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A window for reversibility in pulmonary arterial hypertension

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A window for reversibility
in pulmonary arterial hypertension.

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Diederik. E. van der Feen

A window for reversibility in pulmonary arterial hypertension

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1

General Introduction

Clinical challenges and scientific opportunities of pulmonary arterial hypertension in congenital heart disease: *a window for reversibility*.

Pulmonary arterial hypertension (PAH) is a severe pulmonary arteriopathy. This arteriopathy comprises a combination of occlusive vascular remodelling and sclerosis and progressive vascular rarefaction, which may remain clinically silent until pulmonary vascular resistance (PVR) rises above a critical level and symptoms of right ventricular (RV) failure develop(1). Most of the aetiologies of PAH cannot be cured when the disease is diagnosed and have a detrimental prognosis. Contemporary treatment slows down the progression of vascular remodelling at best(2). There are however, a few forms of PAH that know a certain reversible stage, of which PAH in congenital heart disease (CHD) is far and wide the best studied(3). In PAH-CHD, the arteriopathy is triggered by increased haemodynamic load on the pulmonary arterial bed due to a cardiac systemic-to-pulmonary shunt. This haemodynamic load consists of two main components: increased pulmonary blood flow (PBF) and increased pulmonary arterial pressure (PAP). Both of these are a direct consequence of a cardiac systemic-to-pulmonary shunt, where PBF is determined primarily by the size, and PAP by the level of the shunt (atrial: normal PAP; ventricular and arterial: increased PAP) (4). Timely surgical correction of this shunt normalizes the haemodynamic load on the pulmonary arterial bed and can completely and permanently reverse the arteriopathy, thus cure PAH(5). Unfortunately, there is a point of no return after which this curative potential is lost. Beyond this point, when PAH is estimated to be irreversible, complete shunt correction has shown to worsen the prognosis of patients with PAH-CHD, plausibly because this intervention also impairs the right heart to offload suprasystemic pressure through the pre-existent shunt(5). These data imply that early and accurate assessment of the reversibility of PAH-CHD is pivotal in the management of these patients, as it may decide between a complete cure or an accelerated progression towards RV failure. In other words: PAH-CHD knows a unique *window for reversibility* that should not be missed. Two major clinical challenges faced today in the field of PAH-CHD are 1) that no reliable method or biomarker exists to determine the reversibility of the disease, and 2) that, similar to all forms of PAH, contemporary ‘targeted therapy’ can decelerate but not halt or reverse disease progression once the arteriopathy has progressed to its irreversible end-stage.

These clinical challenges however, also bring scientific opportunities. In this thesis, we explored the clinical entity of the reversible and irreversible PAH-CHD phenotype, and used it as a model to unravel the biological mechanisms specifically associated with the transition towards irreversible disease. These mechanisms could also explain the irreversible nature of other forms of PAH. Comprehension of the factors that drive irreversibility, could also provide targets for new pharmacological interventions that may reverse ‘irreversible’ vascular remodelling and treat end-stage PAH. Our aims were:

- 1) to develop a rat model for haemodynamic unloading in shunt-induced PAH to simulate the effects of shunt closure in PAH-CHD.
- 2) to characterize the timing and biological mechanisms associated to the possible reversibility of the pulmonary arteriopathy using that rat model.
- 3) to evaluate new pharmacological treatment strategies aimed at the reversal of advanced pulmonary vascular remodelling in the preclinical setting.

The studies in this thesis were conducted as a part of the PHAEDRA consortium, which aimed to identify new biomarkers and find new treatments for the various forms of PAH. Besides investigating the conundrum of reversibility in PAH-CHD, this research also aimed to translate results from cell biology studies conducted in other PHAEDRA projects, through preclinical validation, into the clinic.

Introduction and thesis outline.

In [Chapter 2](#), we reviewed the current knowledge on the reversibility of PAH-CHD as a clinical basis to design and direct the experimental work in this thesis. We describe the epidemiology concerning reversibility of PAH in paediatric and adult CHD, and other forms of PAH that may also harbour a reversible phase. We reviewed the historic observations of Heath and Edwards, Wagenvoort, Hayworth and Rabinovitch, who provided the first insights in the phenotypical change of the pulmonary vasculature during development of PAH-CHD. Because PAH-CHD is induced by an identifiable and quantifiable trigger (as opposed to other forms), the disease can be followed during all different stages of disease progression using lung biopsies: from disease initiation and the reversible phase in infancy and early childhood, throughout the progression towards advanced, irreversible disease. The earliest changes observed were muscularization of normally non-muscularized arterioles and medial hypertrophy, sometimes accompanied by mild hyperplasia of the intima(6). Haemodynamic unloading by pulmonary arterial banding at this stage was associated with normalization of the vascular morphology and regression of PAH(7). Irreversible PAH on the other hand was characterized by concentric ‘neointimal’ fibrosis, disintegration of the elastic laminae, and the occurrence of plexiform lesions(7, 8). These plexiform lesions, considered to be the hallmark of irreversible PAH, are complex vascular formations that consist of disorganized capillary channels within a fibrotic lesion, typically accompanied by an inflammatory infiltrate(9). The occurrence of neointimal and plexiform lesions on a lung biopsy can thus be used to differentiate reversible from irreversible PAH, as well as PAH from other forms of pulmonary hypertension (PH), such as occurs in chronic lung disease or left-sided heart failure(10). In contrast to advanced PAH, PH, is characterized predominantly by medial hypertrophy, similar to early-stage PAH-CHD, without neointimal lesions, and can usually be managed well with treatment of the underlying disease(11). In [Chapter 3](#), we investigated whether we could differentiate these different types of vascular remodelling

in human PH and PAH(-CHD) using a tissue biomarker: Early growth response-1 (Egr1) (12). Our group has previously shown that Egr1 expression is specifically associated with neointimal remodeling and increases only after the induction of increased PBF in rats with PAH induced by monocrotaline and a shunt(13, 14). Moreover, inhibition of Egr1 was shown to prevent the development of neointimal lesions in that same model(15). In human disease, we show in this thesis that Egr1 expression is increased in both early and advanced PAH-CHD and in advanced idiopathic PAH, while Egr1 expression was absent in remodelled vessels of patients with PH in chronic obstructive lung disease (COPD)(12). These data indicated that Egr1 expression could be used to identify neointimal pulmonary vascular remodelling, and translated the role of Egr1 as a flow-dependent driver of neointimal remodelling, as shown in rats, to the clinic. Additional data on flow-dependent progression of vascular lesions in PAH-CHD are reviewed in [Chapter 4](#). This chapter also discusses alternative methods and biomarkers that may be used to assess the stage and reversibility of pulmonary vascular disease in PAH-CHD(10).

In [Chapter 2](#), we further describe possible pathological mechanisms that could account for the transition from a reversible to an irreversible vascular phenotype and the development of neointimal and plexiform lesions. Different clinical and preclinical evidence suggests that the balance between endothelial cell (EC) apoptosis and apoptosis-resistance is shifting during disease progression. In paediatric PAH-CHD that reversed after haemodynamic unloading, vascular expression of the pro-apoptotic marker p53 was increased, whereas irreversible PAH-CHD was characterized by increased expression of Bcl2 and survivin, both related to apoptosis-resistance(16). Animal models for neointimal PAH show a similar pattern: apoptosis is associated with early development of PAH(15, 17, 18), apoptosis-resistance to end-stage disease(19). Moreover, inhibition of BRD4 (bromodomain-containing protein 4), which results in reduced apoptosis-resistance by reducing expression of survivin, was one of the first drugs shown to reverse advanced neointimal PAH in rats(20). More on the role of BRD4 in PAH is reported in [Chapter 7](#). Proliferation of ECs and smooth muscle cells (SMCs) is a second pathogenic mechanism recognized to contribute to vascular remodelling(21), and both are generally considered to be early disease phenomena. The role of proliferation in the transition from reversible to irreversible PAH however, is unclear. Pro-inflammatory signalling is the third major component involved in vascular remodelling that seems to increase during disease progression(22). Although inflammation is generally considered to peak in advanced disease, it is still unclear if or how it contributes to the irreversible nature of vascular lesions in PAH. [Chapter 2](#) finally describes the role of an imbalance in transforming growth factor- β (TGF β) and Bone morphogenic protein receptor-2 (BMPR2) signalling as a putative pathological driver of vascular remodelling in each aetiology of PAH. Mutations in BMPR2 are the most common genetic cause of hereditary PAH, and TGF β /BMPR2 imbalance has been identified in most other aetiologies of PAH as well(23).

Pharmacological interventions aimed at the restoration of the TGF β /BMPR2 balance have been successful in reversing vascular remodeling and improving pulmonary haemodynamics in several preclinical trials(24–26), underlining the significance of this pathway in the pathobiology of PAH. These drugs however, have not yet reached successful translation into the clinic, an issue that unfortunately has applied to most “successful” preclinical drug trials in PAH.

An important step to enhance the translatability of preclinical research into the clinic is by improving the rigor and reproducibility of animal studies(27, 28). In [Chapter 5](#), we have provided a step-by-step protocol, including an online video, of the rat model for progressive shunt-induced PAH used for over 10 years in our lab and in this thesis(29). This model combines a 60mg/kg injection of the alkaloid monocrotaline (MCT) at day 0 with the surgical construction of an aorto-caval shunt at day 7. Like in human PAH-CHD, MCT+Shunt first leads to muscularization of normally non-muscularized arterioles, medial hypertrophy and mild intimal proliferation (from day 14), followed by the occurrence of neointimal lesions (from day 21) and RV dysfunction and failure (from day 28). In rats treated with 60mg/kg MCT only, neointimal lesions are generally not observed at any stage, and spontaneous reversal has been reported(30). A widely used alternative double hit model for PAH, also used in this thesis, is the Sugen5416-Hypoxia (SuHx) rat. These rats are treated with the vascular endothelial growth factor (VEGF) receptor antagonist Sugen5416, and are then housed in a hypoxic chamber for 3 weeks, followed by 3 weeks of normoxia. In this model, neointimal lesions occur from around the 8-week time point(31, 32). To improve the translatability of trials that investigate new pharmacological strategies, the use of multiple animal models is highly recommended. Additionally, the end-points should be pre-defined, and measured in a blinded and standardised way that is as similar as possible to the human situation(27, 28). The standardised protocols for haemodynamic assessment using closed-chest right heart catheterization and for the qualitative and quantitative assessment of pulmonary vascular morphology, are also reported in [Chapter 5](#).

In [Chapter 6](#), we investigated the effect of haemodynamic unloading (HU) on the reversibility of vascular remodelling in the MCT+Shunt rat model. HU was performed by unilateral transplantation of a left lung with various stages of PAH into a healthy recipient rat. Using this model, we identified a dichotomous reversibility response to HU, similar as in patients with PAH-CHD, with both a reversible and irreversible PAH phenotype, including a zone of no return. We then characterized and compared both disease stages using the traditional morphological approach, and by RNA-sequencing and vascular specific immunohistochemistry. From a clinical-diagnostic point of view, these data could provide new insights and possible biomarkers to identify patients with PAH-CHD who are amenable for shunt closure. From a mechanistic point of view, we found that loss of reversibility coincides with

a loss of a proliferative and gain of a senescent vascular phenotype. Senescence describes the transition of a cell, induced by various forms of cellular stress, from a proliferative state into permanent, irreversible growth arrest(33). We further highlighted the possible role for cellular senescence in the pathobiology of PAH in [Chapter 8](#). To support the concept that cellular senescence could be causal to the irreversible nature of end-stage PAH, we targeted senescence using the clinically available senolytic agent ABT263, as further described in [Chapter 6](#). We demonstrated that ABT263 induced reversal of the hemodynamic and structural changes associated with severe PAH refractory to hemodynamic unloading. Not only did ‘irreversible’ vascular lesions and markers for senescence reverse by targeted senolysis, treated animals also showed evidence for vascular regeneration.

In [Chapter 7](#), we aimed to validate new drugs for the treatment of advanced PAH in multiple animal models for PAH and RV dysfunction. We explored the therapeutic potential of RVX208, a clinically available inhibitor of the Bromodomain and Extra-Terminal motif (BET) family. Bromodomain-containing protein 4 (BRD4), a member of the BET family has been identified as a critical epigenetic driver for cardiovascular disease, including PAH(20). In a collaborative project of the PHAEDRA consortium, we showed that RVX208 normalized the hyper-proliferative, apoptosis-resistant and inflammatory phenotype of ECs isolated from PAH patients, and restored the TGF β /BMP balance. Oral treatment with RVX208 reversed advanced vascular remodelling in both the MCT+Shunt and the SuHx model. To ensure safety of the intervention in the context of RV compromise, we also tested RVX208 in rats with pressure load induced by pulmonary artery banding. We found that RVX208 did not limit the physiological ventricular response to increased afterload, and even enhanced its adaptation. While performing these experiments, we learned that the group of prof. Bonnet in Québec was also evaluating RVX208 for the treatment of PAH in a comparable experimental design and obtained highly similar results. We decided to cooperate rather than compete and unified both independently conducted preclinical trials in a final stage. These data support the establishment of a clinical trial with RVX208 in PAH patients. In fact, a clinical trial is currently taking place: APPRoAcH-p, NCT03655704.

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2

Pulmonary arterial hypertension in congenital heart disease.

*Translational opportunities to
study the reversibility of pulmonary
vascular disease.*

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Abstract.

Pulmonary arterial hypertension (PAH) is a progressive and lethal pulmonary vascular disease (PVD). Although in recent years outcome has improved by new treatments that delay disease progression, a cure has not yet been achieved.

In PAH associated with congenital heart disease (CHD), remodeling of the pulmonary vasculature reaches an irreversible phenotype similar to all forms of end-stage PAH. In PAH-CHD, however, also an early stage is recognised, which can be completely reversible. This reversible phase has never been recognised in other forms of PAH, most likely because these patients are only diagnosed once advanced disease has developed.

We propose that the clinical model of PAH-CHD, with an early reversible and advanced irreversible stage, offers unique opportunities to study pathophysiological and molecular mechanisms that orchestrate the transition from reversible medial hypertrophy into irreversible plexiform lesions. Comprehension of these mechanisms is not only pivotal in clinical assessment of disease progression and operability of patients with PAH-CHD; specific targeting of these mechanisms may also lead to pharmacological interventions that transform ‘irreversible’ plexiform lesions into a reversible PVD: one that is amenable for a cure. In recent years, significant steps have been made in the strive to “reverse the irreversible”.

This review provides an overview of current clinical and experimental knowledge on the reversibility of PAH, focussing on flow-associated mechanisms, and the near-future potential to advance this field.

Introduction.

Pulmonary arterial hypertension (PAH) is an obstructive arterial pulmonary vascular disease (PVD) that is progressive, irreversible and usually fatal, despite current treatment options(1-3). PAH in congenital heart disease (PAH-CHD) is unique in this respect, as it also knows an early phase in which the arteriopathy can generally be reversed(4). The potential for reversibility in PAH-CHD was first identified in the 1950's(5), but mechanisms, timing and identification of such reversibility are still obscure today.

We know that in PAH-CHD, systemic-to-pulmonary shunting can ultimately remodel the pulmonary vasculature to a characteristic irreversible phenotype similar to other forms of PAH(6-8). We also know that *timely* closure of the shunt will generally result in normalisation of pulmonary hemodynamics and vascular morphology, i.a. cure PAH(9). Yet, it appears that closure beyond the reversible phase relates to accelerated disease progression, as these patients have a prognosis substantially worse than those with uncorrected PAH-CHD(10).

Our knowledge is still limited regarding the factors that distinguish reversible from irreversible PVD(11). Identification of these factors would not only be pivotal to improve assessment of operability in PAH-CHD, it could also give clues to comprehend the mechanisms that orchestrate the development of irreversible PVD. Specific targeting of the mechanisms involved could help to *reverse the irreversible*, also in other forms of PAH. This review will discuss features and putative mechanisms associated with reversibility of PAH-CHD.

Reversibility in PAH-CHD: Concepts and observations.

2.1. To normalise versus to stabilise.

The current literature generally refers to *survival* in PAH as disease stability rather than disease reversal. Targeted pharmacotherapy may stabilise or decelerate disease progression, and improve clinical condition, but still does not cure the disease. Here, disease reversibility is defined as complete and permanent normalisation of both pulmonary hemodynamics and vascular morphology. Reversible PAH was first described in children with PAH associated with congenital cardiac shunts(5). Timely correction of the shunt, removing the disease trigger, was found to result in full disease regression(12,13). This unique feature distinguishes PAH-CHD from other forms of PAH. In idiopathic, heritable or PAH associated with connective tissue disease, the trigger cannot be removed, and a normalisation of vascular morphology has never been described. In PAH induced by suppressible triggers, like HIV- or schistosomiasis-associated PAH, treatment may stabilise progression, but complete normalisation remains debated(14). Still, recent cases suggest that reversibility may

not be exclusive to PAH-CHD. For instance, dasatinib, used in the treatment of leukaemia, can induce near systemic rises in pulmonary arterial pressure that may fully reverse after discontinuation of the drug(15). PAH complicating bone marrow transplantation has also shown potential to completely normalise over time(16). These cases support the clinical and histological concepts of reversibility that have been reported extensively in PAH-CHD.

2.2 Epidemiology of regressive and progressive PAH-CHD.

All children born with a systemic-to-pulmonary shunt are at risk for PAH (*figure 2.1*). CHD-type and shunt size are considered principal determinants for the progression of PAH-CHD and the development of irreversible disease(17,18). Increased pulmonary blood flow is considered a prerequisite, combination with high pressure an accelerator(19). Large post-tricuspid shunts (high flow, high pressure) more frequently and quickly induce irreversible PAH than restrictive or pre-tricuspid shunts (high flow, normal pressure) (*figure 2.1*). In contrast, CHD-types associated with normal flow and high pressure, like congenital mitral stenosis, generally do not induce PAH(19).

Figure 2.1. CHD-type and shunt determine the progression of PAH. TA: Truncus Arteriosus, AVSD: Atrio-ventricular septal defect; TGA: Transposition of the great arteries(18,76).

Improved monitoring of CHD has enabled paediatric cardiologists to identify and treat patients at risk for PAH early in life. Currently, around 90% of all children with PAH-CHD are considered surgically correctable(20). In the majority of these children, shunt closure completely normalises the disease: reversible PAH. Unfortunately, the portion of children with disease progression despite early surgery is still significant (2-6%)(20)(*figure 2.2*). This is 5-13% for adults(21,22). While in PAH-CHD, older age at correction is a well-recognised risk factor for irreversible disease, the occurrence of post-operative PAH in infants suggests that also other factors than timing of correction are involved(21). Conditions such as

disturbed perinatal transition or co-morbidities like Down syndrome are often associated with and may contribute to progression of PVD after correction of the cardiac defect in these children(23). Genetic susceptibility could also modify progression of PVD, albeit not through the gene-mutations identified in 'idiopathic' or heritable PAH since these are rarely found in paediatric PAH-CHD(24).

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Figure 2.2 When diagnostic criteria for PAH are met in paediatric left-to-right shunt, 90% evaluated eligible for shunt correction. In a portion of corrected CHD, PAH will progress due to misjudged irreversible disease. When shunt correction is contra-indicated (PAH is evaluated as irreversible or other co-morbidities do not permit surgery), PAH will progress.

Whatever the reason for disease progression after correction, its prognosis is detrimental. Post-operative PAH is associated with lower survival rates both at short- (25) and long-term follow up: 36%, versus 87% for non-operated PAH-CHD(10,20,26). This difference might be explained by the loss of potential for unloading of the right ventricle (RV) after correction of the defect. Whereas RV failure is recognised a major risk factor for mortality in adults(27), RV function in children is generally better preserved at diagnosis(28). Interestingly, pressure unloading of the RV in PAH (e.g. by lung transplantation) is generally associated with a marked reversal of RV function and remodeling, even when severe dysfunction and hypertrophy was present(29).

Of all children with CHD who present with PAH, $\pm 10\%$ is regarded not eligible for shunt closure(20), mostly due to concerns of irreversible PVD in the context of complex CHD and/or co-morbidities that prohibit invasive surgery. The most ominous clinical sign of irreversible PAH is the Eisenmenger syndrome (ES). In ES, pulmonary vascular resistance (PVR) has exceeded systemic values, which reverses the direction of the shunt, causing cyanosis. The mean survival of ES when developed during childhood is 11.4 years(30), with a 4-year survival rate of 77%(31). Currently, indexed PVR >8 Woods Units. m^2 is regarded a

contra-indication for shunt closure, and 6 to 8 WU.m² a 'grey zone'(32). Unfortunately, these cut-off values are mainly based on expert opinions and are not supported by prospective data.

2.3 The vascular morphology in PAH-CHD: *which lesions reverse?*

In end-stage disease, all forms of PAH share a common histology, typically hallmarked by the occurrence of neointimal lesions such as concentric laminar intimal fibrosis and plexiform lesions(1). Plexiform lesions are present in 90% of biopsies in various forms of advanced PAH(1). In early-stage PAH-CHD, these neointimal lesions are generally still absent(9). Early-stage PAH is predominantly characterised by medial hypertrophy of pre-acinar vessels and muscularisation of normally non-muscularised arterioles: the first visible structural change in the vascular remodeling process. This may be accompanied by mild proliferation of intimal cells(*figure 2.3*)(1,7).

Figure 2.3. Schematic representation of the progression of vascular remodeling in PAH-CHD. As the disease advances, the morphological spectrum of vascular lesions also becomes more heterogeneous.

The link of vascular histopathology to disease reversibility dates back to the studies of Wagenvoort, and Heath & Edwards published in the 1950's. They postulated that PAH-CHD might be reversible when histology shows medial hypertrophy only(33,34). In fact, all healthy new-borns display medial hypertrophy directly after birth, which reverses in the first 8 weeks of life. Children with a ventricular septal defect (VSD) however, maintain this medial hypertrophy postnatally and often display proliferative intimal changes within 1 year, and plexiform lesions within 2,5 years after birth(34). The number and size of acinar arterioles in these children were also reported to be reduced at this age(35), suggesting developmental rarefaction that may contribute to disease progression.

Heath & Edwards classified concentric laminar intimal fibrosis, plexiform lesions, dilatation lesions and necrotizing arteriitis as severe lesions, conjoined to irreversible, progressive disease(33,34). Wagenvoort used a descriptive analysis of these vascular lesions to predict outcome after cardiac surgery in 137 PAH-CHD cases. Patients with medial hypertrophy without severe intimal fibrosis were likely to improve after surgery, whereas those with more severe intimal fibrosis or plexiform lesions were likely to deteriorate(12). The concept of vascular reversibility was confirmed in study that assessed morphological change of various lesions in consecutive lung biopsies prior to and several years after pulmonary artery banding: medial hypertrophy reversed, whereas neo-intimal lesions and plexiform lesions did not(4).

Despite these opportunities, pre-operative morphologic evaluation has largely disappeared from clinical practice since the eighties, due to the procedural risks of open lung biopsy (13% morbidity and 20% mortality in children with PAH-CHD)(36). Furthermore, it should be noted that absence of neointimal lesions in a lung biopsy does not ensure post-operative regression of PAH, since the advanced lesions may not be distributed equally through the lungs (13,37). Still, vascular morphology has been confirmed as a useful tool to understand reversibility and should be considered the gold standard when phenotyping PAH in experimental models.

2.4 Reversibility in animal models.

The first animal study that investigated the reversibility of PAH by manipulating pulmonary blood flow, used a rat model that induced over circulation in the left lung by right lung excision. Isolated medial hypertrophy developed in the remaining left lung, which reversed after unloading by lung transplantation into a healthy recipient rat(38). In piglets with shunt-induced medial hypertrophy, shunt closure also decreases medial thickness and PVR(39). These studies confirm the clinical observation that isolated medial hypertrophy has the biological potential to reverse. This also warrants a critical appraisal towards studies that claim reversibility by pharmacological compounds in P(A)H-models characterised by muscularisation only. These animal models have led to a great number of potential therapies that failed to translate to clinical application and should therefore be considered inadequate to investigate the PVD in PAH(42). To more closely resemble human disease, animal models have been developed that lead to rapid progression of the characteristic neointimal lesions associated with human disease: the monocrotalin + increased-pulmonary-flow (MCT+Flow) and Sugen5146 + Hypoxia (SuHx) model, both in rats(6,40,41). At present, the effects of hemodynamic unloading in neointimal flow-induced PAH models are unknown. Investigating these effects could help to answer why and how increased pulmonary flow leads to progression in PAH, and why medial hypertrophy may reverse and why neointimal lesions may not. Approaches to mimic hemodynamic unloading in (neointimal)

PAH models are by pulmonary artery banding, shunt closure or transplantation of a lung with flow-induced PAH into a healthy recipient.

Mechanisms of disease progression in PAH-CHD.

3.1 Triggers for plexogenic remodelling and the unique opportunities of PAH-CHD.

Plexiform lesions have a variety of etiological backgrounds, including genetic mutations, infections, connective tissue diseases or certain drugs(42). In PAH-CHD, increased pulmonary blood flow is regarded the essential trigger for disease development(6). Several factors make PAH-CHD an ideal ‘human model’ to study the mechanisms involved in *early* disease progression, disease reversal and transition to irreversibility: 1) the trigger is known, 2) the onset and magnitude of the trigger can be estimated, 3) the trigger can be removed, 4) *timely* removal of the trigger potentiates disease reversal, and 5) persistence of the trigger leads to progressive PVD that shares many characteristics with other forms of PAH. Moreover, 6) the subgroup of patients that do not reverse despite trigger removal allows to identify conditions and mechanisms specifically associated with (the transition towards) irreversible disease.

Comparison of human reversible to irreversible PAH-CHD could help to identify pathways related to (ir)reversibility(9,43)(figure 2.4a). The ‘liquid biopsy’ could be an innovative, minimally-invasive alternative to ‘solid’ lung biopsy to investigate human PAH. The concept of liquid biopsy originated from oncology and utilises the fact that tumours shed cells and DNA directly into the bloodstream, and tumour-RNA into platelets, which can be analysed to determine stage and treatment response(44). Liquid biopsy may also be applicable in PAH, for instance by performing high-throughput screening on platelets and circulating endothelial cells, which have already been shown detectable in blood samples of PAH(-CHD) patients(45)(figure 2.4b). Specifically, new markers such as Micro-RNAs may be identified in these samples. This knowledge can be confirmed (bedside-to-bench) in animal models that show normalisation of *established* neointimal lesions, either by hemodynamic unloading or pharmacotherapy. We advise the use of multiple, complementary animal models to increase robustness of the data.

The next section describes potential pathways that may be involved in the transition from reversible to irreversible PAH. A proposed mechanistic overview of the relationship of increased flow to neointimal remodeling is presented in figure 2.5.

Figure 2.4. A) The human model of PAH in CHD. A shunt initiates disease progression up to stage A, in which shunt correction either leads to regression of the disease (stage C) or to progression (stage D). Without shunt correction, stage A will progress to stage B: the Eisenmenger Syndrome. B) $A^{R/IR}$: PAH-CHD that becomes reversible or irreversible. Using liquid biopsy combined with high throughput screening, multiple profiles can be compared to investigate mechanisms associated with 1) flow-dependent progression (A^{IR} to B) 2) flow-independent progression (A^{IR} to D) 3) Reversible or Irreversible phenotype before correction, e.g. to judge operability (A^{IR} to A^R), or disease course (C to D) and 4) disease reversal (A^R to C).

3.2 Pathobiology: what could make PAH irreversible?

A) Apoptosis and apoptosis-resistance. The balance between endothelial cell (EC) apoptosis and apoptosis-resistance appears to shift during disease progression. In lung biopsies obtained during shunt correction in paediatric PAH-CHD, the pro-apoptotic marker p53 dominated the tissue of patients that had reversed one year after closure, whereas Bcl-2 (associated to apoptosis-resistance) was expressed only in patients with progressive disease(9). Survivin, another marker for apoptosis-resistance, is expressed abundantly in patients with end-stage PAH and nearly absent in CHD without PAH(46). These observations were

Figure 2.5. Flow in the normal circulation is laminar. In the situation of a large left-to-right shunt, flow and pressure increase. This causes disturbances in local flow, especially at the branching points. Disturbed flow causes endothelial dysfunction, which, together with increased pressure, causes medial hypertrophy. Disturbed flow also induces a disturbance in TGF- β -BMP balance, which up regulates apoptosis, proliferation and inflammation. Misbalance in these cascades cause the endothelial cells to proliferate, which eventually become resistant to apoptosis and form a neointimal layer. This irregular neointimal layer further disrupts flow patterns, which further disturbs signalling balances, causing an irreversible vicious cycle.

confirmed both *in vitro* and *in vivo*. Experiments on human pulmonary ECs confirm that apoptosis-induction combined with high shear stress, ultimately results in hyper proliferation of ECs that are apoptosis-resistant and express survivin(47). In MCT+Flow rats, pro-apoptotic factors peak in early disease, directly after the induction of flow(48). In SuHx rats, anti-apoptotic factors appear late in the disease, specifically in neointimal lesions(49). Recently, in the SuHx model, inhibition of Bromodomain-containing protein 4, an indirect stimulator of survivin and bcl-2, has shown to reverse established neointimal lesions(49). These data underline the role of disturbed apoptosis in the formation of neointimal lesions and suggest potential for drugs that target apoptosis-resistance in the reversal of *irreversible* PAH.

B) Inflammation. Inflammatory cytokines (IL-6, IL-1 β , MCP-1 and TNF- α most importantly) and macrophages have been associated with disease progression not only in inflammation-associated PAH, but also in PAH-CHD(50) and IPAH(51,52). Plexiform lesions are found both in HIV and schistosomiasis-associated PAH (sch-PAH) and in

auto-inflammatory conditions like scleroderma and systemic lupus erythematosus(53). The inflammation hypothesis (reviewed in(53,54)) therefore proposes that inflammation can initiate and sustain vascular remodeling up to the formation of neointimal lesions. Indeed, transgenic mice that over-express IL-6 spontaneously develop neointimal lesions(55), which is particularly remarkable since pulmonary neointimal lesions rarely occur in mice(41). In severe murine sch-PAH, associated with overwhelming vascular inflammation, established neointimal lesions were reversed by anti-parasitic treatment that inhibited the cytokine response(56). In MCT+Flow rats, inhibition of mast cells or chymase, both attenuate vascular remodeling, but do not lead to reversal(57). Considering the emerging evidence for an inflammatory component in PAH and neointimal formation, anti-inflammatory drugs may have potential as (adjuvant) therapy to reverse irreversible PAH.

C) *Tgf- β /BMP-signalling: a common denominator?* It is intriguing that a disease with such diverse triggers, or associated conditions, leads to a rather uniform end-stage vascular phenotype, regardless of aetiology. So could there be a common denominator? Increasing evidence points towards Tgf- β and BMP signalling as respective orchestrators of plexogenic remodeling, or protection and repair of the pulmonary vasculature (reviewed in(58)).

In 2000, mutations in BMP-receptor 2 (BMPR2) were first identified as a cause for hPAH(59). BMPR2-mutations impair vaso-protective BMP signalling and account for 80% of hPAH cases(60). Disturbed Tgf- β /BMP signalling was further identified in IPAH(61), sch-PAH(62), CTD-PAH(63), PAH-CHD(64), drug (cocaine) and HIV-induced PAH(65) and PAH associated with hereditary haemorrhagic telangiectasia(66). Vice versa though, rats with deficient BMPR2-function(67) or mice with a BMPR2-knock-out(68,69) only develop an inconsistent pattern of mild pulmonary hypertension and medial hypertrophy without neointimal lesions. These observations signify that these models may still lack a second hit, or display an early (reversible) phase of BMPR2-deficiency related PAH.

Mechanistically, BMPR2-deficiency can elicit pro-proliferative and anti-apoptotic responses in murine pulmonary smooth muscle cells(70); factors downstream of Tgf- β also induce these responses in human PAECs(71). Also, both BMPR2-deficiency and increased Tgf- β are able to promote the progression of PAH by a hyper inflammatory response through cytokines such as IL-6 and -8(72,73).

Therapeutically, BMP-9, an endogenous stimulator of BMP-signalling, has shown to reverse medial hypertrophy in BMPR2-deficient mice and MCT rats, but also neointimal lesions in SuHx rats(69). Similarly, FK506 (Tacrolimus) showed to 1) restore disturbed BMPR2-signalling and endothelial function in PAECs from IPAH patients, 2) prevent PAH progression in BMPR2-deficient mice and 3) reverse established neointimal lesions in SuHx rats(68).

As a first clinical observation, low-dose Tacrolimus (blood level 1.5-2.5ng/ml) was associated with relieved symptoms of right heart failure and improved WHO functional class in 3 end-stage IPAH patients (74). Finally, Elafin, an endogenous serine protease inhibitor that enhances BMPR2 signalling, has shown to reverse neointimal lesions in SuHx rats and reduce neointimal thickness of pulmonary arteries in cultured sections from lung explants of patients with PAH(75). These recent experimental studies serve proof of the concept that reversibility of advanced PAH, considered irreversible, is feasible in animals and cultured human pulmonary arteries. This concept holds promise for future therapeutic potential in patients.

Conclusion.

PAH is considered a progressive and irreversible arteriopathy and is still lethal. Although advances in the last decade have led to an improvement in outcome, mainly by delaying disease progression, reversal or cure has not yet been achieved.

PAH in congenital heart disease has a vascular end-stage that is universal to other forms of PAH, but also knows a reversible phase. This unique clinical model of PAH-CHD, with an early reversible and advanced irreversible stage, offers opportunities to study pathophysiological and molecular mechanisms that orchestrate the transition from reversible medial hypertrophy into irreversible neointimal and plexiform lesions. Comprehension of these mechanisms is not only pivotal in clinical assessment of disease progression and operability of patients with PAH-CHD; specific targeting of these mechanisms may also lead to pharmacological reversal of *'irreversible'* plexiform lesions into a reversible arteriopathy: one that is amenable for a cure. The recent manipulation of the BMPR2 signalling pathway is an example of the steps to make in the strive to *'reverse the irreversible.'*

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Egr-1 identifies neointimal remodeling and associates to progression in human pulmonary arterial hypertension.

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Abstract

Background. Pulmonary arterial hypertension (PAH) is hallmarked by the development of neointimal lesions. The transcription factor Egr-1 seems to play a critical role in neointimal formation in experimental PAH and was identified as a putative target for intervention. This study investigated whether Egr-1 is also associated with neointimal-type vascular remodeling in different forms of human P(A)H.

Methods. Using immunohistochemistry, we studied Egr-1 expression specifically in a wide morphological spectrum of pulmonary arteries in lung tissue of 72 patients with different forms and stages of PAH: I(diopathic)PAH (n=18), advanced-stage congenital heart disease associated PAH (PAH-CHD) (n=21), early-stage PAH-CHD (n=19); and in non-neointimal hypoxic pulmonary hypertension (PH) (n=4) and controls (n=10).

Results. In PAH, pulmonary vascular expression of Egr-1 protein was abundant, where it was sporadic in non-neointimal (hypoxic) PH and controls. In PAH-CHD, protein expression was more pronounced in patients with advanced vascular lesions compared to those with less advanced lesions, such as medial hypertrophy.

Conclusions. Pulmonary vascular Egr-1 expression is significantly increased in patients with PAH, appears specifically associated with neointimal-type vascular remodeling and correlates with disease progression. These data translate the critical role of Egr-1 in the development of experimental PAH to human pulmonary vascular disease forms.

Introduction.

In Pulmonary Arterial Hypertension (PAH), progressive remodeling of the small pulmonary arteries leads to a vasculopathy hallmarked by characteristic neointimal lesions including plexiform lesions and concentric laminar intimal fibrosis^{1,2}. This specific type of arterial remodeling differentiates PAH from other forms of pulmonary hypertension (PH), such as hypoxic PH or PH due to left-heart disease, in which neointimal lesions do not develop during disease course^{1,2}.

Neointimal remodeling in PAH knows various triggers. In congenital heart disease-associated PAH (PAH-CHD), its occurrence is specifically associated with increased pulmonary blood flow through a systemic-to-pulmonary shunt^{3,4}. PAH-CHD is the only PAH form in which an 'early stage' has been identified, both clinically and histomorphologically^{5,6}. In early-stage PAH-CHD, timely closure of the cardiac shunt may prevent or reverse PAH progression and normalize both pulmonary arterial pressures and vascular histomorphology⁷. In contrast, closure beyond this early stage associates to progression of the remodeling process and has an unfavorable prognosis⁸. The development of neointimal lesions, concentric laminar intimal fibrosis and plexiform lesions particularly, is believed to indicate a completed transition from early-stage, "reversible" to advanced "non-reversible" or progressive PAH^{9,10}. Yet, the pathogenic mechanisms that contribute to this transition and that might account for disease progression in any PAH subclass are currently insufficiently known.

In experimental flow-associated PAH, the progression of vascular remodeling has been well-characterized³. We recently identified the transcription factor Egr-1 to play a critical role in the development of neointimal lesions^{11,12}. In systemic vascular pathobiology, Egr-1 is known to orchestrate a variety of cardiovascular processes, such as intimal thickening, endothelial cell (EC) proliferation, EC migration and vessel inflammation¹³⁻¹⁶. Increased pulmonary blood flow induces pulmonary EC Egr-1 expression and further increases expression during disease progression in both (neo)intimal and medial cell layers. Moreover, inhibition of Egr-1 attenuates neointimal remodeling and PAH progression¹⁷. It is not known whether Egr-1 plays a similar role in human PAH. Therefore, as a first step, the current study aims to assess pulmonary vascular expression of Egr-1 in human PAH and its selectivity for neointimal development when compared to (non-neointimal) PH. For this purpose, we describe pulmonary vascular Egr-1 expression in lung explants of patients with advanced PAH-CHD, I(diopathic)PAH and hypoxic PH, and in lung biopsies from patients with early and advanced-stage PAH-CHD. We hypothesized that 1) Egr-1 expression is up-regulated in human PAH compared to control and hypoxic (non-neointimal) PH, and 2) vascular Egr-1 expression correlates with vascular disease progression.

Materials and Methods.

Study population and tissue preparation.

