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Pathogenesis of Pulmonary Hypertension

Rajamma Mathew

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1. Introduction

Pulmonary arterial hypertension (PAH), although rare, is a progressive disease with a high morbidity and mortality rate. In 1981, Ernst von Romberg, a German physician described pulmonary vascular lesions as “pulmonary vascular sclerosis”, the first description of histological changes in PAH [Fishman 2004]. The average survival time for untreated patient is around 2.8 yrs [D’Alonzo 1991]. Despite remarkable progress made since then, the pathogenesis of PAH, however, is not yet well understood; because a large number of cardiopulmonary and systemic diseases can lead to PAH, and in addition, multiple signaling pathways have been implicated. Current advances in therapy, have improved the quality of life and delayed the progression of the disease, but have not provided a cure. Lack of cure in PAH is further underscored by a recent study showing persistent large plexiform lesions and inflammatory infiltrates in patients despite having been on a long term prostacyclin therapy [Pogoriler 2012]. One of the main reasons for the failure of therapy is that the diagnosis is often made late because of vague symptoms; and by the time the diagnosis is made extensive pathologic changes have already taken place in pulmonary vasculature. From experimental studies, it is clear that pathological changes in the vasculature occur before the onset of PAH [Huang 2010]. Another problem is that a large number of signaling molecules implicated in PAH may not be relevant in all patients; and the activation of some of these molecules may depend on the stage of the disease.

The current clinical classification updated in 2008 maintains five major groups [Simonneau 2009]. *Group 1*: Pulmonary arterial hypertension (PAH): Included in this group are idiopathic (IPAH) and heritable PAH (HPAH), PAH associated with congenital heart defects (CHD), connective tissue diseases, portal hypertension, infection, chronic hemolytic anemia, drug toxicity and persistent pulmonary hypertension of the newborn (PPHN). Pulmonary veno-occlusive disease and pulmonary capillary hemangiomatosis are included in this group as a subcategory. Approximately 70% of HPAH and 26% IPAH exhibit heterozygous germline

mutations in BMPRII, a member of TGF β superfamily; however, only about 20% of people with BMPRII mutation develop PAH. The incidence of HPAH is reported to be 3.9% of IPAH. [Thompson 2000, Machado 2006, Cogan 2006, Sztrymf 2007, Humbert 2006]. It has recently been shown that in BMPRII \pm mice, a “second hit” such as inflammation or serotonin increases the susceptibility to develop PAH [Song 2008, Long 2006]. In addition, altered metabolism of estrogen resulting in low production of 2 methylestradiol is also thought to be a “second hit” for the development of PAH in females with BMPRII mutation [Austin 2009]. Interestingly, a short exposure to fenfluramine, a diet suppressant, is enough to induce PAH in patients with a BMPRII mutation [Humbert 2002]. Thus, the “second hit” is almost a requirement for the development of PAH in patients with BMPRII mutations. Approximately 6% of adults and children with congenital heart defect CHD and PAH also exhibit BMPRII mutations [Roberts 2004]. In addition, mutations of activin-like receptor kinase 1 (ALK1) and endoglin, both belonging to the TGF β superfamily have been reported in patients with hereditary hemorrhagic telangiectasia, and some of these patients develop PAH [Trembath 2001]. Recently mutation of SMAD 8, belonging to another member of the TGF- β family, and thrombospondin-1 (TSP-1) were found in patients with PAH and HPAH respectively. Interestingly, TSP-1 is known to regulate the activation of TGF- β [Shintani 2009, Maloney 2012]. Thus, the TGF- β /BMP signaling pathway has an important role in pulmonary vascular health and disease. Polymorphisms of other genes have been described in PH such as serotonin (LL allele), TRPC6 gene promoter, and Norrie disease with deficiency of monoamine oxidases, which degrades serotonin have been reported in patients with PH [reviewed in Mathew 2011]. In addition, polymorphism of the *KCNA5* gene with altered expression and function of Kv1.5 channels has been observed in pulmonary vascular smooth muscle cells (SMC) from IPAH patients [Remillard 2007].

Among adults, PAH occurs more frequently in women than men. The French national registry revealed female to male ratio to be 1.9:1 and in a recent report from the US registry the ratio was reported to be around 4:1 with better survival rate among the females. The higher incidence of PAH in females in the US was thought to be related to the higher incidence of obesity [Humbert 2006, Shapiro 2012]. In HIV-PAH, however, there is higher incidence in males (M:F 1.5:1), because more male patients have HIV infection [Cicalini 2011]. PAH is the leading cause of death in patients with scleroderma, and the estimated prevalence of PAH in this group is 8-12% [Mathai 2011]. *Group 2*: PH due to left heart diseases such as mitral valve disease, systemic hypertension, ischemic heart disease and cardiomyopathy are included in this group. These diseases lead to LV diastolic overload, impaired function and passive congestion in capillaries. Sustained elevated pressure in pulmonary venous circulation results in structural and functional damage of pulmonary arteries, and endothelial dysfunction leading to PH. Heart failure with preserved ejection fraction (HFpEF) is recognized as the major cause of PH associated with left heart disease. In one study, female preponderance (58%) was observed in HFpEF + PH group. These patients have higher LV end-diastolic pressure. It is important to distinguish this group from PAH (group 1), because the therapy used in PAH is not effective in patients in this group [Guazzi 2010, Thenappan 2011, Hill 2011]. *Group 3*: This group encompasses PH due to chronic obstructive pulmonary disease (COPD) and other

parenchymal lung diseases associated with hypoxia. Major components in this group are vasoconstriction and vascular remodeling. Inflammation plays a significant role in the pathobiology of lung diseases. Recent studies suggest that the pathological changes seen in COPD and idiopathic pulmonary fibrosis are related to oxidative stress and aging as evidenced by increased expression of senescence markers in lungs and enhanced tissue destruction [MacNee 2009, Faner 2012]. Furthermore, senescent pulmonary artery SMC exhibit telomere shortening and increased production of cytokines, thus, contributing to the progression of the disease [Nouredine 2011]. *Group 4*: Included in this group is PH resulting from an incomplete resolution of chronic pulmonary thromboembolism. The incidence of PH in this group is reported to be approximately 4% at 2 yrs. About 10% of patients develop PH even after satisfactory thrombo-endarterectomy. Worsening of the disease is thought to occur because of recurrent thromboembolism, or in situ thrombosis and pulmonary vascular remodeling. Reduction in the expression of eNOS and impaired endothelium-dependent, NO-mediated relaxation response in pulmonary arteries distal to ligation was recently reported in a porcine pulmonary artery ligation model. Importantly, histological features include pulmonary vascular remodeling and plexiform lesions indistinguishable from PAH [Moser 1993, Pengo 2004, Darteville 2004, Fadel 2000]. *Group 5*: This group includes a large number of miscellaneous diseases such as PH secondary to other systemic diseases such as sarcoidosis, myeloproliferative diseases, metabolic and hematological disorders, Thyroid diseases, Gaucher's disease and chronic renal failure requiring dialysis.

