The role of disturbed blood flow in the development of Pulmonary Arterial Hypertension: Lessons from preclinical animal models

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Abstract

Pulmonary Arterial Hypertension (PAH) is a progressive pulmonary vasoproliferative disorder characterized by the development of unique neointimal lesions including concentric laminar intima fibrosis and plexiform lesions. Although the histomorphology of neointimal lesions is well described, the pathogenesis of PAH and neointimal development is largely unknown.

After three decades of PAH pathobiology research the focus has shifted from vasoconstriction towards a mechanism of cancer-like angioproliferation. In this concept the role of disturbed blood flow is seen as an important trigger in the development of vascular remodeling. For instance, in PAH associated with congenital heart disease, increased pulmonary blood flow (i.e. systemic-to-pulmonary shunt) is an essential trigger for the occurrence of neointimal lesions and PAH development. Still, questions remain about the exact role of these blood flow characteristics in disease progression.

PAH animal models are important for obtaining insight in new pathobiological processes and therapeutical targets. However, as for any preclinical model the pathophysiological mechanism and clinical course has to be comparable to the human disease that it mimics. This means that animal models mimicking human PAH ideally are characterized by: a hit recognized in human disease (e.g. altered pulmonary blood flow), specific vascular remodeling resembling human neointimal lesions, and disease progression that leads to RV dysfunction and death.

A review that underlines the current knowledge of PAH due to disturbed flow is still lacking. In this review we will summarize the current knowledge obtained from PAH animal models associated with disturbed pulmonary blood flow and address questions for future treatment strategies for PAH.
Introduction

Pulmonary arterial hypertension (PAH) is a fatal and progressive form of pulmonary hypertension with, so far, unknown origin (37, 56). PAH is characterized by a typical form of pulmonary vascular remodeling, i.e. plexogenic arteriopathy (Figure 1)(71). Where thickening of the medial and adventitial layer of the pulmonary arterioles is seen in many forms of PH (e.g. PH associated with hypoxemia), PAH is additionally characterized by the formation of complex cellular and fibrotic lesions with at the end of the spectrum the formation of concentric laminar intima fibrosis and plexiform lesions (Figure 1)(71, 88). These neointimal lesions cause intraluminal obstruction characterized by apoptotic dysregulation and proliferation of endothelial cells, smooth muscle cells, fibrosis, and inflammation.(36, 67)

PAH is considered irreversible when these neointimal lesions have formed(67), resulting in increased pulmonary vascular resistance, increased right ventricular (RV) workload and eventual death due to RV failure(7, 11, 39). The complex pathogenesis of PAH needs to be further explored in order to identify potential therapeutic targets and improve future treatment possibilities.

According to the typical histopathology, the progressive clinical course, and therapeutic responses, PAH is classified in group I of the clinical classification (Dana Point) for PH(70).

PAH is subdivided in idiopathic, heritable, drug-induced, and associated with other conditions including congenital heart disease (CHD). Although the histomorphology of PAH is well described, the pathogenesis of these progressive angioproliferative lesions remains largely unknown.

After three decades of research on the pathobiology of PAH, the focus has shifted from vasoconstriction(38) towards a cancer-like mechanism of angioproliferation(58, 67) in which the role of disturbed pulmonary blood flow is re-appreciated as an important trigger in the development of pulmonary vascular remodeling (Figure 2)(81). In general, disturbed blood flow can be defined as any pattern of flow that is nonuniform and irregular. Examples are recirculation or swirling flow, reciprocating flow and turbulent flow. These patterns can occur
naturally at specific regions in the vascular system (e.g. branching points) or due to (pathological) changes in either vascular geometries (vessel stenosis or obstruction) or hemodynamic forces (17, 20). Under pathophysiologica conditions these changes can lead to specific vascular and cellular responses resulting in remodeling of the vascular wall(17, 20).

Clinical observation has shown that in patients with CHD who develop PAH, characteristic vascular lesions occur almost exclusively in defects associated with increased pulmonary blood flow, qualifying this increased pulmonary blood flow as a trigger for the induction of neointimal development. Here, increased pulmonary blood flow is regarded an inductor of disturbed blood flow patterns in the pulmonary vasculature. In these patients with increased pulmonary blood flow, additional increased pulmonary arterial pressure, as in non-restrictive post-tricuspid shunts, accelerates the progression of pulmonary vascular remodeling, as an apparent second trigger(81). Disturbed blood flow patterns are likely to be one of the triggers in PAH. Also other triggers, such as genetic predisposition due to BMPR2-signaling defects, drugs, toxins, inflammation have been associated with induction or aggravation of the vascular disease process in PAH(36, 55). Apart from the initiating trigger, once PAH has developed, the elevated pulmonary arterial pressure in itself leads to altered hemodynamic forces and flow patterns, that subsequently may propagate the vascular disease process. Still, questions remain about the exact role of blood flow disturbances in neointimal development and disease progression.

During the past decades animals models have had played a crucial part in this process and will continue to give people new insight through PH research in the future. Although much insight has been gained from “historical” PH models, such as hypoxic PH or monocrotaline-induced PH, most of these studies are hampered by the fact that these models do not truly reflect the typical features of PAH, i.e. the histopathology and progressive course(73). Indeed, while it is possible to “cure” many forms of PH in animal models, the clinical profile of patients with PAH is more resistant to therapy. Hence, there is an increasing interest in models that more reliably reflect the complex disease PAH. This review will summarize and discuss current
knowledge about models of PAH. Since increased pulmonary blood flow appears to be a crucial factor in disease development, particular emphasis will be put on models of increased pulmonary blood flow and the current knowledge of the role of disturbed flow patterns in the development of pulmonary vascular remodeling in PAH.