A total of 72 patients were selected from two institutions, that both serve as referral centers for patients with congenital heart disease and pulmonary hypertension. From the University Medical Center Groningen, The Netherlands, 30 PAH patients were included who underwent lung transplantation for either end-stage PAH-CHD (n=12) or IPAH (n=18) between 1991 and 2009 and from whom representative lung explant tissue was available (*explant cohort*). For comparison with PAH patients, we included 4 patients with hypoxic pulmonary hypertension associated with lung disease (Chronic Obstructive Pulmonary Disease) who underwent lung transplantation due to end-stage disease. As a control group, we included 10 patients with disease free lung parenchyma from whom lung tissue specimens were available from autopsy or lobectomy.

From the Hôpital Necker des Enfants Malades Paris, France, 28 patients with PAH-CHD were included who underwent open lung biopsies in the lower superior lobe of the left lung as isolated procedure or during either complete cardiac repair or palliative surgery (*biopsy cohort*). These patients were divided in two categories based on posteriori evaluation of clinical outcome and echocardiography one year after biopsy. We obtained lung biopsies from a) 19 patients who showed complete clinical and hemodynamic regression of PAH (early stage PAH-CHD) and from b) 9 patients who showed clinical and hemodynamic progression of PAH (advanced PAH-CHD).

Clinical patient data collected from hospital medical records included diagnosis, invasive hemodynamic measurements (when available) and treatment. From each patient, we used 2 blocks of formalin fixed, paraffin embedded lung tissue and obtained 10 serial sections of 5 µm thickness per block. For morphometric identification, we then stained sections with both hematoxylin/eosin and Verhoeff's Stain. Pulmonary vascular vessels were identified as either pre-acinar (large; >150 µm) or intra-acinar (small; <150 µm). The study protocol was approved by the Institutional Medical Ethics Committees, who waived the need of patient consent in this retrospective study because individual patients were not identified.

Immunohistochemistry: qualitative and semi-quantitative analysis.

We chose for an approach using immunohistochemistry (IHC) to assess the spatio-temporal expression of Egr-1 protein specifically in the pre- and intra-acinar pulmonary arteries of patients with different forms of pulmonary hypertension, and to compare with controls. IHC uniquely allows for simultaneous qualitative assessment of both vascular morphology as well as protein expression patterns and localization in the vessel wall. Further, when appropriately applied, it allows for semi-quantitative estimation of protein expression by

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objective scoring protocols, counting the number or proportion of positively stained cells in a vessel wall layer, as described previously^{12,17} and below.

IHC was performed according to a protocol reported previously^{12,17}. Paraffin-embedded lung sections were microwave-heated for antigen retrieval. To reduce non-specific staining, sections were preincubated in 0,3% hydrogen peroxide and were then embedded for 1h at room temperature with primary antibody of Egr-1 (Abcam, 1:100 dilution). We used species-specific immunoglobulin G coupled to peroxidase as a secondary antibody (DAKO, 1:100 dilution). The sections were stained with diaminobenzidine for 10 min and counterstained with hematoxylin. Prostate adenocarcinoma samples served as a positive control for Egr-1 staining. Negative control (by replacing the primary antibody with phosphate buffered saline) showed no staining for all secondary antibodies. To further assess cell-type selective localization of Egr-1 in neo-intimal lesions, we stained a selection of sections for Egr-1, Von Willebrand factor (ECs) and α SMA (smooth muscle cells), using immunofluorescence.

For qualitative assessment of Egr-1 expression, we assigned pulmonary arteries to two groups: pre-acinar vessels ($>150\ \mu\text{m}$) or intra-acinar vessels ($<150\ \mu\text{m}$). For each vessel, the intimal layer and the medial layer were assessed separately. The intimal layer was defined as the cell layer between the vessel lumen and the lamina elastica interna. The medial layer was defined as the layer between the lamina elastica interna and the lamina elastica externa. We then semi-quantitatively scored the proportion of Egr-1 positive cells in the layers of the pulmonary vessels in 10 pre-acinar and 10 intra-acinar vessels according to a standardized protocol previously described¹². 0: no cells positive for Egr-1; 1: less than 50% of cells positive for Egr-1 per vessels layer, 2: 50-80% of cells positive for Egr-1 per vessel layer, 3: more than 80% positive for Egr-1 per vessel layer).

Statistics.

Data are presented as mean $\pm$ standard error of the mean. Differences between groups were determined by one-way analysis of variance (ANOVA) with Bonferroni post-hoc test. A p-value of less than 0.05 was considered to be significant. Statistical analyses were performed using SPSS (version 20).

Results.

Patient characteristics and vascular morphology.

We enrolled a total of 72 patients, forming the following study groups: Advanced IPAH (n=18 lung explants); Advanced PAH-CHD (n=21, 12 lung explants, 9 biopsies); Early-stage PAH-CHD (n=19 biopsies); Hypoxic PH (n=4 explants); Control patients without

lung disease (n=10 explants). The clinical and hemodynamic characteristics of the patients are listed in Table 1. Available hemodynamic variables did not differ significantly between PAH-patient groups or disease stage. Anatomical diagnoses of the patients in the PAH-CHD groups are shown in table 2.

Table 3.1. Clinical and hemodynamic characteristics. Data are presented as mean $\pm$ 1SD. Con: control; PH: Pulmonary Hypertension; (I)PAH(-CHD): (Idiopathic) Pulmonary Arterial Hypertension (-Congenital heart disease); Early: early-stage PAH-CHD; Advanced: Advanced-stage PAH-CHD; BMI: body mass index; 6mWT: 6 minute walk test; mPAP: mean pulmonary artery pressure; Qp: pulmonary blood flow; Qs: systemic blood flow; PVR: pulmonary vascular resistance. SVR: systemic vascular resistance. Numbers between brackets represent the total number of patients when not all data for that characteristic could be acquired.

Characteristics	Lung explants			Lung biopsies (PAH-CHD)		
	Con	Hypoxic PH	IPAH	PAH-CHD	Early	Advanced
<i>n</i>	10	4	18	12	19	9
Gender (% male)	50	100	44	33	47	67
Age (y. at explant/biopsy)	63 $\pm$ 3	58 $\pm$ 2	42 $\pm$ 3	32 $\pm$ 3	3 $\pm$ 6	2 $\pm$ 3
BMI	25 $\pm$ 1	25 $\pm$ 2	22 $\pm$ 1	20 $\pm$ 1	16 $\pm$ 2	14 $\pm$ 2
WHO 3 / 4	-	-	13 / 5	4 / 8	-	-
6mWD (m)	-	-	333 $\pm$ 140	226 $\pm$ 78	-	-
Systemic systolic pressure (mmHg)	-	-	111 $\pm$ 16	116 $\pm$ 12	75 $\pm$ 18	87 $\pm$ 7
Systemic diastolic pressure (mmHg)	-	-	71 $\pm$ 3	66 $\pm$ 3	41 $\pm$ 19	46 $\pm$ 7
mPAP (mmHg)	-	-	64 $\pm$ 13 (18)	69 $\pm$ 16 (8)	47 $\pm$ 15 (14)	56 $\pm$ 12 (8)
Cardiac Index	-	-	2.4 $\pm$ 0.1	2.8 $\pm$ 0.5	-	-
Qp	-	-	4.2 $\pm$ 1.5 (18)	4.2 $\pm$ 2.3 (6)	-	-
Qp/Qs	-	-	1.0 $\pm$ 0.13 (18)	0.8 $\pm$ 0.1 (10)	1.7 $\pm$ 0.8 (11)	1.6 $\pm$ 0.4 (7)
PVR (dyn/s/cm ⁵)	-	-	1024 $\pm$ 428 (18)	899 $\pm$ 462 (6)	-	-
PVR/SVR	-	-	-	-	0.4 $\pm$ 0.2 (8)	0.7 $\pm$ 0.4 (6)

In advanced PAH-CHD and IPAH, intra-acinar vessels were characterized by vessels with media hypertrophy, intimal proliferation, laminal concentric intimal fibrosis and plexiform lesions (Verhoeff Figure 3.1B and 2.2). Pre-acinar vessels were mainly characterized by increased muscularization combined with intimal proliferation and fibrosis (Verhoeff Figure 3.1A). In early-stage PAH-CHD, both pre- and intra-acinar vessels were characterized by increased muscularization without neointimal development. Patients with hypoxic PH similarly showed increased muscularization of both pre- and intra-acinar vessels without neointimal lesions (Verhoeff Figure 3.1A and B). In control patients, intra-acinar vessels were mainly non-muscularized (Figure 3.1B). Pre-acinar pulmonary vessels in control subjects were elastic with a single layer of endothelial cells (Figure 3.1A).

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Table 3.2. Anatomical Diagnoses PAH-CHD Cohort. PAH: Pulmonary Arterial Hypertension; D: Diagnosis; LTx: lung transplantation; VSD: ventricular septum defect. PDA: persistent ductus arteriosus; TGA: transposition of the greater arteries; AVSD: atrioventricular septum defect; SV: single ventricle; TAPVD: total anomalous pulmonary venous drainage.

Early-stage PAH-CHD				Advanced stage PAH-CHD				
Patient	Age at biopsy (y)	Anatomical diagnosis	Surgical procedure	Patient	Age at biopsy (y)	Anatomical diagnosis	Surgical procedure	
1	3.2	AVSD	Closure	1	1.3	VSD	Banding	
2	0.8	ASD	Closure	2	4.0	VSD	Closure	
3	0.5	AVSD	Closure	3	9.3	AVSD	Banding	
4	0.6	AVSD	Closure	4	0.2	VSD	Closure	
5	0.3	TA	Closure	5	0.7	VSD + TGA	Senning	
6	1.8	VSD	Closure	6	0.3	AVSD	Closure	
7	0.0	VSD	Closure	7	4.7	PDA	Closure	
8	0.4	AVSD	Closure	8	0.2	ASD + TAVR	Correction	
9	2.1	VSD + PDA	Closure	9	1.0	ASD + PAVR	Correction	
10	0.3	Ao-pulm window	Closure	Patient	Time Dx to LTx (y)	Anatomical diagnosis	2nd gen PAH drugs	Anticoa- gulants
11	0.7	VSD	Closure	1	33	VSD+PDA	0	1
12	3.8	VSD + PDA	Banding	2	31	TGA+AVSD+SV	0	1
13	0.3	VSD	Closure	3	10	ASD	0	1
14	0.2	AVSD	Closure	4	21	VSD+PDA	0	0
15	0.5	AVSD + PDA	Closure	5	33	ASD	0	1
16	10.9	AVSD	Banding	6	24	TGA+AVSD	0	1
17	1.5	AVSD + TGA	Senning	7	41	TGA	1	1
18	0.6	AVSD	Closure	8	16	VSD+PDA	1	1
19	26.8	VSD	Closure	9	2	ASD+TAPVD	0	1
				10	2	ASD	0	1
				11	9	VSD	0	0
				12	22	TGA+SV	0	0

Qualitative analysis of Egr-1 expression.

In hypoxic PH and controls, Egr-1 expression was found only sporadically throughout the vessel walls. Patients with advanced PAH-CHD and IPAH showed Egr-1 expression in a wide morphological spectrum of remodeling, ranging from isolated muscularization to neointimal lesions (Egr-1 Figure 3.1-2). In advanced PAH-CHD and IPAH, Egr-1 expression was generally found in all cells of the the intimal and more than half of the cells in the medial layer of both pre- and intra-acinar vessels. In early PAH-CHD, Egr-1 expression was present in nearly all cells of the intimal layer of the intra-acinar pulmonary vessels. Egr-1 staining in the media of these vessels was positive in less than half of the cells (Figure 3.3).

Figure 3.1. Representative pulmonary vascular arteries in lung explants. A) Pre-acinar vessels (>150µm) in the different groups. Note the marked intimal thickening (arrow) in PAH-CHD and the typical cushion-like eccentric intimal thickening (as described previously in (1)) in IPAH. In these vessels Egr-1 expression was observed throughout the vessel wall. In control and hypoxic PH neointimal formation was not seen. B) Intra-acinar vessels (<150µm) with characteristic neointimal lesions in PAH-CHD and IPAH. In these vessels Egr-1 expression (arrows) was seen both in the endothelial and medial layer. The intimal layer showed the strongest Egr-1 expression.

Pre-acinar vessels in this group generally show Egr-1 expression in less than half of cells in the intimal layer, and only sporadic expression in the media.

In both PAH-CHD and IPAH, Egr-1 expression was strongest in plexiform lesions, where expression was especially pronounced in endothelial cells lining the plexus channels (Figure 3.4A-B). In vessels with severe concentric intimal fibrosis, Egr-1 was found throughout the vessel wall in cells that co-express either endothelial (vWF), or smooth muscle cell (α -SMA) markers, or both. In vessels with only mild intimal proliferation, Egr-1 was predominantly found in endothelial cells bordering the vessel lumen (Figure 3.4C-D).

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Figure 3.2. Different forms of pulmonary vascular remodeling in PAH-CHD. Egr-1 expression is seen in all these different forms of vascular remodeling (black arrow). Note the occasional negative cells in the medial layer of vessels with media hypertrophy and in neointimal lesions (dotted arrow) whereas in the intimal layer, almost all cells stain positive for Egr-1.

Figure 3.3. Egr-1 in PAH-CHD progression. Representative examples of Egr-1 expression in both early-stage and advanced PAH-CHD. Early stages show moderate medial hypertrophy with mainly intimal Egr-1 staining (black arrow). In advanced stage, medial hypertrophy is more pronounced and accompanied by neointimal formation, with intense Egr-1 staining (black arrows). Dotted arrows annotate Egr-1 negative cells. Scale bars: 100 μ m.

Figure 3.4. Localization of Egr-1 in different forms of vascular remodeling. A) Representative Egr-1 expression in a plexiform lesion in PAH-CHD. Note the strong staining of the cells bordering the plexus channels. Scale bars: 150 μ m. B) Representative Egr-1 expression in advanced pulmonary vascular lesions in IPAH, note that the pattern is to be comparable to PAH-CHD (4A). Scale bars: 150 μ m. C) Tripple immunofluorescence for DAPI (blue), von Willebrand factor (vWF, green), α -Smooth Muscle Actin (α -SMA, red) and Egr-1 (white) in a severely occluded vessel with concentric laminar intimal fibrosis. Egr-1 expression is found throughout the vessel wall and is strongest in the neo-intimal layer within the internal elastic lamina. Egr-1 is found both in cells that express vWF or α -SMA or both. Also note the expression of α -SMA within the neo-intimal layer, suggestive of endothelial-to-mesenchymal-transition. Elastic laminae show aspecific staining for the secondary antibody coupled to vWF. Scale bars: 150 μ m. D) Tripple immunofluorescence alike figure 3.4C in a vessel with mild intimal proliferation. In these vessels, Egr-1 expression is predominantly co-localized with vWF, in cells bordering the vessel lumen. Scale bars: 150 μ m

Semi-quantitative analysis.

Results of the semi-quantitative analyses of vascular Egr-1 expression are shown in Figure 3.5. In the tissue of the biopsy cohort, pre-acinar vessels were scarce (plausibly due to a peripheral location of the biopsy) and therefore excluded from semi-quantitative analysis on Egr-1 expression. In all vessel sizes and layers, Egr-1 expression was significantly higher in patients with PAH-CHD and IPAH, compared to both controls and hypoxic PH ($p < 0.001$). Egr-1 expression did not differ between controls and hypoxic PH ($p = 1.000$). In the intimal layer of the intra-acinar vessels, Egr-1 expression was lower in IPAH as compared to early

Egr-1 identifies neointimal remodeling and associates to progression in human pulmonary arterial hypertension.

Figure 3.5. Semi-quantitative scoring of Egr-1 in both the intimal layer and the medial layer of pre- and intra-acinar vessels in control, hypoxic PH, PAH-CHD and IPAH patients. * $P < 0.05$ vs. Con and Hypoxic-PH. # $p < 0.05$.

or advanced PAH-CHD ($p = 0.034$). In the media of intra-acinar vessels, Egr-1 expression was lower in early PAH-CHD as compared to advanced PAH-CHD ($p = 0.007$). Egr-1 scores of advanced PAH-CHD in lung explant and lung biopsy cohorts did not differ ($p = 1.000$).

Discussion.

This study shows that pulmonary vascular expression of the transcription factor Egr-1 is increased in patients with PAH, and is specifically associated with neointimal-type vascular remodeling when compared to non-neointimal remodeling as found in hypoxic PH and disease-free controls. In early-stage PAH-CHD, expression of Egr-1 is predominantly located in the endothelial layer of the smallest pulmonary arterioles, while it expands throughout the vessel wall as vascular lesions aggravate and the disease advances. This suggests a role

for Egr-1 both in the initiation of remodeling in response to disturbed hemodynamics and associates to disease progression in patients with congenital heart disease.

The findings in the current study are of particular interest in the context of recently reported data on Egr-1 in experimental PAH-models. In rat models of different types of pulmonary hypertension, it was demonstrated that pulmonary vascular expression of Egr-1 specifically associated to PAH-characteristic vascular remodeling, in particular with the occurrence of complex neointimal lesions^{3,11,12}. Experimental studies show that *in vivo* selective down-regulation of Egr-1 attenuates early-stage vascular proliferation rates and increases apoptosis in advanced disease¹⁷. This was associated with reduced expression of vascular transforming growth factor- β 1 (TGF- β 1), platelet-derived growth factor-B, interleukin-6, p53 and tissue factor: all effectors known to play a role in the pathogenesis of PAH²⁰⁻²⁴. Egr-1 inhibition concurrently attenuated neointimal development, improved pulmonary vascular resistance and reduced right ventricular hypertrophy¹⁷. A proposed mechanism for Egr-1-dependent neointimal formation is displayed schematically in Figure 3.6. These preclinical results indicate that Egr-1 governs pulmonary neointimal development and could be a putative target for treatment in patients with PAH. Treatment with pioglitazone, a clinically used peroxisome proliferator-activated receptor- γ -ligand (PPAR- γ), has shown to inhibit Egr-1 expression *in vivo*^{25,26} and positively affect vascular remodeling both in flow-PAH and in other models for pulmonary hypertension^{17,27,28}. Experimental studies have also suggested that Egr-1 targeting may improve right ventricular adaptation to increased afterload²⁹⁻³¹. Hence, targeting Egr-1 may be one of the first therapies that favors both the pulmonary vasculature as well as the collaterally affected right ventricle.

In PAH-CHD patients, we have described distinct expression patterns of Egr-1 throughout disease development. Egr-1 is known to be triggered by disturbed flow and shear stress, via the extracellular regulated kinase 1/2 pathway³². Pulmonary vascular Egr-1 localization and expression patterns in flow-associated PAH rats resemble current findings in human PAH-CHD progression. Both show predominantly endothelial expression of Egr-1 in early-stage disease, while expression increases and migrates throughout the vessel wall as the disease advances to its neointimal form. Pathophysiologically, these data suggest that besides the ability to initiate vascular remodeling in PAH, Egr-1 could also further orchestrate the transition from reversible to non-reversible pulmonary vascular disease and exacerbate neointimal development.

We also demonstrate prominent Egr-1 expression in IPAH. This is in line with the observations of Kwapiszewska *et al.* who found increased Egr-1 mRNA and protein expression in remodeled arteries of IPAH patients³⁴. The stimulus for increased Egr-1 expression in IPAH is less evident. The vascular inflammation and auto-immunity that are observed in

Egr-1 identifies neointimal remodeling and associates to progression in human pulmonary arterial hypertension.

Figure 3.6. Proposed mechanism of Egr-1-dependent neointimal formation. Induced by specific stimuli, Egr-1 is able to up regulate different effector proteins that cause endothelial (EC) and smooth muscle cell (SMC) proliferation, inflammation and dysregulated apoptosis. Prolonged elevated Egr-1 expression eventually leads to neointimal lesions, with plexiform lesions at the end of the spectrum. BMPR: bone morphogenic protein receptor; PDGF: platelet derived growth factor; PPAR- γ : peroxisome proliferator-activated receptor- γ TGF- β : Transforming growth factor β ; IL-6: interleukin-6; TF: tissue factor.

IPAH, may induce Egr-1 expression through e.g. TGF- β or Rho-ROCK signaling³⁵⁻³⁷. Also, mutations in the bone morphogenic protein receptor-2 (BMPR2) gene, pathognomonic for heritable PAH and associated with sporadic forms of IPAH³⁸, may induce Egr-1 expression via down-regulation of PPAR- γ ³⁹. Egr-1 expression may subsequently be maintained by local flow disturbances³².

In hypoxic, non-neointimal PH patients, Egr-1 was expressed only sporadically. In contrast, Yan and colleagues showed in mice that Egr-1 was increased in lungs subjected to a various range of hypoxia⁴⁰. However, in that study, the investigators quantified Egr-1 expression levels in whole lung lysate and not selectively in the pulmonary vessels. In addition, the oxygen levels in this study (6-10%) were far lower than in human disease settings which hinders translation of these experimental results to human hypoxic PH.

The limitations of this study attribute to the semi-quantitative nature of immunohistochemical analysis. In pulmonary (vascular) pathology, immunohistochemistry is the gold

standard in the assessment of structure or cell (layer) specific protein expression. In the current study, this enabled us to investigate precisely the spatio-temporal patterns of Egr-1 expression in the development of neointimal lesions. To maximize objective semi-quantification, Egr-1 expression was both stained and scored according to a standardized protocol, previously reported in experimental flow-PAH¹². In that study, semi-quantification of immunohistochemical Egr-1 expression corresponded with mRNA quantification on laser-dissected microvasculature in lung cryo-sections of rats with PAH¹². The difference in Egr-1 expression between groups in the present study was large enough to vindicate objective comparison. This study was also limited by the small number of samples available in the hypoxic PH group, and the restricted availability of hemodynamic data in all study groups. The lack of statistical difference regarding hemodynamic variables prohibited correlation with Egr-1 expression. This may be due to a lack of power, or could reflect current clinical practice, where the distinction of early or advanced PAH-CHD based on hemodynamic status, still proves difficult.

In conclusion, this study shows that the transcription factor Egr-1 is progressively expressed in pulmonary vascular neointimal lesions of patients with PAH-CHD. Also in patients with IPAH, similarly associated with pulmonary vascular neointimal lesions, the expression of Egr-1 is abundant. This in contrast with the scarce Egr-1 expression found in the remodeled pulmonary vessels in patients with non-neointimal, hypoxic PH. These data add to the accumulating evidence that Egr-1 plays a critical role in the development of neointimal lesions in human PAH and identifies Egr-1 as a potential target for PAH therapy.

Egr-1 identifies neointimal remodeling and associates to progression in human pulmonary arterial hypertension.

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Assessment of pulmonary arterial hypertension and congenital heart disease.

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Abstract.

Pulmonary arterial hypertension (PAH) in congenital heart disease (CHD) can be reversed by early shunt closure, but this potential is lost beyond a certain point of no return. Therefore, it is crucial to accurately assess the reversibility of this progressive pulmonary arteriopathy in an early stage. Reversibility assessment is currently based on a combination of clinical symptoms and haemodynamic variables such as pulmonary vascular resistance. These measures however, are of limited predictive value and leave many patients in the grey zone. This review provides a concise overview of the mechanisms involved in flow-dependent progression of PAH in CHD and evaluates existing and future alternatives to more directly investigate the stage of the pulmonary arteriopathy. Structural quantification of the pulmonary arterial tree using fractal branching algorithms, functional imaging with intravascular ultrasound, nuclear imaging, putative new blood biomarkers, genetic testing and the potential for transcriptomic analysis of circulating endothelial cells and educated platelets are being reviewed.

Introduction.

Pulmonary arterial hypertension (PAH) is a lethal syndrome characterized by increased pulmonary artery pressure (mPAP), pulmonary vascular resistance (PVR), and normal pulmonary capillary wedge pressure. The diagnosis is essentially based on assessment of these hemodynamic values, and clinical presentation predominantly comprises symptoms of resulting right heart failure¹. These symptoms however, are preceded by a progressively obstructive arteriopathy that may be clinically silent for many prior years. At diagnosis, most PAH aetiologies such as idiopathic PAH, have already progressed to an irreversible stage in which current targeted therapy may stabilize or decelerate progression, but cannot cure the disease².

PAH in congenital heart disease (PAH-CHD) presents with unique features in this aspect. In these patients, the arteriopathy is triggered by increased pulmonary blood flow resulting from a left-to-right shunt due to an intra- or extracardiac defect. Early identification of the cardiac defect allows detection of the pulmonary arteriopathy in an early stage, and timely shunt closure can permanently reverse, thus cure the disease³. However, the beneficial effects of shunt closure seem lost after a certain point of no return, after which even accelerated PAH progression may occur months to years after surgery⁴. These observations underscore the critical importance of early and accurate detection of this '*window for reversibility*' in patients with PAH-CHD. The assessment of reversibility however, is nowadays primarily based on clinical judgement and measurements of hemodynamic variables, which have limitations as surrogates for the stage of the arteriopathy^{1,5}. Techniques able to directly assess the pulmonary vasculature are still absent from clinical practise today. Reliable assessment of the vascular disease stage may not only improve identification of reversibility in PAH-CHD, but could also help to detect disease development early in patients at risk for other forms of associated PAH (e.g. in familial or HIV-, or connective tissue disease-PAH).

This review will evaluate the possibilities and limitations of the contemporary assessment of reversibility in PAH-CHD. First, we will provide a brief overview of the temporal structural, functional and cellular changes that occur during PAH progression in CHD. We will then provide a conceptual and practical approach to assess the pulmonary vasculature in PAH-CHD, using existing and new imaging modalities and biomarkers.

Flow-induced remodeling of the pulmonary vasculature: a sequence of events.

The normal pulmonary arterial (PA) tree (*figure 4.1A*) has a fractal branching structure that equally divides blood flow to the alveoli. The distal intra-acinar arterioles are non-muscularized and consist of a smooth endothelial cell (EC) layer (*figure 4.2*). More proximal pre-acinar arteries have a thin muscular medial vessel layer that becomes thicker and more elastic towards the PA trunk. The blood flow in the normal acinar pulmonary circulation is laminar: well-ordered and streamlined, pulsatile in the proximal pulmonary arteries, more continuous in the arterioles and capillaries, with a small pressure difference in the arterial and venous compartments^{6,7}.

Blood flow and pressure are essential triggers for pulmonary vascular remodelling in CHD⁸. Increased pulmonary blood flow seems a prerequisite for the neointimal type remodelling, where increased pulmonary artery pressure functions as an accelerator. Non-restrictive, post-tricuspid shunts such as a ventricular septal defect (high flow / high pressure) induce advanced PAH-remodelling frequently and rapidly, usually within a few years. In contrast, pre-tricuspid high flow / normal pressure lesions like atrial septal defects induce advanced remodelling only in 5-10% of the patients and generally only after 2 to 4 decades². Increased flow, especially in combination with increased pressure, disturb blood flow throughout the PA tree⁶, leading to up regulation of flow-sensitive genes via mechanotransduction, such as early growth response-1⁹ or p53¹⁰. These in turn induce pro-apoptotic, pro-proliferative and inflammatory signalling² and cause endothelial dysfunction. Morphological changes in cell surface, swelling and cohesion of endothelial cells are one of the first structural changes visible¹¹, followed by neomuscularization of the normally non-muscularized acinar arterioles and medial hypertrophy of pre-acinar arteries⁷. Proximal medial hypertrophy increases proximal PA stiffness, reducing physiologic proximal flow damping¹². As a result, peak flow velocity and flow velocity variance are increased, amplifying the flow disturbance in the distal PAs¹². (*figure 4.1C, E, figure 4.2*)

Neointimal remodelling, the pathohistological signature for any form of progressive PAH, occurs when blood flow remains disturbed. Neointimal lesions are found primarily at branching points in the distal PAs¹³. This process involves intimal hyperplasia, degradation of the elastic laminae, infiltration of pericytes into the intima and encroachment of smooth muscle cells into the lumen, and is driven by a prolonged misbalance in proliferation and apoptosis, up-regulation of anti-apoptotic signalling and on-going inflammation¹⁴⁻¹⁶. Neointimal remodelling disrupts the vascular luminal surface, which further disturbs local flow patterns, causing a vicious cycle of disturbed flow on a progressively remodelling intimal layer⁶, which ultimately leads to the development of a fibrotic neointimal layer and luminal occlusion (*figure 4.2*).

Figure 4.1. Schematic representation of the pulmonary arterial tree in the normal situation (A) and in PAH (B). PAH is characterized by proximal arterial dilatation, loss of fractal branching structure, distal arterial narrowing and pruning, and peripheral vessel loss. (C) Increased hemodynamic force increases medial thick- and stiffness, this causes turbulent flow mainly at the branching points. Turbulent flow induces endothelial dysfunction and proliferation, which lead to neointimal lesions that occlude the vascular lumen and further disturb flow. (D) Logarithmic relationship of proximal arterial dilatation and distal occlusion in health and PAH. In PAH, the slope of this line increases. Adopted from²⁸. (E) Proposed sequence of hemodynamic (blue) and structural (red) events following increased flow.

Figure 4.2. Concise scheme of the progression of PAH in response to flow at the intra-acinar level of the PA tree. The normal arteriole has a smooth single layer of endothelial cells (EC) and no medial layer. Increased flow leads to the up regulation of numerous flow-sensitive genes and induces EC apoptosis and EC dysfunction, and neomuscularization. Reversible PAH is characterized by medial hypertrophy and EC proliferation. The shifting balance between EC proliferation and apoptosis induces intimal hyperplasia. Irreversible PAH is characterized by neointimal fibrotic lesions and adventitial fibrosis and inflammation. ECs have become resistant to apoptosis and progressively occlude the vascular lumen. Plexus channels start to form within the vessel, eventually leading to the characteristic plexiform lesion.

These local distal arteriolar changes affect the structure and function of the whole pulmonary arterial tree, the most marked being reduced branching complexity, occlusion of supernumerary arteries, pruning and loss of distal vasculature, and dilatation and stiffening of the proximal arteries¹⁷ (figure 4.1B). The *human model* of PAH-CHD offers the unique opportunity to study these structural, functional and molecular changes in all different stages of disease progression: from disease initiation and the reversible phase in infancy and childhood, throughout the progression towards advanced, irreversible disease².

Contemporary haemodynamic assessment of PAH-CHD and its utility for reversibility.

The assessment of reversibility is a crucial part in the decision for shunt closure (often referred to as ‘operability’) in patients with PAH-CHD. An algorithm for the assessment of reversibility is presented in figure 4.3. In essence, operability of CHD associated with PAH involves the possibility to successfully perform the operative correction of the heart defect and for the patient to survive that procedure, where reversibility of PAH-CHD refers to the normalisation of the pulmonary vasculature, directly but also long term after correction of the heart defect. At present, no gold standard exists for the assessment of reversibility. Contemporary assessment of reversibility relies on the integration of clinical variables (including age, type of CHD, central cyanosis at exertion, clubbing, right ventricular heave, accentuated pulmonary second heart sound component, fading of a ventricular murmur, associated co-morbidities such as genetic or chromosomal abnormalities), laboratory variables (including haemoglobin, haematocrit) and hemodynamic evaluation by echocardiography and heart catheterization (HC)^{5,18}. Currently, HC is regarded the cornerstone in the diagnosis and prognostication of PAH-CHD.

According to current guidelines, assessment of reversibility is limited to hemodynamic variables: those in favour of reversible PAH-CHD are a left-to-right shunt and a PVR index $<4\text{WUm}^2$. Shunt closure is contra-indicated when the net shunt is directed right-to-left, and is discouraged when PVR index is $>8\text{WUm}^2$. When the PVR index is between 4 and 8 WUm^2 “individual patient evaluation in tertiary centres” is advised¹⁹. These recommendations however, are predominantly based on expert opinion and are hardly supported by data. In fact, in PAH-CHD, no prospective studies have yet identified reliable hemodynamic cut-offs that predict reversal of pulmonary vascular disease and normalisation of hemodynamics after cardiac correction². Available retrospective studies are seriously hampered by selection bias, lack of pre-operative characteristics or incomplete follow up.

Acute pulmonary vasodilator tests (AVT) are performed during HC to test the effect of short-acting pulmonary vasodilators (inhalation of nitric oxide or epoprostenol) on PVR, PAP and shunting. Although the use of AVTs to estimate reversibility prior to corrective surgery is widespread in current clinical practice, no hemodynamic cut offs have shown sufficient accuracy in predicting reversal after shunt correction. The use of AVTs to assess reversibility/operability in PAH-CHD should not be confused with AVT’s in idiopathic PAH, which are indicated to predict prolonged beneficial effects of calcium channel blocker therapy and where specific criteria for acute responders have been defined²⁰. However, these response criteria cannot be extrapolated to assess reversibility in PAH-CHD²¹. Considering the heterogeneity in CHD and the complexity and limitations of hemodynamic measure-

ments (especially when performing AVTs) in the situation of a shunt²², it might be an illusion to expect a single hemodynamic parameter to distinguish reliably between reversible and irreversible PAH-CHD.

Figure 4.3. Algorithm for the assessment of reversibility in PAH-CHD. Currently, a large grey zone exists for PAH-CHD patients with a $PVRi > 4 \text{ WUm}^2$. Table 1 provides a summary of possible methods to discern reversibility, but more research is needed to accurately predict a reversible response to shunt closure. 2-6% of children and 7-13% of adults with PAH-CHD develop post-operative PAH after shunt closure, which is associated with a significantly worse prognosis than uncorrected Eisenmenger patients. TA: Truncus Arteriosus, AVSD: Atrio-ventricular septal defect; TGA: Transposition of the great arteries; VSD: Ventricular septal defect; ASD: Atrial septal defect; RV: right ventricular; WU: Woods Units $\times \text{m}^2$; Qp/Qs: Pulmonary to systemic blood flow ratio; PVR/SVR: pulmonary to systemic vascular resistance ratio.

Structural, functional and molecular assessment of the pulmonary vasculature in PAH.

The following paragraphs present an overview of methods to analyse structural, functional and molecular changes at the different anatomical levels of the PA tree in PAH, and their potential to assess reversibility. We summarized the indices evaluated in this review, and the current indices for reversibility in Table 1.

Structural markers.

The assessment of vascular morphology via lung biopsy has long been considered the gold standard for phenotyping pulmonary vascular disease². In PAH-CHD patients, a lung biopsy showing neointimal fibrosis and plexiform lesions predicts progression, also after shunt closure, whereas isolated medial hypertrophy and mild intimal proliferation are likely, but not sure, to improve^{3,23}. Although this morphological approach to reversibility is still widely accepted as a concept, its practical limitations have led to its abolishment from clinical practice. Morbidity and mortality in PAH-CHD has been reported as high as respectively 13 and 20%²⁴. The patchy distribution of advanced vascular lesions in the PAH lung further limits the reliability of judging reversibility from a single or multiple biopsy specimen. Hence, less invasive methods to assess the structural changes of the PA tree have been developed.

Main PA dimensions can be readily visualized by MRI, CT, plain radiography and echocardiography. A longitudinal CT study in PH patients showed that main PA dilatation already occurs at an mPAP of 21-24mmHg²⁵, and correlates with mPAP and PVR during disease progression, indicating applicability for early PH detection. However, the value of main PA dimension as a predictor of reversibility in PAH-CHD is likely limited, as not only pressure but also volume overload is involved, and the dimensions change with age²⁶.

The distal PA tree with advanced PAH shows progressive reduction in fractal branching structure (pruning) and arteriolar cross sectional area (vascular loss, see figure 4.1). These features can be visualized by wedge angiography and quantified by calculating tapering (rate of reduction in vessel diameter), background haze (contrast fluid intensity in peripheral lung fields) and circulation time²⁷. At present, reduction in fractal branching can be calculated using a fractal dimension algorithm on CT angiography¹⁷. A reduction in fractal branching correlates with PVR and functional class in IPAH and PAH-CHD¹⁷.

Main PA dilatation and fractal branching have limitations as separate indices in the context of reversibility, but combined they justify further investigation. A power-law model integrated both parameters showing a logarithmic linear correlation: small increments in main

PA diameter correspond to relatively large changes in the distal PA tree; and the slope of this line (X) decreases during disease progression (*see figure 4.1D*). Comprehensive analysis showed that the largest decrements in X happen in the earliest phases of the PAH development, before any elevation in resting PVR, indicating X as a sensitive marker for early remodelling of the distal pulmonary vasculature²⁸. These insights rationalize prospective studies in animal models and human PAH to validate their predictive value with regard to reversibility.

Functional markers.

Vascular stiffness indices such as distensibility and compliance are functional parameters for the PA tree that can be assessed by intravascular ultrasound (IVUS) or by integrating dynamic MRI and HC data. In PAH, arterial stiffening was shown to occur early in the disease, when mPAP and PVR are still within normal range²⁹. In 41 children with various stages of PAH-CHD, distensibility and compliance, measured by IVUS at baseline, correlated with progressive PAH and mortality after a 20-year follow up. Interestingly, IVUS could also predict disease progression in patients with a presupposed favourable hemodynamic profile at HC³⁰. IVUS has been shown safe and applicable even in small children with PAH-CHD³¹. These data support a role for PA stiffness indices in the assessment of reversibility.

Molecular markers.

The increasing comprehension of the molecular biopathology associated with early progression, reversal or irreversibility of PAH, provides a theoretical basis to stage PAH using nuclear imaging, circulating biomarkers and profiling of the transcriptome or metabolome.

Nuclear imaging. Nuclear imaging techniques like positron emission tomography (PET) and single photon emission computed tomography (SPECT) allow in vivo assessment of pathophysiologic processes and responses. Nuclear imaging could identify PAH patients with molecular profiles favourable for reversibility using a variety of tracers such as ¹⁸F-FLT (proliferation); ¹⁸F-fluciclatide (neovascularization); and annexin-tracers (apoptosis).

The first trial with nuclear imaging in PAH, involved a PET study with fluorine-18-labeled 2-fluoro-2-deoxyglucose-tracer (¹⁸F-FDG). ¹⁸F-FDG uptake is increased in cells with high aerobic glycolysis, which has also been observed in ECs of PAH patients³². In rats with PH, ¹⁸F-FDG signal positively correlated with PA muscularization³². ¹⁸F-FDG signal was also increased in end-stage IPAH patients compared to controls³³. A subsequent study however, showed that among IPAH patients, ¹⁸F-FDG-uptake is highly heterogeneous³⁴. Hurdles of pulmonary nuclear imaging in PAH include the influence of target site perfusion on tracer uptake and the limited spatial resolution of current nuclear imaging techniques. In addition, the radiation exposure limits its applicability in pediatric PH.