1.1. PH in pediatric age group

PH in pediatric age group has several different features compared with the adult patients. In children, medial hypertrophy is the main feature; with increasing age other pathological features such as intimal proliferation, concentric fibrosis and subsequently dilatation and plexiform lesions appear [Wagenvoort 1970]. A recent study revealed that females comprised 46% of all PH and 51% of all PAH group patients [van Loon 2011]. The major causes of PH in children are CHD, PPHN, lung diseases such as respiratory distress syndrome (RDS), bronchopulmonary dysplasia (BPD), and congenital defects associated with hypoplasia of the lungs [Mathew 2000]. Antenatal and perinatal problems have adverse effects on vascular and alveolar development. Preterm delivery disrupts normal pulmonary vascular and bronchoalveolar development which leads to reduced cross sectional area of the pulmonary vasculature resulting in increased pulmonary vascular resistance and PH [Farquhar 2010]. Another interesting difference from the adult group is that >80% of pediatric patients have transient PH. These include resolution of PPHN and in the majority of the cases after surgical correction of CHD [van Loon 2012]. However, poor outcome has been reported in children with IPAH or HPAH associated with BMPRII mutation [Chida 2012]. A new classification for pediatric PH has been proposed that is comprised of 10 major groups and includes prenatal and developmental anomalies. The main categories are: 1. Prenatal or developmental pulmonary hypertensive vascular disease, 2. Prenatal pulmonary vascular maladaptation, 3. Pediatric cardiovascular disease, 4. Bronchopulmonary dysplasia, 5. Isolated pediatric PAH, 6. Pulmonary hypertensive vascular disease in congenital malformation syndrome, 7. Pediatric lung

disease, 8. Pediatric thromboembolic disease, pediatric hypobaric hypoxic exposure, 10. Pediatric pulmonary vascular disease associated with other systemic disorders [del Cerro 2011]. Irrespective of the underlying pathology, patients usually present with similar changes in the lungs including endothelial dysfunction, impaired vascular reactivity, activation of inflammatory processes, vascular remodeling, with subsequent neointima formation and eventually right heart failure.

2. Pulmonary vascular physiology

Monolayer formed by endothelial cells (EC) is a critical interface between the circulating blood and underlying SMC and provides a non-thrombogenic barrier. EC transform mechanical stimuli into biological responses and depending on the stimuli, EC secrete several transducing molecules that participate in a number of biological functions such as vascular tone, cell proliferation, apoptosis, inflammation and thrombosis. EC maintain vascular tone by activating cGMP and cAMP pathways. Nitric oxide (NO) is synthesized from L-arginine through the catalytic activity of endothelial NO synthase (eNOS). NO activates soluble guanylate cyclase (sGC) that catalyzes guanylate triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), which via cGMP-dependent protein kinase (PKG) induces vascular relaxation, inhibits cell proliferation and modulates inflammation. Subsequently cGMP is metabolized and inactivated by Phosphodiesterase 5. Prostacyclin (PGI_2), an arachidonic acid metabolite is produced by the enzymatic activity of cyclooxygenase and PGI_2 synthase. The prostacyclin receptor found on EC and platelets belongs to the family of G-protein coupled receptors. PGI_2 binds to the receptor and stimulates adenylyl cyclase which catalyzes the conversion of ATP to second messenger cAMP. In addition, the cAMP/PKA pathway activates NO production via phosphorylation of eNOS. Both cGMP and cAMP mechanisms induce vascular relaxation and inhibit platelet aggregation and DNA synthesis. Juxtaposition of EC and SMC facilitates crosstalk, and EC maintain SMC in a quiescent state. [Mathew 2011a]. SMC inhibits flow-mediated activation of the mammalian target of rapamycin (mTOR) in EC, and SMC also participate in altering the expression of the factors involved in coagulation and fibrinolysis [Balcells 2010].

Caveolae, a subset of specialized microdomains (omega shaped invaginations, 50-100 nm) are found on a variety of cells including EC, SMC, fibroblasts and epithelial cells. They serve as a platform and compartmentalize a number of signaling molecules that reside in or are recruited to caveolae. They are also involved in transcytosis, endocytosis and potocytosis. Caveolin-1 (22kD) is the major scaffolding protein that supports and maintains the structure of caveolae. It interacts and regulates a number of proteins including Src family of kinases, G-proteins (α subunits), G protein-coupled receptors, H-Ras, PKC, eNOS, integrins and growth factor receptors such as vascular endothelial growth factor-receptor (VEGF-R), and epidermal growth factor-receptor (EGF-R). Caveolin-1 stabilizes these signaling proteins, and negatively regulates the target proteins within caveolae, through caveolin-1-scaffolding domain (CSD, residue 82-101). For optimal activation, eNOS is targeted to caveolae. Although it is negatively regulated by caveolin-1, caveolin-1 is essential for NO-mediated angiogenesis. In addition, the

