung innervation may also influence the response. The mechanisms underlying the sensing of low oxygen levels by cells have been the subject of discussion. Both mitochondria and nicotinamide adenine dinucleotide (phosphate) (NAD[P]H) oxidases have been suggested as oxygen sensors. A change in the levels of ROS is thought to be important but there is lack of agreement about whether the signal is an increase or decrease in ROS. Hypoxia-inducible factor (HIF)-1 and HIF-2 that transactivate a number of common as well as distinct downstream target genes are clearly candidates for explaining variability in the pulmonary vascular response to chronic hypoxia[67]. In addition to differing in terms of downstream targets, their α subunit expression levels are also known to vary in cells and tissues. Recently, a comparison of two rat strains, F344 and WKY, that differ in their response to chronic hypoxia has highlighted the importance of zinc in several cell types, and in particular in PA-SMCs, in regulating pulmonary vascular homeostasis[68]. A mutation in the *Solute carrier family 39 member 12* (*Slc39A12*,) gene, which encodes the zinc transporter ZIP12, predicts a truncated and inactive protein in the F344 rat strain. Introduction of a similar mutation in the WKY rat strain reduced responsiveness of the WKY strain to hypoxia. Upregulation of ZIP12 expression in the lungs of humans living at high altitude (and patients with idiopathic pulmonary arterial hypertension (PAH)) has now stimulated a search for small molecule inhibitors of ZIP12, and interest in the role of intracellular labile zinc in pulmonary vascular disease.

5. From a dynamic to an unadapted pulmonary vascular remodeling

The pulmonary circulation responds to alveolar hypoxia by increasing pulmonary vascular resistance and HPV is responsible for the initial rise in pulmonary pressures[4, 52, 53]. With continued exposure to hypoxia, other mechanisms which drive structural changes in resistance vessels contribute to the elevated pressures, thus explaining why after two or three weeks of hypoxia there is little response to rebreathing 100% oxygen. However, pulmonary pressures decrease progressively with time on re-exposure to a normal oxygen environment, suggesting that the pulmonary vascular remodeling-induced by chronic hypoxia is reversible. Histological examination of lungs from mammals, including rat, cow and humans, exposed chronically to hypoxia demonstrate structural remodelling of pulmonary arterioles[69]. All layers of the vascular wall, including fibroblasts, are involved in the remodeling, but the hallmark of the vascular response to chronic hypoxia is increased muscularisation of distal vessels with extension of muscle into previously non muscularised arterioles **(Figure 2)**. The relative contributions of HPV and vascular remodeling to chronic hypoxia-induced PH are the subject of debate[70]. Histological studies in the rat contest the extent to which remodeling narrows the vascular lumen and so provides an obstruction. A recent consideration in this debate is vascular stiffness. The changes in vascular structure lead to an increase in stiffness which alters the transmission of pulse waves along affected vessels and their reflection from branch points. The right heart then senses an increased pressure load from the combination of advancing and reflected waves. The role of haemodynamic forces and shear stress in initiating and sustaining pulmonary vascular remodeling in hypoxia has been highlighted recently by studies in the rat. Pulmonary artery banding reduces haemodynamic shear stress and not only prevented the development of vascular remodeling but also reversed occlusive lesions and perivascular inflammation in the Sugen-hypoxia model[71]. It is easy to speculate that haemodynamic stress and occlusive vascular lesions could form a vicious cycle. If so, these observations suggest that mechanisms to reduce shear stress, for example with vasodilator therapies, should not be ignored in the search for treatments for PH.