Blood biomarkers. Blood biomarkers currently incorporated in clinical PAH guidelines are brain natriuretic peptide (BNP) and NT-proBNP³⁵. NT-proBNP correlates with hemodynamic parameters and survival, and parallels a treatment effect with vasodilator therapy³⁵. These data confirm the utility of the BNPs as a biomarker for the pressure loaded RV, but being markers of cardiac stretch, their role in the assessment of pulmonary vascular disease is limited.

Biomarkers for EC damage. In PAH, damaged pulmonary ECs detach and become detectable in the peripheral blood³⁶. In PAH-CHD, Circulating EC (CEC) count in peripheral blood was 10-fold higher in irreversible versus reversible PAH and controls³⁷. Both in PAH-CHD and IPAH, clinical deterioration was associated with increased CEC count, while treatment with PAH-targeted therapy was associated with a decrease³⁶. The number of circulating endothelial progenitor cells, recruited from the bone marrow (indicating reduced capacity to maintain EC homeostasis), is significantly reduced in Eisenmenger PAH compared to healthy controls³⁸, but did not differentiate in reversibility in a cohort of reversible and irreversible PAH-CHD³⁷. Other suggested circulating biomarkers for EC damage are asymmetric dimethylarginine (ADMA) and ghrelin. ADMA levels were significantly higher in end-stage versus early PAH-CHD³⁹. Conversely, ghrelin levels were increased in paediatric early PAH-CHD versus CHD without PH, and decreased in end-stage versus early PAH-CHD⁴⁰.

Other Biomarkers. A recent study compared the metabolomics blood profiles of 10 early, reversible and 4 patients with advanced, irreversible PAH using mass spectrometry⁴¹. In this small cohort, 4 candidate proteins were found with discriminative potential between groups: caveolin-1, filamin-1A, cathepsin-D (increased in irreversible PAH) and glutathione S-transferase mu1 (GSTM1; decreased in irreversible PAH). Seemingly contradictory, Caveolin-1 is known to amplify beneficial BMPR2 signalling in ECs⁴², and mutations or loss of caveolin-1 in ECs are generally associated with the development of PAH. However, caveolin-1 expression was indeed found to increase with disease progression in the media of remodelled arteries in end-stage iPAH and PAH-CHD⁴¹. This temporal expression pattern makes caveolin-1 a potential marker to distinguish reversibility. Filamin-1A is associated to apoptosis-resistance, and cathepsin D to elastin and collagen degradation, and to vascular remodelling: processes all consistent with end-stage PAH pathology. GSTM1 finally is associated with protection against reactive oxygen species (ROS)⁴¹. ROS induces DNA damages which propagates the progression of PAH⁴³. Loss of GSTM1, as found in the blood of irreversible PAH patients, may therefore be an appropriate biomarker for PAH progression. Larger prospective clinical studies, ideally with incorporation in a therapeutic algorithm, are needed to confirm the value of these biomarkers for clinical practice.

Next generation sequencing of circulating cellular RNA and educated platelets.

Recent observations in lung cancer indicate that lung tumours shed cells and ctDNA into the bloodstream and “educate” platelets with tumour-RNA as they scrape past the tumour⁴⁴. These factors can be isolated from a standard peripheral blood sample. Next generation sequencing (NGS) then allows to identify tumour profiles that can be used for staging, to determine treatment strategy and to predict treatment response⁴⁴. Pulmonary ECs also shed into the bloodstream in PAH (see §4.3.3) and platelets from PAH patients are significantly altered compared to controls as well⁴⁵. These observations rationalize NGS studies of circulating ECs and platelets in PAH, to detect transcriptomic profiles that are associated to reversible or irreversible disease.

Genetic evaluation.

An increasing number of genetic mutations is associated with PAH (also see table 1). If this mutation leads to PAH, the phenotype is usually severe and progressive⁴⁶. Genetic mutations are not common in PAH-CHD⁴⁷, and available data are controversial. Nevertheless, the presence of a mutation in the context of PAH-CHD could predispose for or accelerate progressive, thus irreversible disease. Mutations in *BMPR2*^{48,49}, and recently in the transcription factor *Sox17*⁵⁰, have been associated with PAH in CHD specifically. Whether this provides sufficient rationale to screen for these variants in the assessment of PAH reversibility remains to be determined.

Conclusion.

Early and accurate detection of *the window for reversibility* is critical in patients with PAH-CHD, but up to the present day there is no evidence, nor consensus, how to define this window. The current clinical tools including invasive hemodynamic evaluation do not suffice and are prone for improvement. Insights in the structural, functional and the molecular changes that occur in the PA tree during the progression of PAH, open windows for less invasive imaging techniques and exploration of new biomarkers. Extensive mapping of the metabolomic or transcriptomic profile of the peripheral blood in PAH-CHD patients, enables a promising opportunity to determine a signature for reversibility that aids therapeutic decisions in the clinic. Prospective trials to address this clinical need are highly warranted.

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5

Shunt Surgery, Right Heart Catheterization and Vascular Morphometry in a Rat model for Flow-induced Pulmonary Arterial Hypertension.

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Abstract.

In this protocol, PAH is induced by combining a 60 mg/kg monocrotalin (MCT) injection with increased pulmonary blood flow through an aorto-caval shunt (MCT+Flow). The shunt is created by inserting an 18 G needle from the abdominal aorta into the adjacent caval vein. Increased pulmonary flow has been demonstrated an essential trigger for a severe form of PAH with distinct phases of disease progression, characterized by early medial hypertrophy, followed by neointimal lesions and progressive occlusion of the small pulmonary vessels. To measure right heart and pulmonary hemodynamics in this model, right heart catheterization is performed by inserting a rigid cannula containing a flexible ball-tip catheter via the right jugular vein into the right ventricle. The catheter is then advanced into the main and the more distal pulmonary arteries. The histopathology of the pulmonary vasculature is assessed qualitatively by scoring pre- and intra acinar vessels on the degree of muscularization and the presence of a neointima; and quantitatively by measuring wall thickness, wall-lumen ratios and occlusion score.

Introduction.

The goal of this method is to create a reproducible model for severe, flow-induced pulmonary arterial hypertension in rats and measure its principle hemodynamic and histopathological end points. Pulmonary arterial hypertension (PAH) is a clinical syndrome that encompasses a progressive increase in pulmonary vascular resistance leading to right ventricular failure and death. Within the superordinate disease spectrum of pulmonary hypertensive diseases (PH), PAH is the most severe form and one that remains without a cure¹. The underlying arteriopathy in PAH is characterized by a typical form of vascular remodeling that occludes the vessel lumen. Muscularization of normally non-muscularized vessels and hypertrophy of the medial vessel layer are regarded an early disease phenomenon in PAH, are also seen in other forms of PH², and are thought to be reversible³. As PAH advances, the intimal layer begins to remodel, eventually forming characteristic neointimal lesions². Neointimal-type pulmonary vascular remodeling is exclusive to PAH and currently regarded to be irreversible⁴.

As PAH is a rare disease, advances in its pathobiological comprehension and development of novel therapies have relied heavily on animal models. The monocrotalin (MCT) model in rats is a simple *single hit* model that has been, and still is used frequently. MCT is a toxin that causes injury to the pulmonary arterioles and regional inflammation⁵. 60 mg/kg MCT leads to an increase in mean pulmonary artery pressure (mPAP), pulmonary vascular resistance (PVR) and right ventricular hypertrophy (RVH) by 3-4 weeks⁶. The histomorphology is characterized by isolated medial hypertrophy, without neointimal lesions⁵. The MCT rat model thus represents a moderate form of PH, and not PAH, although it is commonly presented as the latter.

In children with PAH associated with a congenital left-to-right shunt (PAH-CHD), increased pulmonary blood flow is regarded as the essential trigger for the development of neointimal lesions⁷⁻⁹. In rats, increased pulmonary blood flow can be induced by the creation of a shunt between the abdominal aorta and vena cava, a technique first described in 1990¹⁰. Alternatives to create increased pulmonary flow are by unilateral pneumonectomy or subclavian to pulmonary artery anastomosis¹¹. Conceptual disadvantages of these models consists of potential compensatory growth of the remaining lung and adaptive pathway activation induced by pneumonectomy; or of iatrogenic injury of the pulmonary vasculature due to pulmonary artery anastomosis, both confounding the mere effects of increased pulmonary blood flow.

When an aorto-caval shunt is created and increased pulmonary blood flow is induced as a second hit in MCT-treated rats, characteristic neointimal lesions occur and a severe form of PAH and associated right ventricular failure (RVF) develops in 3 weeks after increased flow¹². The hemodynamic progression of PAH in this model can be assessed *in vivo* by

echocardiography and right heart catheterization. The vascular histomorphology, vessel wall thickness, degree of arteriolar occlusion and parameters for right ventricular failure form the pillars of the *ex vivo* characterization of PAH.

This method describes detailed protocols for the aorto-caval shunt (AC-shunt) surgery, right heart catheterization and qualitative and quantitative assessment of the vascular histomorphology.

Protocols.

Procedures involving animal subjects have been approved by the Dutch Central Committee Animal Experiments and the Animal Care Committee at University Medical Center Groningen (NL). Both Wistar and Lewis rats with weights between 180 and 300 g were used.

1. Housing and Acclimatization.

1.1 After arrival at the central animal facility, house rats in groups of 5 per cage. During a 7-day acclimatization period, accustom the rats to human handling, but do not perform any experimental procedures.

2. Preparation and injection of Monocrotalin.

2.1 For 1 ml of 60 mg/ml monocrotalin (MCT) solution, weigh 60 mg monocrotalin in a 2 ml tube. Add 700 μ l NaCl 0.9 %. Add 200 μ l 1M HCl. Warm the solution in the tube in hot running tap water and vortex. Use 6 N NaOH to bring the pH towards 7.0.

2.2 Inject 1 ml of 60 mg/ml MCT solution per kg, subcutaneously in the neck (0.3 ml 60 mg/ml MCT for a rat of 300 g).

NOTE: We prefer not to use smaller volumes due to the greater chance that the injected dose will not be appropriate.

3. Aorta-caval shunt surgery.

3.1 Anesthesia.

3.1.1 Fill the induction chamber with 5 % isoflurane/ 100 % O₂ (flow 1 L/min) and place the rat in the chamber. Check adequate depth of the anesthesia by hind toe pinch. Weigh the rat.

3.1.2 Shave and clean the abdomen over an area of approximately 8 cm length and 3 cm width. Place the rat on its back on a heat mat (37 °C), covered by a sterile mat.

3.1.3 Place the snout in a ventilation mask/hood with 2-3 % isofluorane/100 % O₂ (flow 1 L/min). Check depth of anesthesia by hind toe pinch. Apply eye ointment to prevent dryness while under anesthesia.

3.2. *Shunt Surgery.*

3.2.1 Scrub the skin with chloride-hexidine for disinfection Inject 0.01 mg/kg Buprenorphin subcutaneously, for post-operative analgesia.

3.2.2 Make an incision with a #10 scalpel blade in the abdomen on the midline, starting 1 cm below the diaphragm down to just above the genitalia.

3.2.3 Lift up the intestine with a cotton swab, cover intestines in a sterile wet gauze (0.9 % NaCl) and place to the left side of the animal.

3.2.4 Use cotton swabs to separate the membranes that attach the abdominal aorta and the vena cava inferior to the surrounding tissues.

NOTE: Do not dissect the membranes between the aorta and the vena cava.

3.2.5 Using splinter forceps remove the perivascular aortic fat just above the bifurcation only on the right side of the aorta, only on the site where the needle will be inserted.

3.2.6 Use cotton swabs to separate the aorta and vena cava from 2 mm superior to the site where the needle will be inserted, to create space for a Biemer clamp.

3.2.7 At this area, first place a loose ligature (5-0 suture) around the aorta. Create tension on the ligature by placing a Kocher clamp on it, then place the Kocher superior to the incision (figure 5.1A). Place the Biemer clamp just superior to the ligature (figure 5.1A).

3.2.8 Using a cotton swab, compress the vena cava as distally as possible, to obstruct the flow (figure 1A). Bend a needle (18 G in this protocol) into an angle of 45 degrees, with the orifice pointing towards the outside (figure 5.1A).

3.2.9 At an angle of 90 degrees, insert the needle in the aorta, just above the bifurcation, with the orifice of the needle pointing to the left (figure 5.1A). Manipulate the tip of the needle to the left and insert in the vena cava.

NOTE: The needle tip should now be visible in the vena cava (figure 5.1B).

Figure 5.1. Schematic representation of the aorto-caval shunt surgery. A) the aorta is tensioned and clamped superior to the insertion site. The vena cava is compressed inferior to the insertion site. The needle (bent in 45° with the orifice to the outside), is inserted into the aorta in a 90° angle. B) The needle positioned in the aorta, the tip is inserted into the vena cava.

3.2.10 Use a second cotton swab to push the remaining blood in the aorta out of the insertion site to prevent thrombosis. Dry the area around the shunt with a sterile gauze, in order for the glue to stick adequately.

3.2.11 Pull back the entire needle out of the aorta and immediately apply a drop of tissue glue on the puncture site in the aorta. Make sure not to glue the cotton swab to the tissue. Unclamp the aorta.

3.2.12 Verify the shunt manually by pulling on and releasing the ligature on the aorta proximal to the shunt. Loosening should color the vena cava distal to the shunt in bright red and create turbulence at the shunt site.

NOTE: Tightening will turn the blood in the vena cava back to dark red.

3.2.13 Place the intestines back in the animal. Close the muscle layer and skin with resorbable 4-0 sutures. Ventilate the animal with 100 % O₂ to recover from anesthesia.

NOTE: Do not leave an animal unattended until it has regained sufficient consciousness to maintain sternal recumbency.

3.3 Sham Surgery.

3.3.1 Perform all of the above, except insertion of the needle into the aorta.

3.4 Post-surgical care.

3.4.1 Place the rat in a single cage and into an incubator at 37 °C until the next morning.

3.4.2 Around 6 hours after surgery, inject 0.01 mg/kg Buprenorfin subcutaneously, for post-operative analgesia. Repeat next morning if the rat shows signs of discomfort.

NOTE: The first 3 days after surgery, rats tend to eat and drink less (this is particularly important when chow or drinking water are mixed with drugs). Most rats show normal behaviour from 3 days after surgery. If not, monitor closely. Weight loss exceeding 15 % in 1 week is considered abnormal and such rats will be euthanized, by extraction of the circulating blood volume while under anesthesia.

4. Development of PAH.

NOTE: In this protocol the animal is euthanized by extraction of the circulating blood volume while under anesthesia.

4.1 Sacrifice 1 day after shunt (MF8) for the early cellular and functional responses to increased pulmonary blood flow, e.g. gene up-regulation or early transcription factors.

4.2 Sacrifice 1 week after shunt (MF14) for an early-stage PAH vascular phenotype (medial hypertrophy without neo-intimal lesions).

4.3 Sacrifice 2 weeks after shunt (MF21) for an advanced-stage PAH vascular phenotype (marked medial hypertrophy and neointimal formation) with mild elevation in RVP and mPAP.

4.4 Sacrifice 3 weeks after shunt (MF28) for an end-stage PAH vascular phenotype (marked neo-intimal occlusion) and strong elevation in RVP and mPAP. Clinical signs of right ventricular failure are common in this stage.

4.5 Sacrifice after day 28 (MF-RVF) for PAH-associated right ventricular failure (RVF), clinically defined as dyspnea, severe lethargy and weight loss (<10 % in 1 week). Terminate rats when one of these signs is present. Frequently, rats develop these symptoms between day 28 and 35 and if left unguarded rats die spontaneously during this time interval.

5. Right heart catheterization.

5.1 Anesthesia

5.1.1. Fill the induction chamber with 5% isoflurane/ 100 % O₂ (flow 1 L/min) and place the rat in the box. Check adequate depth of the anesthesia by hind toe pinch. Weigh the rat.

5.1.2 Shave and clean the neck at the right-ventral side of the rat and (for the echocardiography protocol) the thorax and upper abdomen.

5.1.3 Place the rat on its back on a heat mat (37 °C), place the snout in a ventilation mask/hood with 2-3% isofluorane/100% O₂ (flow 1 L/min). The snout should be faced towards the researcher.

5.1.4 Check depth of anesthesia. Be careful with rats with severe PH, if heart rate decreases, reduce depth of anesthesia. Perform all measurements preferably within 20 minutes. Apply eye ointment to prevent dryness while under anesthesia.

5.2 Echocardiography protocol.

5.2.1 Perform the echo according to the protocol described by Brittain et al in JoVE¹³.

5.3 Catheterization protocol.

NOTE: This protocol uses a rigid *cannula* with a preformed tip bent in 20 degrees to guide the 15 cm silicon *catheter* with a ball at 2mm from the tip. A 20 G *needle* with its orifice slightly bent to the inside, is used to insert the cannula into the right jugular vein (see list of materials). Rats in any phase of PAH progression and control can be used in this protocol.

5.3.1 Disinfect the neck with chloride-hexidine. Make an incision of 1.5 cm with a #10 scalpel blade in the right-ventral side of the neck, from the right collar bone to the mandibular bone.

5.3.2 Spread the tissue using scissors. Using tweezers, gently pull the tissue apart until the jugular vein appears. Dissect the membranes around the jugular vein using splinter forceps.

5.3.3 Put tension on the jugular vein by placing a loose ligature (5-0suture) around the vessel. Tension and tape the ligature onto the ventilation mask (figure 5.2A).

5.3.4 Downstream of the insertion site, place a loose ligature around the vessel to tighten after the cannula is *in situ*, in order to prevent leakage and loss of pressure.

5.3.5 Using the handles of a forceps, slightly bend the tip of a 20 G needle with the orifice to the inside, to conduct the cannula with the catheter.

5.3.6 Introduce the tip of the 20 G needle in the vein, and quickly place the cannula containing the catheter inside the vessel. Pull out the needle, then close the ligature that was prepared in step 5.3.4.

5.3.7 Conduct the cannula containing the catheter in the jugular vein. The tip of the cannula is preformed in a curve of 20 degrees. Maneuver the cannula under the collar bone and advance a little to enter the right atrium (figure 5.2C).

5.3.8 To enter the right ventricle, point the tip of the cannula to the left, towards the heart (figure 5.2D). On the bedside monitor, an RV pressure curve should appear, matching figure 5.2D.

5.3.9 When the RV pressure curve is constant, write down systolic and diastolic right ventricular pressure 1 (sRVP1/dRVP1).

5.3.10 Manipulate the tip of the cannula to the left and upwards. Now advance the catheter within the cannula (figure 5.2E).

5.3.11 Advance the catheter into the main pulmonary artery (PA). No resistance should be felt when passing the pulmonary valve.

NOTE: When the catheter enters the main pulmonary artery, the diastolic pressure will rise. On the bedside monitor, a PA pressure curve should appear matching figure 5.2E.

5.3.12 When the PA pressure curve is constant, write down systolic, diastolic and mean PA pressure 1 (sPAP1,dPAP1,mPAP1).

5.3.13 Further advance the catheter within the cannula, until the ball at the tip of the catheter gets wedged in a pulmonary artery. Observe the pressure curve on the bedside monitor drop and match the wedge pressure curve in figure 5.2F.

5.3.14 When the Wedge pressure curve is constant, write down systolic, diastolic and mean Wedge pressure.

5.3.15 Now pull back the catheter slowly and measure and write down subsequently the values for sPAP2,dPAP2,mPAP2 and sRVP2, dRVP2 as displayed on the bedside monitor.

5.3.16 When in the RV, slightly pull back the cannula and catheter to measure mean right atrial pressure (RAP). The curve should match the RAP curve in figure 5.2A.

NOTE: In this protocol, rats are euthanized after the catheterization protocol by extraction of the circulating blood volume while under anesthesia.

Figure 5.2. Right heart catheterization procedure and representative pressure curves. A) The right jugular vein is tensioned with a ligature and taped onto the ventilation mask. The catheter is placed into the jugular vein. B) Bed side monitor displaying a Right ventricular pressure wave. C) Catheter within the cannula placed in the right atrium after introduction in the right jugular vein. Below: a typical right atrial pressure wave. D) Catheter within the cannula placed in the right ventricle. Below: a typical right ventricular pressure wave in end-stage PAH. E) Catheter is advanced in the cannula to enter the main pulmonary artery. Below: a typical pulmonary arterial pressure wave. F) Catheter is advanced into the pulmonary arteries until a wedge pressure wave is displayed on the monitor. Below: a typical pulmonary wedge pressure wave.

6. Morphology assessment and morphometry.

NOTE: In this protocol the animal is euthanized by extraction of the circulating blood volume while under anesthesia. Rats in any phase of PAH progression and control can be used in this protocol.

6.1 After sacrifice, take out the lungs by cutting the trachea around 5 mm above the bronchial bifurcation and the vessels that connect the lungs to the heart. Put the lungs in cold saline. Dissect the left lung. Cut the left main bronchus at the bifurcation.

6.2 Fill a 50 mL syringe with 4 % paraformaldehyde, attach a tube with a cannula to the syringe and hang the syringe about a meter above the working table. Fit the cannula in the left main bronchus to passively fill the lung with paraformaldehyde. Handle paraformaldehyde with caution.

6.3 Incubate the left lung in paraformaldehyde for 48 hours.

6.4 Dehydrate the left lung by incubating consecutively in 70 % ethanol (1h), 80 % ethanol (1h), 90 % ethanol (1h), 100% ethanol (3h), xylol (2h), and paraffin (2h).

6.5 Embed the left lung in paraffin, with the hilum of the lung facing the cassette.

6.3 Stain paraffin embedded 4 μ m lung sections using a Verhoeff or Elastica-van Gieson staining. Make sure the elastic laminae are well differentiated (as in figure 5.3). Scan the stained sections at 40 x magnification.

6.4 Divide the lung into 4 quadrants. Per quadrant, find 10 vessels with an outer diameter <50 μ m (intra-acinar), and 10 vessels with an outer diameter >50 μ m (pre-acinar) and take a picture (2 x 40 pictures per lung). Zoom in randomly up to 20x magnification and photograph every vessel in this field of view to minimize selection bias.

6.5 Exclude vessels that have a longest / shortest diameter ratio of >2, an incomplete circular shape or a collapse of more than on quarter of the vessel wall. NOTE: An example of an excluded vessel is shown in figure 5.3b Make each picture on the same magnification (40 x) and include a scale bar.

6.6 Open image-J, and the first picture. Draw a straight line on the scale bar in the picture to set the scale via *Analyse, Set scale*. For 'known distance', use the value on the picture's scale bar. Use micrometers (μ m) as the unit of length. Set the scale to global.

6.7 Using 'freehand selections' draw a line on the inner border of the luminal area (figure 5.3), use *measure (ctrl m)* to measure this area. Then draw a line around the outer elastic lamina (figure 5.3) to measure total vessel area.

6.8 Calculate the luminal and outer diameter ($\varnothing$) using $2 \times \sqrt{\text{area}/\pi}$.

Figure 5.3. Vascular morphology and morphometry in control and PAH rats. A. Normal, non muscularized vessel, occlusion score 3.7 %. B. Totally muscularized arteriole, occlusion score 24.3 %. C. Neointimal lesion, occlusion score 54.1%. D. Excluded vessel (longest / shortest diameter ratio of >2, and incomplete circular shape). E. Measurement of total vessel and luminal area (in a vessel with a schematic representation of a neo-intimal lesion), including calculations. Bars represent 50 μm.

6.9 Calculate wall thickness using $(\text{outer } \varnothing - \text{lumen } \varnothing)/2$.

6.10 Calculate wall/lumen ratio using $\text{wall thickness}/\text{lumen } \varnothing$.

6.11 Calculate occlusion score using $100 \times ((\text{outer } \varnothing - \text{lumen } \varnothing)/\text{outer } \varnothing)$.

6.12 Score the vessel on muscularization (no, partial or total muscularization). (figure 5.3B).

NOTE: Vessels with a double elastic lamina for more than half its circumference are defined totally muscularized. Vessels with a double elastic lamina less than half its circumference are defined partially muscularized.

6.13 Score the vessel on having a neointima (yes or no). (figure 5.3C).

NOTE: Vessels without a clearly defined internal elastic lamina combined with (often eccentric) luminal occlusion are defined as neointimal lesions.

Representative results.

Representative results are presented in figure 5.4. The presented results show characteristics of MCT+FLOW in Lewis rats in the following groups: Control (n=3), MF8 (n=5), MF14 (n=5), MF28 (n=5) and MF-RVF (n=10). Statistical analyses were performed using the one-way ANOVA with bonferroni correction.

60 mg/kg MCT and increased pulmonary blood flow lead to a mean rise in systolic right ventricular pressure (sRVP) (23 ± 6 to 56 ± 11 mmHg); systolic pulmonary artery pressure (sPAP) (20 ± 4 to 54.0 ± 10 mmHg) and mean pulmonary artery pressure (mPAP) (16 ± 3 to 36 ± 4 mmHg) at 28 days (MF28) and remain equally high up to the stage when right ventricular failure develops (MF-RVF) (figure 5.4). At the early PAH stages (MF8 and MF14) no rise in sRVP, sPAP and mPAP is observed. Diastolic PAP and right atrial pressure increase in the late phases, but not significantly. Wedge pressures do not change significantly during disease progression.

Right ventricular to left ventricular and septal weight ratio increases significantly from MF14 to MF-RVF, indicating right ventricular hypertrophy. The liver's wet to dry weight ratio is significantly increased at the MF-RVF stage, indicating liver edema and congestive right ventricular failure.

Muscularization of intra-acinar vessels $< 50 \mu\text{m}$ increases progressively during PAH progression. Vessels of this size normally do not have a muscular medial layer in control rats. At MF14, almost half of these vessels (43 ± 17 %) has a total muscular media (as in figure 5.3B). At MF28 and MF-RVF, nearly every arteriole is muscularized (98.7 ± 2.5 % and 100 ± 0 %). Neointimal lesions first occur at MF21, while at MF28 and MF-RVF around 65% of all arterioles has a neointimal layer (as in figure 5.3C). Arteriolar wall to lumen ratio and

occlusion scores both increase significantly from MF14 to MF28 (respectively 10.4 ± 3.9 to 71.5 ± 30 (con: 7.1 ± 0.2) and 20.0 ± 2.8 to 54.7 ± 10.6 (con: 12.2 ± 0.3)). The hemodynamic and histomorphologic characteristics of PAH progression in MCT+FLOW in Wistar rats are similar¹⁴.

Figure 5.4. Representative results of pulmonary hemodynamics and vascular morphology / morphometry. Statistical analyses were performed using the one-way ANOVA with bonferroni correction. Values are represented as mean $\pm$ SEM. con: control; MF (monocrotalin + flow); RVF: right ventricular failure; s: systolic; d: diastolic; m: mean; RVP: right ventricular pressure; PAP: pulmonary arterial pressure; RAP: right atrial pressure. RV: right ventricle; LV: left ventricle; IVS: interventricular septum. BW: body weight.

Discussion.

This method describes the surgical procedure of an aorto-caval shunt in rats pre-treated with MCT to create flow-induced PAH and the techniques to assess the principle hemodynamic and histopathologic end points that characterize PAH and this model.

Critical steps within the protocol and troubleshooting.

Surgery and post-surgery. During the aorto-caval shunt surgery, the most critical step is the dissection of the aorta and vena cava. The membranes that enclose the aorta and vena cava should be dissected enough to create 1) good visibility of the aortic area where the needle will be inserted, and good visibility of the position of the needle in the vena cava after insertion and 2) sufficient space to clamp the aorta above the insertion site. The same membranes, however, are also used to conduct the aortic blood through the puncture site between both vessels (figure 5.1). Dissecting the membranes too much will cause the shunt to leak. Tissue glue may solve the leakage, but may then also seep into the shunt, compromising its size. When the glue has restricted flow through the shunt or either of the vessels, the glue can be removed gently, but rupture of the vena cava or the membranes that conduct the shunt may occur. The size or adequacy of the shunt can be estimated by comparing the color difference and the degree of turbulence of the blood in the vena cava during compression and decompression of the proximal aorta with a cotton swab.

An 18 G needle has shown to create an adequate shunt, that results in a consistent and reproducible form of PAH progression in Lewis (this article) and Wistar rats (see¹⁴) and right ventricular volume overload¹⁵. An 18 G needle size created the most well-balanced shunt with significantly increased pulmonary flow on the one hand and a low post-operative complication rate on the other hand.

The most common post-surgical problem is weight loss. Weight loss up to 10 % in 1 week occurs in all rats after surgery, presumably due to lower intake the first days postoperatively. Rats are euthanized when the weight loss exceeds 15 % in 1 week, as this is considered a sign of being unwell. Liquid chow can improve feeding in the first week after surgery. Rare post-operative complications are hind leg paralysis and bowel ischemia, which also result in euthanasia. In total, less than 5 % of the rats has to be euthanized post-operatively.

Catheterization.

Critical steps during the catheterization protocol start with the regulation of anesthesia. The depth of the anesthesia should be as minimal as required (1,5-2 % isoflurane in this protocol) as an increase in anesthetic depth appears to decrease right ventricular and

pulmonary artery pressures, especially in rats with right ventricular failure. Measurements have a tendency to become unreliable when the protocol exceeds 20 minutes in duration.

The next critical step is the manipulation of the catheter in the RV and into the main pulmonary artery. This can be challenging. Flushing the catheter can help to curve the catheter in the outflow tract when the tip is stuck in the RV's trabeculae. The manipulation itself can cause RV dyskinesia, which shows irregular pressure curves on the bed side monitor. The introduction of the catheter in the right ventricle and the pulmonary artery should run smoothly. When the tip gets stuck at the pulmonary valve, a resistance is felt. Pressing through this resistance may cause the pulmonary valve to rupture, which limits reliability of subsequent measurements.

In present protocol, rats are sacrificed after the catheterization procedure. In theory however, the jugular vein and surgical wound can be closed after the catheter is pulled out, as animals can live with the remaining left jugular vein only.

Morphometry. In the assessment of vascular wall thickness and occlusion scores, the most critical step is to identify the elastic laminae. From experience, the likelihood of success to this end is greatest with a well-differentiated Verhoeff or elastica-van Gieson staining. While the lumen can usually be discerned easily from the intima (to measure internal vascular area), distinction of the media from the adventitia may require a closer look (to measure external vascular area). Some protocols measure intimal and medial thickness separately, defining the intima as the layer between the lumen and the internal elastic lamina, and the media as the layer between the internal and outer elastic lamina. This is usually possible in early-stage MCT+FLOW PAH. However, arterioles in advanced disease, neointimal lesions particularly, may display multiple elastic laminae and often lose integrity of the elastic laminae (figure 5.3C). In advanced lesions, the internal lamina elastica is therefore often difficult to identify, hindering distinction between media and (neo-) intima. This reticulation of the intimal and medial layer prohibits separate layer thickness assessment. Therefore, the use of total vessel wall thickness (to lumen ratio) and occlusion scores are preferred as an end point, instead of separate intimal and medial thicknesses.

Advantages and limitations of adding flow as a trigger.

The use of increased pulmonary blood flow to create PAH in rats has several advantages, the most prominent being that it is a known (patho-)physiological trigger for the disease, which favors translation to human PAH-CHD (Eisenmenger physiology), but also to other forms of PAH⁹. The model allows for regulation of the flow by varying the size of the needle when creating the AC-shunt.

In human PAH-CHD, closure of the shunt will lead to reversal of PAH in the early phase of disease, but to progression of PAH in advanced disease stages. Closure of the shunt *in vivo* would allow to investigate the effect of removal of the trigger at different time points of disease progression and thus to investigate the mechanisms of (non-) reversal of PAH. Unfortunately, at present, shunt closure is not feasible in the current model. The effects of hemodynamic normalization (e.g. removal of excess flow and normalization of pulmonary artery pressure) in rats with flow-associated PAH, can be investigated by transplantation of the affected left lung into a recipient rat with a normal circulation. It has been shown previously that hemodynamic normalization, in rats by lung transplantation and in human PAH-CHD by closure of a cardiac shunt, leads to regression of medial hypertrophy in early-stage PAH²¹. The effects of hemodynamic normalization in advanced stages of experimental flow-PAH are currently unknown.

Significance with respect to alternative models.

The single hit MCT model. A subcutaneous injection of 60 mg/kg MCT is a simple and effective way to create a model for pulmonary hypertension in rats. MCT induces pulmonary arterial endothelial cell injury, followed by hypertrophy of the muscular layer of the pulmonary arteries⁵. Although the exact mechanisms remain unclear, various pathways and growth factors have been identified that participate in medial hypertrophy following MCT. Pharmaceutical intervention upon these pathways has often successfully reduced medial hypertrophy and mPAP in MCT-rats. However, since medial hypertrophy is known to have a natural tendency to reverse in humans³, and has also been described to reverse spontaneously in MCT-rats¹⁶, the effect of these treatments should be appraised critically.

The double hit MCT+FLOW model. The addition of increased pulmonary blood flow 7 days after MCT injection critically alters the (vascular) phenotype in a characteristic time-dependent fashion. At MF14 (7 days after induction of increased flow), normally non-muscularized vessels start to develop a muscular medial layer; At MF21, medial thickness increases and the first neointimal lesions occur; at MF28 a neointimal layer has developed in the majority of vessels; between MF28 and MF35 most rats develop right heart failure and die of its sequelae. Previous studies in MCT+Flow rats have shown that the addition of flow to MCT leads to activation of specific clusters of genes. In some clusters, flow opposed the effects induced by MCT; in others, flow enhanced these effects, and one cluster contained genes that were specifically up regulated after flow¹⁷. One of those flow-specific genes is the early growth response-1 gene¹⁴ (Egr-1). Early inhibition of Egr-1 resulted in attenuation of PAH and neointimal formation in MCT+Flow rats¹⁸. Egr-1 was also associated with neointimal remodeling in human PAH (PAH-CHD and idiopathic PAH)¹⁹. These observations add to the evidence that increased or disturbed pulmonary blood flow is an essential trigger for neointimal formation.

The single hit Flow-only model. In rats with an aorto-caval shunt without MCT-injection, pulmonary hypertension (mPAP > 25 mmHg) develops between 10 and 20 weeks after shunt induction²⁰. At 20 weeks, the pulmonary vascular histology is dominated by medial hypertrophy of the pre-acinar arteries and neo-muscularization of intra-acinar arterioles. Although some neointimal lesions have also been described in this model²⁰, development of these lesions needs to be confirmed and quantified.

The Sugen-Hypoxia model. Another common model for PAH with neointimal lesions is the Sugen5416-Hypoxia (SuHx) rat. Sugen5416 blocks the vascular endothelial growth factor (VEGF) receptor. This induces endothelial cell damage and a signaling cascade that, in combination with hypoxia, evokes endothelial apoptosis and proliferation²². After Sugen5416 injection, the rat is placed in a hypoxic chamber for 4 weeks, upon which PAH develops. The rat is then re-exposed to normoxia for 4 weeks. Pharmacological compounds that target endothelial apoptosis-resistance or the signaling cascades of TGF- β and BMP have shown potential to reverse neointimal lesions in this model²³⁻²⁵. A new variant of the SuHx model is the Sugen-pneumectomy model, which also results in severe PAH with neointimal lesions²⁶. This model has, however, not been fully characterized yet. A novel genetic method to induce PH in rats involves a mutation in the BMP-receptor-2 gene, which results in significant muscularization (PH), but no neointimal formation (PAH)²⁷.

Comparable results have been reported regarding the number of neointimal lesions and the degree of luminal occlusion in the end-stage of untreated SuHx and MCT+Flow rats²⁸. The main differences between both models are 1) that the mPAP in MCT+Flow progressively increases, whereas in SuHx, the mPAP has shown to decrease gradually after re-exposure to normoxia²⁸; 2) that the MCT+Flow model knows an early disease stage, characterized by medial hypertrophy and endothelial dysfunction, 3) the time it takes both models to reach an end-stage where right ventricular failure begins to develop (4 weeks in MCT+flow, 8 weeks in SuHx)²⁸; and 4) that Sugen5416 interferes in a molecular pathway (VEGF) which role in the pathogenesis of PAH is still unclear. This may hinder translation to human PAH.

Future applications or directions.

The distinct disease phases of the MCT+Flow model allow 1) to test mechanisms of disease progression (human tissue in general is only available from post-mortem or explant procedures) and 2) to test interventions in different strategies. A preventive strategy could be initiated at the construction of the shunt (MF7). An early intervention can be initiated at MF14. This may be relevant as a treatment strategy prior to shunt closure in children with a congenital cardiac shunt and associated PAH that has progressed into the grey zone between reversible and irreversible disease. Reversal strategies can be initiated at MF21 or MF28. Latter stages both show neointimal lesions, a manifestation of end-stage PAH.

Conclusion.

In conclusion, the addition of increased pulmonary flow to MCT in rats, creates a model of progressive and severe PAH that mimics human disease development. Right heart catheterization and the qualitative and quantitative assessment of the vascular histopathology form the cornerstones of the disease characterization in this and other models for PAH.

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Cellular senescence impairs the reversibility of pulmonary arterial hypertension.

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Abstract.

Pulmonary arterial hypertension (PAH) in congenital cardiac shunts can be reversed by hemodynamic unloading (HU) through shunt closure. However, this reversibility potential is lost beyond a certain point in time. The reason why PAH becomes irreversible is unknown. In this study we used MCT+shunt-induced PAH in rats to identify a dichotomous reversibility response to HU, similar to the human situation. We compared vascular profiles of reversible and irreversible PAH using RNA sequencing. Cumulatively, we report that loss of reversibility is associated with a switch from a proliferative to a senescent vascular phenotype, and confirmed markers of senescence in human PAH-CHD tissue. In vitro we showed that human pulmonary endothelial cells of PAH patients are more vulnerable to senescence than controls in response to shear stress, and confirmed that the senolytic ABT263 induces apoptosis in senescent, but not in normal endothelial cells. To support the concept that vascular cell senescence is causal to the irreversible nature of end-stage PAH, we targeted senescence using ABT263 and induced reversal of the hemodynamic and structural changes associated with severe PAH refractory to HU. The factors that drive the transition from reversible to irreversible pulmonary vascular phenotype could also explain the irreversible nature of other PAH etiologies and provide new leads for pharmacological reversal of end-stage PAH.