downstream effector of NO, sGC has been shown to compartmentalize in caveolae to facilitate its activation. In caveolin-1 knockout mice, the loss of caveolin-1 is associated with the hyper-activation of eNOS, and increased cGMP production. The hyper-activation of eNOS subsequently leading to PKG nitration-induced stress is considered responsible for PH in these mice; and re-expression of endothelial caveolin-1 restores vascular and cardiac abnormalities [reviewed in Mathew 2011b]. Caveolin-1 functions as an antiproliferative molecule; it negatively regulates proliferative pathways such as mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK), tyrosine- phosphorylated signal transducer and activator of transcription (PY-STAT) 3, EGF and platelet-derived growth factor (PDGF). Caveolin-1 also regulates cell cycle and apoptosis. In addition, caveolin-1 interacts with major ion channels such as Ca²⁺ -dependent potassium channels, voltage-dependent K⁺ channels (Kv1.5), and a number of molecules responsible for Ca²⁺ handling such as inositol triphosphate receptor (IP₃R), heterodimeric GTP binding protein, Ca²⁺ ATPase and several transient receptor potential channels in caveolae. Through these interactions, caveolin-1 modulates cell proliferation and cell cycle progression. In SMC, caveolin-1 regulates Ca²⁺ entry and enables vasoconstriction. The localization of Ca²⁺ regulating proteins in caveolae and the proximity to the sarcoplasmic reticulum suggests an important role for caveolae/caveolin-1 for Ca²⁺ homeostasis [reviewed in Mathew 2011b]. RhoA interacts directly with caveolin-1, and the translocation of RhoA to caveolae is essential for myogenic tone. The CSD peptide of caveolin-1 has been shown to inhibit the agonist-induced redistribution of RhoA and PKC- α . Caveolin-1 blockage results in impaired formation of capillary tubes, and the overexpression of caveolin-1 accelerates EC differentiation and tube formation [Santibanz 2008, Liu 2002]. Furthermore, caveolin-1 modulates inflammation. It has recently been shown that caveolin-1 inhibits HIV replication through NF- κ B [Wang 2011].

BMPRII is predominantly expressed in EC, and a part of BMPRII colocalizes with caveolin-1 in caveolae and also in golgi bodies. BMPRII signaling, essential for BMP-mediated regulation of vascular SMC growth and differentiation also protects EC from apoptosis [Yu 2008, Teichert-Kuliszewska 2006]. BMPRII directly modulates proteins involved in cytoskeletal organization, possibly through Mas1 (G-protein-coupled receptor) interaction with Rho GTPase. Recently discovered angiotensin converting enzyme (ACE) 2, an endogenous inhibitor of ACE, is endothelium-bound. ACE2 cleaves angiotensin (Ang) I to Ang 1-9 which is an inactive compound. ACE2 metabolizes Ang I to produce Ang 1-7 which is a physiological antagonist of Ang II. ACE2/Ang (1-7) pathway antagonizes Ang II acting through Mas1, increases NO production via the Akt-dependent pathway, releases PGI₂ and it inhibits Ang II-induced reactive oxygen species (ROS) formation within the cell nucleus. Loss of ACE2 causes increased vascular permeability, pulmonary edema and worsening lung function. The over-expression of Ang-(1-7) has a protective effect on MCT-induced PH and bleomycin-induced lung fibrosis. Interestingly, inhibition of Rho kinase has been shown to activate the ACE2/Ang-(1-9) pathway resulting in increased eNOS expression and amelioration of hypertension [Johnson 2012, Burton 2011, Lovern 2008, Mathew 2011, Ocaranza 2011, Shenoy 2010]. Thus, under normal conditions EC maintain homeostasis by producing cell protective factors and inhibiting inflammation and cell proliferation.

3. Pathobiology of pulmonary hypertension

Endothelial dysfunction associated with an impaired endothelium-dependent vascular relaxation response is an important feature of clinical and experimental models of PH. It is the EC that bear the major brunt of injury regardless of the underlying disease, and the loss of the vascular dilatation mechanism associated with the activation of proliferative and anti-apoptotic pathways are the hallmarks of PH [reviewed in Mathew 2011]. Genetic susceptibility may make the effects of injury to occur earlier and to be more severe. It is becoming clear that epigenetics has a significant role in the pathogenesis of PH. Epigenetics is the study of changes in phenotype or gene expression not caused by any alterations in the underlying DNA sequence. Epigenetic mechanisms include 1) DNA methylation, 2) modification of histone proteins and 3) microRNAs [Kim 2011].

3.1. Loss of vasodilatation mechanisms

Impaired endothelium-dependent, NO-mediated relaxation and reduced cGMP levels are well documented in PH. Monocrotaline (MCT)-induced PH is associated with progressive disruption and loss of endothelial proteins. At 2 weeks post-MCT, the expression of eNOS is not significantly lower compared with the controls, but is associated with the loss of the eNOS activating molecules, HSP90 and Akt, thus leading to uncoupling of eNOS, and ROS generation. ROS generation returns to normal by 3-4 wks as the eNOS levels diminish. In PH patients, the expression of eNOS in the lungs is reported to be either low or increased. This is not surprising because the disease does not progress uniformly; thus, the expression of eNOS may depend on the stage of disease in a given lung section. Increased eNOS expression and PKG nitration have been shown in caveolin-1 null mice and also in the lungs of patients with IPAH, contributing to worsening of PH. In PH, the initial loss of EC is followed by the appearance of apoptosis resistant EC. These neointimal EC have increased expression of eNOS and reduced expression of caveolin-1, leading to uncoupling of eNOS and oxidant and nitration injury. The expression of PGI₂ synthase is reduced in the lungs of patients with PH, and the release of PGI₂ is decreased in these patients. Interestingly, mice with over-expression of PGI₂ synthase are protected from hypoxia-induced PH. In addition, PGI₂ synthase expression is reduced in the lungs of patients with PH, and the release of PGI₂ is decreased in these patients. Interestingly, overexpression of PGI₂ synthase protects mice from hypoxia-induced PH [reviewed in Mathew 2011 and Mathew 2011a]. Loss of cGMP and cAMP mechanisms leads to loss of endothelium-dependent vasodilatation, elevation of pulmonary artery pressure, platelet aggregation, increased mitogenic activity and negative modulation of inflammation.

3.2. Activation of proliferative pathways

During the development of PH, several proliferative and antiapoptotic pathways are activated. Endothelin-1 (ET-1) was discovered in 1980s as a potent vasoconstrictor predominantly produced by vascular endothelial cells from the inactive big endothelin-1 by catalytic activity of endothelin-converting enzyme (ECE)-1. ET1 is involved in several physiological and pathological processes such as vascular contraction, wound healing, cancer and vascular

diseases [Khimji 2010]. ET-1 has mitogenic and inflammatory properties; and it acts in paracrine and autocrine fashion. The effects of ET-1 are mediated through ETA and ETB receptors. Endothelial cells possess ET-B receptors which induce NO upon stimulation. ETA and a subpopulation of ETB receptors cause vasoconstriction in SMC. Increased levels of ET-1 have been reported in the lungs of patients with PH and in pulmonary arteries in the MCT-model of PH. Interestingly, higher levels of ET-1 and both its receptors have been reported in pulmonary arteries of the patients with irreversible PAH associated with CHD compared with the reversible ones. The reversible PAH had higher expression of ET-1 and ET receptors compared with the controls [Huang 2011, Mathew 2011].