**Animal models of PAH: general concept**

Animal models remain to play a crucial role in studying both new biomolecular pathways as well as investigating new treatment effects in PAH. Although material obtained from patients, – usually when the lungs are explanted – is valuable, the use of animal models allows for evaluation of disease development and analysis of processes in early stages of the disease, as well as testing of reversibility. However, as for any preclinical model, the pathophysiological mechanisms and clinical course have to be comparable to the human disease that it is supposed to mimic.

For PAH this means that an ideal model would include the following:

i. An initiation or trigger(s) of pulmonary vascular remodeling that mimics the human situation, e.g. an increase in or redistribution of pulmonary blood flow.

ii. Pulmonary vascular remodeling that represents the development of complex obliterative neointimal lesions of the small intra-acinar arteries, and media hypertrophy of the smaller pre-acinar arterioles.

iii. A progressive disease development that leads to RV dysfunction and eventually death.

In addition, the model has to be viable in an experimental setting regarding both functional (hemodynamic and histological assessment) and biomolecular analysis, as well as having a workable period to disease progression.

To date no such ideal model for PAH exists. However, some recent animal models may resemble more closely both pathophysiological mechanisms and clinical course of human PAH compared to more historical animal models of PH.
Development of pulmonary vasculature: how do animals compare to humans?

In choosing a proper animal model for PAH research, first consideration has to be given to possible anatomical and developmental differences between specific animal species and humans. These differences could have consequences for disease progression and severity, and therapeutic response.

In humans the pulmonary parenchym undergoes 6 developmental stages: embryonic, pseudo-glandular, canalicular, saccular, alveolar, and vascular maturation(93-95). Of these changes only alveolar development and vascular maturation continue after birth(93, 94). Comparable with humans, rats and mice have little alveolar development at birth, whereas for instance lambs have already well developed alveoli at birth. Such differences are likely to be of importance when studying changes in pulmonary blood flow and vascular remodeling perinatally. Rat lungs on the other hand show quite similar alveolar and capillary surface development after birth compared to humans (95). Mice have less pulmonary blood vessel walls and more alveolar space compared to rats (24, 95), and presumably less distal supernumerary arteries, which might explain the mild forms of vascular remodeling found in most murine PH models.(28).

The pulmonary vasculature is lined by endothelial cells, which are the first structure to perceive changes in hemodynamic forces. The endothelium has an important function in controlling vasomotor tone, regulating permeability, maintenance of homeostatic balance and immunity. As the pulmonary vascular tree branches and the size of the pulmonary arteries decreases, the endothelial cell phenotype progresses from pulmonary artery endothelial cells towards pulmonary microvascular endothelial cells, which differ in their permeability and mechanical properties(17) These differences may reflect in the location-specificity of the pulmonary vascular histomorphological changes observed in pulmonary PAH. In the larger pre-acinar arterioles (100-500µm) only increased medial wall thickness and intima proliferation is observed, whereas complex neo-intimal lesions progressively develop in the normally non-muscular arterioles (<100 µm), the so-called intra-acinar arteries (Figure
Laminar concentric intimal fibrosis and plexiform lesions are predominantly formed at distal dichotomous branching points and branching points of supernumerary arteries, respectively (18, 70, 91) (Figure 2).

**Historical PH models**

Historically the most widely used animal models of PH have been chronic hypoxia and the monocrotaline (MCT) induced PH rodent models. Although these models have added enormously to the understanding of the mechanisms of pulmonary vascular remodeling in PH, they are truly limited by the lack of typical complex vascular neointimal lesions and severe disease phenotype seen in PAH. (73) Still, these models form a basis for other newer animal models where additional hits (triggers) result in characteristic pulmonary vascular lesions resembling human disease, e.g. the monocrotaline/increased flow model (28, 73) or the hypoxia/sugen model (79). For more knowledge about these models the readers is referred to reviews published recently (29, 57, 73).

**Models of Increased pulmonary blood flow**

Early chronic studies in large animals have shown that when either one lung (i.e. left pulmonary artery) (19, 24, 33) or a single lobe (13, 59, 69) is directly connected to the aorta, which induces both high pulmonary blood flow and increased pulmonary arterial pressure, complex neointimal lesions develop comparable with human disease (Figure 4). Unfortunately, since extensive morphometric analysis was not conducted in these studies it is difficult to interpret the exact magnitude of this remodeling.

Many later animal studies inducing increased pulmonary blood flow have used restrictive shunts resulting in increased muscularization of the pulmonary arteries, but no complex neointimal lesions. (10, 25, 26, 60, 62, 89).