Introduction.

Pulmonary arterial hypertension (PAH) is a progressive vasculopathy of the pulmonary arteries that has no cure. The disease is characterized by a process of occlusive arteriolar remodeling that causes increased pulmonary vascular resistance culminating in symptoms of right ventricular (RV) failure(1). Contemporary vasodilator therapies may improve pulmonary hemodynamics and decelerate progression by alleviating RV afterload, but cannot reverse the arteriopathy(2).

PAH associated with congenital heart disease (CHD) with a left-to-right shunt has a distinct reversible stage(3) during which shunt closure [hemodynamic unloading (HU)] restores normal pulmonary blood flow and reverses the vasculopathy. However, there is a point beyond which the potential benefit of HU is lost, and pathologic remodeling and the elevation in pulmonary vascular resistance persist or progress(3). These observations have led to the recommendation that CHD associated with PAH (PAH-CHD) should be repaired in early infancy. However, not all children with PAH-CHD are referred for a surgical intervention in a timely manner, and for many of those -- even for some infants -- it has been difficult to clearly distinguish those with irreversible from reversible PAH on the basis of hemodynamic assessment(4). The specific factors that drive the transition from reversible to irreversible PAH-CHD could also explain the irreversible nature of other PAH etiologies, potentially providing new leads for pharmacological reversal of end-stage PAH(3).

Multiple preclinical studies have thus far confirmed that HU can reverse vascular changes associated with PAH(5–7). Our study design was based on previous work, in which PH was induced in rats by pyrrolizidine alkaloid monocrotaline (MCT, 60 mg/kg), and HU was carried out 28 days after MCT injection by transplanting the left lung with MCT-PAH into a normotensive recipient(5). Latter study showed that the vascular changes induced by MCT at 28 days, medial hypertrophy and endothelial cell proliferation, are reversed 14 and 28 days after HU.

Here, we used an established PAH rat model that combines an MCT (60 mg/kg) injection with an aorto-caval shunt, which doubles pulmonary blood flow(8, 9), leading to a neointimal-type of vascular remodeling that is similar to human PAH. HU was carried out at different time points during the progression of PAH (14, 21, and 28 days after MCT injection) by transplanting the left lung with PAH into a normotensive recipient without a shunt (Fig. 6.1A). In partial contrast with aforementioned preclinical research(5–7), but similar to clinical observations in PAH-CHD(3, 10), we found that HU could reverse the early, but not the late stages of MCT+shunt PAH. This enabled us to characterize transcriptomic and vascular expression profiles that distinguish a reversible from an irreversible response to

HU. RNA sequencing indicated that a pattern of cellular senescence was associated with HU-irreversibility of PAH. By targeting senescence using the senolytic ABT263, we reversed MCT+shunt-induced PAH that was refractory to HU.

Results.

MCT+Shunt-induced PAH and hemodynamic unloading in rats.

According to a previously published protocol (9), MCT+shunt-induced PAH was created in 35 adult male syngeneic Lewis rats (200 g) by a subcutaneous injection of MCT (60 mg/kg, T0), followed by aorto-caval shunt surgery 7 days later (8, 9). We used 3 time points of PAH progression previously defined (11) to establish the following groups: MCT+shunt (MS) day 14 (MS14) (n=10), MS21 (n=15), and MS28 (n=10). 6 rats were injected with saline and served as sham-operated controls. For full hemodynamic characterization of these time points, 5 PAH rats per group and 3 controls were evaluated by echocardiography (echo) and right heart catheterization before sacrifice (9). The other MS14, MS21, and MS28 rats (n=5, 10, and 5, respectively) and controls (n=3) served as donors for a unilateral orthotopic left lung transplantation (12) (LTx) into a syngeneic recipient rat without a shunt (Fig. 6.1A). Recipient rats are referred to as hemodynamically unloaded (HU) by their transplanted lung disease stage at LTx: HU14 (n=5), HU21 (n=10), HU28 (n=5), and HUcon (n=3).

First, an echo was performed in the donor rats. Then the left donor lung with PAH was transplanted into a healthy recipient Lewis rat (250 g). The middle lobe of the right donor lung was used to assess baseline vascular morphology at HU. One recipient rat of the HU14 group was sacrificed 1 week after LTx due to surgical complications. This rat and its donor were excluded from all analyses. All other recipients fully recovered within 2 days after LTx. 21 days after LTx, all recipients underwent hemodynamic evaluation by echo and heart catheterization, followed by sacrifice. The left transplanted lung was used to assess the effect of HU on vascular morphology.

Hemodynamic unloading reverses early but not late stages of MCT+Shunt-induced PAH.

MCT+shunt progressively increased systolic right ventricular pressure (sRVP) and mean pulmonary artery pressure (mPAP). Pulmonary artery acceleration time (PAAT) was decreased at MS21 and MS28, also indicating pulmonary hypertension (Fig. 6.1B). At MS14, cardiac output (CO) and tricuspid annular plane systolic excursion (TAPSE) were increased compared to controls, indicating adequate ventricular adaptation to volume overload. CO decreased after MS21, which, combined with the increased sRVP and mPAP, reflects elevated pulmonary vascular resistance. TAPSE also decreased after MS21, reflecting RV dysfunction. The relative RV weight and liver wet-dry ratio progressively increased dur-

ing PAH progression, indicating RV hypertrophy and decompensation. Severe dyspnoea, fatigue, pleural oedema and ascites were observed in 60 % of the rats at day 28. All recipient rats (without a shunt and with a left lung with different PAH stages) had normal sRVP, mPAP, and CO at sacrifice, 21 days after HU. PAAT and TAPSE were decreased and relative RV weight was increased at HU28 versus HU14, which might reflect residual pulmonary hypertension (Fig. 6.1B).

In control donors, most intra-acinar vessels were non-muscularized. LTx did not affect the normal aspect of the vascular morphology. In MS14-donors, vascular occlusion was increased compared to control, most intra-acinar vessels (83 ± 13 %) were muscularized, but neointimal lesions were scarce (5 ± 7 %). HU at day 14 reversed vascular occlusion in the transplanted lung, which was associated with a decreased percentage of muscularized vessels (Fig. 6.1, C to D). In MS21-donors, vascular occlusion further increased compared to MS14. 67 ± 15 % vessels showed isolated muscularization, and in 30 ± 17 % a neointima was observed. The effect of HU at day 21 was dichotomous. In 7/10 HU21-recipients, vascular occlusion and the percentage of vessels with muscularization or a neointima decreased, indicating reversal in this subgroup. Conversely, in the other 3/10 HU21-recipients, vascular occlusion and the percentage of vessels with a neointima increased, indicating progression of PAH. In MS28-donors, vascular occlusion and the percentage of vessels with a neointima further increased compared to MS21. The percentage of vessels with isolated muscularization decreased compared to MS21, which can be attributed to internal elastic laminae disintegration and lack of a separate, discernible media layer. HU at this stage did not reverse vascular remodeling: Rather, the percentage of vessels with a neointima further increased (Fig. 6.1, C to D). In conclusion, HU led to partial reversal or complete normalisation in all MS14- and 7 out of 10 MS21-donors. HU did not lead to reversal in all MS28- and 3 out of 10 MS21-donors.

Vascular profiles associated with the reversibility of MCT+Shunt-induced PAH.

To study profiles associated with PAH reversibility, rats were reassigned on the basis of response to HU, leading to the following groups: normal controls ($n=5$), reversible PAH at HU (right donor lung, $n=11$), irreversible PAH at HU (right donor lung, $n=8$), reversed PAH 21 days after HU (transplanted lung $n=11$), and non-reversed/progressed PAH after HU (transplanted lung, $n=8$). Next generation RNA-sequencing (RNAseq) was performed on all donor right lung tissue. 1 irreversible PAH sample was excluded after quality control. Principle component analysis (Fig. 6.2A) showed differential clustering of reversible versus irreversible PAH in component 1, and clustering of controls versus both PAH stages in component 2. At cut-offs of >1 log₂ fold change (LogFC) and $q < 0.05$, a total of 751 genes were differentially expressed, of which 82 were increased and 9 were decreased in reversible PAH only; 150 were increased and 298 decreased in irreversible PAH only; and 181 were

Fig. 6.1. Study design and hemodynamic and vascular morphologic evaluation. (A) Experimental design. Step 1: PAH was induced by an injection of monocrotaline [MCT, 60 mg/kg, day 0 (T0)] and creation of an aorto-caval shunt, which increases cardiac output (CO) twofold (see Fig. 6.1B). Step 2: Donor rats developed early, advanced, and end-stage PAH after 14, 21, and 28 days, respectively. Step 3: The left lung of a normotensive, healthy recipient rat was explanted (LR). Step 4: To mimic hemodynamic unloading (HU), the left donor lung

(LD) with different PAH stages was transplanted into the recipient. Step 5: The right donor lung (RD) was explanted, and used to assess the PAH stage at HU. The donor rat was sacrificed. Step 6: 21 days after transplantation, the recipient rat was sacrificed. The transplanted lung (LTx) was used to assess the effect of haemodynamic unloading (HU) of lungs with the different stages of PAH. RV: right ventricular; RR: right lung of recipient rat. (B) Hemodynamic evaluation. The variables measured in the donor rat at the time point of HU are indicated in black, those in the recipient 21 days after HU are indicated in teal. N=5 per PAH group, n=3 for controls. sRVP: systolic right ventricular pressure; mPAP: mean pulmonary artery pressure; CO: cardiac output; TAPSE: tricuspid annular plain systolic excursion; PAAT: pulmonary artery acceleration time; RV/LV+IVS: right ventricular to left ventricular plus septal weight ratio. Red dotted line in mPAP panels indicates pulmonary hypertension (C) Quantification of vascular remodeling and (D) representative examples of Verhoeff staining. Left: Representative examples of the intra-acinar vessels at the different time points of PAH progression, showing progressive medial hypertrophy (MS14, MS21) and the development of a neointima (MS21, MS28). Right: Representative examples of the effect of HU after 21 days, showing normalization of the MS14 and some MS21 stages, and progressive remodeling, leading to acellular neointimal fibrosis of the other MS21 and all MS28 PAH stages. Data are reported as mean $\pm$ SD. Statistics by one-way ANOVA with Bonferroni correction or Kruskal-Wallis with Dunn's correction. Relevant significant differences are indicated with a black bar and asterisk. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001

increased and 31 decreased in both PAH stages compared to control (Fig. 6.2B). Pathways and biological processes associated with reversible PAH included those related to proliferation and DNA-repair. Pathways and biological processes associated with irreversible PAH included those related to immunologic responses, tissue remodeling, aging, and cell survival-associated pathways such as the *Rap1*, *PI3k-Akt*, and *HIF1* signalling (Fig. 6.2C). Pathways related to inflammatory responses, chemokine signalling, *AGE-RAGE*, and *p53* pathways were observed in reversible as well as in irreversible stages of PAH.

Using cellular deconvolution -- a method to assess the proportion of specific cells in mixed-cell tissue(13) -- we found a decrease in the endothelial cell (EC) proportion in reversible PAH versus control, which further decreased in irreversible PAH, indicating endothelial cell death. The smooth muscle cell (SMC) proportion was increased in both PAH stages versus control, indicating neomuscularization and medial hypertrophy. The proportion of fibroblasts was increased in reversible PAH and increased further in irreversible PAH, indicating progressive fibrosis (Fig. 6.2D). Deconvolution for immune cells showed an increased CD4+/CD8+ T-cell ratio in reversible PAH, which inverted in irreversible PAH (Fig. 6.2E). CD4+/CD8+ inversion is associated with HIV infection, auto-immunity, and immunosenescence(14, 15). There were no significant differences in proportions of other immune cells.

Pathway analysis indicated that reversible PAH is characterized by increased proliferation and DNA repair. Cellular deconvolution additionally indicated increased endothelial apoptosis in reversible PAH. The differentially expressed genes (DEGs) ($q < 0.05$; $> 1.5\log_2FC$) associated to these processes (cell proliferation GO:0008283; apoptotic processes GO:0006915;

Fig. 6.2. RNA-sequencing analysis and cellular deconvolution of rat lung tissue. (A) Principle component analysis showing clustering of reversible versus irreversible PAH in component 1. (B) Venn diagram of differentially expressed genes (DEGs) at $q < 0.01$ and $> 2\text{Log}_2\text{FC}$ cut-offs. (C) List of 20 most enriched biological processes associated to reversible or irreversible PAH only (pathway analysis). (D) Deconvolution for vascular cells, analyzing endothelial cell (EC) versus smooth muscle cell (SMC) and fibroblast (Fibrobl) proportions. Con: N=5, Reversible: N=11, Irreversible N=7. (E) Deconvolution for immune cells, showing CD4^+ T cells versus CD8^+ T cells (left) and $\text{CD4}^+/\text{CD8}^+$ T cell ratios (right). Con: N=5, Reversible: N=11, Irreversible N=7. Statistical analyses [A-C] performed on raw reads using pathway analysis algorithm in R. Cellular deconvolution analyses [D-E] performed on raw reads using the support vector regression algorithm in R, also see cellular deconvolution (materials and methods). Data are reported as mean $\pm$ SD. Statistical differences in [D-E] were assessed by one-way ANOVA with Bonferroni correction. Relevant significant differences are indicated with a black bar and asterisk. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$

DNA repair GO:0006281) are visualized in (Fig. 6.3, A-C). The expression of *BMPR2*, a hallmark gene of PAH, was downregulated upon transition towards irreversible PAH (Fig. 6.3D). We confirmed and quantified the roles of proliferation, apoptosis, and DNA damage by immunohistochemistry (Fig. 6.3, E-F). The expression of Ki67 (proliferation) and cleaved-caspase3 (apoptosis) was increased in the endothelium of reversible PAH compared to control and irreversible PAH, reflecting high EC turnover. Survivin, a protein associated with apoptosis-resistance, was increased in irreversible versus reversible PAH. Survivin expression was also located in the endothelium. Rad51, the top DEG for DNA repair, was increased in reversible PAH compared to control and irreversible PAH. Nuclear Rad51 foci were present in the endothelium of reversible PAH and absent in irreversible PAH.

Fig. 6.3. Vascular profiles associated to the reversibility of PAH in MCT+Shunt rats. (A-D) Functional categorization of differentially expressed genes at $q < 0.01$ and $> 1 \text{Log}_2 \text{FC}$ cut-offs in lungs from controls and rats with reversible or irreversible PAH, made using the heatmap algorithm in R. (E) Representative immunohistochemistry of the intra-acinar vessels stained for Ki67, cleaved caspase3, survivin, and rad51 in lungs from controls and rats with reversible PAH and irreversible PAH at HU and 21 days after HU. (F) Quantification of vascular expression from (E). Black bars represent the expression at HU, green bars represent the expression 21 days after HU. Vascular localisation of expression per group is represented in the pie charts [white: no expression; light grey: intima (i); dark grey: media (m); black: perivascular/adventitia (a)]. con=n=3, reversible n=7, irreversible n=7. Data are reported as mean $\pm$ SD. Statistics by one-way ANOVA with Bonferroni correction or Kruskal-Wallis with Dunn's correction. Relevant significant differences are indicated with a black bar and asterisk. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$

In reversible PAH, all proteins that were induced during PAH development were normalized by HU, except for cleaved-caspase3, which was decreased but remained higher than controls. Cleaved-caspase3 was located predominantly in the media (Fig. 6.3, E-F), possibly reflecting regression of medial muscularization. In irreversible PAH, HU did not affect the expression of the proteins that were assessed.

This transition from a hyper proliferative, pro-apoptotic phenotype to a growth arrested, apoptosis-resistant, and pro-inflammatory milieu in the context of DNA damage suggested a role for cellular senescence in the irreversibility of PAH. DEGs associated with cellular senescence (GO:0090398) and the senescence-associated secretory phenotype (SASP)(16,

Fig. 6.4. Vascular profiles associated to the irreversibility of PAH. (A-B) Functional categorization of differentially expressed genes at $q < 0.01$ and $> 1\text{Log}_2\text{FC}$ cut-offs in lung tissue from controls and rats with reversible or irreversible PAH, made using the heatmap algorithm in R. SASP: senescence-associated secretory phenotype. (C) Representative immunohistochemistry of the intra-acinar vessels stained for expression of p16^{ink4A}, p21^{cip1}, MMP2, IL6, and TNFα in lungs of controls and rats with reversible PAH and irreversible PAH at HU and 21 days after HU. (D) Quantification of vascular expression from (C). Black bars represent the expression at HU, green bars represent the expression 21 days after HU. Vascular localisation of expression per group is represented in the pie charts [white: no expression; light grey: intima (i); dark grey: media (m); black: perivascular/adventitia (a)]. con n=3, reversible n=7, irreversible n=7. Data are reported as mean $\pm$ SD. Statistics by one-way ANOVA with Bonferroni correction or Kruskal-Wallis with Dunn's correction. Relevant significant differences are indicated with a black bar and asterisk. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$

17) were upregulated in irreversible PAH (Fig. 6.4. A-B). We confirmed this by immunohistochemistry for the senescence markers p16^{ink4A} and p21^{cip1}, and SASP markers MMP2 and IL6, which were all increased in irreversible compared to reversible PAH (Fig. 6.4.

Fig. 6.5. A senescent vascular profile in human irreversible PAH-CHD. Immunohistochemistry of Ki67, cleaved caspase3, RAD51, survivin, p21^{CIP1}, and p16^{INK4A} expression in human pulmonary arteries (<150 µm) in explant tissue of a patient without PAH (control 1), a paediatric patient with end-stage PAH-CHD (PAH 1), and an adult patient with end-stage PAH-CHD (PAH 2). Positive (pos) controls (human tissue) were added when no vascular expression was expected. Arrowheads in RAD51 denote positive RAD51 nuclear foci. inset shown at higher magnification.

C-D). p21 was located in the ECs and adventitia. p16 was mainly located in the adventitia. MMP2 was located in the endothelium and the adventitia. IL6 was mainly located in the adventitia and showed diffuse staining in the media/neointima. Additionally, we assessed the expression of TNFα, as both vascular remodeling in PAH and EC senescence are known to be driven by TNFα(18–20). TNFα was increased in the endothelium of reversible PAH compared to control. Expression further increased in irreversible PAH where it was located in the neointima and adventitia (Fig. 6.4, C-D).

A similar senescent vascular profile in human irreversible PAH-CHD.

The vascular profile of irreversible shunt-induced PAH in rats was corroborated in human lung explant tissue from patients with irreversible PAH-CHD versus controls (Fig. 6.5). Patient 1 is a 16-year-old female with repaired ventricular septal defect (VSD) (post-operative PAH-CHD); patient 2 is a 30-year-old male with unrepaired atrial septal defect (ASD) (Eisenmenger PAH-CHD). Ki67 and cleaved caspase-3 were not present in the vessel wall in controls, nor in patients. Rad51 was not observed in controls but Rad51 foci were present in the luminal cells of mild lesions in PAH (Fig. 6.5) and in more severe lesions. Survivin

Fig. 6.6. Pro-senescent effects of shear stress on human pulmonary MVECs. Pulmonary microvascular endothelial cells (MVECs) isolated from idiopathic PAH patients (PAH, $n=5$) and controls (con, $n=5$) (A) BMPR2 expression in control and PAH MVECs in static and low (2.5 dyn/cm^2) and high 15 dyn/cm^2 shear stress conditions. (B) Quantification (left) and representative images (right) of DNA damage ($\gamma\text{H2A.X}$ staining) in control and PAH MVECs cultured under static and shear stress conditions. (C) Effect of shear stress on the expression of senescence and DNA damage markers in control and PAH MVECs. Data are reported as mean $\pm$ SD. Statistics by two-sided t-test or Kruskal-Wallis test. Relevant significant differences are indicated with a black bar and asterisk. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$

was largely absent in controls but stained intensely in the luminal cells of severe lesions in PAH. In PAH patients, p21 was expressed in plexiform lesions lining the plexus channels. whereas p16 localised predominantly in the luminal cells and in the adventitia. Vascular p21 and p16 expression was not observed in controls.

Shear stress induces DNA damage in PAH endothelial cells and a phenotypic switch in smooth muscle cells.

To investigate the effect of disturbed blood flow on the possible development of senescence in PAH, we cultured pulmonary microvascular endothelial cells (pMVECs), isolated from patients with idiopathic PAH ($n=5$) or controls patients ($n=5$), under static conditions or low (2.5 dyn/cm^2) (LSS) and high (15 dyn/cm^2) (HSS) shear stress. In static conditions, BMPR2 expression was twice as low in isolated PAH pMVECs compared to control cells (Fig. 6.6A). Staining for $\gamma\text{H2A.X}$, a marker for DNA double strand breaks, showed increased pMVEC DNA damage under high shear stress in control and even more in PAH cells compared to static conditions (Fig. 6.6B). In PAH pMVECs, HSS increased *p16* and *PAI1* mRNA. These markers were largely unaffected by shear stress in control cells (Fig. 6.6C), whereas *KLF2*, a positive control for shear stress, was increased by shear stress in both PAH and control pMVECs. These data indicated that disturbed blood flow can induce DNA damage and *p16*

Fig. 6.7. Effects of the human MVEC SASP on SMCs and targeted apoptosis of senescent MVECs. Pulmonary microvascular endothelial cells (MVECs) isolated from idiopathic PAH and control patients (A) Experimental schematic (left), mRNA expression analysis (middle), and microscopy (right) of human PAH and control pulmonary MVEC senescence, induced by irradiation or TNFα. Experiments were repeated 3 times. (B) Proliferation of naïve SMCs cultured with medium preconditioned by senescent or non-senescent control or PAH MVECs. Experiments were repeated 5-7 times. (C) IL6 and CNN1 expression in SMCs from (B) assessed by PCR. Experiments were repeated 3 times (D) MTS assay of MVEC viability with or without ABT263. MVECs were isolated from controls and PAH, with or without irradiation or TNFα-induced senescence, experiments were repeated 3 times. Data are reported as mean ± SD. Statistics by two-sided t-test or Kruskal-Wallis test. Relevant significant differences are indicated with a black bar and asterisk. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001

expression, possibly leading to senescence, and that PAH pMVECs are more vulnerable to this than control cells.

We further aimed to assess the possible effect of the SASP of senescent pMVECs on SMCs. To this end, we first induced senescence in pMVECs from controls (n=3) and PAH patients (n=3) by i) 10 Gy γ-irradiation and ii) a 10-day stimulation with 10 μM TNFα(19). Sham-irradiated, unstimulated cells served as a control. After 4 and 11 days respectively, irradiation and TNFα both increased *p16*, *p21*, and *IL6* mRNA and caused the cells to become multinucleated, flat, and enlarged, indicating senescence (Fig. 6.7A). Cell medium preconditioned by senescent versus non-senescent pMVECs was then used to culture naïve pulmonary vascular SMCs. We observed that the conditioned medium from senescent pMVECs increased proliferation of SMCs (Fig. 6.7B). *IL6* mRNA was also increased in SMCs cultured with SASP medium, whereas *CNN1*, a marker of SMC contractile function was

Fig. 6.8. Targeted senolysis by ABT263 reverses established experimental PAH. (A) Characterization of rat EC senescence induced by irradiation, measured by mRNA expression (left) and microscopic phenotype (right) including EdU. Experiments were repeated 3 times (B) MTS assay of rat EC viability with or without irradiation-induced senescence, treated with or without ABT263. Experiments were repeated 3 times (C) Experimental design. ABT263 (10 mg/kg) or Vehicle (5 % DMSO + 4 % Tween in PBS) was administered to MCT+Shunt rats (n=10 per group) daily via a 10mg/kg intraperitoneal injection from day 25-32. (D) Hemodynamic parameters in rats treated with vehicle (n=7) or ABT263 (n=8). sRVP; systolic right ventricular pressure; mPAP: mean pulmonary artery pressure; CO: cardiac output; TAPSE: tricuspid annular plain systolic excursion; PAAT: pulmonary artery acceleration time; RV/LV+IVS: right ventricular to left ventricular plus septal weight ratio. (E) Verhoeff staining of remodeled vessels in ABT263-treated rat lung (right) versus vehicle-treated lung (left). (F) Semi-quantification of (C). (G) Vascular p16 and p21 mRNA and protein expression in ABT263- and vehicle-treated rat lungs. Representative immunohistochemistry images are shown below. (H) Vascular IL6, MMP2,

BMPR2, and Klotho mRNA in ABT263- and vehicle-treated rat lungs. Data are reported as mean $\pm$ SD. Statistics by one-way ANOVA with Bonferroni correction or Kruskal-Wallis with Dunn's correction. Relevant significant differences are indicated with a black bar and asterisk. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$

decreased (Fig. 6.7C). These data indicated a role for the endothelial SASP in SMC function and vascular remodeling.

As proof of the concept that cellular senescence is causally involved in the transition toward irreversible PAH, we investigated specific elimination of senescent cells using the senolytic agent ABT263. ABT263 treatment specifically decreased cell viability in the senescent control and PAH pMVECs, whereas viability remained intact in the sham-irradiated pMVECs (Fig. 6.7D). ABT263 treatment (2.5 μ M) also normalized the hyper-proliferative phenotype in sham-irradiated PAH pMVECs, which was 1.5x higher than controls at baseline.

Reversal of shunt-induced PAH by targeted apoptosis of senescent cells.

To confirm the selectivity of ABT263 for senescent cells in vitro and that cellular senescence is causally involved in the transition toward irreversible experimental end-stage MCT+Shunt-PAH, we first performed a dose-response study in rat ECs. Senescence was induced in rat ECs using 10 Gy γ -irradiation as described above. Irradiation increased *p21* and *IL6* mRNA, decreased proliferative potential (laminB1, EdU staining), and caused multinucleation, and cellular flattening and enlargement (Fig. 6.8A). *p16* was not detected in these cells. 4 days after irradiation, treatment with ABT263 (1.25, 2.5 and 5 μ M for 24 h) selectively decreased cell viability up to 20 % in the senescent cells, whereas viability remained intact in control ECs (Fig. 6.8B). These data indicate that ABT263 both effectively eliminates senescent cells and allows for normal, but not hyper proliferation, of pMVECs.

To investigate whether ABT263 can reverse MCT+Shunt PAH in vivo, we induced MCT+Shunt PAH in 20 Wistar rats and administered daily IP injections with 10mg/kg ABT263 (n=10) or vehicle (n=10) (5 % DMSO + 3.5 % Tween20) from day 25 to 32, an advanced-stage PAH that we characterized as HU-irreversible (Fig. 6.8C). The shunts in these rats remained functional during treatment, driving disease progression during the treatment period. One rat died post-operatively due to a leaking shunt. In both groups, two rats died of severe right heart failure that occurred before or 1 day after treatment initiation at day 25 (indicating severe end-stage PAH), and were therefore excluded from all analyses. Dyspnoea, pleural edema, and ascites occurred in 5/7 vehicle-treated rats versus 0/8 in the ABT263 group. sRVP, mPAP, PVR, and relative RV weight were decreased and CO, PAAT, and TAPSE were increased by ABT263-treatment ($p < 0.05$) (Fig. 6.8D), indicating disease regression. ABT263 significantly decreased luminal occlusion, reduced the percentage of vessels with a neointima, and increased those with a normal morphology

Fig. 6.9. Working model for the role of cellular senescence in vascular remodeling in PAH. (A) Healthy pulmonary arteriole. (B-C) Disturbed flow causes DNA damage. (D) Persistence of disturbed flow permits DNA damage accumulation, leading to apoptosis or apoptosis resistance. (E) Endothelial cells hyper proliferate, leading to intimal hyperplasia; the media becomes muscularized and smooth muscle cells (SMCs) proliferate and migrate into the intima. (F) Hyper proliferation and accumulating DNA damage induces growth arrest. Inflammatory cells are recruited to the vessel. The adventitia becomes fibrotic. (G) Vascular cells become senescent and acquire a senescence-associated secretory phenotype (SASP), which promotes inflammation, degradation and fibrosis (H). Figure references to the data in this study are cited in teal; references to the literature used for this model are cited in the figure in red.

Table 6.1. Characteristics human PAH-CHD

	Age at explantation	Sex	Cardiac Lesion	Repaired	PAP pre Tx (s/m/d)
PAH-CHD	16 y	F	Ventricular Septal Defect	Yes	88/50/64
PAH-CHD	30 y	M	Atrial Septal Defect	No	128/60/85
No PAH	12 y	M	-	-	-
No PAH	46 y	F	-	-	-

($p < 0.05$), indicating reversed remodeling or potential vascular regeneration (Fig. 6.8, E-F). ABT263 treatment decreased *p16* and *p21* mRNA, the number of p16-positive adventitial cells, and the number of p21-positive endothelial and adventitial cells, indicating clearance of senescent cells (Fig. 6.8G). The SASP marker *IL6* was also decreased by ABT263. *MMP2* mRNA however, was increased by ABT263. In this context of regressing PAH, this may indicate a physiological role for *MMP2*, which is also implied in vascular development or

Figure 6.10. Matrix of vascular cell type-specific genes. Per cell type, a minimum of 5 signature genes was included to reach maximum specificity for each lineage, which together form a matrix that distinguishes 3 vascular cell types

neovascularization(21). We therefore also assessed *BMPR2* and *Klotho*, both associated with vascular regeneration, which were also increased by ABT263 treatment (Fig. 6.8H). Together, these data show that ABT263 reduces the senescence burden in vivo, which is associated with reversed vascular remodeling, evidence for a pro-regenerative milieu, and improved pulmonary hemodynamics.

Table 6.2. Human and rat primer sequences for PCR.

Gene	Human Primer	Gene	Rat Primer
p16ink4A	Fw: CATAGATGCCCGCGGAAGGT Rv: CCCGAGGTTTCTCAGAGCCT	p16ink4A	Fw: GAGCAGCATGGAGCCTTC Rv: CGTAACTATTCGGTGCCTTG
p21Cip1	Fw: GCACTTTGATTAGCAGCGGA Rv: AGCCGAGAGAAAACAGTCCA	p21Cip1	Fw: TCACTGTCTTGTACCCTTGTGC Rv: GGCCTTTGGAGTGCTAGAAA
IL6	Fw: CCCACACAGACAGCCACTCA Rv: CCGTCGAGGATGTACCGAAT	LMNB1	Fw: GTGCTGCGAGCAGGAGAC Rv: CCATTAAGATCAGATTCCTTCTTAGC
CNN1	Fw: GGGCTGCAGATGGGCACCAA Rv: CTGTGCCAGCTTGGGGTCG	IL6	Fw: CAGGAGCCCAGCTATGAACT Rv: GAAGGCAGCAGGCAACAC
BMPR2	Fw: GTCCTGGATGGCAGCAGTAT Rv: CCAGCGATTCAGTGAGATGA	MMP2	Fw: TGAGCTCCCGAAAAGATTG Rv: CATTCCCTGCGAAGAACACA
KLF2	Fw: CAAGACCTACACCAAGAGTTCTG Rv: CATGTCCGTTTCATGTGC	BMPR2	Fw: TCTGTGGGAGAAATCAAAAAGGG Rv: GCAGCAAAAACGGTATGTTC
RAAd51	Fw: GCTGCGGACCGAGTAATG Rv: CCAGCTTCTTCCAATTTCTTCAC	s-Klotho	Fw: TGGCATTGATGACGATCCCC Rv: CGTCCAAACAGTAGGCTTTC
BRCA1	Fw: GTCCCATCTGTCTGGAGTTGAT Rv: AAAGGACACTGTGAAGGCC	rTUB1A	Fw: GCAACCATGCGTGAGTGT Rv: ATTGCCGATCTGGACACC
PAI1	Fw: CAATCGCAAGGCACCTCTGA Rv: TTCACCAAAGACAAGGGCCA		
GAPDH	Fw: GGTCTCTCTGACTTCAACA Rv: AGCCAAATTCGTTGTCATAC

Discussion.

Using experimental shunt-induced PAH, here we showed that hemodynamic unloading (HU) can reverse pulmonary vascular remodeling, but not beyond a certain point of no return. The dichotomous response to HU enabled us to identify and compare vascular profiles of reversible and irreversible PAH using RNA sequencing. We found that loss of reversibility is associated with a switch from a proliferative to a senescent vascular phenotype. To support the concept that vascular cell senescence is causal to the irreversible nature of end-stage PAH, we targeted senescence using ABT263 and induced reversal of the hemodynamic and structural changes associated with severe PAH refractory to hemodynamic unloading. This class of drugs may represent a new line of treatment for advanced PAH.

The pulmonary vascular histomorphology associated with the (ir)reversibility of PAH-CHD has been described extensively in clinical studies(22). Several decades ago, it was shown in patients with open-shunt PAH-CHD that HU by banding of the main pulmonary artery can reverse structural changes like medial hypertrophy and intimal hyperplasia. Acellular neointimal fibrosis, degradation of the elastic laminae, and the formation of plexiform lesions were associated to disease progression(10). Several preclinical studies have since then confirmed that medial hypertrophy can be reversed by HU. In rats with PH induced by MCT and right middle and lower lobectomy, both medial hypertrophy and increased EC proliferation were normalized by HU through transplantation into a normotensive recipient at 28 days after MCT-injection(5). Note that pulmonary blood flow in latter study is increased because about 1/3 of lung tissue is removed, but not to the same magnitude as in our shunted rats, in which pulmonary blood flow is increased two- to threefold, explaining the difference in PAH severity between MCT+Pneumonectomy vs. MCT+Shunt in rats. Pigs with an aorto-caval shunt that was closed after 5 weeks show a similar pattern of reversed medial hypertrophy and normalized EC proliferation(7). These two studies performed HU in a stage of PH wherein neointimal lesions are not yet present. A recent study in the Sugen5416+Hypoxia rat model, however, showed that HU by left pulmonary artery banding at 5 weeks can also reverse established neointimal lesions(6). Our study describes both a reversible and an irreversible stage of vascular remodeling in a PAH model that has features of neointimal vascular remodeling that do not change after HU and may progress, consistent with a severe rise in PAH. These observations suggest that the reversibility of PAH is governed not merely by the presence but also by the molecular characteristics of neointimal lesions.

We described that the progression from reversible to irreversible PAH with HU coincides with three distinct changes in the pulmonary vasculature that are consistent with senescence: i) a switch from a proliferative and/or regenerative state into growth arrest; ii) a

shift from pro-apoptosis to apoptosis-resistance, as also previously described in human PAH-CHD(23); and iii) a marked increase in pro-inflammatory cytokines accompanied by inversion of the CD4+/CD8+ T cell ratio in the lung. Furthermore we observed an initial increase followed by a substantial reduction in genes associated with DNA damage repair. We confirmed senescence by demonstrating increased pulmonary vascular expression of p16^{ink4A} and p21^{Cip1} both in rats and in human end-stage PAH-CHD. The vascular expression pattern of p16 in PAH-CHD is consistent with previous observations in idiopathic PAH(24). In vitro, we showed that pulmonary MVECs isolated from PAH patients are more prone to develop senescence via increased sensitivity to DNA damage in response to high shear stress compared to control cells. We hypothesize that this effect may be due to decreased expression of BMPR2 in PAH MVECs, which was recently shown to compromise genomic integrity of ECs(25). We also found that the SASP of senescent MVECs stimulates proliferation and causes a phenotypical switch of SMCs in culture, providing insight into mechanisms of vascular remodeling beyond the endothelial cell. Moreover, we showed that low-dose treatment with the senolytic ABT263 selectively induces apoptosis in senescent MVECs in vitro and reversed advanced vascular remodeling and shunt-induced PAH in rats. Combined, these data serve proof of the concept that cellular senescence contributes critically to the transition from reversible to irreversible PAH. After this transition has occurred, we hypothesize that the senescence associated secretory phenotype (SASP) may exacerbate the end-stage vascular sclerotic phenotype by promoting medial hypertrophy, suppressing vascular regeneration, maintaining inflammation, promoting fibrosis and degrading elastin (fig. 6.9).

A central role for cellular senescence in the pathology of various degenerative vascular and pulmonary pathologies has become apparent in recent years. In atherosclerosis, senescent ECs and SMCs were shown to accumulate in plaques, which typically occur in areas where flow is disturbed(26). Senescent ECs in plaques produce less nitric oxide and prostacyclin, and develop a pro-inflammatory SASP, leading to vascular dysfunction, constriction and sclerosis(27). Clearance of senescent cells improved vascular function in atherosclerotic mice(28). Senescence was also described in degenerative pulmonary diseases like idiopathic pulmonary fibrosis (IPF)(29) or chronic obstructive pulmonary disease (COPD)(30), both of which are also associated with vascular remodeling and pulmonary hypertension(31). Accumulating evidence, including this study, indicates that DNA damage and insufficient DNA repair are early and central phenomena in PAH as well(32). Many triggers linked to the development of PAH are also genotoxic, such as treatment with dasatinib, chronic inflammation, or oxidative stress. Moreover, it was shown recently that reduced *BMPR2* signaling, a critical feature of all PAH etiologies as well as a genetic cause, impairs normal DNA damage repair mediated by *BRCA1 and 2*(33) and *Rad51*(25). DNA damage was also shown to cause *BMPR2* loss itself, which suggests a vicious cycle that could account for

DNA damage accumulation(33). These data are consistent with the decrease in *BRCA1/2* and *Rad51*, as well as in *BMPR2* that we observed upon transition from reversible to irreversible PAH in vivo, and the increased DNA damage in PAH MVECs with halved *BMPR2* expression upon shear stress in vitro. Excessive DNA damage can eventually lead to either apoptotic cell death, reflected here in the decreased proportion of ECs in reversible PAH, or survival of cells with genomic abnormalities, causing dysfunction, hyper-proliferation or finally senescence(17). Disturbed blood flow can further regulate the development of EC senescence via *p53*, a context-dependent mediator for cell fate, that decides between apoptosis or senescence(26). The presence of increased TNF α in this context, appears to shift this balance towards senescence(26). The heightened expression of neointimal TNF α observed in this study indicates that this process may also play a role in the induction of vascular cell senescence in PAH.