Platelet-derived growth factor (PDGF) was identified in 1970s as a serum growth factor for cells including fibroblasts and SMC; it induces proliferation of SMC and fibroblasts. PDGF is synthesized by a variety of cells including SMC and EC, and functions in both paracrine and autocrine manners. PDGF receptors are more pronounced in SMC compared with EC. Increased expression of PDGF receptor β (PDGFR β) has been reported in patients with IPAH and also in MCT and hypoxia models of PH. Furthermore, inhibition of PDGF receptor with imatinib, a tyrosine kinase inhibitor, reverses MCT and hypoxia-induced PH [Schermyly 2005, Perros 2008]. Both PDGF and VEGF belong to the same superfamily of signaling molecules. Interestingly, VEGF-A can stimulate both PDGF α and β receptors, and both receptors mediate VEGF-A and PDGF signaling; inhibition of either receptor significantly attenuates VEGF-A-induced cell migration. Thus, both participate in promoting recruitment and proliferation of vascular SMC both under physiological and pathological conditions [Mathew 2012]. After vascular injury, increased expression of PDGF ligand and its receptor occur. PDGF down regulates SMC genes, altering SMC phenotype from a contractile to an undifferentiated synthetic type, which is required for vascular repair. After repair, SMC revert to a contractile phenotype; whereas the deregulated synthetic phenotype leads to vascular disease. Micro (mi) RNA-221 is considered essential for PDGF-induced cell migration. MiRNA-221 is thought to promote proliferation through binding to the 3'-untranslated region of cell cycle inhibitor, and inhibiting p27/kip1 expression. Importantly, miRNA-221 reduces c-Kit mRNA. The inhibition of c-Kit leads to PDGF-mediated downregulation of SMC gene expression resulting in an undifferentiated synthetic phenotype and leading to cell proliferation. In several cell systems, activation of STAT3 is required for PDGF-induced cell proliferation; furthermore, inhibition of the PDGF receptor suppresses cell proliferation via inactivation of PY-STAT3 signaling [Mathew 2012].

STAT3 belongs to a family of cytoplasmic proteins that functions as extracellular effectors of cytokines and growth factors, and plays a role in a number of biological processes. Phosphorylation of STAT3 at tyrosine 705 residue leads to dimerization, nuclear translocation to nucleus, DNA synthesis and transcription of genes that mediate survival and cell proliferation. PY-STAT3 plays a critical role in cell growth, anti-apoptosis, survival, and immune function and inflammation; and it is a downstream effector of cytokines such as IL-6 and also growth factors. PY-STAT3 is activated by the JAK family of receptor-associated tyrosine kinases, and also by non-receptor tyrosine kinases such as Src, EGF and PDGF. Persistent phosphorylation of STAT3 associated with a number of primary tumors confers resistance to apoptosis. A role for STAT3 in vascular diseases including PH is emerging. Inhibition of the activated PY-STAT3

in the carotid arterial injury model prevents neointima formation. In addition, progressive activation of PY-STAT3 has been reported in several forms of experimental PH including MCT and hypoxia models, and in EC obtained from patients with IPAH. The downstream effectors of PY-STAT3 are cyclin D1 (cell cycle regulator), survivin and Bcl-xL (anti-apoptotic factors), and they are upregulated in PH. Inhibition of PY-STAT3 reduces the expression of cyclin D1, survivin and Bcl-xL, and attenuates PH. STAT3 also plays a significant role in stabilizing hypoxia inducible factor (HIF) 1 α , a pivotal event in hypoxia-induced PH, and its interaction with HIF1 α mediates transcriptional activation of VEGF promoter. Abundant expression of HIF1 α and VEGF has been reported in plexiform lesions in the lungs of the patient with PH [reviewed in Mathew 2010].

Rho kinase (ROCK), an effector of small GTPase binding proteins was identified in 1990s. The RhoA/ROCK pathway plays pivotal role in the organization of actin-cytoskeletons, cell cycle progression, cell proliferation and migration. ROCK isoforms (I and II) are expressed in vascular tissue. Rho/ROCK signaling modulates a number of cellular functions such as inflammation, vascular tone, barrier function, vascular remodeling, atherogenesis and cell transformation. Furthermore, it promotes endothelial repair and maintains SMC differentiation [Rolfe 2005]. Interestingly, Rho is required for STAT3 activation; and Rho-mediated cell proliferation and migration occur via STAT3. In some cell systems, IL-6 increases the expression of active RhoA in a time and dose-dependent manner, and promotes cell migration and invasiveness. Rho activation is well established in PH, and inhibition of Rho kinase has been shown to attenuate PH in experimental models, such as hypoxia, bleomycin and MCT, and in a shunt model of PH with increased pulmonary blood flow. Furthermore, inhibition of STAT3 or Rho-associated kinase suppresses neointima formation in balloon-injured arteries [Mathew 2010, Mathew 2011].

Serotonin or 5 hydroxytryptamine (5HT) is synthesized in enterochromaffin cells and stored in platelets and is also synthesized in pulmonary artery EC. Serotonin through 5HT transporter (5HTT) is involved in pulmonary artery SMC and fibroblasts proliferation. Interestingly, 5HT-induced ROCK activation is essential for 5HT-induced SMC proliferation. Serotonin through 5HT transporter (5HTT) is internalized in pulmonary artery SMC and is linked to RhoA by intracellular type 2 transglutaminase leading to constitutive RhoA activation also known as RhoA serotonylation. Enhanced RhoA serotonylation associated with increased RhoA and Rho kinase activity has been observed in IPAH. In addition, EC from patients with PH exhibit increased expression of tryptophan hydroxylase, a rate limiting enzyme responsible for the synthesis of 5HT and increased production of 5HT. These patients have increased plasma levels of 5HT. Both 5HT transporter (5HTT) and 5HT receptors promote pulmonary artery SMC proliferation and migration, vasoconstriction and local microthrombi, considered to be dependent on RhoA/ROCK. In hypoxia-induced PH, 5HT receptors are thought to contribute to RhoA/ROCK-mediated Ca²⁺ sensitization. In addition, 5HTT transactivates PDGF β receptors in pulmonary artery SMC, indicating crosstalk between 5HT and PDGF pathways, both of which are implicated in the pathogenesis of PH. Mice with over-expression of 5HTT develop PH spontaneously and on exposure to hypoxia or MCT, they exhibit a significantly increased pulmonary artery pressure compared with the wild type mice. The MCT model of PH revealed an early and sustained increase in the expression of 5HTT in the rat lungs, and the inhibition of 5HTT but not the inhibition of 5HT receptors significantly attenuated MCT-

induced PH. The 5HT pathway is thought to have played an important role in anorexigen drugs-induced PAH; these drugs have been shown to be 5HTT substrates [Mathew 2011, Guilluy 2009]. Prenatal exposure to selective serotonin uptake inhibitors has been shown to increase the risk of developing PPHN in infancy [Chambers 2006]. Thus, serotonin alone or in interaction with other mitogens participates in the pathogenesis of PH.