An explanation for the relatively mild form of vascular remodeling found in these ‘increased-flow only’ models is that the shunts used in these models were restrictive shunts with no
significant flow-induced rise in pulmonary arterial pressure.(10, 60, 89) This is in analogy to the clinical observation, that patients with untreated pre-tricuspid shunts, such as atrial septal defect, in general develop advanced pulmonary vascular remodeling only in 5-15% of the cases and only after 2-3 decades. In contrast, patients with untreated non-restrictive, post-tricuspid shunts, such as ventricular septal defects, develop advanced complex neointimal lesions in 1-2 years in 80-100% of the cases.(81)

Whether longer duration of the increased pulmonary blood flow in these animal models (months/years in stead of weeks) or an associated increase in pulmonary arterial pressure by a non-restrictive shunt, would have induced advanced pulmonary vascular lesions, can only be speculated. However, the absence of advanced vascular remodeling in these restrictive-flow models makes them less suitable to study the typical features of human PAH and to extrapolate the therapeutic effects to patients with PAH.(64, 78)

Despite the lack of relevant endpoints, the high-flow low-pressure models allow to study the role of various pathways known in human PH. First, several studies have reported that systemic-to-pulmonary shunting in young lambs and piglets results in alterations of the endothelin pathway (Table 1)(9, 10, 53, 60, 63). In these models Endothelin-1 and Endothelin A receptor expression is first up regulated followed by increased Endothelin-B receptor expression after chronic (>8 weeks) shunting which were found on the smooth muscle cells of the pulmonary vessels. Chronic increased flow has also been shown to increase endothelial nitric oxide synthesis (eNOS), but not inducible nitric oxide synthesis (iNOS), activity in lung tissue and the pulmonary arteries subjected to increased flow(49, 60). Interestingly, NO availability itself was reported to decrease after increased flow exposure, which has been suggested to be the result of peroxynitrite production that leads to scavenging of NO and decreased production of NO by eNOS. In addition to biomolecular investigation, several treatment effects have been reported in these models, including beneficial effects on pulmonary vascular resistance and vascular wall thickness after preventive treatment with...
PAH specific drugs endothelin receptor antagonists (ERAs)(61, 62) phosphodiesterase-5 inhibitors(61), prostacyclin and inhaled NO(89).

**Increased pulmonary blood flow combined with an additional trigger**

*A second hit needed?*

Tanaka and colleges were the first to show that combining vascular injury (via monocrotaline administration) with increased pulmonary blood flow (via an anastomosis of the left subclavian artery to the distal left pulmonary artery(74) or by unilateral pneumonectomy (52)) resulted in an extended neointimal pattern of pulmonary vascular remodeling in the lung after 5 weeks (Figure 4). In addition, increased pulmonary artery pressure and RV hypertrophy were reported. In this study either monocrotaline or anastomosis alone did not result in severe neointimal vascular remodeling.(74) In a different model van Albada et al have shown that increased pulmonary flow via an aortacaval (av-) shunt, when combined with monocrotaline administration, results in 1) neointimal obliteration of the intra-acinar vessels staining positive for eNOS and smooth muscle actin, 2) increased systolic pulmonary artery pressure and RV hypertrophy and 3) increased mortality after 5 weeks (Figure 4).(84)

Cumulative data from this model confirmed that increased pulmonary blood flow, preceded by endothelial injury via MCT, is a prerequisite for the development of pulmonary neointimal lesions.(8, 21, 84) Interestingly, in an experimental study Nishimura and colleagues showed that when monocrotaline was administered after induction of the av-shunt, pulmonary vascular remodeling was less pronounced. This could possibly be explained by the fact that due to the increased blood flow through av-shunt the concentration of metabolized monocrotaline pyrrole (after activation in the liver) is lower in the venous system reaching the pulmonary circulation compared to non-shunted rats.

*Neointimal lesions in models with increased pulmonary blood flow*
Are the vascular lesions seen in “double-hit” models comparable with those in human disease (Figure 5)?

In human PAH irreversible neointimal lesions are comprised of both ECs and SMCs with a reduction in apoptotic markers and increase in inflammatory cells (43). In flow models, these obliterative lesions are comprised of cells that stain positive for the endothelial cell markers VEGF-R2 (90), eNOS (84), von Willebrand Factor (59), CD31+ (47) and for SMCs staining positive for \( \alpha \)-smooth muscle actin (Figure 4, Table 1) (21, 47, 84, 90). Longitudinal studies using the av-shunt or unilateral pneumectomy combined with monocrotaline in rats have shown that these neointimal lesions start to form 1-2 weeks after increased pulmonary blood flow (Figure 4) (21, 51, 52, 90). In these experimental models the exact role of apoptotic or pro-proliferative state of these vessels during vascular remodeling is fairly unknown. In addition, questions still remain where in the smaller pulmonary vasculature these occlusive lesions are most prominent (i.e. at branching points of larger arteries or at more distal locations).

Interestingly, White and colleagues have reported in their model that when monocrotaline administration is combined with unilateral pneumonectomy in younger rats, more complex plexiform-like lesions are formed (Figure 4) (90). The authors suggested that younger rats were more prone to vascular proliferation after injury. These plexiform-like lesions stained positive for vWF, VEGFR-2 and \( \alpha \)-SMA and, as shown by microangiography, to be part of the pulmonary vasculature. However in this monocrotaline / pneumonectomy model, unlike the monocrotaline / av-shunt model, the effects of unilateral pneumonectomy have to be taken into consideration, including compensatory proliferation (up to 35%) of the remaining lung parenchyma itself (15).

**Increased pulmonary blood flow and inflammation**

Inflammation has been suggested to play a role in PAH as well as in the development of flow-induced neointimal formation in PAH patients with systemic-to-pulmonary shunts (32, 80, ...
Proinflammatory cytokines and other inflammatory cells including mast cells, macrophages, lymphocytes, and dendritic cells have all been linked to flow and non-flow PAH (Table 1). Accumulation of inflammatory cells in experimental PAH has also been linked to increased oxidative stress, which is also seen in pulmonary vessels of PAH patients. In animal models of increased pulmonary blood flow inflammation has also been suggested to play a role in neointimal development. Clearly, the effect of monocrotaline administration in vascular inflammation has to be taken into account in these experimental models and questions remain whether inflammation can be seen as the additional trigger for neointimal development in the increased pulmonary blood flow / monocrotaline models. Still, reducing inflammation such as recently shown by mast cell stabilization or inhibition of chymase can attenuate the development of pulmonary vascular remodeling. These results justify future exploration of the possible role of anti-inflammatory therapy for PAH.