In MCT+Shunt rats, senescence did not appear to be limited to the endothelial cells, as we also observed senescence markers in adventitial cells, likely fibroblasts or immune cells. The role of fibroblast senescence is well described, for example, in IPF(29) and systemic sclerosis(34) -- pathologies also both associated with PAH. Immunosenescence, induced by aging or oxidative stress, is characterized by chronic low-grade inflammation(35) and a reduced CD4/CD8-ratio(36). These hallmarks are also consistent with our observations in the transition towards irreversible PAH. In macrophages, p16 expression is associated with proliferation and differentiation towards the pro-inflammatory M1 macrophage(37) leading to increased IL6 secretion. Senolytic drugs can eliminate these p16-positive macrophages(38) as well as senescent vascular cells, indicating a possible dual mode of action to reverse vascular remodeling. Moreover, senolytics such as ABT263 or Foxo4-DRI have shown to promote tissue regeneration after clearance of senescent cells(39). The increased expression of *BMPR2*, *Klotho*, and *MMP2* after ABT263 treatment in this study suggests that regeneration of lung vasculature occurs after clearance of senescent cells. This aspect could additionally benefit PAH patients in whom vascular loss or the inability to recruit vessels during exercise contributes to the severity of the disease. Given a favorable safety profile, these agents may have a place in the future treatment of PAH-CHD and perhaps other forms of PAH as well. ABT263 has already been evaluated in multiple phase 2 clinical trials for different malignancies (small cell lung cancer, NCT00445198). Senescence is associated with a distinct secretome that could be investigated as a peripheral blood biomarker to identify PAH that is refractory to HU and select patients that may respond to senolytics.

A limitation of this study is that the individual contribution of either MCT or the shunt to the development of irreversible PAH cannot be discerned. Single hit models such as MCT-only, shunt-only, or hypoxia-only, however, are characterized only by medial hypertrophy-type vascular remodeling and lack the neointimal lesions reminiscent of PAH. All of these models

are deemed reversible, and therefore not suitable to investigate the (ir)reversibility of severe, neointimal PAH(40–42). It is likely the combination of hits (MCT and shunt, Sugen5416 and hypoxia) causes the occurrence of neointimal lesions. Likewise, in human PAH-CHD, it is also the combination of increased flow and increased pressure (as in a VSD) that leads to irreversible PAH within 2 years, whereas increased flow alone (as in an ASD) causes irreversible PAH in only 20 % of patients after more than 30 years (3). A second limitation is that LTx, used here as a surrogate for HU, may also contribute to the reversibility of vascular lesions because it places a diseased lung into a healthy, non-inflammatory environment. Still, it is apparent from the rat groups that were not reversible by LTx (which also showed the most inflammation), that this effect is not leading. The fact that MCT+Pneumonectomy is reversible by LTx at day 28 (5), while MCT+Shunt at day 28 is not, does make it plausible that volume loading/offloading is important, as the degree of pulmonary overflow (1.5- versus 2- to 3-fold increase) is the only variable that is changed between these set-ups. Human and animal studies that use methods of HU without transplantation, such as shunt closure or pulmonary artery banding (PAB), also support that pulmonary vascular lesions can be reversed just by reducing pulmonary blood flow (6,7,10). Unfortunately, shunt closure is not technically feasible in this model, nor is performing PAB surgery during advanced PAH (due to RV dysfunction) (6). Another question that remains uncertain is in which cell type senescence is first induced, or which senescent cell type contributes primarily to irreversibility in MCT+Shunt-PAH. We chose to focus on the ECs because of their sensitivity to disturbed flow, but senescence in tissue usually involves multiple cell types, as was also observed in the pulmonary vasculature of rats with irreversible PAH.

Here, using hemodynamic unloading, we identified a reversible and an irreversible stage of vascular remodeling in experimental shunt-induced PAH. We report that loss of PAH reversibility by HU is associated with a senescent vascular phenotype. Targeted senolysis reversed vascular remodeling and improved pulmonary hemodynamics in the end-stage of experimental shunt-induced PAH. This serves proof of the concept that senescence contributes critically to the transition of reversible to irreversible disease, and indicates senolytic class drugs as a new line of treatment for end-stage PAH.

Materials and Methods

Study design.

The goal of this study was to identify histological and molecular characteristics related to the reversibility of severe PAH, and to elucidate the mechanisms associated to the transition from reversible to irreversible disease. The study design was based on the clinical observation that PAH in congenital cardiac shunts can be reversed by hemodynamic unloading

(HU) through shunt closure, but that this potential is lost beyond a certain point of no return. To reproduce this clinical phenomenon, we used an established shunt-associated rat model for severe PAH, that combines a 60 mg/kg MCT injection with an aorto-caval shunt, which doubles pulmonary blood flow and leads to a neointimal-type of vascular remodeling. HU was carried out at different time points during the progression of PAH (14, 21, and 28 days after MCT injection) by transplanting the left lung with PAH into a normotensive recipient without a shunt. Similar to clinical observations in PAH-CHD, we found that HU could reverse the early, but not the late stages of MCT+Shunt PAH. We could then characterize transcriptomic and vascular expression profiles that distinguish a reversible from an irreversible response to HU using RNA sequencing and immunohistochemistry, which indicated that a pattern of cellular senescence was associated with HU-irreversibility of PAH. In vitro, we further investigated the role of cellular senescence in vascular remodeling and evaluated the senolytic drug ABT263 in its selectivity to induce apoptosis in senescent versus healthy pulmonary microvascular endothelial cells. Finally, to support the concept that vascular cell senescence is causal to the irreversible nature of end-stage PAH in vivo, we treated rats with advanced MCT+Shunt PAH with ABT263 and induced reversal of the hemodynamic and structural changes associated with severe PAH refractory to HU.

All experimental protocols were approved by the Dutch national, and institutional animal care and use committee of the University of Groningen, the Netherlands, and were conducted according to published guidelines for animal and translational research in PAH (47). Prospectively selected primary and secondary end points for all animal studies were vascular occlusion score and mPAP respectively. Animal experiments were terminated at the defined time point or when severe dyspnoea developed, as assessed twice a day by ABCDE criteria defined previously (11). Sample sizes were based on previous research in our labs, reporting occlusion scores around 60 ± 10 % in end stage PAH, and an effect size of ± 20 points reduction in vascular occlusion (associated with hemodynamic improvement). Rats were randomly assigned to each experimental group and were housed randomly in group cages to ensure blinding for the researcher when conducting the in vivo analyses. Ex vivo, al samples were renumbered randomly for further blinding. The MS21/HU21 group ($n = 5$) was replicated once. All in vitro experiments were replicated three times.

MCT+Shunt PAH in rats.

MCT+Shunt-associated PAH was induced in adults male rats (200-250 g) by a subcutaneous 60 mg/kg injection of monocrotalin at T0 (MCT, Sigma Aldrich), followed by aorto-caval shunt surgery 7 days later, described in detail previously (9). Control rats were injected with saline and sham-operated (NaCl+Sham). Syngeneic Lewis rats (Envigo) were used for the transplantation experiments; Wistar rats (Charles River) for the ABT263 experiment.

MCT+Shunt rats are referred to as MS followed by the time point of PAH progression in days after MCT.

Hemodynamic unloading by single lung transplantation.

Lewis rats in which PAH was induced by MCT+Shunt up to the predefined time points of PAH progression (MS14, MS21, and MS28) served as donors for a syngeneic unilateral orthotopic left lung transplantation (12) (LTx) into a healthy recipient Lewis rat without a shunt (Fig. 6.1A). NaCl+Sham donors were used as control. Recipient rats are referred to as hemodynamically unloaded (HU) by their disease stage at LTx. Recipient rats (250-300 g) were anaesthetized using 3 % isoflurane, intubated and ventilated mechanically at 21 % O₂. The left lung was explanted via a lateral thoracotomy. Simultaneously, the donor rats were intubated and anaesthetized using 3 % isoflurane. After echocardiography a sternal thoracotomy was performed. The lungs were heparinized and flushed with cold saline via a cannula in the right ventricle. The left donor lung wrapped in foil with 4 °C saline, explanted and transplanted into the recipient. Cold ischaemic time to reperfusion was 30-40 min. The lungs were re-inflated using positive pressure ventilation. The donor rat was sacrificed after left lung explantation. The lower and middle lobes of the right donor lung were infused with 4 % paraformaldehyde and paraffin-embedded for histomorphology: to assess baseline disease stage at HU. The upper right lobe was snap frozen for RNA extraction. The heart and liver of the donor were harvested for evaluation of PAH-associated sequelae. 21 days after LTx, all recipients underwent hemodynamic evaluation by echocardiography and right heart catheterization, followed by sacrifice. The left transplanted lung was infused with 4 % paraformaldehyde and paraffin-embedded for histomorphology to assess the effect of HU.

Hemodynamic evaluation.

Hemodynamic evaluation was performed using echocardiography and closed-chest right heart catheterization, using a standardized protocol described previously (9,11).

Quantitative and qualitative vascular morphologic assessment.

4-μm paraffin-embedded lung sections were stained according to the Verhoeff protocol and scanned on 40x magnification (Hamamatsu nanoviewer). All slides were masked, blinding the investigator for the experimental group. 40 vessels (diameter <50 μm) per lung were analysed according to a standardised pulmonary vascular morphometry protocol, described in detail previously (9,11).

RNA sequencing and analysis.

From all donor rats, RNA was extracted from whole upper right lobular lung tissue, in 3 biological replicates of 500 ng each. After quality control, the RNAseq library was prepared using the Quantseq 3'mRNA-seq FWD kit (Lexogen). The library was sequenced on

3 HiSeq4000 lanes (Illumina). Adapter and polyA sequences and low quality nucleotides were removed using BBDuk. Trimmed reads were mapped against the Rnor_6.0 rat genome using STAR, and htseq-count was used to determine read counts. After quality control with FASTQC, biological replicates were averaged, followed by differential expression analysis using DESeq2. Differentially expressed genes were included at cut-offs of >1 log2 fold change (LogFC) and FDR-adjusted p-value (q) <0.05 . KEGG pathway and gene ontology analysis were conducted by Goseq. Raw read files were submitted to the NCBI GEO online database (GEO accession number: GSE149899).

Cellular deconvolution.

Within the mixed-cellular lung samples, we assessed individual cellular proportions of all immune cells using ImmunoStates, a matrix of immune cell type specific genes that can be used to estimate proportions of 20 immune cell subsets in a mixed tissue sample (PBMC, whole blood, or a tissue) by statistically deconvolving a transcriptome profile of the given sample (13). Briefly, ImmunoStates consists of 317 genes and their expression across 20 immune cell types estimated using 166 gene expression datasets (GEO) of different cell-sorted immune cells. We applied the same principals to create a second matrix was constructed for rat endothelial cells (EC), smooth muscle cells (SMC) and fibroblasts (Fig. 6.10) using the same strategy. Then, deconvolution was performed on the raw reads of the present study using the support vector regression algorithm in R.

Immunohistochemistry and vessel-specific expression quantification.

4- μ m paraffin-embedded lung sections were stained using the following primary antibodies: Ki67 (1:75, Abcam), cleaved caspase-3 (1:150, Cell Signaling), TNF- α (1:100, Abcam), Rad51 (1:100, Abcam), IL-6 (1:200, Abcam), MMP2 (1:100, Abcam), survivin (1:125, Santa Cruz), p21 (1:100, Abcam), and p16 (1:100, Abcam). Species-specific immunoglobulin G coupled to peroxidase were used as secondary or tertiary antibody and then stained by diaminobenzidine. All slides were masked and scanned on 40x magnification. 20 vessels per lung were analysed and quantified according to a standardised pulmonary vascular immunohistochemistry protocol, described previously (11).

Human PAH lung tissue.

Sections from lung explant tissue of patients with end-stage PAH-CHD and controls were acquired from the biobank at the pulmonary vascular research institute at Stanford University, Palo Alto, CA, USA. Patient characteristics are listed in **table S1**. IHC was carried out for Ki67 (1:75), cleaved caspase-3 (1:150), Rad51 (1:100), survivin (1:50), p21 (1:100), and p16 (1:100). Species-specific G coupled to peroxidase were used as secondary or tertiary antibody and then stained by diaminobenzidine. Slides were scanned on 40x magnification and used for qualitative analysis.

Human pulmonary MVEC isolation.

Human microvascular endothelial cells (MVECs) were isolated from pleura-free peripheral lung tissues, as described previously (49). The lung specimens were obtained from end-stage PAH patients undergoing lung transplantations or from autopsies. Lung tissues for the isolation of control cells originated from lobectomies for suspected or proven non-small cell lung cancer (NSCLC) without clinical or histological signs of pulmonary hypertension. Cell isolations were approved by the IRB of the VU University Medical Center (Amsterdam, the Netherlands) and consent was given.

Fluid shear stress experiments in human MVECs.

Cells were either cultured on 0.1 % gelatin-coated 8-well μ -Slides at static conditions or in 0.6 I Luer μ -Slides under conditions of fluid flow (both ibidi GmbH). Cultures were maintained in ECM medium with 1 % pen/strep, 1 % endothelial cell growth supplement, 5 % FCS (all ScienCell), and 1 % non-essential amino acids (Biowest) until confluency was reached. Pulsatile (1 Hz), low shear stresses (LSS) of 2.5 dyn/cm² or high shear stresses (HSS) of 15 dyn/cm² were applied with the ibidi Pump System. RNA was isolated 24 h after onset of HSS or LSS with the miRNeasy mini kit (Qiagen). cDNA synthesis was performed with the iScript cDNA synthesis kit, and rt-PCR carried out on a C1000 Thermo Cycler with iQ SYBR green supermix (all Bio-Rad) following manufacturer's instructions.

γ H2A.X-staining and analysis for DNA double strand breaks on human MVECs.

After static culture or the application of HSS for 5 days, cells were fixed in warm 4 % para-formaldehyde for 20 min at room temperature (RT), permeabilized with 0.2 % Triton X-100 for 10 min at RT, blocked with 5% BSA for 30 min, labelled with γ -H2AX antibody (1:100, Abcam 26350) overnight and Hoechst nuclear counterstaining for 1 h, and fixed in ibidi mounting medium. Imaging was done on a Nikon A1 confocal laser microscope. Z-stacked images (1024 $\times$ 1024 px) were acquired with a step size of 0.3 μ m on an Apo 60x oil lenses. Image analysis was performed with Fiji (ImageJ, NIH). Maximum intensity projections of the z-stacks were generated for representative figures. For the analysis of DNA damage, 4x4 stitched scans at random locations were generated. First, nuclei were automatically detected by running a bandpass filter, followed by application of a threshold, and the analyse particle command in Fiji. Second, each nucleus was individually background corrected and the mean nucleic γ -H2AX staining intensity was quantified per nucleus.

Irradiation and TNF α -induced senescence and ABT263 treatment in human MVECs.

Control and PAH MVECs were irradiated with 10 Gy γ -radiation using a ¹³⁷Cesium source, or sham irradiated. Medium was refreshed every 2 days. Cells were harvested after 5 days. Additionally, PAH and control MVECs were treated with 10 μ M TNF α for 10 days, as described previously (19). Senescence was assessed morphologically, and by PCR for

p16, p21, and IL6. Irradiated and sham-irradiated ECs were exposed to treatment with ABT263 from at day 4 after irradiation. Cells were treated with 1.25, 2.5, and 5 μ M ABT263 or vehicle (DMSO) in triplicate wells for each concentration. On day 5, cell viability was assessed using the MTT assay.

MTT assays.

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assays were performed on human and rat cells. Briefly, cells were seeded in a 96-well plate at a density of 3×10^3 cells/well and incubated overnight. Cells were made quiescent by incubation in medium without FCS for 6 h and then incubated overnight with FCS (10 % [vol/vol]) and vehicle or ABT263 [1.25, 2.5, 5 μ M] for 24 h. After the incubation, cells were incubated with 10 μ l of MTT reagent (5 mg/ml) for 3 h at 37°C. 100 μ l of isopropanol was added to each well and incubated for 15 min. Colorimetric analysis was performed with an enzyme-linked immunosorbent assay (ELISA) plate reader. Cells were also counted using an automated cell counter (Biorad). Each experiment (in quadruplicate) was repeated at least three times.

PASMC growth in cultured medium of senescent MVECs.

Senescence was induced in PAH MVECs by 10 Gy γ -irradiation and stimulation with 10 μ M TNF α for 10 days as described above. 5 days after irradiation, and 24h after the last TNF α dose, conditioned medium from the senescent MVECs (theoretically containing SASP factors) was collected and added to serum-starved healthy control PASMCs and incubated for 48 hours. SMC proliferation was measured by automated cell counting (Biorad) and a PCR for IL6 and CNN1 was performed.

Irradiation-induced senescence in rat ECs and ABT263 treatment.

To acquire a senescent phenotype, rat endothelial cells of endocardial origin (cell line) were irradiated with 10 Gy γ -radiation using a ¹³⁷Cesium source. Sham-irradiated ECs served as a control. Medium was refreshed every 2 days. Cells were harvested after 5 days. Senescence was assessed morphologically, by loss of EdU staining and by PCR for p16, p21, IL6, and loss of LaminB1. Experiments were repeated 3 times. For the dose-response study with ABT263, irradiated and sham-irradiated ECs were exposed to treatment with ABT263 from at day 4 after irradiation. Cells were treated with 1.25, 2.5, 5 μ M ABT263 or vehicle (DMSO) in triplicate wells for each concentration. On day 5, cell viability was assessed using the MTS assay (Promega).

Rat EC EdU staining.

Cells were cultured for 24 hours in the presence of EdU, and stained using the Click-iT EdU Alexa Fluor 488 Imaging kit; Thermo Fisher Scientific). Images were acquired at 400X magnification and quantified using ImageJ.

Real time-PCR.

Total RNA was extracted from MVECs using NucleoSpin RNA II (Machery Nagel, Düren) and from rat lung tissue using TRIPURE (Roche) and cDNA was synthesized using iScript (Bio-Rad). Real time qPCR was performed with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and run in triplicate on a QuantStudio 7 Flex Real-time PCR System (Applied Biosystems). Analysis of qPCR results was done using the $\Delta\Delta C_t$ method. GAPDH and rTUB1 were used as internal controls. Amplification specificity was controlled by post-amplification melting curve analysis. In addition, qPCR products were electrophoresed on 2 % agarose gels (w/v) to confirm the presence of a single band of expected size. Primer sequences are listed in **table S2**.

In vivo intervention in MCT+Shunt PAH with ABT263.

20 Wistar (WU) rats were acquired from Charles River (France). PAH was induced by MCT+Shunt. From day 25 to day 32, daily IP injections were given with either vehicle (5 % DMSO + 4 % Tween in PBS) or 10 mg/kg ABT263 in vehicle (n=10 per group). Rats were sacrificed at day 32, or in pairs upon development of severe dyspnoea. Haemodynamic and histopathologic evaluation was carried out as described above.

Statistical analyses.

Statistical analyses were performed using SPSS. For all variables, normality was assessed using the the Shapiro-Wilk test. $\alpha < 0.05$ was considered significant. To assess differences between time-points within the pre- or post-HU groups we used One-way ANOVA with Bonferroni correction for parametric and Kruskal-Wallis for nonparametric variables. We used a two-sided T-test or Mann-Whitney-U test to assess the effects of HU on lung morphology, or the effect of ABT263 versus Vehicle. Transcriptomic data were analysed using R. All data are reported as mean $\pm$ SD. Individual data for animal experiments are provided in data file S1.

Supplementary Materials:

Fig. 9. Working model for the role of cellular senescence in vascular remodeling in PAH.

Fig. 10. Deconvolution matrix for vascular cells.

Table S1. Characteristics human PAH-CHD.

Table S2. Human and rat primer sequences for PCR.

Data file S1 Individual data tables for all animal experiments.

References

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Multicenter preclinical validation of BET inhibition for the treatment of pulmonary arterial hypertension.

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Abstract.

Rationale. Pulmonary arterial hypertension (PAH) is a degenerative arteriopathy that leads to right ventricular (RV) failure. Bromodomain-containing protein 4 (BRD4), a member of the Bromodomain and Extra-Terminal motif (BET) family, has been identified as a critical epigenetic driver for cardiovascular diseases.

Objectives. To explore the therapeutic potential in PAH of RVX208, a clinically available BET-inhibitor.

Methods. Microvascular endothelial cells (MVECs), smooth muscle cells (SMC) isolated from distal pulmonary arteries of PAH patients, rats with sugen5416+Hypoxia (SH)- or monocrotalin+shunt (MS)-induced PAH, and rats with RV pressure overload induced by pulmonary artery banding (PAB) were treated with RVX208 in three independent laboratories.

Measurements and main results. BRD4 is upregulated in the remodeled pulmonary vasculature of PAH patients, where it regulates FoxM1 and PLK1, proteins implicated in the DNA damage response. RVX208 normalized the hyper-proliferative, apoptosis-resistant and inflammatory phenotype of MVECs and SMCs isolated from PAH patients. Oral treatment with RVX208 reversed vascular remodeling and improved pulmonary hemodynamics in two independent trials in SH-PAH and in MS-PAH. RVX208 could be combined safely with contemporary PAH standard of care. RVX208 treatment also supported the pressure loaded RV in PAB rats.

Conclusion. RVX208, a clinically available BET-inhibitor, modulates pro-proliferative, pro-survival and pro-inflammatory pathways, potentially through interactions with FoxM1 and PLK1. This reversed the PAH phenotype in isolated PAH-MVECs and -SMCs *in vitro*, as well as in diverse PAH rat models. RVX208 also supported the pressure loaded RV *in vivo*. Together, these data support the establishment of a clinical trial with RVX208 in PAH patients.

Introduction.

Pulmonary arterial hypertension (PAH) is characterized by occlusive vascular remodeling, vascular rarefaction and sclerosis. This ultimately causes pulmonary vascular resistance (PVR) to rise and induces right ventricular (RV) failure. Loss of bone morphogenic receptor 2 (BMPR2) and accumulating DNA damage(1) are early pathogenic phenomena that appear to act in a vicious cycle(2). In different cardiovascular diseases and cancers, Bromodomain-containing protein-4 (BRD4), a member of the Bromodomain and Extra-Terminal motif (BET) family, has been identified as a critical transcriptional modulator in the context of DNA damage(3–6). BRD4 can inhibit apoptosis, the physiological response to accumulating DNA damage, by promoting cell survival(7), and stimulate hyper-proliferation(8). BRD4 can further regulate a cellular switch into a pro-inflammatory phenotype via increased transcription of cytokines like IL6 and IL8(9) (fig.7.1). In previous studies, we have shown that BRD4 is also increased in PAH (7) and associated with an aberrant DNA damage response (DDR)(10) mediated by FoxM1, a downstream BRD4 effector(11). Specific BRD4 inhibition by JQ1 and siRNA has shown to reverse vascular remodeling and improve pulmonary hemodynamics in Sugen5416-Hypoxia rats, which was associated with reduced PAH-SMCs apoptosis-resistance and proliferation(7). However, neither JQ1 nor siRNA can be used clinically. We therefore explored the therapeutic potential of RVX208, a clinically available BET inhibitor and BRD4 antagonist. The experiments were initiated and conducted independently by the *Pulmonary Hypertension and Vascular Biology Research Group* in Quebec, Canada and by the *PHAEDRA consortium* in the Netherlands. Because of high similarity in experimental set up and outcome, the Canadian and Dutch preclinical trials were later unified.

Methods.

Human cell culture and treatments.

Pulmonary microvascular endothelial cells (MVECs) and smooth muscle cells (SMCs) were isolated from idiopathic PAH (IPAH), and normal lung explant tissue. Cells were treated with vehicle (5% DMSO), or RVX208, Thiostrepton (FoxM1 inhibitor) or BI6727 (PLK1 inhibitor) in the indicated doses for 16 hours, or with siRNA against BRD4, FoxM1 or PLK1. Cell proliferation, apoptosis, inflammation and BMPR2 signalling were assessed. Detailed culture procedures and all assays are described in the supplement.

Rat models for PAH and RV pressure overload and treatments.

All experiments were carried out according to recently published standards for methodological rigor in preclinical PAH studies(12), as described in the supplement. RVX208 was

Figure 7.1. Hypothesis. Hypothetical representation of the role of BRD4 in PAH. DNA damaged, induced by various genotoxic triggers associated with PAH, leads to recruitment of BRD4 to the DNA, where it induces hyper proliferation, cell survival and inflammation, which lead to vascular remodeling.

evaluated in two established rat models for progressive PAH: Sugen5416/Hypoxia (SH-PAH) and Monocrotalin/Shunt (MS-PAH). The Canadian and Dutch in vivo experiments were initiated simultaneously, conducted and analyzed independently, and finally unified in this work. In the Canadian study (fig.7.4a), SH-PAH was induced in male Sprague-Dawley rats by a 20mg/kg Su5416 injection followed by 3 weeks in 10% hypoxia and 3 weeks in normoxia. Saline-injected, normoxia rats (n=6) served as controls. From day 42 to 70, SH-PAH rats received daily oral treatment with vehicle (formulation EA006(13)) (n=14), 90mg/kg RVX208 (n=8) or RVX208+contemporary care (CC, Tadalafil 10mg/kg + Macitentan 10mg/kg, n=7). In the Dutch studies, SH-PAH was induced similarly (fig.7.4d). Rats received daily oral treatment from day 42 to 70 with vehicle (5%DMSO in drinking water, n=12) or 100mg/kg RVX208 (n=12). In the MS-PAH study, PAH was induced in 27 male Wistar rats by a 60mg/kg monocrotalin injection followed by aorto-caval shunt surgery at day 7 (fig.7.4g) as described previously(12,13). Daily oral treatment with vehicle (n=12) or 100mg/kg RVX208 (n=10) was given from day 21 to 35 (MS35Veh or MS35RVX). The remaining 5 MS-PAH rats were sacrificed at day 21 to allow a baseline comparison at treatment initiation (MS21). RV pressure overload was induced in rats by pulmonary artery banding (PAB), as described previously(17,18). Rats received daily oral treatment from day 28 to 56 with vehicle (PAB56Veh, n=5) or 100mg/kg RVX208 (PAB56RVX, n=8) (fig.7.6a). The in vivo effects of RVX208 on hemodynamics, vascular morphology, proliferation, apoptosis, inflammation and BMPR2, FoxM1 and PLK1 signaling were assessed as described in the supplement. Tissue exposure levels of oral 100 mg/kg RVX208 treatment were measured in the lung and heart of the PAB rats.

Figure 7.2. BRD4 regulates the DDR protein FoxM1 and its transcriptional target PLK1 in vascular cells in human PAH. (a) Western blot showing increased BRD4 protein in MVECs and PSMCs isolated from IPAH patients vs. controls. (b) Western blot showing increased PLK1 in IPAH-SMCs. (c) increased expression of PLK1 (red) in the IPAH vessel wall. SMCs are marked by α SMA in green. (d) Western blot showing the effect of BRD4 and FoxM1 siRNA on PLK1 in PAH-SMCs. (e) Western blot showing the effect of RVX208 of PLK1 in FoxM1 in PAH-SMCs. (f) FoxM1 and PLK1 mRNA is reduced dose-dependently by RVX208 in PAH-SMCs. Data is represented as mean $\pm$ SD. Statistical differences were assessed by Mann-Whitney-U or Kruskal-Wallis test. Significant differences are indicated with an asterisk (* p <0.05; ** p <0.001; *** p <0.0001). Abbreviations. MVEC: microvascular endothelial cell (pulmonary origin), SMC: pulmonary smooth muscle cell (pulmonary origin), AB: Amido black, Si-Scrm: Scrambled siRNA; siBRD4/FoxM1: siRNA against BRD4/FoxM1. NT: No treatment, Veh: Vehicle (DMSO).

Results.

BRD4 regulates the DDR protein FoxM1 and its transcriptional target PLK1 in vascular cells in human PAH.

We confirmed increased BRD4 protein expression in MVECs and SMCs isolated from IPAH patients compared to healthy controls (fig.7.2a). In IPAH, BRD4 localized in the media of larger pulmonary arteries (PAs) with medial hypertrophy, in neointimal fibrotic lesions, and lining the plexus channels within plexiform lesions. In controls, BRD4 localized predominantly in the endothelium (fig.7.7E1). We then investigated the functional relationship of BRD4 with FoxM1 and PLK1, respective downstream targets of BRD4(11), in IPAH SMCs. In previous research, we have shown that expression of FoxM1 is increased in PAH-SMCs(10). FoxM1 is known to up regulate PLK1, an oncogene implied in hyperproliferation and apoptosis-resistance in cancer(15). PLK1 expression was increased in IPAH, both in isolated PAH-SMCs (fig.7.2b) and in the vessel wall (fig.7.2c). To confirm that BRD4 regulates PLK1 via FoxM1 in PAH-SMCs, we inhibited both BRD4 and FoxM1 by siRNA, which both decreased PLK1 protein expression (fig.7.2d). Inhibition of BRD4

by RVX208 also decreased FoxM1 and PLK1 on the protein (fig.7.2e) and transcriptional level in PAH-SMCs (fig.7.2f). Finally, we showed that inhibition of FoxM1 by Thiostreptone dose-dependently decreased PLK1 protein expression (fig.7.7E2). In conclusion, we report that BRD4 expression is increased in MVECs and SMCs of patients with PAH, and induces PLK1 via FoxM1.

Reversal towards a healthy vascular cell phenotype by RVX208.

Next, we assessed the effect of BRD4-FoxM1-PLK1 inhibition on principle features of vascular remodeling in PAH: proliferation, apoptosis-resistance, TGF β /BMP-imbalance and inflammation (fig.7.3a/g/k). We found that 10 μ M RVX208 significantly reduced cell viability, a product of reduced proliferation and increased apoptosis, in control as well as PAH-MVECs and PAH-SMCs (fig.7.3b). RVX208 reduced proliferative signalling in PAH-MVECs by decreasing Cyclin-D1 promotor activity, and normalized the level of Cyclin-D1 mRNA, which had been increased by stimulating healthy MVECs with 10nM TNF α (fig.7.3c). We further showed that medium conditioned by PAH-MVECs induced proliferation of normal SMCs, while 10 μ M RVX208 treatment of PAH-MVECs resulted in conditioned medium that decreased SMC proliferation (fig.7.3d), signifying that RVX208 modulates the pro-proliferative crosstalk of ECs to SMCs. In isolated PAH-SMCs, siBRD4 or 50 μ M RVX208 significantly reduced proliferation and apoptosis-resistance, as shown by decreased Ki67 and increased Annexin V expression (fig.7.3e/f). Similar effects were observed in PAH-PASMCs exposed to siPLK1 or the PLK1 inhibitor BI6727 (fig.7.7E3). Next, we assessed the effect of RVX208 (10 μ M) on TGF β /BMP imbalance, using ID1 and PAI1 as the respective readouts of BMP- and TGF β -mediated signalling. In PAH-MVECs, RVX208 reduced the activity of the PAI1-derived TGF β -responsive CAGA-Luc reporter and the ID1-derived BMP-responsive BRE-Luc reporter (fig.7.3h). In control MVECs, RVX208 reversed the decrease in ID1 and increase in PAI1 mRNA that were induced by TNF α stimulation (fig.3i). The increase in ID1 protein levels was confirmed in PAH-MVECs, likely explained by a concurrent increase in BMPR2 protein upon RVX208 treatment (fig.7.3j). Seeing the regulatory effects of an inflammatory stimulus such as TNF α on the aforementioned processes, we finally assessed the effect of RVX208 on cytokine production. In control MVECs, RVX208 reversed the TNF α -mediated increase in IL8, MCP1 and CCL5 mRNA (fig.7.3l). RVX208 also reduced IL6 and MCP1 secretion by PAH-MVECs (ELISA) (fig.7.3m), and reduced NF κ B promoter activity (fig.7.3n). In conclusion, we report that RVX208 amended the TGF β /BMP imbalance and reversed the pro-proliferative, apoptosis-resistant and inflammatory PAH vascular cell phenotype.

Figure 7.3. Reversal towards a healthy vascular cell phenotype by RVX208. (a) The effect of BRD4-FoxM1-PLK1 inhibition on cell proliferation and survival (hypothesis). (b) MTT cell viability assays with 10 μM RVX208 in control- and PAH-MVECs and PAH-SMCs. (c) Luciferase assay showing decreased activity of the Cyclin-D promoter by RVX208 in PAH-MVECs, and PCR for Cyclin-D1 under TNFα stimulation and RVX208 treatment. (d) SMCs cultured in medium conditioned by PAH-MVECs treated with vehicle or RVX208. (e) Ki67 staining and quantification showing the effect of BRD4 inhibition by siBRD4 and RVX208 on PAH-SMC proliferation. (f) Annexin V staining and quantification showing the effect of BRD4 inhibition by siBRD4 and RVX208 on PAH-SMC apoptosis. (g) The effect of BRD4 inhibition on the TGFβ/BMP imbalance in PAH (hypothesis). (h) Luciferase assay for the BMP reporter (BRE-Luc) and TGFβ reporter (CAGA-Luc) in PAH-MVECs after respectively BMP9 (1 ng/ml) or TGFβ (1 ng/ml) stimulation in the absence or presence of RVX208 treatment. (i) PCR for ID1 and PAI1 under TNFα stimulation and RVX208 treatment. (j) Western blot showing increased BMPR2 and ID1 protein under RVX208 treatment in PAH-MVECs. (k) The effect of BRD4 inhibition on inflammation (hypothesis). (l) PCR for IL8, MCP1 and CCL5 under TNFα stimulation and RVX208 treatment in normal MVECs. (m) ELISA for IL6 and MCP1 in PAH-MVECs. (n) Luciferase assay for the NFκB promoter in PAH-MVECs with RVX208 treatment. Data is represented as mean ± SD. Statistical differences were assessed by Mann-Whitney-U or Kruskal-Wallis test. Significant differences ($p < 0.05$) are indicated with an asterisk.

RVX208 reverses pulmonary vascular remodeling and improves hemodynamics in vivo.

In the Canadian SH-PAH study all rats survived until the end-point. Vascular occlusion, systolic right ventricular pressure (sRVP) and mean pulmonary artery pressure (mPAP) were increased in vehicle-treated rats compared to controls. Treatment with either RVX208 alone or RVX208+CC in SH-PAH rats decreased sRVP, mPAP and pulmonary vascular resistance (PVR) and vascular occlusion by decreasing intimal thickness (fig.7.4b/7.8E4). Medial thickness was only decreased by RVX208+CC (fig.7.8E4). The RV to LV+septal weight ratio (RV/LV+IVS) was increased in SH-PAH compared to controls, but was not significantly decreased by treatment (fig.7.8E4).

Results were largely similar in the Dutch SH-PAH study. 1 rat died in each group (fig.4). RVX208 significantly reduced sRVP, PVR and vascular occlusion, also by reducing intimal thickness. No effects on the RV/LV+IVS weight ratio were observed (fig.7.4e/7.8E5). Pulmonary artery acceleration time (PAAT), an echocardiographic parameter that decreases early during development of pulmonary hypertension(16), was increased by RVX208 treatment (fig.7.8E5).

In the MS-PAH study, 3 rats died before the endpoint at day 35 in the vehicle group versus 2 in the RVX208 group. Vascular occlusion score and intimal thickness were comparable in the MS21 and MS35veh rats, indicating initiation of treatment during end-stage pulmonary vascular disease. RVX208 significantly reduced vascular occlusion and intimal thickness (fig.7.4h). Lung fibrosis, as assessed by Masson staining and quantified in the total lung (fig.7.4j), was decreased by RVX208. Specifically, RVX208 treated lungs showed less fibrosis in the vessel wall (fig.7.4k). While mean PAAT was significantly improved by RVX208, the reduction in sRVP, mPAP and PVR did not reach statistical significance and the RV/LV+IVS weight ratio was not affected by RVX208 (fig.7.4h/7.8E6).

In conclusion, we report that in three independent research groups, oral treatment with RVX208 reversed vascular remodeling in both the SH- and the MS-PAH rat, and improved pulmonary hemodynamics, particularly in the SH-PAH rat.

In vivo effects of RVX208 on FoxM1, PLK1, proliferation, apoptosis, inflammation and BMP signalling.

In the Canadian SH-PAH study, total lung PLK1 mRNA was increased in vehicle-treated rats versus normal controls, and was decreased by RVX+CC (fig.7.5a). FoxM1 expression in PASMCs was increased in SH-vehicle rats versus controls, and decreased significantly by RVX+CC (fig.7.5b). Cell proliferation was increased in SH-vehicle rats versus controls, which was reversed by RVX+CC (fig.7.5c). Apoptosis was not affected in SH-PAH, nor by RVX treatment (fig.7.5d). Finally, mRNA levels of IL6 and MCP1 were increased in SH-PAH versus controls and were decreased by RVX+CC (fig.7.5e/7.8E4).

Figure 7.4. RVX208 reverses pulmonary vascular remodeling and improves hemodynamics in vivo. (a) Experimental design for the Canadian in vivo intervention study with RVX208 ($\pm$ contemporary care) in Sugen-Hypoxia rats (SH-PAH). (b) Quantification of vascular occlusion and hemodynamics. (c) Elastica-van Gieson (EvG) staining, representative examples of vascular lesions. Black bar indicates 20 μ m. (d) Experimental design for the Dutch in vivo intervention study with RVX208 in SH-PAH. (e) Quantification of vascular occlusion and hemodynamics. (f) EvG staining. Left: representative examples of vascular lesions. Black bar indicates 50 μ m. Right: overview of the SH-PAH lung, showing fewer occluded vessels and inflammatory infiltrates in RVX208 treated rats. Black bar indicates 500 μ m. (g) Experimental design for the in vivo intervention study with RVX208 in MCT+Shunt rats (MS-PAH). (h) Quantification of vascular occlusion and hemodynamics (i) EvG staining. Left: representative examples of vascular lesions. Black bar indicates 50 μ m. Right: overview of the MS-PAH lung, showing fewer occluded vessels in RVX208 treated rats. Black bar indicates 500 μ m. (j) Quantification of fibrosis in the whole lung. (k) Masson staining showing increased fibrosis in the vessel wall of vehicle-treated rats. Black bar indicates 50 μ m. Data is represented as mean $\pm$ SD. Statistical differences were assessed by Mann-Whitney-U or Kruskal-Wallis test. Significant differences are indicated with an asterisk (* p <0.05; ** p <0.001). Abbreviations. Veh: vehicle (5% DMSO), CC: contemporary care (macitentan/tadalafil). sRVP: systolic right ventricular pressure. PVR: pulmonary vascular resistance, RV/LV+IVS: right ventricular to left ventricular + intraventricular septal weight ratio.