Evidence is emerging to suggest that Notch and mTOR signaling pathways may have a role in the pathogenesis of PH. Notch3 in SMC regulates cell proliferation and antiapoptotic activity and interestingly, increased expression of Notch3 is reported in the lungs of patients with non-familial PAH, and in the MCT and hypoxia-induced PH [Li 2009]. mTOR, a signaling protein for cell proliferation via the Akt pathway is well studied in cancer and interestingly, mTOR/Akt pathway may play a key role in hypoxia-induced adventitial fibroblast proliferation [Gerasimoskaya 2005]. Furthermore, ET1, 5HT and PDGF are known to enhance mTOR activation. Inhibition of mTOR significantly reduces 5HT-induced cell proliferation; and in PASMC from CTEPH patients, mTOR inhibition attenuates store-operated Ca^{2+} entry into cells [Ogawa 2009, Liu 2006]. Huang et al in 2011 reported increased expression of mTOR in the pulmonary artery medial layer in the reversible form of PAH associated with CHD, but increased expression in both medial and intimal layers in the irreversible form, further supporting a role for mTOR in PAH..

3.3. TGF- β /BMP signaling pathway

TGF- β is a large family with 3 isoforms, activins and BMPs. They play an important role during embryogenesis and are involved in vasculogenesis and cardiac development. TGF- β participates in cell proliferation, transformation, apoptosis and matrix deposition, and it maintains homeostasis. TGF- β is stored in the extracellular matrix (ECM) in an inactive form. Several mediators including plasmin, TSP1 and integrins are known to cause stromal release of TGF- β . TGF- β binds to its receptor T β RII leading to the formation of a complex with ALK5 or ALK1. BMPs (2, 4, 6 and 7) stimulate heterodimerization and the activation of BMP receptors (I and II) and initiate phosphorylation of Smad proteins (1, 5 and 8) which combines with Smad4, translocates to the nucleus and binds to genes to activate or repress their transcription. Similarly, TGF- β /T β RII/ALK5 complex initiates phosphorylation of Smad2/3 which combines with Smad4 and translocates to nucleus to affect gene transcription. BMP2 has been shown to inhibit SMC proliferation after balloon-induced arterial injury in rats and also to attenuate hypoxia-induced PH. BMP-mediated regulation of vascular SMC growth and differentiation occur via BMPRII signaling [Goumans 2009, Eickelberg 2007, Davies 2012].

TGF- β , also referred to as fibrotic cytokine, decreases caveolin-1 expression in fibroblasts. Caveolin-1 suppresses TGF- β -mediated fibrosis, and regulates TGF- β /SMAD signaling through an interaction with the receptor T β RI [Wang 2006, Razani 2001]. Interestingly, TGF- β inhibits proliferation of pulmonary artery (PA) SMC from normal and patients with PAH; however, it has no antiproliferative effects on PASMC harvested from patients with IPAH or HPAH. BMPRII maintains vascular integrity and dampens inflammatory signals and its dysfunction results in TGF- β -induced secretion of pro-inflammatory cytokines such as IL-6 and IL-8, and the loss of TGF- β -mediated antiproliferative effects [Morrell 2001, Davies 2012]. Furthermore, loss of BMPRII has been shown to increase the expression of CXCR1/2 endothelial receptor for a proinflammatory cytokine IL-8. Mice with specific endothelial loss

of BMPRII exhibit increased expression of CXCR1/2, leukocyte migration and PH; and the blockade of CXCR1/2 receptor attenuates PH [Burton 2011].

There is a significant interaction and crosstalk between the BMP system and IL-6/STAT3 pathway; therefore, a reduction in the expression of BMPRII may exacerbate the inflammatory response in PH. Furthermore, persistent activation of PY-STAT3 leads to a reduction in the BMPRII protein expression, and BMP2 induces apoptosis by inhibiting PY-STAT3 activation and down-regulating Bcl-xL. Interestingly, persistent activation of STAT3 leads to a strong upregulation of mature miR-20a, a microRNA that reduces the expression of BMPRII [Hagen 2007, Brock 2009, Kawamura 2000]. In addition to the association of BMPRII mutation and PAH, reduction in the expression of BMPRII has been reported in patients with IPAH without BMPRII mutation, and to a lesser extent in patients with secondary PH [Atkinson 2002]. Both MCT and hypoxia models of PH have been reported to be associated with the reduction in the expression of BMPRII. Both these models exhibit PY-STAT3 activation [Murakami 2010, Mathew 2011b]; therefore, it is likely that the STAT3 activation by itself may have a negative effect on BMPRII expression. In addition, BMPRII mutation may lead to PAH through the loss of its normal inhibition of inflammation

Recent studies have drawn attention to the role of gremlin in PH. Gremlin; a glycoprotein constitutively expressed in EC has been identified as an antagonist of BMP2, BMP4 and BMP7. It also functions as an angiogenesis factor independent of its action on BMPs. Increased expression of gremlin confined to EC has been reported in the lungs of patients with IPAH and HPAH. In hypoxia-induced PH, there is an increased expression of gremlin, and haplo-deficiency of gremlin attenuates PH [Costello 2010, Cahill 2012], further underscoring the importance of a balance between TGF- β /BMP and the antagonist, gremlin in maintaining vascular health.

TGF- β 1 is also an immunomodulator; it plays an important role in the mechanism of regulatory T cells (CD4+CD25+). Regulatory T cells (Treg) are thought to be a primary source of TGF- β 1 and it maintains self tolerance and prevents autoimmune diseases. It is further suggested that IL-6 counteracts TGF- β 1 effect on Treg generation [Bommireddy 2007, Redstake 2009]. In scleroderma, Treg function has been shown to be compromised. Interestingly, Treg levels were low in mice prone to develop autoimmune diseases. In IPAH, several T cell subset abnormalities were noted, however, Treg levels were increased in this group compared with the controls. All these patients were on PH therapy. It is not certain whether the increased levels of Treg reflect an attempt to control inflammation or whether their function is impaired [Austin 2010].