In summary, in flow-models that combine a trigger such as vascular injury (monocrotaline) with either pneumonectomy or an av-shunt, increased pulmonary blood flow specifically induces severe, characteristic pulmonary vascular remodeling, including neointimal development, perivascular inflammation and plexiform like lesions with remarkable similarities with human plexogenic arteriopathy (ii) (Figure 4,5). In addition increased flow leads to more pronounced increases in pulmonary artery pressure, RV hypertrophy and mortality (iii).

Role of disturbed flow on vascular remodeling: lessons to be learned from the systemic circulation

In systemic vessels, blood flow, and the shear stress it inflicts, has been demonstrated to play a critical role in vascular homeostasis and vessel remodeling. Shear stress imposed upon the luminal surface of the endothelial cell is defined as the tangential force per unit area caused by flowing blood. Here, the concept is that physiological laminar flow (seen in the
straight part of a healthy vessel) has athero-protective effects due to a physiological shear stress with a definite direction resulting in endothelial homeostasis\cite{696 Chiu,J.J. 2011; 828 Davies,P.F. 2009}. Changes in flow patterns (e.g. swirling flow, turbulent flow or low flow) lead to changes in shear stress (e.g. reciprocating shear stress, a high shear stress gradient or reduced shear stress). These changes in shear stress are able to induce endothelial changes, specifically at predilection points (e.g. arterial branching points) where blood flow patterns are disturbed rather than laminar\cite{4, 16, 17, 20}.

In pathophysiological settings (such as increased blood flow, vascular stenosis or surgical intervention) disturbed blood flow at these predilection sites is amplified resulting in vascular remodeling due to upregulation of pro-atherogenic and pro-thrombogenic genes in ECs, EC proliferation and inflammation and SMC modulation\cite{16, 17, 20}. A vicious cycle is made when the consequential changes in vessel wall geometry at these sites (due to vessel wall proliferation) further disturb flow patterns (and thus shear stress) resulting in propagation of vascular remodeling\cite{17, 20}. In addition, besides changes in local flow pattern, other triggers such as oxidative stress, inflammation, hypercholesterolemia, and advanced glycation end products can result in endothelial dysfunction thereby accelerating the process of vessel wall remodeling in the systemic vasculature\cite{17}.

Can these concepts of vascular remodeling triggers in systemic vessels be translated to the pulmonary vasculature? Of course, one needs to take into account that the pulmonary endothelium differs from its systemic counterpart in several aspects; for instance 1) angiogenic rather than vasculogenic development, 2) a transition from a low flow/high resistance environment towards a high flow/low resistance environment at birth and 3) the molecular heterogeneity of pulmonary endothelial cells within the pulmonary vascular bed\cite{3, 4}. Nevertheless, several concepts of hemodynamic changes in systemic vessels may also apply to the pulmonary circulation, supported by various observations.

First, pathophysiological triggers that result in disturbed flow patterns in systemic vascular remodeling (e.g. increased pulmonary blood flow and/or increased pressure) are also seen to
induce pulmonary vascular remodeling in PAH. For instance, as in systemic vessels, increased blood flow induces characteristic vascular lesions in pulmonary arterioles, as illustrated in patients with CHD and systemic-to-pulmonary shunt. Also as in systemic vessels, in PAH pulmonary vascular remodeling is specific found at specific branching points where flow is disturbed (swirling or reciprocating flow) and shear stress is altered (18, 91). Conceptually, different branching points (dichotomous vs. supernumerary arteries) could then induce different flow pattern alterations, thereby inducing different forms of pulmonary vascular remodeling. This would explain why laminar concentric intimal fibrosis is described to form predominantly at dichotomous branching points and plexiform lesions predominantly at the origin of supernumerary arteries (Figure 2) (18, 91).

Finally, as in systemic vessels, additional triggers (such as increased pressure, genetic predisposition, inflammation) may add to endothelial cell injury thereby accelerating the process of vascular remodeling.

Still, it is yet unclear how changes in flow patterns (i.e. shear stress) exactly induce endothelial changes in the pulmonary vasculature ultimately leading to advanced pulmonary vascular remodeling in PAH. Several mechanotransduction pathways linked to changes in pulmonary blood flow have been suggested to be involved in the pathobiology of PAH. These include direct induction of proliferation (through VEGF(27), PDGF(79), TGF-β(56) and pathways), apoptosis-resistance of endothelial cells(65), induction of inflammatory processes like mast cells(8), macrophages or lymphocytes(32) recruitment, differentiation of endothelial cells into mesenchymal cells(6), direct damage to the endothelial cell barrier and autocrine effects(77).

This concept of disturbed flow patterns leading to neointimal development may also play a role in other animal models in which characteristic neointimal lesions have been reported. These models will briefly be described below.
Other neointimal animal models

In addition to flow-associated PAH animal models, other models (mostly rodents) have also been reported to develop specific obliterative lesions, some with great resemblance to neointimal lesions in human PAH (figure 7). Murine models are of interest since they allow one to investigate the relevance of single genes using genetic manipulation. The main disadvantage of murine models however, is the mere mild degree of PH and RV dysfunction that usually is achieved in mice compared to other animal models(28). In addition, possible differences in the vascular bed of mice and humans have to be taken into consideration when comparing these data, as described above.