Figure 7.5. In vivo effects of RVX208 on FoxM1, PLK1, proliferation, apoptosis, inflammation and BMP signaling. (a-e) Canadian SH-PAH study. (f-k) Dutch MS-PAH study. (a) PCR for PLK1 mRNA in whole lung lysates. (b) FoxM1 staining (pink) and quantification of pulmonary vascular FoxM1 expression. SMCs are stained in green. (c) Quantification of pulmonary vascular Ki67 expression (proliferation). (d) Quantification of pulmonary vascular TUNEL expression (apoptosis). (e) PCR for IL6 mRNA in whole lung lysates. (f) Western blots and quantification for FoxM1 and PLK1 protein in total lung lysates. (g) PCR for PLK1 mRNA in whole lung lysates. (h) FoxM1 and PLK1 expression in rat pulmonary arterioles, bar represents 50µm. (i) Staining (brown, arrows) and quantification of pulmonary vascular Cleaved Caspase-3 expression (apoptosis). (j) Western blot and quantification for BMPR2 and ID3 in whole lung lysates. (k) PCR for IL6 mRNA in whole lung lysates. Data is represented as mean±SD. Statistical differences were assessed by Mann-Whitney-U or Kruskal-Wallis test. Significant differences are indicated with an asterisk (*p<0.05; **p<0.001; ***p<0.0001).

In the MS-PAH study, FoxM1 and PLK1 protein expression tended to be higher in the total lung lysate of MS35Veh compared to normal control. RVX208 significantly decreased FoxM1 protein, and tended to decrease PLK1 protein (fig.7.4gf). Total lung PLK1 mRNA was decreased by RVX208 (fig.7.5g). FoxM1 mRNA was not detected. Vascular FoxM1 expression, located mainly in the media, was increased in MS35Veh versus controls, which was blunted by RVX208 treatment (fig.7.5h). Vascular PLK1 expression was largely absent in healthy controls, was increased in the endothelium of MS35Veh, and was reduced by RVX208 (fig.7.5h). Contrary to the SH-PAH study, apoptosis (assessed by cleaved-caspase-3 staining which located mainly in the intima), was increased by RVX208 treatment in MS35-PAH (fig.7.5i). Ki67 staining is usually scarce in the vasculature of MS35Veh(14), and was therefore not assessed here. Furthermore, RVX208 treatment resulted in increased BMPR2 and ID3 protein (fig.7.5j). IL6 and MCP1 mRNA level was low in all MS35RVX rats, while high expression was observed in 4/9 MS35Veh rats, and CCL5 mRNA was significantly reduced by RVX208 (fig.7.5k/7.8E6). In conclusion we report a consistent down regulation of FoxM1 and PLK1 and a decrease in cytokine mRNA levels after RVX208 treatment in both the SH- and MS-PAH model. Model-specific effects of RVX208 treatment were observed regarding proliferation and apoptosis.

RVX208 supports right ventricular function during increased pressure load.

We finally studied the effects of RVX208 treatment in RV pressure load induced by PAB in rats(16,17) to confirm safety of the drug during RV compromise. 3 rats died during surgery. At day 14, we measured PAB pressure gradient using echocardiography to confirm effective and equal pressure load at baseline. In PAB56RVX rats, the pressure gradient increased from day 14 to 56, indicating adaptation to pressure load (fig.7.6b). In line with this observation, CO, RV stroke volume, stroke work, and RV power were increased by RVX208 as well (fig.7.6b). TAPSE was normal in both groups. The relatively higher contractile force in PAB56RVX was also reflected in an increase in RV/LV+IVS mass. RV cardiomyocyte cross sectional area showed a 2-fold increase compared to sham conditions (fig.7.8E7), but was similar in PAB56RVX and PAB56Veh (fig.6c), indicating that the increased RV mass in PAB56RVX could be due to a proportional increase in the length of cardiomyocytes, which is associated to higher contractility(19). The increased RV mass in RVX208-treated rats was not accompanied by evidence of adverse remodeling, as indicated by a favourable capillary/myocyte ratio and a low percentage of fibrosis (fig.7.6d)(20). Furthermore, β -Myosin heavy chain (MHC) to α -MHC ratio, typically increased in pathologic cardiac hypertrophy, tended to be lower in PAB56RVX. Together, these data indicated that RVX208 stimulates RV adaptation in response to increased pressure load without signs of adverse remodeling, suggesting a favourable safety profile or even therapeutic benefit for patients with RV dysfunction in advanced PAH. FoxM1 and PLK1 mRNA levels were also increased in the PAB56Veh RV compared to sham-operated animals, but were not decreased by RVX208

Figure 7.6. RVX208 supports right ventricular function during increased pressure load. (a) Experimental design for the in vivo intervention study with RVX208 in the rat pulmonary artery banding (PAB) model for isolated RV pressure load. (b) Hemodynamic and histologic evaluation. (c) Wheat germ agglutinin staining for cardiomyocyte cross sectional area measurement (CCSA). (d) Masson staining for fibrosis measurement. (e) PCR for FoxM1 and PLK1 mRNA in whole RV lysates. (f) Staining for FoxM1 and PLK1 (both in green) in RV tissue of PAB rats. (g) Tissue exposure levels for RVX208 at 100mg/kg concentration administered via drinking water in 5% DMSO. The desired clinical dose is between 1 and 2 μM . Data is represented as mean $\pm$ SD. Statistical differences were assessed by Mann-Whitney-U or Kruskal-Wallis test. Significant differences (p<0.05) are indicated with an asterisk. Abbreviations. CO: Cardiac Output; TAPSE: tricuspid annular plane systolic excursion; Cap: Capillary. MHC: myosin heavy chain.

(fig.7.6e/f). In conclusion, we report that RVX208 treatment support RV function in the context of increased RV pressure load.

As an additional verification of oral RVX208 bioavailability at the 100mg/kg dose, we used pulmonary and left-ventricular tissue of the RVX-treated rats in the PAB study to determine tissue exposure levels. Mean pulmonary exposure was $2.1 \pm 1.8 \mu\text{M}$, mean left ventricular exposure was $0.82 \pm 0.4 \mu\text{M}$ (fig.7.6g), which is in the same range as the maximum plasma concentration (Cmax) at the desired clinical dose.

Fig E1 BRD4 expression in the vasculature of control and PAH patients

Fig E2 Effect of FoxM1 inhibitor Thiostreptone on PLK1 expression in PAH-SMCs

Fig E3 Proliferation of PAH-SMCs during PLK1 inhibition

Apoptosis of PAH-SMCs during PLK1 inhibition

Figure 7.7 E1. Localization of BRD4 (red) in IPAH and controls. ECs are marked by PECAM in green. Nuclei are marked by DAPI in blue. Plexus channels are marked with an arrow. E2. Western blot (WB) showing the effect of the FoxM1 inhibitor Thiostreptone (Thio) on PLK1 expression in PAH-SMCs. E3. Ki67 and Annexin V staining and quantification showing the effect of PLK1 inhibition by siPLK1 and BI6727 on PAH-SMC proliferation and apoptosis respectively. Data is represented as mean $\pm$ SD. Statistical differences were assessed by Mann-Whitney-U test. Significant differences ($p < 0.05$) are indicated with an asterisk. Abbreviations. PA: Pulmonary artery; NT: no treatment, SiScr: Scrambled SiRNA, SiPLK1: SiRNA against PLK1. Veh: Vehicle. BI6727: PLK1 inhibitor.

Fig E4: SH-PAH, Québec

Fig E5: SH-PAH, Amsterdam

Fig E6: MS-PAH, Groningen

Figure 7.8 E4. Additional hemodynamic evaluation of the Canadian Sugen-Hypoxia PAH (SH-PAH) study with RVX208 and RVX208 combined with contemporary care (CC, macitentan and tadalafil, both 10mg/kg/day). Vascular occlusion scores correlated with systolic right ventricular pressures (sRVP). MCP1 cytokine mRNA levels in vivo. E5. Additional hemodynamic evaluation of the Dutch SH-PAH study. Vascular occlusion scores correlated with sRVP. E6. Additional hemodynamic evaluation of the Dutch Monocrotalin+Shunt (MS)-PAH study, including in vivo levels of MCP1 and CCL5 mRNA. E7. Cardiomyocyte cross sectional area (CCSA) in sham-operated rats compared to Pulmonary Artery Banding (PAB) rats. Statistical differences were assessed by Mann-Whitney-U or Kruskal-Wallis test. Significant differences are indicated with an asterisk (* $p<0.05$; ** $p<0.001$; *** $p<0.0001$).

Table 7.1. Characteristics of pulmonary arterial hypertension patients for MVEC isolation(E4)

mPAP (mmHg)	PAH Etiology	Sex	Age
43	IPAH	F	42
89	IPAH	F	22
85	IPAH	M	21
94	IPAH	F	30

Discussion.

We described the union of two independent preclinical trials in three independent research labs that explored the therapeutic potential of the clinically available BET inhibitor RVX208 in PAH. Together, we confirmed that BRD4, the best studied member of the BET family, is upregulated in the remodeled pulmonary vasculature of PAH patients, where it regulated the expression of the oncogene *PLK1* and *FoxM1*, a DDR protein implied in vascular remodeling in PAH(10,19,20). BET inhibition by RVX208 normalized the hyper-proliferative, apoptosis-resistant and pro-inflammatory phenotype of MVECs and SMCs isolated from PAH patients. At a clinically relevant dose, RVX208 reversed vascular remodeling in multiple complementary preclinical models of PAH, and could be combined safely with current PAH-therapy. Finally, we showed that RVX208 supports the pressure loaded RV in rats, indicating a beneficial, dual mode of action for patients with PAH-associated RV pressure overload.

BET proteins such as BRD4 can be recruited to the DNA in a variety of pathological circumstances, such as in cancer or inflammatory and cardiovascular diseases(5,6,21,22). BRD4 recruitment has a common association with compromised DNA integrity(3) or genotoxic conditions such as replicative, oncogenic or oxidative stress(8). As an epigenetic reader, BRD4 is able to sense double strand breaks in the DNA where it elicits a DNA damage response (DDR) by stimulating γ H2AX signalling(3), leading to survival rather than apoptosis of the cell. While an effective DDR is crucial in normal physiology to repair DNA and prevent apoptosis after DNA damage, persistent DDR activation (such as by BRD4) during chronic genotoxic conditions can lead to aberrant DNA repair and to survival of cells with genomic abnormalities(2). In these cells, BRD4 can further modulate the chromatin landscape(3), and enable transcription of a context-dependent, yet characteristic profile of pro-proliferative, pro-survival(25) and/or pro-inflammatory factors(9). These data indicated BRD4 as an essential driver of DNA damage-associated pathologies. Substantial evidence indicates that DNA damage and prolonged DDR activation are also implied in PAH(26). DDR proteins such as γ H2AX, 53BP1 and PARP1 are up regulated in the pulmonary vasculature of PAH patients(1), and many of the known triggers for PAH cause genotoxic stress. These include acute genotoxic hits such as alkylating chemotherapy, toxins or irradiation, or chronic genotoxicity as in inflammatory disease like systemic sclerosis, schistosomiasis or HIV. Persistent BMPR2 down regulation, like in BMPR2-mutant PAH or as occurs in situations of chronically disturbed shear stress(27), also compromises DNA integrity through loss of normal DNA repair mechanisms(2). Taken together, these data suggested that modulating the DDR by BRD4 inhibition could be beneficial for PAH patients. This was first established by inhibiting BRD4 specifically via JQ1 and siRNA in the Sugen-Hypoxia rat(7). JQ1 and siRNA both improved pulmonary hemodynamics and reversed vascular remodeling, which was associated with reduced apoptosis-resistance and proliferation(7).

In other work, we showed that inhibition of FoxM1, another master regulator of the DDR associated with ineffective DNA repair(28), reversed Sugen-Hypoxia and MCT-PAH in a similar fashion(10). This underlined the importance of the BRD4-FoxM1 axis in PAH. Here, we confirmed once more that BRD4 and FoxM1 are up regulated in PAH. As reported in cancer cells(11), and for the first time in PAH, we show that BRD4 and FoxM1 regulate the expression of PLK1, an oncogene involved in the proliferation-apoptosis (im)balance (fig.7.2d). In cancer, where PLK1 is commonly overexpressed(15), it is thought that PLK1 promotes tumorigenesis by stimulating survival, cell-cycle re-entry and proliferation(29). We hypothesized that this characteristic could also be relevant to the PAH-context, and confirmed PLK1 overexpression in isolated PAH-SMCs and the in the remodeled PAH vasculature (fig.7.2b/c). We further demonstrated that specific inhibition of PLK1 by siRNA or the PLK1 inhibitor BI6727 decreased the cancer-like phenotype of SMCs in PAH (fig.E3).

PAH patients are in critical need of new, directly applicable treatment strategies that are able to reverse advanced vascular remodeling and are safe in context of right ventricular failure. Contemporary treatment is aimed mainly at vasodilation and slows down the progression of vascular occlusion at best. The BRD4 inhibitor JQ1 held promise for treatment of end-stage PAH(7), but is not suitable for clinical use. We therefore chose RVX208 as a clinically relevant, orally available inhibitor of the BET-family that includes BRD4. In vitro, RVX208 reduced PAH-SMC proliferation and survival as effectively as BRD4- and PLK1-siRNA, or PLK1 inhibition by BI6727 (fig.7.3e/f). RVX208 had a comparable effect on proliferation and survival in PAH-MVECs (fig.7.3b-d). Additionally, RVX208 was able to normalize the pro-inflammatory phenotype observed in PAH-MVECs (fig.7.3l-n), which is in line with the results of RVX208 treatment in pro-inflammatory ECs and SMCs in atherosclerosis(30). Interestingly, inflammation and BRD4 were also recently implicated in coronary artery remodelling in PAH patients, suggesting that BRD4 inhibition may have beneficial effects beyond the pulmonary circulation in PAH(31). A novel finding in this study was that RVX208 also increased BMPR2 signalling in PAH-MVECs, or restored the TGF β -BMP balance in healthy MVECs after disturbance by TNF α , a genotoxic hit known to downregulate BMPR2 in ECs and predispose to PAH(32) (fig.7.3h-j). This was an unexpected positive contribution of RVX208 in restoring the PAH phenotype on top of the more commonly known actions of BRD4 inhibition.

In vivo, we have shown that a 4-week treatment with RVX208 at a clinically relevant dose (fig.7.6g) significantly reduced vascular occlusion and improved hemodynamics in two independent rat studies with Sugen-Hypoxia PAH (fig.7.4a-f), which was as effective as treatment with JQ1 or BRD4-SiRNA in previous work(7). To improve translatability to the clinic, we showed that RVX208 can be combined safely with contemporary care (Tadalafil and Macitentan). However, RVX208 contributed to the most profound beneficial effects,

particularly with regard to vascular remodeling (fig.7.4b), indicating that RVX208 alone might also suffice. The MCT-Shunt rat studies showed that short-term treatment with RVX208 already improves severe neointimal vascular remodeling, even with the shunt still in place (fig.7.4h). Importantly, together these data demonstrated that the beneficial effects of RVX208 were not PAH model-specific.

We finally investigated the effect of RVX208 treatment on the RV during pressure load, primarily to ensure safety of the drug in the context of RV dysfunction. Interestingly, FoxM1 and PLK1 mRNA were also significantly increased by pressure load in the rat RV, indicating a potential role for BRD4-FoxM1-PLK1 signalling in RV pathology as well (fig.7.6e/f). We found that RVX208 treatment stimulated physiological RV adaptation, which increased RV power (fig.7.6b), suggesting that RVX208 treatment could be beneficial in patients with PAH-associated RV dysfunction, in addition to the effects on the pulmonary vasculature. Longer term studies may determine whether RVX208 can prevent or reverse the development of RV failure during pressure load. Our data are consistent with the results of BRD4 inhibition by JQ1 in physiological left ventricular remodeling, where treatment did not suppress LV adaptation due to high-intensity exercise in mice. In mice with severe LV failure due to pressure load and myocardial infarction, JQ1 treatment improved LV function, reduced LV fibrosis, and repressed a spectrum of heart failure and fibrosis-associated genes, including TGF β and NF κ B(6). Overall, interfering with the BRD4 pathway does not limit the ventricular response to increased afterload, and may even enhance its adaptation.

The present study is the first to implement the methodological rigor preconized in PAH preclinical confirmatory studies to increase the reproducibility and translatability of pre-clinical research, including independent replication, use of multiple animal models, treatment on top of contemporary care and assessment of direct effects on the RV, as published recently(12).

Conclusion.

In conclusion, we describe the unified results of the first multicentre randomized preclinical trial performed in three independent labs that confirmed the therapeutic potential of the clinically available BET inhibitor RVX208 in various PAH rodent models. We also report that RVX208 modulates the pro-proliferative, pro-survival and pro-inflammatory pathways by direct interaction with FoxM1 and PLK1. This reversed the PAH phenotype in isolated PAH-MVECs and SMCs, reversed vascular remodeling in two complimentary preclinical PAH models, and supported the pressure loaded RV. Taken together, these data support the establishment of a clinical trial with RVX208 in PAH patients.

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Supplemental Methods.

Cell culture and treatments.

Pulmonary microvascular endothelial cells (MVECs) were isolated from idiopathic PAH patients and normal lung explant tissue as described before(E1). See table 1 for the patient characteristics. MVECs were treated with vehicle or RVX-208 (10 μ M) for 16 hours and performed 1) cell proliferation (MTT) assays to measure cell growth, 2) luciferase reporter assays (CyclinD1-luc, BRE-luc, CAGA-luc and NF κ B-luc), 3) Quantitative RT-PCR, 4) western blotting as described previously(E2, E3).

PAH-PASMCs (n=10 cell lines) were isolated from small PAs (<1mm diameter) from PAH patients. Controls PASMCs (n=8 cell lines) were either purchased from Cell Application or isolated from non-PAH patients. Cells were used at passages 5 to 9 for experiments. PASMCs were grown in high-glucose DMEM supplemented with 10% Fetal Bovine Serum (FBS, Thermo Fisher Scientific) and 1% antibiotic/antimycotic (Thermo Fisher Scientific). RVX-208 (Resveralogix Corp.), Thiostrepton (Santa Cruz Biotechnology), and BI6727 (ApexBio) were dissolved in dimethylsulfoxide (DMSO). siBRD4 (ID# s23903, Thermo Fisher Scientific), siPLK1 (ID# HSS108118, Thermo Fisher Scientific) and scrambled control siRNA (ID# 4390847, Thermo Fisher Scientific) were transfected at a final concentration of 20nM with Lipofectamine RNAiMAX (Thermo Fisher Scientific).

Quantitative RT-PCR.

Total RNA extraction from cultured PAH-PASMCs was performed using TRIzol reagent (Invitrogen). cDNA was synthesized with the qScriptFlex cDNA kit (Quanta Bio) using 200ng of total RNA and random primers following the manufacturer's instructions. Total RNA was extracted from MVECs was extracted using NucleoSpin RNA II (Machery Nagel, Düren) and from rat lung and right ventricular tissue using TRIPURE (Roche) and cDNA was synthesized using iScript (Bio-Rad). Real time qPCR was performed with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and run in triplicate on a QuantStudio 7 Flex Real-time PCR System (Applied Biosystems). Analysis of qPCR results was done using the delta delta Ct method. 18S and GAPDH were used as internal controls. Amplification specificity was controlled by post-amplification melting curve analysis. In addition, qPCR products were electrophoresed on 2% agarose gels (w/v) to confirm the presence of a single band of expected size.

Protein expression.

Proteins were extracted from cell pellets using a 2% Chaps protein extraction buffer supplemented with a protease-inhibitor cocktail (Roche) and quantified as previously described(5). Equal amount of proteins was resolved on SDS-polyacrylamide gels and

transferred to PVDF membranes. Membranes were subsequently blocked with either 5% non-fat dry milk or 10% goat serum in TBS-T buffer, and incubated with primary antibodies in 3% BSA overnight at 4°C. Rinsed 3 times with TBS-T buffer, membranes were incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour at RT in 5% non-fat milk or in 3% BSA. Antibodies were revealed using ECL reagents (Perkin-Elmer) and labeled proteins were detected with the imaging Chemidoc MP system (Bio-Rad Laboratories). Protein expression was quantified using the Image lab software (Bio-Rad Laboratories) and normalized to Amido black or GAPDH or Vinculin as previously described(E5). The following antibodies directed against BRD4 (Abcam, ab128874), FOXM1 (Santa Cruz Biotechnology, sc-376471), PLK1 (Abcam, ab17056), BMPR2 (Thermo Scientific, MA5-15827), and ID3 (Santa Cruz, sc-490) were used.

MTT assays.

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assays were performed as described previously(E6). Briefly, cells were seeded in a 96-well plate at a density of 3×10^3 cells/well and incubated overnight. Cells were made quiescent by incubation in medium without FCS for 6 h and then incubated overnight with FCS (10% [vol/vol]) and vehicle or RVX-208 (10uM) for 24 h. After the incubation, cells were incubated with 10 μ l of MTT reagent (5 mg/ml) for 3 h at 37°C. 100 μ l of isopropanol was added to each well and incubated for 15 min. Colorimetric analysis was performed with an enzyme-linked immunosorbent assay (ELISA) plate reader. Cells were also counted using an automated cell counter (Biorad). Each experiment (in quadruplicate) was repeated at least three times.

PASMC growth in cultured medium of MVECs.

PAH MVECs were pretreated with vehicle or RVX-208 (10uM) for 24 h. Conditioned medium from these cells was added to control PASMCs and incubated for 48 hours.

In vitro proliferation and apoptosis measurements in PASMCs.

PASMC were cultured for 48 hours in 10% FBS (a condition that promotes proliferation) or 0.1% FBS (a starvation condition that promotes apoptosis) in presence or absence of RVX-208, siBRD4 or their respective controls. PASMC proliferation and resistance to apoptosis were measured by Ki67 labeling or ApoAlert Annexin V-FITC assay (Millipore), respectively, as previously described(5). The proliferative and apoptosis indices were calculated as the ratio of immunoreactive cells to the total number of DAPI-stained cells per field. For each cell line, experiments were performed in triplicate and at least 750 cells per condition were counted.

Luciferase assays.

Primary MVECs were transfected using Lipofectamine 3000 transfection reagent (Thermo Scientific, L3000001) as per the instructions of the manufacturer. Renilla plasmid was co-transfected as an internal control. 48 hours after transfection, the cells were treated with vehicle or RVX-208 (10uM) for the indicated time points and lysed for measuring the luciferase activity using luciferase reporter assay system from Promega by a Perkin Elmer luminometer Victor3 1420. The amount of plasmid DNA in each well was equalized with an empty vector when necessary and each experiment (in duplicate) was repeated at least three times. MVECs were transduced with two reporter construct containing either TGF β responsive elements isolated from the PAI-1 promotor (CAGA-Luc or BMP responsive elements isolated from the ID1 promotor (BRE-Luc). To be able to determine the effect of RVX-208 on the signal transduction pathway, the CAGA-Luc transduced cells were stimulated with TGF β 3 (1ng/ml) while the BRE-Luc cells were stimulated with BMP9 (1 ng/ml) for 16h in the absence or presence of RVX-208.

In vivo experiments.

All experimental protocols were approved by the Dutch national, and institutional animal care and use committees of the University of Groningen (NL), the VU University Amsterdam (NL), and the Laval University Quebec (CAN), and were conducted with rigor and care that is now routine for clinical studies, including incorporation procedures to ensure blinding and random allocation of animal to therapeutic groups as recommended(E7). Vascular occlusion score was the primary end-point for all in vivo studies in PAH models. Sample sizes were based on previous research in our labs, reporting occlusion scores around $60\pm 10\%$ in end-stage PAH, and an effects size of ± 20 points reduction in vascular occlusion, which was associated with haemodynamic improvement (E8, E9). RV Stroke volume and work were the primary end-points for the PAB studies. Sample size was based on previous studies in our lab reporting that RV stroke work decreases with ± 20 points in PAB rats upon development of RV failure, from 30 ± 8 to $10\text{mmHg}\cdot\text{mL}$ (E10).

Treatment with RVX208 in Sugen-Hypoxia rat PAH and combination with contemporary care (Québec).

Sugen-Hypoxia-PAH was induced in 36 adult male Sprague-Dawley rats ($\pm 250\text{g}$, Charles River Laboratories) by a subcutaneous 20mg/kg injection of SU5416 (Sigma) and were exposed to hypoxia ($10\% \text{O}_2$) for 3 weeks, followed by 7 weeks of normoxia. Saline-injected rats kept at normoxia served as controls. From day 42 to day 70 rats were randomly assigned to either oral gavage with vehicle (EA006, as described previously(E11)), vehicle + 90mg/kg RVX208, or vehicle + 90mg/kg/day RVX208 + contemporary care (CC, 10mg/kg/day tadalafil + 10mg/kg/day macitentan). Before sacrifice, all rats underwent haemodynamic

evaluation by echocardiography and closed chest right heart catheterization. The lungs and heart were collected for histopathologic evaluation.

Treatment with RVX208 in Sugen-Hypoxia rat PAH (Amsterdam).

Sugen-Hypoxia-PAH was induced in 24 adult male Sprague-Dawley rats (± 200 g, Charles River, FR) by a subcutaneous 20mg/kg injection of SU5416 (25 mg/kg, Tocris) and were exposed to hypoxia (10% O₂) for 3 weeks, followed by 7 weeks of normoxia. From day 42 to day 70 rats were randomly assigned to either oral gavage with vehicle (5% DMSO in drinking water) or vehicle + 100mg/kg RVX208. Before sacrifice, all rats underwent haemodynamic evaluation by echocardiography and right heart catheterization. The lungs and heart were collected for histopathologic evaluation.

Treatment with RVX208 in MCT+Shunt rat PAH (Groningen).

Flow-induced PAH was induced in 25 adult male Lewis rats (± 200 g, Envigo, UK) by a subcutaneous 60 mg/kg injection of monocrotalin (MCT, Sigma) at T0 days, followed at T7 by aorto-caval shunt surgery, which doubles pulmonary blood flow. MCT+Shunt induces neomuscularization and medial hypertrophy from T7 to T14, neointimal lesions from T14 to T21 and RV failure from T21 to T28(E9, E12). Rats were randomly assigned to 3 groups: 1) MCT+Shunt sacrificed at T21 (MS21) as a baseline group; 2) treatment with vehicle (5% DMSO in drinking water) from T21, with sacrifice at T35 (MS35Veh); 3) treatment from T21 with 100mg/kg/day RVX208 in vehicle with sacrifice at T35 (MS35RVX). Before sacrifice, all rats underwent haemodynamic evaluation by echocardiography and right heart catheterization. The lungs and heart were collected for histopathologic evaluation.

Treatment with RVX208 in pulmonary artery banding RV pressure load rats (Groningen).

RV pressure load was created in 16 adult male Wistar rats (± 200 g, Charles River, Fr) by main pulmonary artery banding (PAB) surgery at T0, with a tight constriction at the size of an 18G needle(E10, E13). PAB gradient was assessed at T14. Rats were randomly assigned to 1) treatment with vehicle (5%DMSO in drinking water) from T28 with sacrifice at T56 (PABveh56), or 2) treatment with 100mg/kg/day RVX208 in vehicle from T28 with sacrifice at T56. Before sacrifice, all rats underwent haemodynamic evaluation by echocardiography (echo). The heart was collected for histopathologic evaluation.

Quantitative pulmonary vascular morphometry.

4 μ m paraffin-embedded lung sections were stained according to the Elastica-Van Gieson protocol and scanned on 40x magnification (Hamamatsu nanoviewer, Japan). All slides were masked, blinding the investigator for the experimental group. 40 vessels (diameter

<50 μm) per lung were analysed according to a blinded, standardised pulmonary vascular morphometry protocol, described in detail previously(E9).

Pulmonary and RV fibrosis and quantification.

For measurement of tissue fibrosis, 4 μm paraffin-embedded lung sections were stained according to the Masson protocol and scanned on 40x magnification (Hamamatsu nanoviewer, Japan). Then, % fibrosis was calculated using the positive pixel count algorithm (V9) on image scope V12.3.

Immunofluorescence (IF) and immunohistochemistry (IHC).

Paraffin-embedded lung or RV tissues were sectioned at 5 μm , deparaffinized, rehydrated in graded ethanols, and then subjected to heat-induced epitope retrieval using citrate buffer. Following antigen retrieval, the sections were blocked with normal goat serum (2%) in PBS for 2h at room temperature. Then, sections were incubated with primary antibodies at 4°C overnight. Mouse monoclonal anti-PLK1 (1:100, Abcam, ab17056), mouse monoclonal anti-FOXM1 (1:100, Santa Cruz Biotechnology, sc-376471), rabbit polyclonal anti-Ki67 (1:400, Millipore, ab9260), rabbit polyclonal anti-cleaved Caspase 3 (1:100, Cell Signaling, #9661), mouse monoclonal anti-alpha smooth muscle actin (αSMA , 1:200, Sigma, A2547), rabbit polyclonal anti- αSMA (1:200, Abcam, ab5694), or rabbit monoclonal anti-PECAM (1:1000, Santa Cruz), were used as primary antibodies. For IF, proteins were detected using appropriate fluorescent-dye conjugated secondary antibodies (Thermo Fisher Scientific). IF slides were analyzed under the Zeiss AxioObserver Z1 inverted microscope. For IHC, species-specific immunoglobulin G coupled to peroxidase were used as secondary antibody and then stained by diaminobenzidine. All slides were masked and scanned on 40x magnification. 20 vessels per lung were analysed and quantified according to a standardised pulmonary vascular immunohistochemistry protocol, described previously(E9).

Cardiomyocyte Cross-sectional Area, Capillary Density and Capillary/Myocyte ratio.

Wheat Germ Agglutinin (WGA) and PECAM stained RV tissue sections were scanned under the Zeiss AxioObserver Z1 inverted microscope on 40x magnification. Cardiomyocyte cross-sectional area (CCSA), Capillary Density and Capillary/Myocyte ratio were analyzed according to standard protocol described previously (E10).

Statistical analyses.

Statistical analyses were performed using SPSS. Statistical differences were assessed using the Mann-Whitney-U or Kruskal-Wallis non-parametric tests with Dunn's post-hoc correction for multiple comparisons, when appropriate. $P < 0.05$ was regarded significant.

Supplementary Table 2. Human and primer sequences for PCR.

Gene	Human Primer	Gene	Rat Primer
FOXM1	Fw: CTTCTAGACACCTGGAGC Rv: AGCCAGTCCATCAGAACTC	Foxm1	Fw: CTCCTTCTGGACCATTACCC Rv: TCTGCTGTGATTCCAAGTGC
CCL5	Fw: CGCTGTCATCCTCATTGCTA Rv: TGTACTCCCGAACCCATTTC	IL6	Fw: CCGGAGAGGAGACTTCACAG Rv: ACAGTGCATCATCGCTGTTC
PLK1	Fw: ATTCCCAAGCACATCAACCC Rv: GGAGACGGGCAGGGATATAG	Plk1	Fw: CAGGAAGCCTCTCACAGTCC Rv: ACTAAGGTGGCACTCGATGG
PAI-1	Fw: CACAAATCAGACGGCAGCACT Rv: CATCGGGCGTGGTGAAGTTC	Mcp-1	Fw: ATGCAGTTAATGCCCCACTC Rv: TTCCTTATTTGGGGTCAGCAC
IL8	Fw: CTGGCCGTGGCTCTCTTG Rv: CTTGGCAAACTGCACCTTCA	IL8	Fw: CCCCCATGGTTCAGAAGATTG Rv: TTGTCAGAAGCCAGCGTTTAC
MCP-1	Fw: CCTAGCTTTCCCCAGACACC Rv: CCCAGGGGTAGAACTGTGG	Gapdh	Fw: GGTGGACCTCATGGCCTACA Rv: CTCTCTTGCTCTCAGTATCCTTGCT
ID1	Fw: CTGCTCTACGACATGAACGG Rv: GAAGGTCCCTGATGTAGTCGAT		
CyclinD1	Fw: CTTCTAGACACCTGGAGC Rv: AGCCAGTCCATCAGAACTC		
18S	Fw: CGCACGGCCGGTACAGTGAA Rv: GGGAGAGGAGCGAGCGACCA		
GAPDH	Fw: AGCCACATCGCTCAGACAC Rv: GCCCAATACGACCAATTC		

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8

Converging paths of pulmonary arterial hypertension and cellular senescence.

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Abstract.

Cellular senescence is recognized as a crucial contributor to the pathobiology of various degenerative and cardiovascular diseases, like idiopathic pulmonary fibrosis and atherosclerosis. We describe the potential link between cellular senescence and the degenerative character of neointimal pulmonary vascular disease in pulmonary arterial hypertension (PAH). Senescence markers have been described in remodeled pulmonary arteries, triggers that induce senescence also induce PAH, pathways known to be disturbed in PAH, such as TGF β /BMP and TNF α , are also causally associated to senescence and interventions that target a senescence phenotype, also target pulmonary vascular remodeling in vivo. These data provide a basis for further exploration of the role for senescence in the pathobiology of PAH and for preclinical trials with senolytic class drugs.

Introduction.

Pulmonary arterial hypertension (PAH) is a severe arteriopathy in which a characteristic form of neointimal vascular remodeling progressively occludes the pulmonary arteries. The vascular lesions observed in the various PAH etiologies typically consist of dysfunctional endothelial and smooth muscle cells that have formed a neointima and a hypertrophied media. In severe lesions, the elastic laminae are disintegrated and the vessel wall and perivascular area are inflamed and fibrotic(1). No drugs yet exist in the clinic that can reverse this sclerotic end-stage vascular sclerotic phenotype, which is commonly referred to as irreversible(2). Primary pathologic features of vascular remodeling in PAH are hyper proliferation and apoptosis-resistance of vascular cells, accompanied by the expression of an extensive profile of pro-inflammatory cytokines(3). Recently, a role for DNA damage has also been established(4). Combined, these mechanisms could also indicate a process of cellular senescence, which has thus far remained relatively unexplored in the context of PAH(5). The last decades, a fundamental role has been established for senescence in the pathogenesis of many degenerative and cardiovascular diseases like idiopathic pulmonary fibrosis (IPF) (6), chronic obstructive pulmonary disease (COPD) (5,7–9), and atherosclerosis (10), which all share many parallels with the pathogenesis of PAH. In this review, we discuss the potential role for cellular senescence in PAH as a pathologic contributor to neointimal vascular remodeling, and as a novel target for treatment.

Senescence and the senescence-associated secretory phenotype.

Cellular senescence describes the transition of a cell, induced by various forms of cellular stress, from a (potentially) proliferative or regenerative state into permanent, irreversible growth arrest (11). This transition is typically accompanied by context- and cell type-dependent phenotypical changes in morphology, chromatin structure and secretome(12). Senescent cells also up regulate specific senescent cell anti-apoptotic pathways (SCAPs) like survivin and bcl2(13), which makes them resistant to the pro-apoptotic environment in which they usually occur. The development of senescence is essentially regulated by the tumour suppressor protein p53 and the cell cycle inhibitors, p16^{ink4A} and p21^{cip1}(14), however various senescence-inducing programs are being identified(11). Senescence occurs in biological aging and is associated with many degenerative diseases of the elderly such as Parkinson's, arthrosis and IPF(6). Specific triggers for cellular senescence, some of which are not necessarily associated with old age, include replicative stress, telomere attrition, oncogene activation, and prolonged exposure to γ -irradiation, reactive oxygen species (ROS), or inflammation(15). The common denominator for these pathogenic triggers for senescence is that they are all genotoxic, i.e. converge to DNA damage(15). Still, the occurrence of senescence in response to DNA damage is primarily physiological. In cancer, senescence is recognized as an important tumour-suppressive mechanism that protects

cells with accumulating DNA damage from further proliferating(12). Yet, the presence of senescent cells in tumours was also linked to a pro-inflammatory senescence-associated secretory phenotype (SASP) that paradoxically seemed to promote tumour progression and tissue degeneration, and contributed to chemotherapy-resistance(16–18). This implied that senescent cells, although unable to replicate, can still be metabolically active and as such influence their microenvironment in a cell non-autonomous fashion (19). The SASP has since been identified as a critical driver of tissue degeneration and functional decline in various other senescence-associated pathologies(15). A SASP typically consists of numerous interleukins (e.g. IL 1 α / β , 6 or 8), cyto- and chemokines (e.g. TNF α , MCP1), proteases (e.g. MMPs, cathepsins) and angiogenic growth factors (e.g. VEGF, PAI1). The exact secretome however, is dynamic and depends on cell type, cause of senescence, microenvironment and the biological pathway by which the SASP is activated(19). Activation of the SASP can be regulated by transcription factors such as NF κ B, mTOR and GATA4, or by epigenetic “super enhancers”, like BRD4 and HMGB2, which reorganize (open up) the chromatin structure to facilitate transcription of SASP genes(19). Combined, these data have established a role for senescent cells and the SASP as a driver for disease, which has also made them an opportune target for intervention in degenerative pathologies. Local clearance of senescent cells has, in multiple degenerative disease models, already shown to attenuate disease progression by decreasing the SASP and creating a pro-regenerative environment(20–22).

Vascular senescence.