PH, defined as a mean PAP $\geq$ 25 mmHg at rest as assessed by right heart catheterization, can present in a number of clinical scenarios, including several with a high mortality rate [72, 73]: pulmonary arterial hypertension (PAH or group 1), PH due to lung diseases and/or hypoxia such as COPD and emphysema (group 3), chronic thromboembolic pulmonary hypertension (CTEPH or group 4) and PH with unclear multifactorial mechanisms, such as hematological disorders (group 5)[74]. In all these PH subgroups,

pulmonary endothelial dysfunction contributes to pulmonary vascular remodeling [75, 76]. In particular, major functional alterations in the pulmonary vascular endothelium in PAH have been demonstrated, including among others: 1) a transition from a quiescent state (without adhesion capacity) to an activated state with adhesive capacity[77]; 2) an aberrant pro-proliferative and apoptosis resistant phenotype[78]); 3) a pro-inflammatory phenotype characterized by an excessive release of various key cytokines and chemokines: interleukin (IL)-1α, IL-6, IL-8, IL-12, CCL2/monocyte chemotactic protein (MCP)-1[77]; 4) an excessive production and secretion of various key growth factors including fibroblast growth factor-2[78, 79], angiotensin-II (Ang II)[80], and leptin[81-83]. Additional insights into the altered pulmonary EC phenotype and the endothelial communication with both resident vascular cells (PA-SMCs, myofibroblasts) and immune cells are a prerequisite for a better understanding of PAH pathogenesis that could lead to novel therapeutical strategies.

More recently, an endothelial communication was reported with pericytes, a key progenitor cell-type that are found around pre-capillary arteries, capillaries, and post-capillary venules and ~~that~~ occupy a strategic position at the interface between circulating blood and interstitial space, and at close proximity to ECs and PA-SMCs. Ricard and coauthors ~~We~~ have demonstrated an excessive pericyte coverage in distal pulmonary arteries in human PAH (a 2- to 3-fold increase), a phenomenon that contributes to pulmonary vascular remodeling as a source of smooth muscle-like cells[84]. Pericytes are central regulators of vascular development, stabilization, maturation, and remodeling modulating: (i) EC growth, proliferation, differentiation and migration; (ii) PA-SMC contraction and capillary blood flow; (iii) immune cell functions; (iv) and their possible differentiation in SMC-like cells and dendritic cells, acting as progenitor cells.

Deciphering the intracellular pathways that modulate pulmonary vascular remodeling has paved the way for precise molecular targeting strategies with new treatments for PH[85]. For example, one can now choose between specific and nonspecific ET-1 receptors, or stimulation of synthesis or inhibition of degradation of the key intracellular, second messenger cyclic guanosine monophosphate (cGMP) to alter pulmonary vascular tone in diseased states. Even though the prognosis remains poor, these additional pathways may represent~~ent~~ innovative tools for the treatment of PH in the future.

In PH due to lung disease, such as COPD, pulmonary vascular impairment is highly prevalent: about 40% of patients with COPD GOLD 4 and 27% of GOLD 3 stages display an increased mPAP[86]. Among patients w~~h~~ithout ~~o do not present~~ PH at rest, a significant proportion of them develop it during exercise[87, 88]. These findings concur with the frequent

observation of histological changes in small pulmonary arteries, mainly consisting of intimal hyperplasia, in patients with end-stage disease who underwent lung transplantation[5], but even in patients with mild-to-moderate disease[6, 89]. In addition to vascular remodeling, dysregulated vascular tone is a major contributor to pulmonary hypertension in COPD: the expression of eNOS[90], sGC[16] and PGI_2 synthase[36] are reduced in the pulmonary arteries of COPD patients, thus decreasing their vasodilator and antiproliferative capacity **(Figure 3)**.

With this background it is interesting to note that some patients with extensive emphysema develop severe PH[91], suggesting a link between the emphysematous destruction of lung parenchyma and the severity of pulmonary vascular impairment. In fact, in explanted lungs of COPD patients who underwent lung transplant, the severity of emphysema was significantly higher in those patients who had PH[92]. Accordingly, the combination of vascular remodeling, endothelial dysfunction and parenchymal derangement might explain the development of severe PH in a subset of patients with COPD[93].