Sugen / hypoxia rat model

In both iPAH as well as PAH associated with congenital systemic-to-pulmonary shunts, VEGF is strongly expressed in plexiform lesions in end stage disease.(27) However, the exact role of VEGF in pulmonary vascular remodeling in PAH remains unclear.

Taraseviviene-Stewart et al have shown that by combining VEGF receptor 2 (VEGFR2) inhibition, using the compound Sugen-5416, with chronic hypoxia results in PAH with characteristic obstructive neointimal lesions in the arterioles, increased mPAP and right ventricular hypertrophy(75, 76). In addition, when these rats were subjected to normoxia for a longer period of time (up to 14 weeks), more complex neointimal lesions did form(1). However, unlike in irreversible human PAH, the survival rate of Sugen-5416 / hypoxia rats has been shown to be close to 100 percent even during long follow-up.(1) Apparently the degree and magnitude of arteriopathy in these rats is unable to induce RV failure which can raise the question whether the type of arteriopathy is representative for the human setting.

In addition, it is unclear how in these rats VEGFR2-inhibition, as additional “trigger” to hypoxia, leads to the induction of neointimal lesions. Sugen-5416 injection or hypoxia alone both do not result in obliterative lesions of the intra-acinar vessels.(75). Unfortunately, little is known what the exact role is of Sugen-5416 in this process of SMC and EC proliferation and
vascular occlusion. For instance, besides VEGF-R2 inhibition Sugen-5416 is also known for
its inhibitory effect on other tyrosine kinases(66, 92). It is possible that other kinases also may
play a role in the development of vascular remodeling in the 5416/hypoxia model. In other
words, in this model the trigger is already interfering with the pathobiological mechanisms of
PAH. This hampers biomolecular analyses of PAH and evaluation of treatment effects using
this Sugen/hypoxia(/normoxia) model. Indeed, Moreno-Vinasco et al have shown that when
comparing gene expression of Sugen/hypoxia rat lungs with human PAH lung tissue, only one
similar hit comes up (Fyn: a protein-tyrosine kinase oncogene)(45).

Endothelin-B receptor deficient rats

Ivy et al have reported in rats that Endothelin-B (ETB) receptor deficiency combined with
monocrotaline results in the development of neointimal lesions, increased RV hypertrophy
and reduced cardiac output compared to monocrotaline alone(40). The vascular lesions in this
model are comprised of cells staining positive for both EC and SMC markers and show
similarities with human PAH lesions as well as those in other PAH models. However, when
ETB receptor deficient rats were subjected to hypoxia instead of monocrotaline (41) these
neointimal lesions were not seen even though PH did occur.

Other models of potential interest are the cell-specific BMPR-2 loss of function models in
mice(35), Il-6 transgenic mice(72) and mice overexpressing a calcium binding protein
S100A4Mts(30) (Figure 7). In these models, however, incomplete disease penetrance and the
need for additional hits, may limit its use.

Treatment effects in neointimal animal models

Currently PAH patients are treated, in addition to supportive medication, with either 1)
calcium channel blockers or 2) one or a combination of the following agents: prostacyclin-
analogues, endothelin receptor antagonists and phosphodiesterase-5 inhibitors. Despite
improvements with these treatments, PAH still remains a fatal disease(39). The general thought is that this is related to the severity of pulmonary vascular remodeling, which is considered irreversible once neointimal lesions have formed. Therefore, future treatments should focus targeting on reversal of these so-called irreversible neointimal lesions, something that has not been accomplished so far.

In general, although some positive effects have been described (predominantly in prevention studies), to date no cure for animals with established advanced lesions have been described. Nevertheless, several interesting “proof of principle” studies may direct future research for development of novel therapies (Table 2).

In monocrotaline / flow PAH rats inhibition of the vasoconstrictor tromboxane, a key player of vascular remodeling in PAH, using the prostacyclin analogue treprostinil, resulted in a reduction in pulmonary artery pressure, although neointimal development was not reduced(85). Similar resistance to vascular remodeling improvement was seen when using aspirin or iloprost(82), suggesting a vasodilatory effect rather than an anti-proliferative effect of the drug.

Other studies have shown that ACE inhibition using Quinapril attenuated neointimal formation of the intra-acinar vessels.(50) In the future, novel treatments in PAH may shift more towards targeting angiogenesis and inflammation. Indeed, in monocrotaline / flow PAH models simvastatin (also known for its anti-inflammatory properties)(47), mast cell stabilization (via Cromolyn)(8), Dehydroepiandrosterone (known to have antioxidant and anti-inflammatory effects)(34), rapamycin (an anti-angiogenic agent)(48) and EPO (erythropoietin; known for endothelial repair by endothelial progenitor cell mobilization)(83) have shown to attenuate, or in part reverse, neointimal formation and/or increased pulmonary artery pressure. However, caution has to be taken when directly extrapolating these data to the human setting as illustrated a recent randomized controlled trial in PAH patients, in which simvastatin as add on therapy did not show a positive effect on 6 minute walking
distance. Unfortunately, the clinical endpoint of this study, 6-minute walk test, was not assessed in animal models. Indeed, most studies in animal models of PAH lack the use of clinical relevant endpoints, such as exercise capacity, although these end-points have been proven useful in studies addressing RV therapeutic strategies. (7, 12)

Possible novel pathophysiological pathways in PAH

The pathogenesis of the complex pulmonary vascular lesions is likely a multifactorial process, necessitating system biology approaches to identify novel targets (2). Using a microarray analysis, van Albada et al showed that increased pulmonary blood flow specifically induced the expression WNT-signaling genes and several other transcription factors including activating transcription factor-3 and early growth response factor-1 (Egr-1) (21, 80). Also, in the last years many new putative pathways have been discovered in non-neointimal models that could potentially affect PAH development. Examples are the PPAR gamma pathway in PAEC in mice (5), tyrosine kinase inhibitors in hypoxic PH or monocrotaline induced PH (68), soluble GCs (23) and the possible role of microRNAs in PH (54). Although promising pathways, their roles have yet to be established in more clinically relevant neointimal models of PAH (67, 73).