Aging is a risk factor for the vast majority of cardiovascular diseases, and many cardiovascular diseases are, vice versa, associated with premature vascular aging and vascular cell senescence(23). Typical age-related changes in the systemic vasculature include endothelial dysfunction, intimal thickening, medial hypertrophy, increased collagen deposition and fractured elastin(23). In atherosclerosis especially, it is becoming evident that senescent cells play a deleterious role in disease development and progression(24). The principle risk factors for atherosclerosis are biological aging, smoking and diabetes. Accumulating DNA damage and vascular cell senescence are most likely a consequence of these risk factors due to a combination of telomere attrition, oxidative stress, and glycation(10). During the development of atherosclerosis, senescent endothelial (EC) and smooth muscle cells (SMC) were shown to accumulate particularly in areas of plaque formation(25). Senescent ECs in these lesions produce less nitric oxide and prostacyclin, and both ECs and SMCs develop a pro-inflammatory, pro-fibrotic SASP(26). Altogether, this leads to a dysfunctional vessel that is constricted and intrinsically inflamed, which further exacerbates plaque formation, creates a pro-thrombotic milieu and leads to stiffening and systemic hypertension(23). Senescent ECs also lose their ability to form new vessels and seem to impair normal angiogenesis in their vicinity(27). The hypothesis that DNA damage-induced vascular senescence may be causal to the development and progression of arteriopathies (rather than a consequence of

pre-existent vascular disease) is underscribed by rapid-aging syndromes like Hutchinson-Gilford Progeria (HGP) (28). In HGP, a mutation in laminin-A compromises normal DNA repair and mitosis, which rapidly leads to a senescence phenotype with thorough effects on the cardiovascular system. These patients usually die from cardiovascular events during adolescence. The vascular morphology of HGP patients is characterized by fibrotic acellular neointimal lesions, extensive adventitial fibrosis and diffuse inflammation throughout the vessel wall(29). The senescence-associated cellular and vascular abnormalities found in atherosclerosis or biological and premature aging in the systemic vasculature are in many ways similar to those found in the pulmonary vasculature in PAH.

A role for senescence in PAH: conceptual model and first observations.

PAH is a progressive arteriopathy. The end-stage pulmonary vascular phenotype is characterized by neointimal formation, intimal, medial and adventitial fibrosis, degradation of the internal elastic laminae and perivascular inflammation, culminating in the manifestation of plexiform lesions: the hallmark of irreversible PAH(3). The occurrence of this progressive, plexogenic-type of vascular remodeling has been associated with various pathogenic triggers. These include mutations in the BMPR2 pathway, auto-immune diseases, schistosomiasis or HIV infections, alkylating chemotherapy or anorexigens, increased blood flow and pressure like in congenital heart disease, and irradiation(30). A common denominator for these triggers is that they induce a form of genotoxic stress that causes accumulating DNA damage to vascular cells(31). The evidence for an early role of DNA damage in the pathology of PAH has been growing over recent years(4,32,33) (fig 8.1c). The two main cellular responses to DNA damage beyond repair are 1) apoptosis or 2) survival with genomic abnormalities(13), which may happen simultaneously in the situation of genotoxic stress. This phenomenon is also observed in pulmonary ECs treated with Sugeng5416, a compound used for the induction of PAH in rats(34,35). Widespread EC and SMC apoptosis, caused by inadequately compensated genotoxic stress, could thus lead to the vascular remodeling and rarefaction (fig 8.1f) that is observed already early in the development of PAH(36,37) (fig 8.1d). Increased proliferation in this case, could be viewed as a physiological response of the surviving cells trying to restore the injured vasculature. Survival of cells (resistant to apoptosis) with genomic abnormalities however, are also known to provoke a cancer-like hyper-proliferative phenotype(35) (fig 1e). Both type of this hyper-proliferative response could create a form of replicative or oncogenic stress and are prone to lead to senescence eventually (fig 8.1g-h). Like in atherosclerosis, senescent cells could contribute to vascular remodeling, degeneration and sclerosis in PAH (fig 8.1j) by 1) promoting inflammation and fibrosis in and around the vessel wall through the SASP, which inhibits regenerative mechanisms, promotes further senescence and leads to vascular degeneration and 2) by physically obstructing the vascular lumen due to their intrinsic resistance to apoptosis (fig 8.1i). p16-expressing cells have already been described in plexiform lesions in end-stage

idiopathic PAH(9,38). We have summarized further evidence for a role for senescence in PAH in the following paragraphs: 1) triggers that induce senescence also induce PAH, 2) pulmonary vascular remodeling occurs in chronic lung diseases associated with cellular senescence 3) pathways disturbed in PAH, are also causally associated to senescence (see also fig. 8.2) and 4) interventions that target senescence, also target pulmonary vascular remodeling in vivo.

Figure 8.1. Conceptual representation of the potential role for cellular senescence in the progression of vascular remodeling in PAH.

Direct triggers of premature senescence also induce PAH.

In experimental settings, common methods to induce senescence are through γ -irradiation and certain classes of drugs. Irradiation and these same drug classes have both also been causally associated with PAH in the clinic.

γ -Irradiation. High-volume, high-dose γ -irradiation of the thorax for e.g. lung cancer can lead to radiation pneumonitis, resulting in serious complications or death(39). A rat study revealed that γ -irradiation also affects the small pulmonary arterioles, which were characterized by medial hypertrophy, adventitial fibrosis and obliterative neointimal lesions with low cellularity, as well as by loss of capillaries. This effect was dose-dependent, with the highest thoracic volume exposures even causing increased mean pulmonary artery pressure and right ventricular hypertrophy(40). The fact that γ -irradiation, one of the most

straight-forward inducers of senescence, also leads to neointimal remodeling, reinforces the hypothesis that these mechanisms could also be causally involved in PAH development or progression.

Figure 8.2. Various triggers associated with PAH: increased $\text{TNF}\alpha$, reduced BMR2, γ -irradiation and alkylating chemotherapy lead to DNA damage via increased reactive oxygen species (ROS) or reduced DNA damage repair signalling mediated by the BRCA family. Disturbed flow can lead to DNA damage via reduced BMPR2 and increases the p53-p21 axis. Unrepaired DNA damage leads to either apoptosis or hyperproliferation of apoptosis-resistant cells. Increased apoptosis can also stimulate proliferation. Hyperproliferation leads to induction of the senescence genes p16 and p21. Senescence is characterized i.a. by persistent up regulation of apoptosis-resistance genes like Bcl2 or Birc5 (survivin) and down regulation of pro-regenerative pathways. After induction of senescence, epigenetic regulators such as BRD4, mTOR and NfκB, induce the senescence associated secretory phenotype (SASP), which stimulates further senescence, and inflammation, fibrosis and degeneration.

Drugs associated with PAH and senescence. Drugs known to cause PAH in the clinical setting include anorexigens, amphetamines, alkylating chemotherapeutics like cyclophosphamide and mitomycin c, and the tyrosine kinase inhibitor Dasatinib(41). The pyrrolizidine alkaloid monocrotaline (MCT) and the VEGF-inhibitor Sugen5416 are the most common substances to induce vascular remodeling in preclinical models for PAH. All these drugs are also known to induce senescence via oxidative stress and ROS, leading to DNA damage and apoptosis(42). The alkylating agent Mitomycin c is associated with senescence in lung cancer and used to induce senescence in vitro(43). Treatment with MCT in dogs was associated with senescence of the endothelial progenitor cell (EPC) population and an overall decrease in EPC number(44), indicating a role for MCT-induced senescence in reduced capacity for vascular regeneration or repair. Low-dose Dasatinib combined with the flavonoid Quercetin, can also serve as a senolytic, selectively killing senescent cells(6). Conversely, higher doses were shown to induce DNA damage leading to p21-dependent senescence, i.a. in non-small cell lung cancer cells(45). In rats, high-dose Dasatinib treatment exaggerates the development of PAH induced by MCT or chronic hypoxia by aggravating EC dysfunction and by inducing apoptosis via increased ROS(46). In patients with chronic myeloid leuke-

mia, high dose Dasatinib treatment can indeed lead to fatal PAH(47). However reversible courses after early discontinuation of the drug have also been described(48).

Pulmonary vascular remodeling in chronic lung diseases associated to senescence.

A central and causal role for cellular senescence has been established in chronic lung diseases (CLD) such as IPF and COPD(6,49), both of which also predispose to vascular remodeling and pulmonary hypertension (PH)(5). This form of PH (classified as WHO group 3) is usually less severe than group 1 PAH, and potentially reversible with treatment of the underlying disease. Still, the vascular phenotype in CLD-PH resembles many of the features found in PAH during the earlier stages of disease development(2). COPD patients show increased pulmonary vascular p21 and p16 expression compared to control patients, and SMCs isolated from COPD patients show accelerated development of senescence and evidence of a SASP in culture(7). Interestingly, this SASP seemed to stimulate hypertrophy, hyperplasia and migration of non-senescent SMCs in culture(7), both of which could contribute to vascular remodeling in PH and PAH(5).

Common pathways that lead to senescence and PAH.

Biological processes fundamentally associated to the development of PAH, like TGF β /BMPR2 imbalance, increased TNF α and mTOR signalling, impaired vascular regenerative mechanisms, and pathways induced in disturbed flow conditions, are also associated to cellular senescence (see also fig. 8.2).

The TGF β /BMP imbalance in PAH and senescence. Mutations in the TGF β /BMP pathway lead to the hereditary form of PAH, and TGF β /BMP signalling imbalance is a central feature in all other PAH etiologies as well. TGF β /BMP imbalance is therefore widely regarded the primary instigator for PAH development. Still, pharmacologic restoration of the imbalance has not yet led to impressive reversal of vascular remodeling in preclinical trials(50–52), and has had variable results thus far in the treatment of patients with end-stage PAH(53). We therefore hypothesize that prolonged TGF β /BMP imbalance (perhaps accompanied by a second hit) induces DNA damage and senescence that, beyond a certain window, cannot be reversed by restoring TGF β /BMP signalling alone.

The role of TGF β in senescence may be both causal as consequential(54). In cancer, TGF β is recognized as an important tumour suppressor capable of causing senescence through direct interaction with p16 and p21(55), and indirectly by exacerbating ROS production in a positive feedback loop via the NOX genes(56). Increased TGF β can however also be a consequence of senescence, since senescent cells are known to secrete TGF β as part of the SASP(16). The TGF β component of the SASP is recognized as an important mediator of fibrosis in age-related disease, like IPF(6).

BMPR2 down regulation has not yet been linked directly to senescence. BMPR2 loss in PAH has however been specifically associated with abnormal DNA damage and repair. Pulmonary ECs with reduced BMPR2 are more susceptible to DNA damage(32), possibly because BRCA1, a DNA repair gene and protector against genotoxic stress, is concomitantly reduced in these ECs(33). Vice versa, DNA damage was also shown to induce BMPR2 down regulation itself, suggesting a vicious cycle of progressively impaired DNA repair(33). One could hypothesize that the interplaying vicious cycles of BMPR2 loss and DNA damage on the one hand, and TGF β and ROS on the other, are highly implicated to lead to senescence, especially when a second genotoxic hit is present.

Anti-regenerative pathways in PAH and senescence. The potential to regenerate declines with age, and deactivation of pathways involved in regeneration, like Klotho, Sirtuin or Wnt, are associated with the development of premature senescence(57). Klotho-deficiency in mice leads to changes in the systemic vasculature similar to those observed in biological aging, like medial hypertrophy and calcification, EC dysfunction and neointimal hyperplasia(58). Klotho expression is also decreased in MCT-PAH rats, while intravenous delivery of mesenchymal stem cells that overexpress Klotho was shown to prevent vascular remodeling, potentially by increasing Sirtuin 1 expression(59). Indeed, Sirtuins are recognized as a protective factor in vascular aging(60) and cardiovascular disease(61), and are also down regulated in PAH(62). Deactivation of Wnt signalling is also associated with many degenerative diseases, including Alzheimer's and atherosclerosis(63), as well as with the onset of cellular senescence(64). In the lung, pro-angiogenic Wnt signalling is essential for vascular homeostasis and regeneration in response to injury(63). Stimulation of vascular regeneration by activating Wnt signalling was therefore proposed as a therapeutic strategy in PAH(63). However, experimental studies aimed at restoration of Wnt have yet to be conducted. This delay may be due to the observation that Wnt signalling is in fact increased in plexiform lesions of PAH patients(65), which could reflect the process of disorganized neoangiogenesis that is thought to lead to plexus channel formation, characteristic of the plexiform lesion(66).

TNF α in PAH and senescence. Inflammatory diseases known to cause PAH, like schistosomiasis, HIV, systemic sclerosis and lupus erythematosus, are all associated with increased levels of TNF α , a key inflammatory mediator(67). Recent evidence indicated that increased TNF α directly promotes pulmonary vascular remodeling by reducing BMPR2 signalling(68). Transgenic mice that overexpress TNF α in the lung, spontaneously develop PAH(69). Therefore, TNF α is recognized as a critical trigger for PAH(70). Prolonged exposure of TNF α is also known to induce senescence and an SASP in fibroblasts(71) and ECs, via increased ROS and activation of the NF κ B pathway(72). In systemic sclerosis, TNF α -associated EC and fibroblast senescence is recognised to exacerbate the progression

of liver and kidney fibrosis (73). The occurrence of PAH in systemic sclerosis could arise in a similar way.

mTOR signalling in COPD-PH, PAH and senescence. The mTOR pathway, an intracellular pathway involved in cell-cycle regulation, is also involved in the induction of cellular senescence and the SASP(74). Recent evidence shows that the mTOR pathway is up regulated in ECs, SMCs and epithelial cells of patients with COPD, along with markers for senescence(8). mTOR signalling was amplified in transgenic mice to confirm a causal role for mTOR-induced senescence in COPD-PH. This up regulated the expression of p16 and p21 and a cytokine profile suggestive of a SASP, as well as a COPD-like pulmonary phenotype with mild elevation in mean pulmonary pressure and medial hypertrophy of the distal pulmonary arterioles. Increased mTOR signalling is also found in the pulmonary arteriolar SMCs of IPAH patients, where it was shown to stimulate apoptosis-resistance and vascular remodeling(76). A relation with senescence however, was not assessed. mTOR inhibition by various drugs (including rapamycin: known to decelerate the development of senescence(77) and reduce the SASP(78)) has shown to attenuate the development of vascular remodeling and improve hemodynamics in multiple PAH animal models, but has thus far not proven effective in reversing the disease(76,79–81). Everolimus was the first mTOR inhibitor evaluated in a small trial with end-stage human PAH (2013). Although a mean decrease of 31% in pulmonary vascular resistance was observed in 8/10 patients after 6 months(82), no follow up trial has been planned, perhaps due to a report of severe Everolimus-induced pulmonary toxicity published that same year(83).

Osteopontin in COPD-PH, PAH and senescence. Senescent pulmonary SMCs were recently shown to secrete high levels of Osteopontin as a key component of their SASP(9). Osteopontin expression was also increased in the pulmonary arterioles of COPD-PH and IPAH patients (9,84) and in patients with advanced, but not early PAH associated with congenital heart disease(85). Osteopontin, secreted by senescent SMCs, was shown to stimulate vascular remodeling by promoting SMC growth and migration in their vicinity(9). Aged mice with an Osteopontin knockout, did not develop medial hypertrophy in response to chronic hypoxia, and had significantly lower vascular expression of p16 and p21 compared to the aged wild-type (9). These data indicate that Osteopontin stimulates age-driven vascular remodeling, and may further promote a pro-senescence vascular phenotype. It remains to be determined whether Osteopontin plays a similar role in PAH, which typically affects a younger patient group.

Disturbed flow in PAH and senescence. In PAH associated with congenital heart disease (PAH-CHD), increased flow due to left-to-right shunting is regarded the essential trigger for neointimal vascular remodeling(75). In the other etiologies of PAH, where pulmonary

flow is initially normal, neointimal lesions still typically occur at branching points where flow is most likely to be disturbed(75). Studies in atherosclerosis have shown a direct relationship between disturbed flow and EC senescence in plaques(86), which may explain the focal nature of these plaques in the systemic vasculature(87). In vitro, disturbed flow alone is able to induce full p21-mediated EC senescence. In vivo, disturbed flow alone induced a moderate senescence response, but the process was strongly exacerbated when the vasculature was sensitized by an inflammatory trigger (TNF α) (86).

Senotherapy in PAH.

The pharmacologic arsenal to target cellular senescence or aging phenotypes, senotherapy collectively, has expanded greatly in recent years. The contemporary 3 classes of senotherapy are: 1) geroprotectors: drugs that prevent DNA damage and senescence by reducing oxidative and other genotoxic stressors, or by protecting normal homeostasis; 2) SASP inhibitors: drugs that reduce the pro-inflammatory phenotype of senescent cells, such as mTOR inhibitors, Nf κ B or BRD4 inhibitors, and 3) senolytics: drugs that selectively induce apoptosis in senescent cells by targeting (anti)apoptotic pathways. Gene and immunotherapies are being explored as well(88). Table 1 provides an overview of the various classes of drugs that have been developed as senotherapy, or were proven to have geroprotective, SASP-modulating or senolytic effects. Some of these drugs have already been evaluated in the context of PAH. However, none of the trials in PAH investigated the effect of the treatments in direct relation to senescence or the SASP. A number of drugs that had positive effects in preclinical trials for PAH, have now also been identified as potent SASP inhibitors. The BRD4 inhibitor JQ1 for instance, significantly improved pulmonary hemodynamics and vascular remodeling in Sugen5416-hypoxia PAH rats(89). Later, BRD4 was also indicated as an essential regulator for the SASP in senescent cancer cells(90). Metformin inhibits the SASP by reducing Nf κ B signalling(91), which also both prevented experimental PAH(92), and improved pulmonary vascular resistance and 6 minute walking distance in patients with PAH-CHD(93). mTOR inhibitors likewise, are both effective in reducing the SASP, and attenuated vascular remodeling in different PAH rat models (76,79–81). Exploration of the SASP phenotypes that are specific to the context of PAH, could help to establish a role for these drugs in attenuating vascular remodeling or preventing further vascular degeneration.

One trial has been conducted in PAH using a drug that has also been identified as a senolytic. Heat shock protein-90 (HSP90) inhibitors both delay the development of senescence and eliminate senescent cells selectively(94), as well as improve vascular remodeling and reduce inflammation in experimental MCT-PAH(95). An interesting feature of other senolytics such as ABT263 or Dri-Foxo4 is that these drugs besides clearance of senescent cells, also seem to stimulate tissue regeneration(20,22). This could additionally benefit PAH patients, where vascular occlusion and rarefaction contribute critically to the severity of the disease.

Together these data indicate that senotherapy may have a place in the treatment of PAH, which merits further exploration in animal models.

Conclusion.

Cellular senescence is recognized as a crucial pathogenic factor in various degenerative and cardiovascular diseases, like idiopathic pulmonary fibrosis and atherosclerosis. Cumulative evidence indicates that senescence could also contribute to the degenerative character of neointimal pulmonary vascular disease in PAH. These data provide a basis for further exploration of the role for senescence in the pathobiology of PAH and for preclinical trials with senolytic class drugs.

Table 8.1. Overview of different types of senotherapy, some of which have been evaluated in the context of PAH. Of note: none of the studies in PH/PAH investigated the effect of the treatments in relation to senescence or the SASP. *Legend and abbreviations:* (C): clinical study; (P): preclinical study. MCT+Shunt rats: progressive PAH induced by monocrotaline and an aortocaval shunt. SuHx rats: progressive PAH induced by Sugén5416 and hypoxia. MCT-rats: A milder form of PAH induced by monocrotaline. PVR: pulmonary vascular resistance.

Drug (class)	Mode of action	Data of trials in PAH (none of the trials has assessed the drugs in direct relation to senescence or SASP)
Geroprotectors (96)		
Any drug that prevents disruption of normal homeostasis, such as: - Antidiabetics - Antihypertensives - Lipid-lowering drugs - Antioxidants - Preventers of telomere shortening	Decelerate the development of senescence or aging phenotypes and prevent genotoxic stress or DNA damage.	Various examples, such as: (C) Vasodilatory therapy decelerates the progression of PAH(97). (P) Antioxidant treatment attenuates the development of PAH in MCT-rats(98). (C+P) Metformin prevents MCT PAH(92), and improved PVR in PAH-CHD patients(93).
SASP inhibitors (88,99,100)		
mTOR inhibitors, such as: - Rapamycin - Everolimus	Suppression of the SASP	(C) Everolimus decreased PVR in 8/10 IPAH patients(82). (P) Rapamycin attenuates the development of PAH in SuHx and MCT rats(79–81).
NFκB inhibitors: - Metformin - Elafin	Suppression of the SASP	(C+P) Metformin prevents MCT PAH(92), and improved PVR in PAH-CHD patients(93). (P) Elafin reverses SuHx PAH(51).
p38 MAPK inhibitors: - GS-444217 - SB203580 - BIRB 796	Suppression of the SASP	(P) GS-444217 attenuates the development of PAH in SuHx and MCT rats by decreasing inflammation via p38 MAPK(101).
BRD4-inhibitors: - JQ1 - Apabetalone/Rvx208	Prevents / reduces the induction of the SASP	(P) JQ1 reverses PAH in SuHx rats by decreasing apoptosis-resistance and inflammation(89).

Table 8.1. Overview of different types of senotherapy, some of which have been evaluated in the context of PAH. Of note: none of the studies in PH/PAH investigated the effect of the treatments in relation to senescence or the SASP. *Legend and abbreviations: (C): clinical study; (P): preclinical study. MCT+Shunt rats: progressive PAH induced by monocrotaline and an aortocaval shunt. SuHx rats: progressive PAH induced by Sugden5416 and hypoxia. MCT-rats: A milder form of PAH induced by monocrotaline. PVR: pulmonary vascular resistance. (continued)*

Drug (class)	Mode of action	Data of trials in PAH (none of the trials has assessed the drugs in direct relation to senescence or SASP)
Glucocorticoids, such as: - Dexametasone	Suppression of the SASP	(P) Dexamethasone reversed PH in MCT rats, associated with decreased IL6(102).
Statins, such as: - simvastatin	Reduces expression of cytokines such as IL6, IL8, MCP1.	(C) No significant effect of statin therapy in PAH patients (meta-analysis)(103).
IL1- α / β (receptor) inhibitors: - Anakinra - Canakinumab - Rilonacept	Neutralize the effects of secreted IL1 α or β .	(P) Anakinra reversed PH in MCT-rats and decreased inflammation(104).
IL6 inhibitors: - Siltuximab - Tocilizumab	Neutralize the effects of secreted IL6.	(P) monoclonal antibody against IL6 receptor attenuated the development of PH in hypoxia(105).
TNF α inhibitors: - Adalimumab - Infliximab - Etanercept	Neutralize the effects of secreted TNF α . Reduces secretion of SASP components e.g. IL6, IL1 α .	(C) Infliximab reduced pulmonary artery pressures in 1 patients with systemic sclerosis-associated PAH(106).
Senolytics (88)		
Dasatinib+Quercetin	Targets multiple anti-apoptotic pathways. Induces senescent cell apoptosis.	(C) high-dose Dasatinib led to fatal PAH in myeloid leukemia patients(47). (P) high-dose Dasatinib treatment aggravates MCT and hypoxic PAH(46).
BCL-inhibitors such as: - Navitoclax (Abt263) - Abt737	Inhibits Bcl2/BclXl anti-apoptotic pathways. Induces senescent cell apoptosis	No data in PAH.
UBX0101	Stimulates p53-mediated apoptosis of senescent cells.	No data in PAH
HSP-90 Inhibitors	Induces apoptosis in senescent cells.	(P) Improved vascular remodeling and reduce inflammation in MCT-PAH, by inducing apoptosis(92).
DRI-FOXO4	Stimulates p53-mediated apoptosis of senescent cells	No data in PAH
Immunotherapy (88)		
Car-T cells	Patient-derived cytotoxic T-cells targeted at senescent cells.	No data in PAH
PDL1 inhibitors	<i>Hypothetical:</i> PDL1 may suppress CD4 ⁺ -T-cell mediated clearance of senescent cells, like is observed in cancer cells(107).	No data in PAH

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9

General discussion and future directions.

Discussion and future perspectives.

The aim of this thesis was to investigate the reversibility of pulmonary arterial hypertension (PAH), a lethal arteriopathy characterized by progressive vascular rarefaction, occlusion, degeneration and sclerosis(1). Most forms of PAH, such as idiopathic PAH (IPAH), are diagnosed at an advanced stage of the pulmonary vascular disease, generally considered to be irreversible: i.e. contemporary PAH-targeted therapy nor treatment of a causally associated co-morbidity has been shown able to reverse the arteriopathy(2). Histopathological examination of lung tissue of these patients may provide information of what constitutes an end-stage vascular phenotype, but does not reveal the processes that have caused the disease to become so. Conversely, the triggers that induce a disease may not be the same as the factors that cause further progression towards an end-stage phenotype. To investigate those factors that could explain the transition from reversible to irreversible pulmonary vascular remodeling, we proposed to use PAH in congenital heart disease (PAH-CHD) as a human model to study reversibility specifically, as described in [Chapter 2](#). PAH-CHD knows an irreversible end-stage phenotype that is similar to PAH of any aetiology, but a unique reversible phase is also recognized in these patients. Timely correction of the congenital cardiac shunt that caused the development of PAH in this patient group, can completely and permanently reverse the arteriopathy. However, this curative potential is lost beyond a certain “point of no return”, after which progressive vascular remodelling will occur despite correction of the shunt(3–5). This observation implies the existence of a biological mechanism that 1) accounts specifically for the transition from a potentially reversible towards a progressive, irreversible phenotype, and that 2) acts independent of the PAH-inducing trigger: the shunt in this case. Therefore, the comparison of reversible and irreversible PAH phenotypes could improve our comprehension of the specific factors that contribute to the irreversible and degenerative nature of PAH-CHD and perhaps also of other aetiologies. The potential clinical merits of this strategy are two-fold. Firstly, these data could direct the discovery of new targets leading to therapies able to reverse vascular remodelling in the end-stage of any PAH aetiology. Such drugs are currently not available in the clinic(6). A second merit, specific for PAH-CHD, is that these data could help to develop non-invasive biomarkers able to identify patients with open-shunt PAH-CHD who could benefit from shunt closure. In PAH-CHD, early and accurate detection of *the window for reversibility* can mean the difference between a complete cure or accelerated progression by shunt closure. However, up to the present day there is no evidence, nor consensus how this window should be defined(7). In [Chapter 4](#), we discussed current and future methods to assess reversibility in PAH-CHD. These methods could also be applied in other aetiologies of PAH to determine an optimal therapeutic strategy, and evaluate treatment response(7).

Reversible and Irreversible PAH-CHD.

A comparison of reversible and irreversible PAH-CHD has been reported in a few clinical studies so far. In 1958, Heath (UK) and Edwards (USA) were the first to relate the structural changes in pulmonary vascular morphology induced by a congenital cardiac shunt to the immediate reversibility of PAH after shunt closure, and provided a histological classification of the severity of those lesions into six different grades. Patients with lung biopsies revealing isolated medial hypertrophy (grade 1) and proliferation of intimal cells (grade 2) were likely to show haemodynamic improvement directly after surgery. This did not occur in patients with severe concentric neointimal fibrotic and plexiform lesions (grade 4-5), or intimal necrosis (grade 6) (8, 9). Dutch pathologist Wagenvoort thought this grading system to be an oversimplification of the complexity of arteriopathy, but did continue to use a descriptive histomorphological approach as the scientific basis to study the reversibility of PAH. In a prospective clinical study in patients with open-shunt PAH-CHD, he compared serial lung biopsies taken during and several years after pulmonary arterial banding surgery (reducing pulmonary blood flow (PBF) and pulmonary arterial pressure (PAP)). Medial hypertrophy and high cellularity intimal lesions generally reversed; severe occlusive intimal fibrosis and plexiform lesions did not regress and had a tendency to progress(4). The observation that medial hypertrophy is able to reverse after normalization of PBF and PAP has been confirmed in multiple animal models(10, 11). In 2007, Lévy et al. were the first to add a cell-biological aspect to the distinction of reversibility. In a database of lung biopsy tissue taken during cardiac surgery in children with PAH-CHD from the *Hôpital Necker* in Paris, it was shown that expression of the pro-apoptotic marker p53 was increased in PAH-CHD that reversed after shunt closure, while markers related to apoptosis-resistance were more prominent in children who appeared to have irreversible disease after closure of the shunt (12). These data are of particular interest in the light of the preclinical studies described later, in [Chapter 6](#).

Early Growth Response 1.

In [Chapter 3](#), we had the opportunity to use biopsy tissues from the *Hôpital Necker* collection mentioned above, including reversible and irreversible PAH-CHD, and could compare these with lung tissues of patients with IPAH or chronic obstructive pulmonary disease (COPD) from our institution, in order to investigate the role of the Early growth response-1 (Egr1) in human PAH. Our group previously identified Egr1 to be up regulated specifically in the pulmonary vessel wall of rats with flow associated PH after the surgical construction of an aorto-caval shunt, but not in rats with PH induced by monocrotaline only (13, 14). In a subsequent study, inhibition of Egr1 was shown to prevent the development of neointimal lesions the MCT+Shunt rat(15), indicating a critical role for Egr1 in neointimal remodeling in shunt-induced PAH. Translating these findings to human PAH-CHD, we showed that Egr1 expression increased progressively from the reversible to the irreversible stage.

Egr1 expression was similarly high in irreversible PAH-CHD and IPAH, but was absent in remodelled vessels of patients with non-neointimal PH associated with chronic obstructive lung disease (COPD)(16), characterized by isolated medial hypertrophy. Together, these data indicated that increased Egr1 expression could identify and drive neointimal pulmonary vascular remodelling in PAH, and perhaps contribute to the transition from reversible to irreversible disease. This hypothesis was reinforced by a recent study reporting that pioglitazone (a peroxisome proliferator-activated receptor- γ agonist shown in our lab to down-regulate Egr1 and prevent neointimal lesions in MCT+Shunt rats) normalized pulmonary haemodynamics and reversed established neointimal lesions also in the Sugen5416-Hypoxia (SuHx) rat(17).

Something about animal models for PAH.

Further studies that aim to investigate the pathobiology of reversibility in human PAH-CHD are scarce. Available lung tissue of patients with PAH usually is of those with end-stage irreversible PAH, since it is acquired from explanted lungs after lung transplantation. In contrast, early or reversible PAH tissue should be acquired via a lung biopsy procedure, e.g. during surgical shunt correction and is therefore scarce. Lung biopsy has largely disappeared from clinical practice due to its procedural risks(18), hindering pathological studies of early human disease. Scientific progress in this field has therefore relied heavily on the use of animal models. Probably the biggest concern in current preclinical PAH research is the translatability of its results to the clinic, which unfortunately has not been satisfactory so far(19). Important steps to enhance translational success are firstly and simply to impose the same methodological standards for clinical studies to preclinical research, such as adequate sample size calculation, randomization and blinding. Experimental protocols should be shared publicly in detail to improve reproducibility. The study end-points should be clinically relevant themselves, measured in a clinically relevant way (e.g. closed rather than open chest catheterization to measure haemodynamics) and should preferably be reported prior to the experiment(20). A stepwise protocol of the MCT+Shunt rat model including the methods for analysis of its primary end-points can be found in [Chapter 5](#), and are further displayed in a video that can be accessed online at: www.ncbi.nlm.nih.gov/pmc/articles/PMC5408705/. Drugs too should be dosed, administered and initiated in the most clinically relevant way and time (e.g. aimed at reversal of established disease rather than prevention). A second, more challenging step is to make the animal model as representative as possible for the human disease. This has improved drastically over recent years with the use of double hit models, such as the MCT+Shunt and Sugen5416+Hypoxia (SuHx) rat(21, 22). Both models are characterized by the progressive development of neointimal lesions, which are lacking in rats subjected to MCT or Hypoxia alone. However, it is still unclear whether the neointimal lesions found in rats are in fact representative of the irreversible, end-stage phenotype that is characteristic for the human disease. Adding to this debate is a

study in the SuHx rat, which showed that established neointimal lesions can be reversed just by reducing PBF and PAP: in other words, by haemodynamic unloading (HU)(23). In latter study, the left main pulmonary artery was banded at the 5-week timepoint of the model, when neointimal lesions are known to have developed. Rats were sacrificed at another 5 weeks after banding surgery. While the right, non-banded lungs showed severe obliterative neointimal lesions and perivascular inflammation, left pulmonary artery banding was able to normalize the vascular phenotype in the left lung. Thus, neointimal PAH triggered by Su+Hx can be reversed by HU at the 5-week timepoint of the SuHx model. Unfortunately, rats with later stages of SuHx-PAH did not survive PA-banding surgery at later timepoints, plausibly due to the fact that a sudden increase in RV-afterload is just too much for an already stressed RV. It therefore remains to be determined whether or not more advanced stages of SuHx-PAH are reversible by HU as well. The observation of reversed neointimal lesions in the SuHx model is discrepant with the pattern of human PAH, and are therefore of particular relevance to the interpretation of pharmacological reversal studies in the SuHx rat. On the other hand, this study did once more highlight the significance of PBF and hemodynamic load in the reversibility or progression of neointimal remodelling(23).

The loss of reversibility, reproduced in rats.

In [Chapter 6](#), our primary aim was to investigate the effect of HU in various stages of PAH in the MCT+Shunt rat: day 14, characterized by neomuscularization and mild endothelial cell proliferation of the intra-acinar pulmonary arterioles; day 21, characterized by medial hypertrophy and intimal hyperplasia; and day 28, characterized predominantly by neointimal lesions and perivascular fibrosis and inflammation. As shunt closure in this model is not feasible, we mimicked HU by transplanting the diseased left lung with PAH at either of the 3 timepoints into a healthy recipient rat without a shunt. The recipient rat was sacrificed 21 days after HU. We found that HU at day 14 normalized the vascular morphology by reducing medial muscularization in all rats. At day 21, 7 out of 10 rats reversed after HU, whereas the remaining 3 showed progressive vascular occlusion and neointima formation. HU at day 28 did not reverse the vascular morphology in any of the animals. These data showed that PAH induced by MCT+Shunt in rats is characterized by a reversible stage in which increased PBF is the essential mediator of disease progression; but also by an irreversible stage where other factors must be involved that cause the vascular remodelling process to be refractory to HU. The differential response to HU in the day 14-reversible and day 28-irreversible rats could be explained using the traditional vascular morphological approach as described by Wagenvoort and others in human PAH-CHD: medial hypertrophy reversed, neointima formation did not(4, 5). However, neointimal lesions were present in all “day 21-reversible rats” (n=7) and no obvious morphological differences were observed between the reversible and irreversible rats at this 21 days timepoint. These observations suggest that the reversibility of PAH is governed not necessarily by the presence or number

of neointimal lesions, but rather by their cellular-molecular characteristics and/or the microenvironment in which they occur.

What makes neointimal lesions irreversible?

We proceeded to investigate this by performing RNA-sequencing on peripheral upper right lobular lung tissue of all rats with reversible and irreversible PAH at the time of HU, and of healthy controls. We began to explore these transcriptomic data using cellular deconvolution, a novel method to assess the proportion of specific cells in mixed-cell tissue(24, 25). We found that the endothelial cell (EC) population was decreased significantly in reversible PAH versus controls and decreased further in irreversible PAH, indicating EC apoptosis and/or impaired EC regeneration. The proportions of smooth muscle cells (SMC) and fibroblasts were increased in PAH, indicating medial hypertrophy and progressive fibrosis, as was expected. Deconvolution for immune cells showed an increased CD4+/CD8+ T-cell ratio in reversible PAH, which inverted in irreversible PAH. CD4+/CD8+ inversion is associated with e.g. HIV Infection, auto-immunity, aging and immunosenescence (26, 27). Pathway analysis further revealed that reversible PAH is characterized by a highly significant increase in pro-proliferative signalling and pathways associated to DNA repair. These two pathways were down regulated in irreversible PAH, while pathways associated to cell survival or apoptosis-resistance, and immune or inflammatory processes were increased. We then stained for markers specific for each of these processes to confirm that the profiles found in the whole lung transcriptome also pertained to the pulmonary arterioles, and found a similar pattern. The processes involved are discussed in the following sections.

The apoptotic switch.

The switch from pro-apoptosis to apoptosis-resistance has been previously reported and related to reversibility in human PAH-CHD(12). Multiple preclinical studies have demonstrated that vascular remodeling often begins with, or because of a pro-apoptotic phase(15, 28–32). Direct clinical evidence for the role of apoptosis as a harbinger of pulmonary vascular remodeling can be derived from patients with PAH due to cytotoxic drugs such as Mitomycin or Dasatinib(33, 34), or PAH after thoracic γ -irradiation(35), both associated with massive pulmonary microvascular endothelial cell death. It is also well established that neointimal and plexiform lesions in end-stage PAH, conversely, are characterized by apoptosis-resistant vascular cells, as is apparent from staining with markers such as survivin and BCL-2(36–38). This pattern was consistent with the findings in our reversible and irreversible PAH tissues.

The two faces of proliferation.

Proliferation is the second big player in the pathobiology of PAH(39–42), at least, that has been the presumption the last decades. Proliferating endothelial and smooth muscle cells

are found in any animal model for PAH(21, 22), including the MCT+Shunt model used in the studies of this thesis. The evidence for proliferation as a pathogenic mechanism in human PAH is largely based on the occurrence of pro-angiogenic growth factors in plexiform lesions (summarized in i.a. (43)) and on a study showing that endothelial cells isolated from PAH patients are hyperproliferative (and apoptosis-resistant)(41). It should be noted, however, that the presence of growth factors does not necessarily indicate that proliferation is in fact (still) taking place(44). Also, the cells that are easiest to grow in culture after cell isolation are the ones that (still) have high proliferative potential, creating a bias in favour of hyperproliferative cells, which may not be representative of the true proliferative cellular state in the lung. In [Chapter 6](#), we showed by RNA-sequencing and immunohistochemistry that proliferation is greatly increased in early stage MCT+Shunt PAH (like in other PH models), but is completely dimmed in end-stage disease. In our study, as well as in SuHx-MCT- or Shunt-induced experimental PAH, the presence of proliferating vascular cells has consistently been associated with a reversible-type of vascular remodeling after HU(10, 11, 23). In human end-stage PAH, neither our study nor the literature shows evidence for proliferation as assessed by Ki67 staining. One could therefore hypothesize that proliferation is a feature of early, reversible stage disease that may not significantly contribute to the pathobiology of end-stage PAH (anymore). This may also help to explain why drugs aimed at reducing proliferation have thus far not been successful in the treatment of end-stage PAH(45). Seeing the massive apoptosis of endothelial cells as a possible inducer of PAH, increased proliferation of the surviving cells could even be viewed as a valuable physiological response to vascular injury: a mechanism of vascular repair or regeneration that should not be inhibited. However, the presence of various cancer-like growth factors indicates that not just the 'good' regenerative kind of proliferation occurs in the development of PAH(43). This brings us to a more recently discovered pathological process in PAH: DNA damage.