3.4. Inflammation

As seen in the preceding sections, loss of cGMP/cAMP mechanisms, deregulated TGF- β and activation of multiple growth factors significantly affect and exacerbate the inflammatory response in PH. The importance of inflammation in PH is further strengthened by the fact that patients suffering from systemic inflammatory and autoimmune diseases, and infectious diseases such as scleroderma [Mathai 2011], sarcoidosis [Nunes 2006], POEMS syndrome [Lesprit 1998] acquired immuno-deficiency syndrome [Cicalini 2011] and schistosomiasis [Kolosienek 2011] develop PH. Furthermore, these patients are shown to have perivascular inflammatory cells, regulated upon activation normal T-cell expressed and secreted

(RANTES), dendritic cells, anti-fibroblast and anti-endothelial antibodies [Tuder 1994, Dorfüller 2002, Perros 2007, Terrier 2008, Tamby 2005]. Elevated plasma levels of proinflammatory cytokines and chemokines such as interleukin (IL)-1, IL-6, IL-8, fractalkine and CC chemokine ligand 2 (CCL2) have been documented in patients with IPAH. Interestingly, IL-6 also contributes to PH in patients with COPD. IL-6 is produced by a variety of cells including EC, SMC, fibroblasts and macrophages. In the MCT model of PH, increased levels and the activity of IL-6 and PY-STAT3 activation occur before the onset of PH, and early treatment with dexamethasone inhibits IL-6 activation and attenuates PH. IL-6 augments hypoxia-induced PH, and not surprisingly, IL-6 knockout mice exhibit less inflammation and attenuated PH in response to hypoxia [reviewed in Mathew 2010]. Importantly, dysregulated cytokines in IPAH and HPAH are associated with negative effects on survival [Soon 2012].

Recent studies have shown the presence of lymphoid neogenesis in IPAH. These are highly organized tertiary lymphoid tissue occurring throughout the pulmonary vasculature possibly sustaining chronic inflammation and contributing to autoimmunity. In addition, expression of CD44, a cell adhesion molecule is reported in the plexiform lesions in the patients with IPAH. Importantly, neither the lymphoid neogenesis nor CD44 expression has been observed in the controls as well as in PAH associated with CHD (Eisenmenger syndrome) [Perros 2012, Ohta-Ogo 2012]. Persistent inflammation observed in IPAH through lymphoid neogenesis may contribute to autoimmunity in IPAH. These differences between IPAH and Eisenmenger syndrome may in part be responsible for the observed better survival in the latter group.

3.5. Caveolin-1

a. Loss/Dysfunction of Caveolin-1: Loss of endothelial caveolin-1 has been reported in several clinical and experimental forms of PH. The MCT model of PH has been extensively studied to understand the pathogenesis of PH. Disruption of endothelial caveolae associated with progressive loss of caveolin-1 accompanied by the activation of PY-STAT3 and increased expression of Bcl-xL occurs as early as 48 hrs post-MCT, before the onset of PH. The downstream effectors of PY-STAT3, survivin, Bcl-xL and cyclin D1 are upregulated in PH. Caveolin-1 functions as a suppressor of cytokine signaling-3, and inhibits PY-STAT3 activation. Importantly, rescue of endothelial caveolin-1 inhibits the activation of proliferative pathways and attenuates MCT-induced PH [reviewed in Mathew R 2011b].

Similar to the MCT model, in hypoxia-induced PH, impaired endothelium-dependent, NO-mediated pulmonary vascular relaxation and low basal and agonist-induced cGMP are present. However, in contrast to the MCT model, there is no reduction in caveolin-1 expression in hypoxia-induced PH. During hypoxia, eNOS forms a tight complex with caveolin-1 and remains dissociated from HSP90 and calmodulin, resulting in eNOS dysfunction. Furthermore, hypoxia-induced PH and pulmonary endothelial cells exposed to hypoxia exhibit hyperactivation of PY-STAT3. PY-STAT3 activation in hypoxia-induced PH despite the unaltered expression of caveolin-1 protein strongly suggests that caveolin-1 is dysfunctional and has lost its inhibitory function. Thus, the disruption of EC membrane and ensuing caveolin-1 loss, or perturbation of EC membrane with mislocalization of caveolin-1 leads to its dysfunction resulting in the initiation and progression of PH [Mathew 2011b].

Caveolin-1 in fibroblasts regulates ECM production. Low levels of caveolin-1 have been reported in fibroblasts from patients with scleroderma and idiopathic pulmonary fibrosis. Loss of caveolin-1 in fibroblasts leads to hyperactivation of MEK, ERK, Akt signaling pathways resulting in enhanced expression of collagen type I and III, tenascin C, reduction in ECM degradation and fibrosis; these processes are inhibited with the re-expression of caveolin-1. In addition, loss of caveolin-1 results in the activation of TGF- β signaling and upregulation of CXCR4 in monocytes resulting in their migration to damaged tissue where CXCL12, its ligand is produced [del Galdo 2008, Tourkina 2012].

b. Enhanced Expression of Caveolin-1 in SMC: Vascular SMC play an important role in the pathobiology of PH. During the progression of PH, SMC change from contractile to synthetic phenotype. These cells then can proliferate and migrate leading to neointima formation. Caveolin-1 functions as an antiproliferative factor in SMC; it keeps mitogens inactive and regulates Ca^{2+} entry in SMC. Recent studies have shown loss of endothelial caveolin-1 and enhanced expression of caveolin-1 in SMC in clinical and experimental PH. Pulmonary vascular SMC from IPAH revealed enhanced caveolin-1 expression, altered Ca^{2+} handling, increased capacitative Ca^{2+} entry and increased $[\text{Ca}^{2+}]_i$, and augmented DNA synthesis. Silencing caveolin-1 mRNA in these SMC has an inhibitory effect on capacitance Ca^{2+} entry and DNA synthesis [Patel 2007]. The enhanced caveolin-1 expression in SMC was reported in a recent case report of a child who presented with severe PH two years after a complete clinical recovery from acute respiratory distress syndrome. Lung biopsy revealed marked endothelial caveolin-1 loss, and the arteries with additional loss of von Willebrand Factor (vWF) exhibited robust expression of caveolin-1 in SMC, ultimately leading to neointima formation and a loss of response to therapy [Mathew 2011c]. Increased expression of caveolin-1 in SMC is also reported in patients with PH and associated COPD [Huber 2009]. MCT-induced PH is associated with progressive disruption of EC and loss of endothelial caveolin-1. During the progression of the disease at 4 wks, 29% of the arteries exhibited loss of vWF, in addition to a further loss of endothelial caveolin-1. Importantly, 70% of the arteries with vWF exhibited enhanced expression of caveolin-1 in SMC. Loss of vWF is indicative of extensive endothelial damage. It is worth noting here that an increased circulating level of vWF or EC is associated with poor prognosis in PH [Huang 2012].