Concluding remarks

Pulmonary arterial hypertension is still a progressive disease with typical lesions characterized by neointimal formation, obliteration and plexiform lesions. The pathogenic mechanisms underlying the formation of these progressive pulmonary vascular lesions is still poorly understood but might be the key to curative treatment.

With PAH research shifting from a concept of mere vasoconstriction towards a mechanism of angioproliferation, the role of disturbed pulmonary blood flow is seen as an important instigator (both clinically and experimentally) in plexogenic arteriopathy and PAH development. Further investigation in the role of disturbed blood flow can provide us with
relevant clues to further unravel the complex pathogenesis of PAH. In this process PAH
models of disturbed pulmonary blood flow – that more closely resemble the human trigger
and histopathology – are well suited for further investigation. Using these models, new
pathways are deemed to emerge which may lead to new therapeutic targets for patients with
PAH in the future.
Addendum legends to figures

Figure 1
Correlate of the Dana Point 2008 clinical classification with the characteristics of the pulmonary vascular arteriopathy. Group 1, PAH, is characterized by formation of concentric laminar intima fibrosis and plexiform lesions. Typical examples of these lesions are presented in the right-sided column. Figure adapted from Wagenvoort and Mooi(87).

Figure 2
Schematic hypothesis of the effects of disturbed pulmonary blood flow patterns on the development of pulmonary vascular remodeling in PAH. (a) Normal thin walled vessels with a supernumerary artery and a dichotomous bifurcation. (b) Increased pulmonary blood flow due to systemic-to-pulmonary shunting results in disturbed blood flow (including reciprocating and recirculating flow) at branching points. This leads to specific pulmonary vascular remodeling at specific sites: plexiform lesions predominantly at the origin of supernumerary arteries vs. laminar concentric intimal fibrosis predominantly at dichotomous branching points. The resulting changes in vessel wall geometry further alter flow patterns, which lead to further vascular modeling. EC: endothelial cell, SMC: smooth muscle cell.

Figure 3
Distribution of the specific lesions seen in pulmonary hypertension throughout the pulmonary vascular tree. The pre-acinar pulmonary arteries mainly display a phenotype of media hypertrophy (a) and neointimal formation (b) whereas the intra-acinar arteries have typical occlusive lesions such as concentric intima fibrosis (c), and plexiform lesions (d).

Figure 4
Effects of increased pulmonary blood flow on the pulmonary vasculature in large animals; differences in type and duration of shunt.

a) A sheep model of increased pulmonary blood flow in the left upper lobe, induced intimal proliferation after 2 months, but developed into more advanced lesions after 1.5 yr. Adapted from Schnader et al(69).

b) A dog model of chronic increased pulmonary blood flow and increased pressure (aorto-pulmonary anastomosis). Chronic shunting (4 years) of one lung resulted in severe pulmonary vascular remodeling including intimal fibrosis and plexiform like lesions. Adapted from Heath et al.(33)

c) Extensive neointimal proliferation in the pulmonary vessels of rats subjected a pneumonectomy combined with monocrotaline. Adapted from Okada et al.(44)

d) Neointimal proliferation in intra-acinar vessels of rats with an aorto-caval shunt combined with monocrotaline (left). Immunofluorescence staining shows proliferation of endothelial cells (green; vWF) and smooth muscle cells (red; αSMA). Immuno-staining shows increased expression of Egr-1 a putative inductor of advanced lesions. Adapted from Dickinson et al(21).

e) Typical examples of plexiform-like lesions in young rats subjected to a pneumonectomy and monocrotaline. These lesions stain positive for vWF (top right) and VEGFR-2 (bottom right). Adapted from White et al(90).

Figure 5

Schematic representation of effects of increased flow on the development of pulmonary vascular lesions in PAH models.

Left side: Triggers such as increased flow, monocrotaline or hypoxia induce media hypertrophy and can induce endothelial cell changes. However, in most animal models, these hits alone do not trigger neointimal development within the time frame studied.
Right side: Double hit models progress from early endothelial cell activation via an initial hit, either increased pulmonary blood flow or monocrotaline, followed by a second hit, increased pressure, (prolonged) increased blood flow that combined triggers neointimal development in these animal models.

Figure 6

Consequences of changes in flow:

a) Schematic hypothesis how disturbed flow at branching points induces changes in shear stress downstream in PAH.

b) A histological example of a branching point lesion from a rat after MCT + increased pulmonary blood flow showing neointimal formation comprised of c) endothelial cells (positive for vWF), and d) in lesser form smooth muscle cells (aSMA).