Pulmonary endothelium represents an interesting therapeutic target in COPD. Studies conducted in experimental models show that targeting the NO-cGMP signaling pathway with sGC stimulators[16] or phosphodiesterase-5 (PDE5) inhibitors[94, 95] prevents the development of PH, pulmonary vascular remodeling and right ventricle hypertrophy. Interestingly, sGC stimulators and PDE5 inhibitors also attenuated the development of pulmonary emphysema[16, 95], an effect that was associated with a reduction in the number of neutrophils and alveolar macrophages in lung tissue, as well as a reduction in leukocyte adhesion[16]. Taken together these findings suggest that therapeutic interventions in the NO-cGMP signaling pathway exert a dual effect: on one hand, relaxation and eventually attenuation of cell proliferation through protein kinase G-dependent mechanisms in smooth muscle cells; on the other, reduction of leukocyte adhesion in ECs, presumably by downregulating P-selectin[96].

These results have been obtained using a preventive experimental design but it remains to be shown to what extent the therapeutic intervention exerts the same effects when lung damage is already established, using a “therapeutic” design. In fact, treatment with PDE5 inhibitors or sGC stimulators in COPD patients with PH has been ineffective in improving exercise tolerance[86]. However, exercise tolerance might not be the most adequate surrogate of the effects of treatment on vascular endothelium. Further experimental and clinical studies

addressing the effects of therapeutic interventions on the disturbed endothelium are warranted to elucidate the significance of this approach in COPD.

In PH due to hematological disorders, such sickle cell disease (SCD)[97-101], the mechanistic linkage between hemolytic anemia and vasculopathy has been the subject of extensive study in pre-clinical animal models, in vascular studies in patients, and in large human cohort studies[99, 102, 103]. Intravascular hemolysis releases cell-free hemoglobin (Hb) in the plasma, which can scavenge NO and generate ROS, impairing redox balance and leading to proliferative systemic and pulmonary vasculopathy[103, 104]. It has been recently appreciated that products released from ~~the~~ red cell during hemolysis can be considered danger associated molecular pattern molecules or erythrocyte "DAMPs" (eDAMPs)[105]. These studies in SCD support a more general pathological role for intravascular hemolysis and cell-free Hb in various human diseases and in transfusion medicine[106].

6. Biological factors to assess pulmonary endothelial dysfunction

In light of the critical role played by the pulmonary endothelium in acute and chronic lung diseases, identification of biological factors to assess its (dys)function is needed in the field, but represents a real challenge for clinicians and researchers. Indeed, a broad appreciation of the numerous functions of the endothelium request the assessment of a large panel of molecules of endothelial origin in circulating blood (Table 1), and a comprehensive prior assessment and validation in large and well-characterized cohorts of patients before routine clinical use. These EC products could include, among others: measures of NO biology, circulating miRNAs, cytokines, chemokines, circulating adhesion molecules, growth factors, regulators of thrombosis, as well as markers of endothelial damage (i.e. detachment of mature ECs, derived microparticles (EMPs)) and repair (i.e., number and functional charasteristics of circulating endothelial progenitor cells).

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Besides changes in vasoactive mediators, COPD patients present structural damage in pulmonary endothelium. Areas of cell denudation and detachment between endothelial cells have been shown in pulmonary arteries of COPD[107]. The presence of endothelial damage in COPD has also been demonstrated by increased numbers of EMPs[108, 109]. Circulating EMPs may originate from endothelial cell apoptosis or activation, which can be identified by the expression of the CD31 and CD62E markers, respectively. The number of circulating EMPs is inversely related to the FEV1[109]. Interestingly, whereas $CD31^+$ EMPs (apoptotic)