Matrix metalloproteinase (MMP) 2 degrades ECM, and facilitates cell proliferation and migration. Caveolin-1 is known to inhibit MMP2 expression and activity. In the MCT model at 4 wks, despite enhanced expression of caveolin-1 in SMC, MMP2 expression and activity are reported to be increased. This indicates that caveolin-1 has lost its inhibitory function. In cultured SMC, caveolin-1 translocates from caveolae to non-caveolar sites within the plasma membrane in response to cyclic strain; the translocated caveolin-1 facilitates cell cycle progression and cell proliferation. Caveolin-1 blockade abolishes the stretch-induced cell proliferation, indicating that caveolin-1 plays a pivotal role in stretch-induced cell proliferation. It is likely that the extensive damage and/or loss of endothelial cells observed in PH may impose wall strain induced by elevated pressure directly on to SMC leading to translocation of caveolin-1 from caveolae and increased expression in SMC, which may cooperate with MMP2 to facilitate further proliferation and cell migration, and, thus, contribute to the worsening of

the disease. Enhanced expression of caveolin-1 and possibly translocation from caveolar sites to non-caveolar sites, switches caveolin-1 from being an anti-proliferative to a pro-proliferative molecule, and thus, may contribute to SMC change from a contractile to a synthetic phenotype [Huang 2012]. Thus, caveolin-1 plays an important role in the pathogenesis of PH and its function depends on the cell context and the disease stage.

3.6. Micro RNAs (miRNAs, MiRs)

MiRNAs are recently discovered small (~22 nucleotides) non-coding RNAs that play a key role in post-translational regulation of a number of genes. Since 1993, close to 1000 miRNAs have been identified in the human genome which regulate one third of all mRNAs. Maturation of miRNA is mediated by RNase III endonucleases, Drosha and Dicer. MiRNAs negatively regulate gene transcription by interacting with the 3' untranslated region of specific mRNA target to repress translation and enhance mRNA degradation. Interestingly, one miRNA can influence several mRNAs and one mRNA is influenced by several miRNAs. They participate in a variety of physiological and pathological functions such as development, cell proliferation, apoptosis, differentiation and inflammation; and they can act as oncogenes or tumor suppressors [Urbich 2008, Zhang 2007].

A number of miRNAs have been reported to participate in cardiovascular pathobiology. Smooth muscle-specific miRNAs miR-143/145, miR-221, miR-222 and miR-26A play a significant role in the regulation of VSMC phenotype. MiR 143 and miR-145 are well expressed in contractile VSMC and are deficient in the synthetic phenotype. In contrast, miR-221 and miR-222, transcriptionally induced by PDGF signaling are over-expressed during neointima formation. Over-expression of miR-221 represses SMC markers via downregulation of c-Kit, and promotes cell proliferation by inhibiting p27Kip1 [Cordes 2009, Song 2010, Bockmeyer 2012]. In addition, miR-26a has been shown to play a significant role in vascular SMC proliferation, inhibit differentiation and apoptosis, and alter TGF- β signaling [Leeper 2011]. Furthermore, induction of miR-1 in SMC inhibits cell proliferation and miR-100 functions as an inhibitor of mTOR and attenuates cell proliferation of EC and SMC [reviewed in Mathew 2011]. Over-expression of miR-17/92 cluster has been shown to reduce BMPRII expression. IL-6 activates 17/92 miR cluster via STAT3 activation, furthermore, it inactivates the TGF- β pathway by inhibiting p21 and BIM (Bcl2 interacting mediator of cell death) [Brock 2009, Petrocca 2008]. The importance of miR-17 and miR-20 in PH is supported by recent studies showing upregulation of p21 and attenuation of MCT-induced PH by antagomiR-17, and restoration of BMPRII function and attenuation of hypoxia-induced pulmonary vascular remodeling and RVH by antagomiR-20a [Pullamsetti 2012, Brock 2012]. IL-6 and PY-STAT3 play a significant role in PH, furthermore, STAT3 activation is thought to suppress the expression of miR-204, which is significantly down-regulated in SMC from PH patients and from experimental models; and treatment with miR-204 significantly inhibits STAT3 activation and attenuates MCT-induced PH [Courboulin 2011].

In cancer, miR-21 induction by IL-6 is dependent on STAT3, and miR-21 is thought to contribute to STAT3-mediated proliferative activity and immune responses [Löffler 2007, Kumaraswamy 2011]. MiR-21 is abundantly expressed in EC, and it modulates eNOS activity

and apoptosis. Over-expression of miR-21 in EC inhibits cell proliferation, cell migration and angiogenesis [Weber 2010, Sabatel 2011]. However, aberrantly expressed miR-21 participates in cell proliferation and neointima formation. Furthermore, there is an increased expression of miR-21 in undifferentiated vascular SMC compared with the differentiated ones [Ji 2007]. In addition to STAT3, TGF- β and BMP4 upregulate miR-21, and it is suggested that it may have a role in profibrotic effects [Kumaraswamy 2011]. Interestingly, downregulation of miR-21 has been reported in the lungs and serum of the patients with IPAH, and also in *SMAD9* mutation-associated HPAH suggesting a role for Smad8 in the processing of miR-21. Interestingly, miR-21 was down-regulated in the MCT-induced PH, but not in the hypoxia model [Caruso 2010, Drake 2011]. Another endothelial-specific miR-126 is thought to activate VEGF signaling and facilitate angiogenesis [Nicoli 2010].