Figure 7

Summary of the main histopathological changes found in several experimental models used to study pathophysiological mechanism of PAH

a) Models with only increased blood flow typically show only increased medial wall thickness in the time frame studied.

b) Double hit models with histology resembling human pathology (e.g. increased flow + additional hit) typically progress into advanced neointimal lesions with obliteration of the vascular lumen within the time frame studied.

c) Severe prolongation of increase pulmonary blood flow can induce plexiform lesions, as is also observed in other double hit models using experimental stimuli.
References:


resembling plexogenic arteriopathy and is increased in human plexogenic arteriopathy. 


81. van Albada ME and Berger RM. Pulmonary arterial hypertension in congenital cardiac disease - the need for refinement of the Evian-Venice classification. *Cardiol Young* 18: 1: 10-7, 2008.


<table>
<thead>
<tr>
<th>Clinical Classification Group</th>
<th>Characteristics of arteriopathy</th>
<th>Histological examples</th>
</tr>
</thead>
</table>
| 1. Pulmonary arterial hypertension  
  *Pulmonary veno-occlusive  
  disease or pulmonary capillary hemangiomatosis* | • Medial hypertrophy  
  • Muscularization of arterioles  
  • Cellular proliferation of intima layer  
  • Concentric laminar intimal fibrosis  
  • Plexiform lesions  
  • Fibrinoid necrosis | ![Histological example](image1.png) ![Histological example](image2.png) |
| 2. PH due to left heart disease | • Medial hypertrophy  
  • Muscularization of arterioles and veins  
  • Non-obstructive intimal fibrosis  
  • Moderate intima fibrosis veins | ![Histological example](image1.png) ![Histological example](image2.png) |
| 3. PH due to lung disease or hypoxia | • Large arteries mostly normal  
  • Medial hypertrophy  
  • Muscularization of arterioles  
  • Similar changes to lesser extent in small pulmonary veins | ![Histological example](image1.png) ![Histological example](image2.png) |
| 4. Chronic Thromboembolic PH (CTEPH) | • Mild medial hypertrophy  
  • Eccentric intimal fibrosis  
  • Recanalization of lumen  
  • Recent thrombi rare | ![Histological example](image1.png) ![Histological example](image2.png) |
| 5. PH with unclear multifactorial mechanisms | • Muscularization of arterioles and veins (fibrotic lung disease, tumors)  
  • Non-obstructive intimal fibrosis (fibrotic lung disease, tumors)  
  • Vascular granulomas (sarcoidosis, tuberculosis)  
  • Enlargement of bronchial arteries (bronchiectasis) | ![Histological example](image1.png) ![Histological example](image2.png) |

Adapted from Wagenvoort and Mooi
• Flow (disturbed shear stress)
• Hypoxia / MCT

• Flow + additional hit (pressure, MCT)

• Prolonged flow (disturbed shear stress)

• Media hypertrophy

• Media hypertrophy + early EC dysfunction

• Normal arteriole

• Media hypertrophy

• Neointimal lesion

• Media hypertrophy + EC dysfunction

• Concentric laminar intimal fibrosis

• Plexiform lesion
Disturbed blood flow—shear stress
<table>
<thead>
<tr>
<th>Experimental model</th>
<th>Characteristics of arteriopathy</th>
</tr>
</thead>
</table>

**Increased flow models:**
- short term or small systemic-to-pulmonary shunt in rats, pigs, sheep
- MCT + pneumonectomy
- Chronic systemic-to-pulmonary shunt in rats, pigs, sheep, dogs

**Other models:**
- hypoxic model rat /mouse
- MCT model in rats

**Increased flow models:**
- MCT + aortocaval shunt in rats
- MCT + pneumonectomy
- Chronic systemic-to-pulmonary shunt in rats, pigs, sheep, dogs

**Other models:**
- Sugen 5416 + chronic hypoxia
- S100A4/Mts1 overexpression in mice
- S100A4/Mts1 overexpression + HSV-68 in mice
- IL6 overexpression + with hypoxia in mice

**Increased flow models:**
- systemic-to-pulmonary shunt (prolonged increased flow + pressure) in dogs, sheep
- MCT + pneumonectomy in young rats

**Other models:**
- Sugen 5416 / hypoxia + prolonged hypoxia

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Image</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media hypertrophy and muscularization of arterioles</td>
<td>Media hypertrophy</td>
</tr>
<tr>
<td>Muscularization + obliterative lesions (SMCs/ECs), perivascular inflammation.</td>
<td>Obliterative lesion (neointima)</td>
</tr>
<tr>
<td>Muscularization + obliterative lesions (ECs)</td>
<td>Obliterative lesion (neointima)</td>
</tr>
<tr>
<td>Muscularization + obliterative lesions (SMCs) + perivascular inflammation</td>
<td>Obliterative lesion (neointima)</td>
</tr>
<tr>
<td>Plexiform lesions with cellular vessel obstruction, dilatation and recanalization</td>
<td>Plexiform lesion</td>
</tr>
<tr>
<td>Plexiform like lesions containing obliterative lesions (SMCs/ ECs and aneurysm like lesions</td>
<td>Plexiform lesion</td>
</tr>
</tbody>
</table>
Table 1  Summary of main findings of animal models mimicking PAH