are already significantly elevated in patients with mild COPD, $CD62E^+$ EMPs (activated) are increased only in patients with severe disease. The number of circulating EMPs further increases during exacerbation episodes[110]. The number of circulating EMPs correlates with the severity of emphysema[109] pointing to a connection between parenchymal destruction and endothelial damage. Furthermore, in COPD, circulating EMPs are also correlated with vascular function of systemic arteries. An increased number of circulating EMPs is associated with impaired endothelial function and increased stiffness in systemic arteries[111]. COPD patients also show reduced numbers of bone marrow-derived circulating progenitor cells (EPCs)[112-115], which play a key role in the repair process of damaged endothelium and are closely related to endothelial function[116]. Studies carried out during the last years have made a great effort in trying to identify, define and characterize cell populations with phenotypic, biochemical, molecular and functional properties of EPCs[117, 118]. EPCs have been suggested as a precious source for generating functionally competent endothelial cells (ECs), candidate for various clinical applications. However, the paucity of these progenitor cells and the technical difficulties for their *in vitro* growth represent a main limitation to their use. Interestingly, recent studies have led to the identification in hematopoietic tissues (blood, bone marrow and cord blood) of very rare EPCs not originating from the bone marrow, called endothelial colony-forming cells (ECFCs) or late outgrowth ECs, ~~and~~ capable of generating a large progeny of phenotypically and functionally competent mature ECs *in vitro* and of sustaining *in vivo* angiogenetic processes[119, 120]. These EPCs are also present at the level of the vessel wall, particularly in the vascular endothelial intima[117, 118].

This methodology can be easily extended to the study of circulating EPCs and can facilitate and improve the functional characterization of EPCs in various pathologic conditions, particularly in various lung diseases.

7. Conclusion

In conclusion, it is now well established that the pulmonary vascular endothelium is involved in most if not all acute and chronic lung disease, either as a primary determinant of these disease processes or as a victim of collateral damage **(Figure 4)**. Although considerable progress has been made in the understanding of pulmonary endothelial cell (dys)function, the triggers, the mechanisms and the consequences of dysfunctional endothelium in lung disease, both acute and chronic, are still not completely understood. In addition, further efforts are also

needed to elucidate the remarkable heterogeneity of the pulmonary endothelium in space and time, in structure and function, as well as in health and disease. An understanding of the molecular basis for these key aspects would help finding new disease biomarkers and/or novel therapeutical targets to preserve homeostasis in response to injury and disease and to reduce the "bench-to-bedside" gap in endothelial biomedicine.

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Table 1 - A partial list of pulmonary endothelium-derived factors

Vasoconstrictors:	Serotonin (5-HT) Endothelin (ET)-1, ET-2, and ET-3 Angiotensin (Ang-II) Thromboxane A2 Oxidative stress
Vasodilators:	Nitric oxide (NO) Prostacyclin (PGI_2) Apelin Adrenomedullin
Cell accumulation:	Fibroblast growth factor (FGF)-2 Vascular endothelial growth factor (VEGF) Platelet derived growth factor (PDGF) Tumor growth factor (TGF)

	Epidermal growth factor (EGF) Nerve growth factor (NGF)
Inflammation:	Interleukin (IL)-1, IL-6, IL-8… Tumor necrosis factor (TNF) Interferon Leptin (Ob) Leukotrienes
Other proteins:	Plasminogen activator inhibitor (PAI)-1 Soluble E-selectin Soluble intercellular adhesion molecule (ICAM)-1 Soluble vascular cell adhesion protein 1 (VCAM)-1 Matrix metalloproteinases (MMP)-2, MMP-9, MMP-10… Tissue plasminogen activator (tPA) Platelet-activating factor Thrombomodulin

Figure legends

Figure 1: Inflammation and the pulmonary microcirculation

Inflammation-mediated events occurring in pulmonary arterioles, capillaries and venules contributes to impaired capillary perfusion. The presented sample was taken from the lungs of a patient with chronic thromboembolic pulmonary hypertension. Note the moderate to severe microvascular inflammation (in the absence of any major vascular wall remodeling)

Figure 2: Vascular response to chronic hypoxia

Previously non muscularized arterioles (A) show "muscularization" with recruitment of smooth muscle cells and myofibroblasts (B). Note the mild inflammatory infiltrate (predominating on the right side of the vessel) which accompanies the remodeling process

Figure 3: Pulmonary artery in a patient with chronic obstructive pulmonary disease

Histological changes of a small pulmonary artery in a patient with chronic obstructive pulmonary disease (COPD). The vessel (here at a dichotomous branching point) presents with important intimal fibrosis. Note the mild perivascular infiltrate (predominating beneath the artery).The surrounding pulmonary parenchyma displays emphysematous remodeling

Figure 4:

Schematic overview of pulmonary endothelial dysfunction main characteristics

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Responses to the Reviewers

We thank the Reviewers for the positive evaluation.
Based on the reviewers' suggestions, we have modified the manuscript.