Significant differences in the miRNAs expression in the concentric and plexiform lesions in the lungs of patients with PAH have been reported. Interestingly, the expression of miR-126, which enhances the VEGF-A signaling pathway for angiogenesis was significantly higher in plexiform lesions compared with the concentric lesions. In contrast, the expression of miR-143/145, which maintains the contractile SMC phenotype, was significantly lower in plexiform lesions, thus, underscoring the presence of deregulated angiogenesis in plexiform lesions [Bockmeyer 2012]. Although, miRNAs are known to play an important role in cancer; the field of miRNAs, however, is in its nascent stage in PH. It is already becoming clear that miRNAs do play an important role in PH.

3.7. Deregulated angiogenesis

The term angiogenesis is applied to the process of new vessels sprouting from the preexisting vessels. Angiogenesis is a tightly controlled process involving a number of signaling pathways including VEGF, bFGF, PDGF-B, TGF- β , BMPs, angiopoietin1/Tie2 and Notch etc. to produce a number of coordinated signals leading to a proper and mature vascular network. Angiogenesis is very active during the embryonic stage, and it becomes active again during adulthood when new vessel formation is required such as during pregnancy and wound healing, and also under various pathological conditions. VEGF is essential for the initiation of angiogenic sprouting [Folkman 1996, Holderfield 2008]. The TGF- β superfamily of proteins plays a significant role in angiogenesis by regulating VEGF expression in a coordinated fashion. TGF- β modulates activities of VEGF and bFGF during wound healing. It is thought TGF- β via Alk5/BMPRII/Smad2 enhances VEGF activity and facilitates angiogenesis, whereas BMP9 via Alk1/BMPRII/Smad1 suppresses angiogenesis. Interestingly, in plexiform lesions, loss of TGF- β family signaling is associated with increased expression of VEGF, highlighting deregulated angiogenesis. The importance of the TGF- β superfamily in angiogenesis is further supported by the observation of the mutation of endoglin as well as Alk1 in hemorrhagic hereditary telangiectasia [David 2009, Shao 2009, Richter 2004, Tudor 2001, Hirose 2000]. RhoA/ROCK signaling is also required for VEGF-mediated angiogenesis; and thrombospondin-1 (TSP1), an endogenous anti-angiogenic factor regulates VEGF signaling by controlling the activation of VEGF-R2 [Bryan 2010]. In plexiform lesions, in addition to increased VEGF expression, increased expression of other signaling molecules such as HIF-1 α , HIF-1 β , MMP9, Notch4 and

TSP-1 has been reported. HIF-1 α and HIF-1 β are hypoxia-inducible factors facilitating hypoxia-induced induction of VEGF. Notch 4 is thought to be involved in reshaping of the local vasculature, via cross-talk with VEGF and TGF- β in EC. MMP9 participates in basement membrane remodeling [Jonigk 2011, Tudor 2001]. Interestingly, increased expression of ET-1 receptors observed in patients with PAH and CHD is also thought to play a role in neointima formation and neo-angiogenesis [Huang 2011]. Thus, the altered expression of several signaling molecules leads to deregulated angiogenesis, a hallmark of plexiform lesion, which facilitates in sustaining the disease and its progression.

3.8. Right Ventricular Failure (RVF)

Because of increased pulmonary vascular resistance, right ventricular hypertrophy (RVH) occurs as an adaptive measure to maintain cardiac output. Right ventricular (RV) contractility is impaired during the progression of the disease, leading to RVF characterized by RV dysfunction, increased filling pressure and low cardiac output. RVF is the major cause of deterioration in PH leading to death. In a recent study, a mortality rate of 41% was found in patients with PH admitted to the intensive care unit with acute right heart failure. Interestingly, patients with Eisenmenger syndrome develop RVF at a much later time compared with IPAH patients with comparable RV afterload. The experimental models of RV pressure overload reveal more cardiac fibrosis, capillary rarefaction, diminished antioxidant protection and oxidative damage. Furthermore, pressure-induced reversal of cardiomyocyte phenotype to fetal stage in chronic PH results in relocation of critical cytoskeletal stress proteins leading to progressive deterioration of RV function; and in PH, these alterations are limited to the RV. Therefore, it is thought that the pressure overload itself may not be sufficient for the RVF in PH [Sztrymf 2010, Bogaard 2009]. Oxidant injury has been thought to play a key role in RVF associated with PH. ROS have a direct effect on cellular structure and function; and can lead to inflammation and apoptosis. Increased generation of ROS can induce mitochondrial DNA damage leading to a reduction in mitochondrial function, thus, facilitating further ROS generation and cellular injury, worsening heart failure. Impaired mitochondrial glucose oxidation and reduction in glucose-based oxygen consumption of RV myocardium have been reported in experimental models of RV pressure overload (MCT-induced PH, and pulmonary artery band-induced RVH). In the MCT model of PH, both NADPH oxidase and mitochondrial ROS generation were shown to be increased associated with reduction in the mRNA expression of both superoxide dismutase (SOD) 1 (cytosolic) and SOD2 (mitochondrial). Treatment with EUK-134, an SOD and catalase mimetic when given at 10 days post-MCT significantly improved RV function and prevented cardiac fibrosis without altering the lung weight [Piao 2010, Redoute 2010]. Thus, oxidant injury may be a key determinant of RV function in PH-associated RVH.

3.9. Reversibility vs irreversibility of PH

Under most circumstances, PH is a progressive disease, ultimately becoming irreversible with a negative impact on survival. Reversibility of PH is seen especially in infants and children with PPHN, PH associated with RDS/BPD or CHD. In the former cases, as the lung vascular/

4. Summary

PH is the result of an imbalance between vasoconstriction and vasodilatation, cell proliferation and apoptosis, and between pro-angiogenesis and anti-angiogenesis. Intricate and delicate intermeshing of a large number of signaling molecules in pulmonary vasculature cooperate to preserve the balance between the opposing signaling and activities, thus, maintain vascular health. Loss or increased expression of a molecule secondary to an injury can derail the delicate network of signaling pathways resulting in deregulated inflammatory response, cell proliferation, cell migration and angiogenesis leading to the initiation and progression of PH. Some of these changes occur before PH becomes clinically manifest, thus, impacting the response to therapy and survival. Genetics, epigenetics, severity of injury and the associated inflammatory response further influence the outcome.

Author details

Rajamma Mathew*

Address all correspondence to: rajamma_mathew@NYMC.edu

Departments of Pediatrics and Physiology, New York Medical College, Valhalla, NY, USA

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