<table>
<thead>
<tr>
<th>Species</th>
<th>Trigger</th>
<th>PAH lesions</th>
<th>Progressive Disease</th>
<th>Findings</th>
<th>ref</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td><em>Trigger flow only</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calf</td>
<td>BT shunt</td>
<td>±</td>
<td>-</td>
<td>Not studied</td>
<td>(24)</td>
</tr>
<tr>
<td>Lamb</td>
<td>mBT shunt</td>
<td>-</td>
<td>-</td>
<td>↑ eNOS, reduced NO availability</td>
<td>(2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>-</td>
<td>Not studied</td>
<td>(71)</td>
</tr>
<tr>
<td>Pig</td>
<td>BT shunt</td>
<td>-</td>
<td>-</td>
<td>↑ ET1, ETA, ETB</td>
<td>(61, 64)</td>
</tr>
<tr>
<td>Rat</td>
<td>AoC shunt</td>
<td>±</td>
<td>-</td>
<td>Not studied</td>
<td>(25, 33, 71)</td>
</tr>
<tr>
<td></td>
<td><em>Trigger Flow + second hit</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>MCT + BT shunt</td>
<td>+</td>
<td>+</td>
<td>TGF-β, ACE</td>
<td>(76)</td>
</tr>
<tr>
<td></td>
<td>MCT + AoC shunt</td>
<td>+</td>
<td>+</td>
<td>EGR1, mast cells</td>
<td>(20, 81, 84)</td>
</tr>
<tr>
<td></td>
<td>mBT shunt + MCT</td>
<td>-</td>
<td>-</td>
<td></td>
<td>(47)</td>
</tr>
<tr>
<td></td>
<td>MCT + pneumectomy</td>
<td>+</td>
<td>+</td>
<td>Tissue Factor</td>
<td>(92)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td>Oxidative stress</td>
<td>(21)</td>
</tr>
<tr>
<td></td>
<td><em>Trigger other</em></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Rat</td>
<td>SU-5416 + hypoxia</td>
<td>+</td>
<td>-</td>
<td>Apoptosis</td>
<td>(1, 77)</td>
</tr>
<tr>
<td></td>
<td>ETBr deficiency</td>
<td>-</td>
<td>-</td>
<td>none</td>
<td>(41)</td>
</tr>
<tr>
<td></td>
<td>ETBr deficiency + MCT</td>
<td>+</td>
<td>-</td>
<td>ETB</td>
<td>(40)</td>
</tr>
</tbody>
</table>

BT shunt = classical Blalock-Taussig shunt (subclavian artery-pulmonary artery anastomosis), mBT shunt = modified Blalock-Taussig shunt = goretex conduit for
subclavian artery to pulmonary artery, AoC shunt = Aortocaval shunt, MCT = monocrotaline, ET = endotheli, TGF = transforming growth factor, ACE = angiotensin converting enzyme, EGR = early growth response protein
<table>
<thead>
<tr>
<th>Species</th>
<th>Trigger</th>
<th>PAH Lesions</th>
<th>Therapy</th>
<th>Strategy</th>
<th>Findings</th>
<th>ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Trigger flow only</strong></td>
<td></td>
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</tr>
<tr>
<td><strong>Pig</strong></td>
<td>mBT shunt -</td>
<td>Bosentan prevent</td>
<td>↓ media</td>
<td>hypertrophy</td>
<td>(62)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Sitaxsentan prevent</td>
<td>↓ media</td>
<td>hypertrophy ↓</td>
<td>PVR</td>
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<tr>
<td></td>
<td>- Sildenafil prevent</td>
<td>↓ pPA</td>
<td></td>
<td>(64)</td>
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<tr>
<td></td>
<td>- Losartan prevent</td>
<td>↓ PVR</td>
<td></td>
<td>(63)</td>
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<tr>
<td><strong>Trigger Flow + second hit</strong></td>
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<tr>
<td><strong>Rat</strong></td>
<td>Mct+AoC shunt +</td>
<td>Cromolyn prevent</td>
<td>↓ occlusion</td>
<td></td>
<td>(10)</td>
<td></td>
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<tr>
<td></td>
<td>+ EPO prevent</td>
<td>↓ mPA</td>
<td></td>
<td>(83)</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>+ Trepostinil prevent</td>
<td>↓ mPA</td>
<td></td>
<td>(85)</td>
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<tr>
<td></td>
<td>+ Prostacyclin, aspirin prevent</td>
<td>↑ RV capillary myocyt ratio</td>
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<td>(82)</td>
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<tr>
<td></td>
<td>MCT+ pneumectomy +</td>
<td>Quinapril prevent</td>
<td>↓ occlusion score, pPA</td>
<td></td>
<td>(51)</td>
<td></td>
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<tr>
<td></td>
<td>+ DHEA prevent</td>
<td>↓ occlusion score, pPA</td>
<td></td>
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<td>(34)</td>
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<td></td>
<td>idem reverse</td>
<td>No effect</td>
<td></td>
<td></td>
<td>(34)</td>
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<tr>
<td></td>
<td>+ Rapamycin prevent</td>
<td>↓ occlusion score, pPA</td>
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<td></td>
<td>+ idem reverse</td>
<td>No effect</td>
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<td>(49)</td>
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<tr>
<td>Trigger Other</td>
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<tr>
<td>Rat</td>
<td>SU-5416 + Simvastatine prevent ↓ pPA (78)</td>
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<tr>
<td></td>
<td>+ hypoxia</td>
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</tr>
<tr>
<td></td>
<td>+ Nifedipine, cyclofosfamide, taxol, thalidomide, VEGF, Ibesartan, PPAR-g agonist, lisinopril No effect (78)</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

mBT shunt = modified Blalock-Taussig shunt = goretex conduit for subclavian artery to pulmonary artery, AoC shunt = Aortocaval shunt, MCT = monocrotaline, PA = pulmonary artery pressure, PVR = pulmonary vascular resistance, EPO = erythropoietin, DHEA = dehydroepiandosterone.