REVIEWER 1

Comments to the Authors

This review of pulmonary vascular endothelial dysfunction and its role in chronic lung disease is a summary of various topics of discussion that were presented at an ERS research seminar. The revised manuscript is well written and discusses the myriad functions that the endothelium plays in regulating pulmonary vascular homeostasis in many chronic lung diseases. The revised manuscript addresses my major concerns. I have only a few minor recommendations:

C1. The statement " Mean systolic PAP at high altitude was 25.3 mmHg, modestly elevated compared to that seen in PH clinics" on page 9 does not seem to be correct and should state instead: " Mean systolic PAP at high altitude was 25.3 mmHg, modestly elevated compared to that seen in healthy individuals from the general population living below 1,200 m"

R1. We thank the reviewer for his comment. We have modified the manuscript accordingly.

C2. As raised in the first review of this manuscript, the statement "Deciphering the intracellular pathways modulating the actin-myosin interaction mechanisms have paved the ways for precise molecular targeting strategies with new treatments for PH " on page 13 does not make sense and would be better stated as something like: "Deciphering the intracellular pathways that modulate pulmonary vascular remodeling has paved the way for precise molecular targeting strategies with new treatments for PH".

R1. We thank the reviewer for his comment and we have modified the manuscript accordingly.

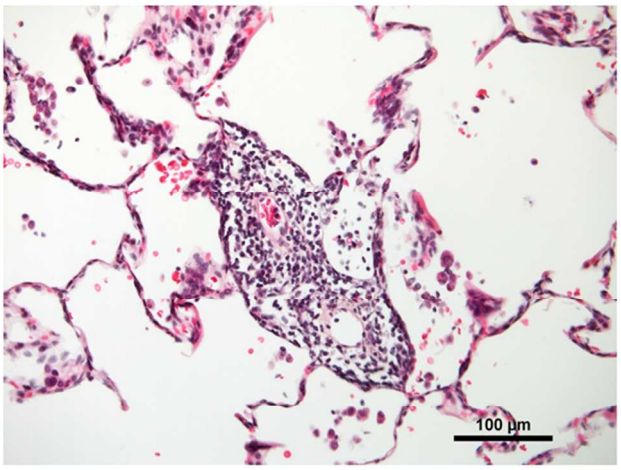


Figure 1

Inflammation and the pulmonary microcirculation.
Inflammation-mediated events occurring in pulmonary arterioles, capillaries and venules contributes to impaired capillary perfusion. The presented sample was taken from the lungs of a patient with chronic thromboembolic pulmonary hypertension. Note the moderate to severe microvascular inflammation (in the absence of any major vascular wall remodeling)

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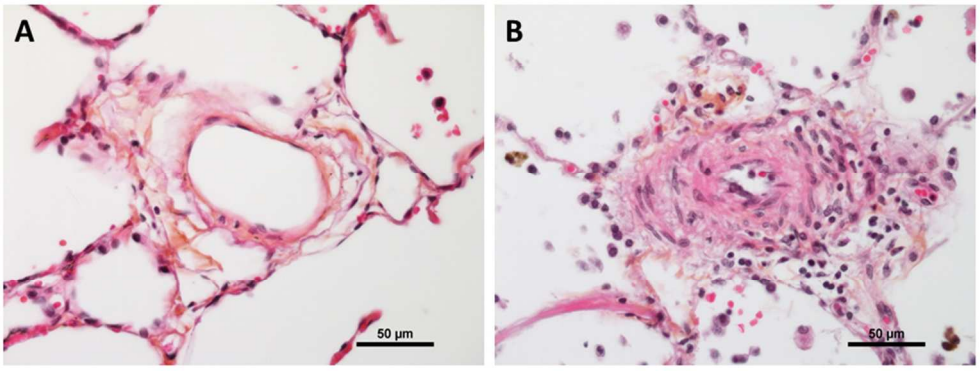