DNA damage and its epigenetic effects.

Our RNA-sequencing analysis in [Chapter 6](#) revealed a strong up regulation of genes and pathways associated to DNA damage and DNA repair in reversible PAH, with one of the most prominent genes being RAD51. The last few years, DNA damage has come up as a new, early and central mechanism in the pathobiology of PAH(46, 47). High levels of DNA damage have been described in animals models and human tissue of PAH(46), and a common denominator for many of the known triggers of human PAH is that they are genotoxic: cause damage to the DNA. Direct genotoxic hits leading to PAH include Dasatinib, cyclophosphamide and mitomycin treatment or γ -irradiation(33, 35). More chronic genotoxicity occurs in chronic inflammatory conditions such as Lupus erythematoses-, schistosomiasis- or HIV-associated PAH, e.g. via prolonged exposure to high levels of TNF α (48). Increasing evidence shows that BMPR2 mutations may also induce PAH through increased sensitivity to DNA damage. In vitro, loss of BMPR2 leads to a decrease in BRCA1 and 2, and RAD51:

all essential mediators for normal DNA repair(49, 50). Vice versa, DNA damage was also shown to induce BMPR2 down regulation itself, suggesting a vicious cycle of progressively impaired DNA repair(49). So, perhaps a second (genotoxic) hit is required to exacerbate this vicious cycle and a PAH phenotype in BMPR2 mutation carriers? Speculatively, this could explain why only 14% of male and 42% of female BMPR2 mutation carriers will end up developing PAH(51, 52), and why BMPR2-mutant rats only develop a mild medial hypertrophy-type of disease(53–55). In PAH-CHD, disturbed flow could lead to DNA damage via BMPR2 down regulation, and due to direct genotoxic effects of shear stress on the endothelium as was shown in vitro in systemic ECs(56). These data are underlined by our finding in Chapter 6 - fig 6.6B, showing that idiopathic PAH microvascular ECs, in which BMPR2 expression is halved, accumulate significantly more DNA damage in disturbed shear conditions than control ECs with normal BMPR2 levels. Constructing an aorto-caval shunt in BMPR2-mutant rats would be an opportune set-up to further investigate this.

So how does DNA damage relate to apoptosis or pathologic hyperproliferation? Put simply, in response to accumulating DNA damage, a cell will either be triggered to go into apoptosis, or will repair the damage via the DNA Damage Response (DDR) program enabling it to survive(57). P53 is an important decision maker in this choice(31). Persistent DDR activation during chronic genotoxic conditions however, can lead to aberrant DNA repair and to survival of cells with genomic abnormalities(58). The genetic and epigenetic changes induced by a persistent DDR most characteristically lead to a hyperproliferative, apoptosis-resistant phenotype(46, 57, 58). The bromodomain and extra-terminal (BET) motif protein BRD4 is one of those epigenetic mediators that is activated upon chronic genotoxic stress(59, 60). BRD4 is recruited to sites of unrepaired DNA damage and once present can modify the structure of the chromatin(61), which enables transcription of an extensive panel of pro-proliferative, pro-survival and pro-inflammatory genes(62), leading to its reputation as a 'super enhancer.' In [Chapter 7](#), we showed together with the group of dr. Bonnet (Université Laval, Québec, Canada) that BRD4 and its downstream DDR effectors FoxM1 and PLK1, are expressed in the vasculature of PAH-CHD and IPAH patients, and that this pathway can be inhibited by RVX208, a clinically available BET-inhibitor. We showed in vitro that RVX208 normalizes the hyperproliferative, apoptosis-resistant phenotype of isolated human PAH ECs. RVX208 reduced the inflammatory response upon stimulation with TNF α , while BMPR2 signalling was increased. In both the Canadian and Dutch in vivo studies, RVX208 treatment reduced neointimal vascular remodeling in the SuHx and MCT+Shunt rat and improved pulmonary hemodynamics. Interestingly, reversal by RVX208 was mainly associated with reduced proliferation in the SuHx model, and by increased apoptosis in the MCT+Shunt rat. We also tested RVX208 in the pulmonary artery banding model, to investigate its effects on the RV during pressure load, primarily to ensure safety of the drug in the context of RV dysfunction. Interestingly, FoxM1 and PLK1 mRNA

were also significantly increased by pressure load in the rat RV, indicating a role for DDR signalling in RV pathology as well. We found that RVX208 treatment stimulated physiological RV adaptation, suggesting that RVX208 treatment could be beneficial in patients with PAH-associated RV dysfunction, in addition to the effects on the pulmonary vasculature.

To reiterate: strong evidence has accumulated that the pulmonary vascular cell DNA of PAH patients is damaged and/or vulnerable to damage, and that persistent activation of the DDR can induce epigenetic changes that lead to pathologic hyperproliferation, apoptosis-resistance and inflammation: a phenotype very similar to the pathobiology of cancer. Not surprisingly, the parallel between cancer and PAH remains a very popular one to draw(43, 63). But there still is something a little off about this analogy...

The not-quite-a-cancer paradigm.

The genotoxic stressors, the DNA damage, the hyperproliferation, the resistance to apoptosis, the hard-to-treat characteristics: all factors that make an easy comparison with cancer. Still, one of the most typical characteristics of cancer is that the proliferation is limitless. This is inconsistent with our findings in [Chapter 6](#), which describe that proliferation genes are in fact switched off upon transition towards irreversible PAH, and inconsistent with the absence of proliferating Ki67-positive cells in end-stage human PAH tissue. Why is proliferation switched off as the disease progresses towards its irreversible stage? A possible answer: Cellular Senescence.

Cellular senescence describes the transition of a cell from a (potentially) proliferative or regenerative state into permanent, irreversible growth arrest (64). Senescence occurs predominantly in response to DNA damage, be it due to a longstanding process of degenerating genomic integrity (also known as ageing), or due to other forms of not necessarily age-related genotoxic hits, such as replicative, oncogenic, inflammatory or oxidative stress. Besides adequate DNA repair or apoptosis of cells with inadequately repaired DNA, cellular senescence is THE physiological defence mechanism against cancer(57), shutting down hyperproliferating cells into permanent growth arrest for good (65). But this service comes with a price: senescent cells undergo a series of changes in their chromatin structure and secretome, making them lose their original physiologic function, making them resistant to apoptosis by up regulating senescent cell anti-apoptotic pathways like survivin and BCL2(66), and making them unable to replicate or regenerate. Another dark-side of senescent cells is their senescence associated secretory phenotype (SASP), which consists of numerous cyto- and chemokines, interleukins, proteases and angiogenic growth factors that together create a pro-inflammatory, pro-fibrotic environment that promotes tissue ageing and degeneration(67). Interestingly, BRD4, the epigenetic DDR super enhancer from [Chapter 7](#), was recently shown to be an important activator of the SASP after oncogenic

stress(68). Senescence and its SASP have now been blamed as critical drivers of various degenerative pathologies like idiopathic pulmonary fibrosis(69), Parkinson's, and atherosclerosis(70), that are currently thought to be incurable. The good news? Senescent cells can be cleared locally by 'senolytic' drugs which have shown to attenuate disease progression in experimental models by decreasing the SASP and by creating a pro-regenerative environment(71–73). Could the occurrence of cellular senescence account for the switch from a hyperproliferative and pro-apoptotic phenotype to one characterized by growth-arrest, apoptosis-resistance and inflammation; rescuing it from becoming cancer, but causing it to evolve into a more degenerative kind of disease? In [Chapter 8](#), we reviewed the ample circumstantial evidence of PAH as a senescence-mediated degenerative vascular disease. In [Chapter 6](#), we continued to test this hypothesis directly on our reversible and irreversible lung RNA-sequencing data and tissue.

Cellular senescence impairs the reversibility of PAH.

When analysing the RNA-sequencing data of reversible and irreversible MCT+Shunt induced PAH lung tissue, we observed a significant increase in pro-senescence and SASP genes in irreversible, compared to reversible PAH. We confirmed this by immunohistochemistry for p16^{ink4A} and p21^{cip1}, both cell-cycle suppressor proteins and markers of senescence(74), showing increased expression in irreversible PAH arterioles. The vascular expressions of SASP proteins IL6 and MMP2 were increased as well. We then subjected microvascular ECs isolated from patients with PAH and controls to increased shear stress to investigate whether disturbed blood flow could contribute to a pro-senescence phenotype. We found that PAH ECs (in which BMPR2 expression is about 50% of normal) accumulate significantly more DNA damage than control ECs in response to increased shear stress. Moreover, only in PAH ECs p16^{ink4A} mRNA was increased by increased shear, suggesting that PAH ECs are more susceptible for senescence in disturbed flow conditions than control ECs. We then induced senescence in PAH and control ECs using verified methods of γ -irradiation(75) and chronic TNF α -stimulation(48). After EC senescence was established, we used the medium of the senescent ECs, theoretically containing SASP factors, to culture naïve smooth muscle cells (SMCs) and observed that SASP of senescent pulmonary ECs stimulates the growth of SMCs, increases SMC IL6 production and decreases CNN1, a marker for SMC contractile function; all indicating that the senescent pulmonary EC can further exacerbate vascular remodeling and dysfunction via cross-talk with neighbouring SMCs.

The next step was to test a drug that selectively kills senescent pulmonary ECs and leaves the healthy ones to proliferate normally. The past decade, a number of drugs with senolytic properties have been developed. Most of these drugs intervene in senescent cell anti-apoptotic pathways such as BCL2-BCL-XL or Foxo4(72, 73). We chose for The BCL2 inhibitor ABT263 (Navitoclax), as a safe and well-tolerated senolytic that has already been

evaluated in phase I and II clinical trials(76). We treated senescent PAH and control ECs with different doses of ABT263 and compared with healthy, non-senescent PAH and control ECs. ABT263 treatment selectively induced apoptosis in the senescent ECs, while cell viability remained intact in the non-senescent cells. Interestingly, 2.5 μ M ABT263 treatment also normalized the hyperproliferative phenotype in the non-senescent PAH ECs, which was 1.5 times higher than controls at baseline. These data indicated that ABT263 both effectively eliminates senescent cells and allows for normal, but not hyperproliferation of non-senescent ECs.

To confirm our hypothesis that cellular senescence is not just associated to end-stage PAH, but may also be a cause of irreversibility, we treated MCT+Shunt rats with low-dose ABT263 during end-stage disease, from day 25 to 32. Compared to placebo, ABT263-treated rats had fewer symptoms of right heart failure, lower pulmonary artery pressures and vascular resistance, less RV hypertrophy and improved RV function. Vascular occlusion score and the percentage of neointimal lesions were decreased significantly. Also, around 30% of the vessels in ABT263-treated rats had a completely normal morphology, perhaps implying that these vessels were newly formed. P16 and p21 were decreased both on the whole lung mRNA and the vascular protein expression level, indicating that PAH reversal was associated indeed with the removal of senescent cells. Also, IL6 mRNA was decreased by ABT263, but MMP2 mRNA, which we had included in our analysis as a SASP factor, appeared to be increased. MMP2 however, is also known to be involved in the formation of new blood vessels(77), which may explain this result. Therefore, we also assessed the expression of BMPR2 and Klotho(78) as proteins associated with vascular regeneration, and found them too to be increased by ABT263 treatment. So, like in other degenerative diseases, senolytic treatment in PAH could improve the pulmonary vascular disease by removing senescent cells and reducing the inflammation associated with the SASP, and by creating a pro-regenerative environment(71–73, 79, 80).

Conclusion.

This thesis aimed to investigate the reversibility of PAH. We extensively reviewed and explored(2, 81, 82) the clinical, haemodynamic and molecular characteristics of reversibility using the ‘human model’ of PAH in CHD, in which haemodynamic unloading (HU) is able to reverse vascular remodeling up to but not beyond a certain point of no return. We then translated this model from humans into rats, by applying HU during different phases of PAH progression in the MCT+Shunt model. We showed that MCT+Shunt PAH, like human PAH-CHD, has a reversible and an irreversible stage, enabling us to identify specific temporal molecular profiles. The progression of PAH was characterized by a distinct bi-phasic process of apoptosis and hyperproliferation, likely driven by DNA damage; followed by senescence-associated growth arrest, apoptosis-resistance and inflammation. Targeted

apoptosis of senescent cells reversed vascular remodeling and improved pulmonary hemodynamics in irreversible MCT+Shunt PAH, serving proof of the concept that cellular senescence contributes critically to the transition of reversible to irreversible disease. In search for a new directly applicable treatment strategy to reverse end-stage PAH, we targeted the BET-bromodomain protein BRD4, known as an epigenetic super enhancer of DNA-damage driven pathologies(83), and as a key activator of the senescence-associated inflammatory phenotype(68). In two independent preclinical trials that were subsequently unified, the clinically available BRD4 inhibitor RVX208 normalized the PAH phenotype in vitro, and reversed vascular remodeling in multiple complementary preclinical models of PAH. These data led to the establishment of a clinical trial with RVX208 in PAH patients, which is currently taking place (APPRoACH-p, NCT03655704).

Future perspectives.

PAH is still a fatal disease that affects a relatively young patient group across its etiological spectrum(6, 84, 85). The two main challenges for PAH in the clinic are: 1) the lack of minimally-invasive methods to assess the stage of pulmonary vascular disease or choose the type of therapy, and 2) the lack of drugs able to reverse advanced neointimal vascular remodeling. A lot of potential to tackle these challenges has risen during the years it took to write this thesis. This has had much to do with the emergence of next generation sequencing (NGS) techniques. Large scale studies are beginning to unveil the genome, transcriptome and metabolome of PAH in humans and preclinical models, and have already provided an abundance of putative biomarkers and targets for intervention(86, 87). The specific biphasic ‘transcriptome of reversibility’ as described in [Chapter 6](#) could be used as a basis for research in humans aimed at staging PAH or determining a treatment strategy. Senescence for example, is associated with a distinct secretome that could be investigated as a peripheral blood biomarker to identify PAH that is refractory to shunt closure, or perhaps to effective vasodilator therapy. As an alternative to classic circulating biomarkers, transcriptomic profiling of circulating cells and so-called ‘educated platelets’ provides a new way to assess disease in a peripheral blood sample. This concept, also known as ‘liquid biopsy,’ was borrowed from lung cancer, and is currently being tested in PAH. The liquid biopsy technology utilises the fact that tumours shed cells and DNA directly into the bloodstream, and tumour-RNA into platelets, which can be analysed to determine e.g. tumour phenotype using NGS profiling(88). A potential advance is also on the horizon regarding the haemodynamic monitoring of PAH. The recent clinical availability of small implantable pressure sensors in the pulmonary artery, currently in trials to prevent hospitalization due to congestive left heart failure, provides a potential new method for continuous measurement of pulmonary artery pressures(89). Such devices can be crucial in further understanding the haemodynamic physiology of PAH, in monitoring disease progression, escalating vasodilator therapy or assess a treatment effect in clinical trials.

Chapter 6 indicated that different stages of PAH may respond differently to different types of therapy, which may have important implications. Limiting apoptosis may be beneficial in early stage PAH, as was demonstrated in the SuHx rat(28, 90), while promoting apoptosis has been associated with disease reversal in more advanced stages(36, 37, 91, 92). The same is true for proliferation. Limiting proliferation may be beneficial in the light of the cancer paradigm that views PAH as a hyperproliferative disease, while stimulating pro-proliferative pathways involved in regeneration would be justified in a paradigm focussed on degeneration(93). In Chapter 6, we have shown that experimental PAH can be both hyper- and hypo-proliferative, again underlining the importance of disease phase or phenotype when opting for an experimental therapeutic strategy. The use of multiple compatible animal models and appropriate timing when testing new interventions can help to prevent translational failure (e.g. due to these opposing effects in proliferation and apoptosis) from preclinical results to the clinic (20, 45). This is illustrated by our findings in Chapter 7, where the treatment effect of RVX208 was associated with reduced proliferation and unaltered apoptosis in SuHx PAH, and with increased apoptosis in MCT+Shunt PAH.

Chapter 6 and 7 both provided new classes of drugs aimed at the reversal of neointimal lesions: Senolytics and modulators of the DNA damage response (DDR) respectively. The DDR modulator and BET-bromodomain inhibitor Apabetalone (RVX208) is now being evaluated in a clinical trial (APPROACH-p, NCT03655704). Various other kinds of BET-bromodomain inhibitors are investigated too that could be promising for PAH patients(94). Other modulators of the DDR that have been shown beneficial in preclinical PAH models include PARP-inhibitors(47), PIM1-inhibitors(95), EYA3 tyrosine phosphatase-inhibitors(96), and FoxM1-inhibitors(97). Senolytics are relatively unexplored in the context of PAH, but have been gaining considerable attention in various other degenerative or oncologic diseases. A few types of senolytic drugs have now been evaluated in clinical trials. In a preliminary report, the senolytic cocktail of Dasatinib (mind you!) plus Quercetin was demonstrated to reduce the number of senescent cells and SASP-associated inflammatory cytokines in patients with idiopathic pulmonary fibrosis. Any treatment effects were not yet mentioned(98). Navitoclax (ABT263), the BCL2 inhibitor used as a senolytic in Chapter 6, was evaluated in a few clinical trials for lung and haematologic malignancies(76, 99–101). These clinical studies did not focus on senescence, but preclinical evidence of the senolytic qualities of BCL2-BCL-XL inhibitors and other senolytics, such as Foxo4-DRI, UBX0101 and HSP-90 is abundant and growing (72, 102, 103). In our studies, we used ABT263 primarily to prove our concept of senescence as a critical biological factor in the transition towards irreversible PAH, and not primarily as a new treatment strategy. Yet, the improvement in both vascular remodeling and pulmonary haemodynamics after just a 7-day low dose senolytic treatment, warrants further exploration in larger scale preclinical trials, ideally with various classes of senolytics(104, 105) in multiple animal models for

PAH(20). The improvement in RV function and lack of any signs of RV failure in end-stage MCT+Shunt rats treated with ABT263, suggests that senolysis may also have had direct effects on the RV, in addition to the reduction in afterload. In left heart failure, experimental data indicate that senescence may also drive pathological cardiac remodeling and fibrosis(106). It is currently unclear whether senolysis can also improve established heart failure. A trial with senolytics in the pulmonary artery banding and transaortic constriction models could explore these questions. Finally, the increase in MMP2, BMPR2 and Klotho mRNA and the high number of completely normal pulmonary arterioles after ABT263 treatment, indicates that vascular regeneration is occurring after senescent cells have been cleared away(78, 107). This is consistent with experimental data from other disease models (71, 73, 80). Besides the de-occlusion of existing branches, regeneration of the pulmonary arterial tree could be the final step in improving the quality of life of PAH patients, who are severely limited by the inability to recruit vessels during exercise.

We are definitely not there yet, but important steps have been taken. I hope this thesis has contributed to a better understanding of PAH and as given a few potential leads in the strive to reverse this disease, that has appeared so irreversible thus far.

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A

Summary in Dutch | Nederlandse samenvatting

Acknowledgements | Dankwoord

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About the author

Nederlandse samenvatting.

De reversibiliteit van pulmonale arteriële hypertensie bij aangeboren hartafwijkingen.

Pulmonale arteriële hypertensie (PAH) is een zeldzame, chronische longziekte: in Nederland hebben zo'n 800 mensen de diagnose. Bij patiënten met PAH groeien de kleine longslagaders progressief dicht. Dit zorgt voor een te hoge bloeddruk in de grote longslagaders. PAH is een dodelijke ziekte, die zich ook op de kinderleeftijd kan presenteren, zoals bij kinderen met een aangeboren hartafwijking (PAH-CHD). Veel aangeboren hartafwijkingen, zoals bijvoorbeeld een wanddefect tussen de linker en rechter kamer, hebben als gevolg dat de bloeddorstroming en de bloeddruk in de longen sterk toeneemt, doordat het bloed van de lichaamscirculatie (hoge druk) naar de longcirculatie (lage druk) stroomt: *een links-rechts shunt*. Het idee is dat deze verhoogde bloeddorstroming en bloeddruk in de longen leiden tot schade van de kleine longslagaders, die als gevolg daarvan remodeleren en verstijven. Gelukkig kan het operatief sluiten van de shunt de progressie van PAH voorkomen en zelfs omkeren. Wanneer de diagnose echter te laat wordt gesteld of de operatie te laat plaatsvindt, zal PAH deze reversibiliteit kwijtraken, groeien de longslagaders verder dicht en stijgt de bloeddruk in de longen verder door. Dit leidt uiteindelijk tot hartfalen op een jonge leeftijd. Momenteel zijn er nog geen medicamenteuze behandelingen die de progressie van PAH volledig kunnen stoppen, laat staan genezen.

In deze thesis proberen we te begrijpen waarom PAH bij aangeboren hartafwijkingen op een gegeven moment niet meer is om te keren door de hartafwijking te corrigeren: welke biologische mechanismen verklaren de progressie van reversibele naar irreversibele PAH? En als we deze specifieke mechanismen hebben ontdekt, kunnen we die dan selectief remmen met medicatie om 'irreversibele' PAH toch nog om te keren?

Om deze vragen te beantwoorden hebben we bij aanvang van ons onderzoek 3 wetenschappelijke doelen geformuleerd:

1. Het ontwikkelen van een proefdiermodel in ratten dat PAH-CHD nabootst, inclusief het corrigeren van de hartafwijking, waarin we de reversibele en irreversibele fase van PAH kunnen onderscheiden.
2. Het karakteriseren van de onderliggende mechanismen die de transitie van een reversibele naar een irreversibele vorm van PAH kunnen verklaren.
3. Het testen van nieuwe medicamenteuze behandelstrategieën gericht op het omkeren van progressieve longslagaderremodellering in het irreversibele stadium van PAH.

In **hoofdstuk 2** hebben we de huidige kennis rondom de reversibiliteit van PAH-CHD samengevat en dient daarmee als klinische basis voor het experimentele onderzoek in de rest van deze thesis. Al sinds de jaren '50 is bekend dat PAH-CHD een reversibele en ir-

reversibele fase kent. PAH-CHD onderscheidt zich daarmee van andere veelvoorkomende vormen van PAH, waarin alleen een irreversibele fase wordt beschreven. Dit komt mogelijk doordat PAH-CHD, in tegenstelling tot de andere vormen, in een vroege fase kan worden ontdekt, namelijk door het constateren van een aangeboren hartafwijking. De ontwikkeling van PAH-CHD kan zodoende gedurende de hele ziekteprogressie worden gevolgd.

Het remodeleren van de longslagaders lijkt zich in een vast patroon te ontwikkelen. Eerst verdikt de spierlaag rondom de slagader (media hypertrofie) en vermenigvuldigen de cellen die de binnenkant van het vat bekleden (intima proliferatie). Daarna verdikt en verstijft de intima zich verder (neointima fibrose), wat leidt tot occlusie van het vat. Eindstadium PAH wordt gekenmerkt door zogeheten plexiforme laesies: ongeorganiseerde vaatstructuren die nauwelijks meer functioneel zijn. Al snel werd beschreven dat PAH met vroege afwijkingen: media hypertrofie en intima proliferatie, reversibel is na corrigeren van de hartafwijking. Vond men gevorderde afwijkingen in het longbiopt, zoals neointima fibrose of plexiforme laesies, dan zou de ziekte waarschijnlijk voortschrijden ondanks sluiting van het hartdefect. Het bestaan van deze twee ziektefasen impliceert dat er ergens in het ziekteproces iets gebeurt: een transitie, een *switch*, wat zorgt dat PAH zijn reversibiliteit verliest. In **hoofdstuk 2** beschrijven we enkele bekende mechanismen die deze transitie zouden kunnen teweegbrengen, zoals *a*) een disbalans in geprogrammeerde celdood (apoptose), en apoptose-resistentie, *b*) een disbalans in de signaalcascades van de eiwitten *TGFβ* en *BMPR2*, *c*) epigenetische mechanismen gereguleerd door het *superenhancer* eiwit *BRD4*, en *d*) verhoogde inflammatie (ontsteking). In **hoofdstuk 6** gaan we op zoek naar nieuwe mechanismen die het verlies van reversibiliteit zouden kunnen verklaren. In **Hoofdstuk 7** onderzoeken we of het remmen van *BRD4* kan bijdragen aan de behandeling van irreversibele PAH.

In **hoofdstuk 3** hebben we onderzocht of de weefsel biomarker *Egr1* onderscheid kan maken tussen een reversibele en irreversibele vorm van longvaatremodellering bij mensen met verschillende vormen van pulmonale hypertensie. Het eiwit *Egr1* is in eerder experimenteel onderzoek van onze groep naar voren gekomen. Bij ratten is *Egr1* nagenoeg afwezig in gezonde longvaten, maar wordt versterkt aangemaakt zodra je een *links-rechts shunt* aanlegt. In diezelfde ratten kon de progressie van PAH zelfs worden voorkomen door *Egr1* specifiek te remmen. In dit hoofdstuk tonen we aan dat *Egr1*, net als bij ratten, verhoogd is bij zowel reversibele als irreversibele PAH-CHD en in idiopathische PAH: een andere irreversibele vorm van PAH. *Egr1* was daarentegen niet aantoonbaar in een mildere, reversibele vorm van pulmonale hypertensie, zoals voorkomt in patiënten met COPD, waarin ernstige vaatafwijkingen (neointima fibrose en plexiforme laesies) nooit ontwikkelen. Hiermee bewezen we dat *Egr1* specifiek geassocieerd is met een ernstige vorm van longvaatremodellering, en hier mogelijk al vroeg aan bijdraagt.

Zoals in **hoofdstuk 2** beschreven, is het goed herkennen van de reversibiliteitsfase bij PAH-CHD essentieel voor de therapiekeuze en prognose van de patiënt. In **hoofdstuk 4** beschrijven we de huidige klinisch-beschikbare methodes om onderscheid te maken tussen reversibele en irreversibele PAH. De conclusie: er bestaat een groot grijs gebied tussen zeker-reversibele en zeker-irreversibele PAH, en huidige methodes kunnen het onderscheid onvoldoende maken. Daarom stellen we een algoritme voor als leidraad voor de benadering van patiënten met PAH-CHD, en evalueren we potentiële nieuwe methodes die kunnen bijdragen aan het vaststellen van hun reversibiliteit.

In **hoofdstuk 5** beschrijven we het proefdiermodel dat de basis is voor het experimentele onderzoek in deze thesis: het *monocrotaline + links-rechts shunt* model (MCT+Shunt) in ratten. In dit proefdiermodel krijgen de ratten eerst een injectie met monocrotaline, wat schade geeft aan de kleine longslagaders. Op dag 7 wordt er een links-rechts shunt aangelegd. Deze 'dubbele hit' leidt tot media hypertrofie en intima proliferatie rond dag 14, neointima laesies vanaf dag 21 en neointima fibrose met rechter hartfalen rond dag 28: een proces dat qua opeenvolging en aard van de vaatafwijkingen overeenkomt met PAH-CHD bij mensen. Dit hoofdstuk bevat een gedetailleerd stapsgewijs protocol, inclusief een video met alle microchirurgische technieken, om het proefdiermodel te realiseren en te analyseren, teneinde de reproduceerbaarheid van onze experimenten te vergroten. De video is openbaar te bekijken via: www.ncbi.nlm.nih.gov/pmc/articles/PMC5408705/.

In **hoofdstuk 6** zijn we opzoek gegaan naar nieuwe mechanismen die de transitie in reversibiliteit van PAH kan verklaren. Eerst ontwikkelden we een nieuw proefdiermodel om het sluiten van de shunt in ratten met MCT+Shunt na te bootsen. Omdat het operatief sluiten van de aangelegde shunt in deze ratten technisch niet mogelijk is, gebruikten we een andere methode om de longen met PAH weer in een milieu te brengen met een normale bloeddoorstroming en bloeddrukken in de long: linker longtransplantatie (LTx). Eerst werd PAH opgewekt in ratten door MCT+Shunt: de donorratten. Vervolgens transplanteerden we in verschillende ziektestadia (dag 14, 21 of 28) de linker donorlong met PAH in een gezonde, genetisch-identieke ontvanger zonder shunt. De rechter donorlong met PAH gebruikten we om het ziektestadium ten tijde van LTx te analyseren. De ontvangende rat werd 21 dagen in leven gehouden. Daarna werd de getransplanteerde (linker donor) long uitgenomen, om te vergelijken met de rechter donorlong en zo het effect van normaliseren van bloeddoorstroming en druk te beoordelen. Wat bleek: de longvaatafwijkingen in alle dag 14-getransplanteerde longen waren reversibel na transplantatie in een rat zonder shunt; alle dag 28-getransplanteerde longen toonde daarentegen progressie van de longvaatafwijkingen. Van de dag 21-getransplanteerde longen was een deel reversibel en een deel irreversibel. Hiermee toonden we aan dat er in ratten met MCT+Shunt PAH, net als in PAH-CHD bij

mensen, een vroege reversibele fase bestaat (dag14), een late irreversibele fase (dag 28), en een transitiefase (dag 21).

Vervolgens konden we de reversibele (dag 14+21) longen vergelijken met de irreversibele (dag 21+28) PAH longen. Hierbij maakten we gebruik van 'Next-generation RNA sequencing', een techniek om de activatie van alle bekende genen van het rattengenoom te kwantificeren. Het genetisch profiel van reversibele en irreversibele PAH bleek essentieel verschillend. Reversibele PAH kenmerkte zich door verhoogde celdeling (proliferatie), verhoogde geprogrammeerde celdood (apoptose) en activatie van de DNA-schade reparatie cascade. Bij irreversibele PAH was er juist sprake van apoptose-resistentie en inflammatie, en waren de genen voor proliferatie en DNA-schade reparatie gedeactiveerd.

Dus, welk proces zou ten grondslag kunnen liggen aan deze *switch*? Een mogelijk verklaring: versnelde cel veroudering, ofwel 'senescence'. Senescence is het biologische proces waarbij snel-delende cellen in groei-arrest gaan, vaak als gevolg van cellulaire stress. Deze cellen verliezen daarbij hun functie en vermogen om te regenereren, worden resistent tegen apoptose, en scheiden continu ontstekingsfactoren uit. Senescence is hét beschermingsmechanisme tegen ongeremde celdeling zoals bij kanker, maar draagt ook bij aan de degeneratie van gezond weefsel door het verlies van functie en regeneratief vermogen en de continue laaggradige inflammatie. Senescence lijkt om die reden aan de basis te staan van vele ouderdomsziekten als longfibrose, artrose, atherosclerose en Parkinson, of ziekten die gepaard gaan met cellulaire stress, zoals bijvoorbeeld COPD (roken) of behandeling met chemotherapie. We beschreven de rol van senescence in PAH uitgebreid in een apart reviewartikel: **hoofdstuk 8**.

In **hoofdstuk 6** toonden we vervolgens aan dat senescente cellen inderdaad in belangrijke mate aanwezig zijn in de vaatwand van sterk geremodelleerde longslagaders in ratten, en ook in mensen met irreversibele PAH. Om onze hypothese te bevestigen dat senescence niet slechts geassocieerd is met irreversibele PAH, maar ook een essentiële rol speelt in de transitie van reversibiliteit, hebben we in een nieuw experiment met ratten met irreversibele MCT+Shunt PAH, de senescente cellen selectief gedood met een experimenteel middel: ABT-263. Behandeling met ABT-263 leidde inderdaad tot een sterke vermindering van het aantal senescente cellen in de longvaten van ratten, en zorgde ook voor '*reversed remodeling*': de longvaatdoorgankelijkheid verbeterde en neointima fibrose nam af. Ook verbeterde de bloeddruk in de longen en de hartfunctie van de behandelde ratten. Er leek zelfs sprake van enige regeneratie van de kleine longslagaders na behandeling met ABT-263. Hierop concludeerden we dat senescence een essentiële rol speelt in het verlies van reversibiliteit in shunt-geïnduceerde PAH. Dit opent de deur voor verder onderzoek naar medicamenteuze

therapieën die senescence tegengaan (senolytica) om irreversibele PAH bij mensen te behandelen.

Hoofdstuk 7. Het implementeren van experimentele medicatie in klinische trials is een tijdrovend proces, dit geldt zeker ook voor de experimentele senolytica. De eerste trials met senolytica in longfibrose zijn momenteel aan de gang, maar voor een trial in PAH is eerst nog meer onderzoek nodig. In **hoofdstuk 7** testten we een nieuwe therapie met snelle klinische toepasbaarheid voor patiënten met PAH. Uit 4 kanshebbers, kozen we voor RVX-208, een *BET-Bromodomainremmer*, die voor andere indicaties al beschikbaar is in de kliniek. RVX-208 grijpt aan op *BRD4*, het *superenhancer* eiwit reeds beschreven in **hoofdstuk 2**. *BRD4* speelt een belangrijke rol in het ontstaan van hart- en vaatziekten, zo ook in PAH. In dit hoofdstuk laten we zien dat RVX-208 *BRD4* effectief remt in cellen van PAH patiënten, en dat dit leidt tot een afname van hun hyperproliferatieve en apoptose-resistente kenmerken. Ook normaliseerde de *TGFβ-BMPR2* balans die verstoord is in PAH, en verminderde het de mate van inflammatie. Interessant genoeg lijkt *BRD4* ook een belangrijke rol te spelen in het inflammatoire profiel dat geassocieerd is met senescence (zie **hoofdstuk 8**). Orale behandeling van ratten met verschillende vormen van progressieve PAH (MCT+Shunt en Sugen5146+Hypoxie) met RVX-208 verbeterde de doorgankelijkheid en de bloeddruk van de longslagaders in beide experimenten. In een derde proefdiermodel voor rechter hartkamerbelasting bleek RVX-208 ook een gunstig effect te hebben op de adaptatie van de rechter kamer aan verhoogde bloeddruk in de longen.

Toen onze experimenten met RVX-208 in een vergevorderd stadium waren, bleek een Canadese onderzoeksgroep onder leiding van prof. dr. S. Bonnet (Université Laval, Québec), zich ook bezig te houden met RVX-208 als behandeling voor PAH, met vergelijkbare resultaten. We besloten uiteindelijk onze experimentele trials samen te voegen tot één ‘multicenter pre-klinische trial’. Deze publicatie heeft geleid tot de eerste trial met RVX-208 in patiënten met PAH (APPRoAcH-p, NCT03655704). De eerste resultaten worden binnenkort verwacht.

Conclusie. Deze thesis had als doel de mechanismen te ontrafelen die een rol spelen bij de transitie in de reversibiliteit van PAH-CHD, en een nieuwe behandeling te vinden die irreversibele PAH kan omkeren. We hebben een proefdiermodel ontwikkeld voor shuntgeassocieerde PAH in ratten, waarbij een reversibele en irreversibele fase kan worden onderscheiden, vergelijkbaar met PAH-CHD in mensen. Vervolgens hebben we factoren onderzocht die bijdragen aan de transitie in die reversibiliteit. We hebben aangetoond dat het verlies van reversibiliteit gepaard gaat met een verlies van proliferatieve en regeneratieve capaciteit, een switch van apoptose naar apoptose-resistentie, en met progressieve DNA schade en inflammatie, allen passend bij versnelde cel veroudering ofwel senescence. Het

selectief doden van senescente cellen bij ratten met irreversibele PAH verbeterde de door-gankelijkheid van eerst dichtgegroeide longslagaders, en normaliseerde de bloeddruk.

Op zoek naar nieuwe, snel toepasbare therapieën voor patiënten met irreversibele PAH, vonden we RVX-208, een remmer van het epigenetische ‘superenhancer’ eiwit *BRD4*. In twee onafhankelijke preklinische trials, normaliseerde het remmen van *BRD4* de verstoorde processen in cellen van patiënten met PAH, en kon bij ratten het dichtgroeien van de long-slagaders worden omgekeerd. Deze publicatie heeft geleid tot de eerste klinische trial met RVX-208 in PAH.

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List of publications.

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About the author.

Diederik Eilard van der Feen was born on the 30th of October 1987 in Warnsveld, the Netherlands. He moved to Haren later, and graduated with honors from high school at the Praedinius Gymnasium Groningen in 2006. That year, he enrolled to study Medicine at the University of Groningen. During his bachelor, he was a board member of the Groningen Medical Student Association 'Panacea', a student member of the 'Prometheus' transplantation research team, and a teacher in basic cardio-pulmonary physiology for international bachelor students. In his masters, he did internships at the University Medical Center Groningen, the Martini Hospital Groningen, the Isala Hospital in Zwolle, the Antonius Hospital in Nieuwegein and a tropical medicine internship in rural Tanzania.

Having always been interested in congenital heart disease and cardiopulmonary physiology, he contacted prof. Rolf Berger for his masters' thesis and started a project on pulmonary arterial hypertension (PAH). During this project, he went to the Necker Hospital in Paris to study histological biopsies of patients with a reversible and irreversible form of PAH. After finishing his masters in 2014, he continued research in the field of PAH as a PhD candidate at the departments of paediatric and experimental cardiology at the University Medical Center Groningen, led by prof. Berger and prof. de Boer respectively. He performed his studies in the context of a multidisciplinary nationwide research consortium: PHAEDRA, which aimed to break the vicious cycle [of disease progression] in PAH and associated right heart failure. Within PHAEDRA, his work is best characterized as translational science. Central questions in his projects were: how can the reversibility of PAH be assessed, what are the mechanisms that cause the transition from reversible to irreversible PAH, and what are possible targets for intervention to reverse irreversible PAH.

In 2017, Diederik was awarded a CVON Young Talent Grant and a KNAW ter Meulen Grant, which enabled him to perform bioinformatic analyses and further mechanistic studies at the lab of prof. Rabinovitch at Stanford University, California, USA.

After his PhD, Diederik moved to Amsterdam. In march 2020, right at start of the COVID19 pandemic in the Netherlands, Diederik started his training to become a pulmonologist at the Onze Lieve Vrouwe Gasthuis Hospital in Amsterdam, the VU medical center in Amsterdam, and Spaarne Gasthuis in Haarlem.