Figure 2

Vascular response to chronic hypoxia.
Previously non muscularized arterioles (A) show "muscularization" with recruitment of smooth muscle cells and myofibroblasts (B). Note the mild inflammatory infiltrate (predominating on the right side of the vessel) which accompanies the remodeling process

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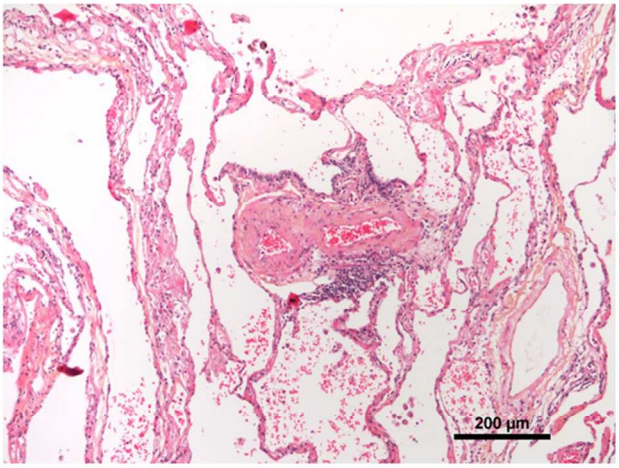


Figure 3

Pulmonary artery in a patient with chronic obstructive pulmonary disease.
Histological changes of a small pulmonary artery in a patient with chronic obstructive pulmonary disease (COPD). The vessel (here at a dichotomous branching point) presents with important intimal fibrosis. Note the mild perivascular infiltrate (predominating beneath the artery). The surrounding pulmonary parenchyma displays emphysematous remodeling

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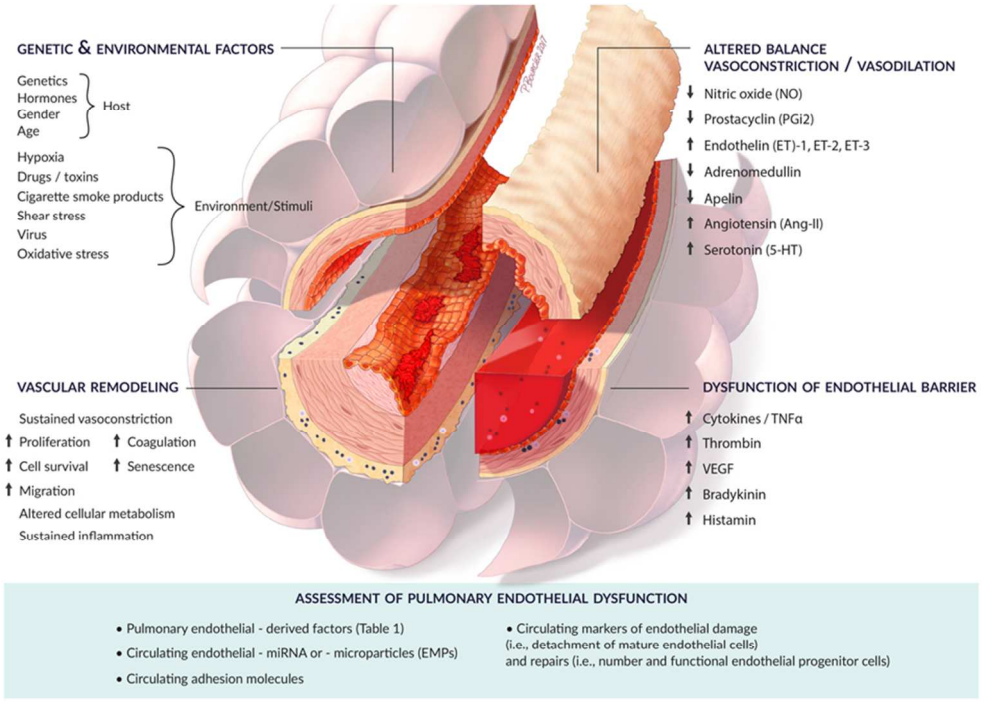


Figure 4

Schematic overview of pulmonary endothelial dysfunction main characteristics

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