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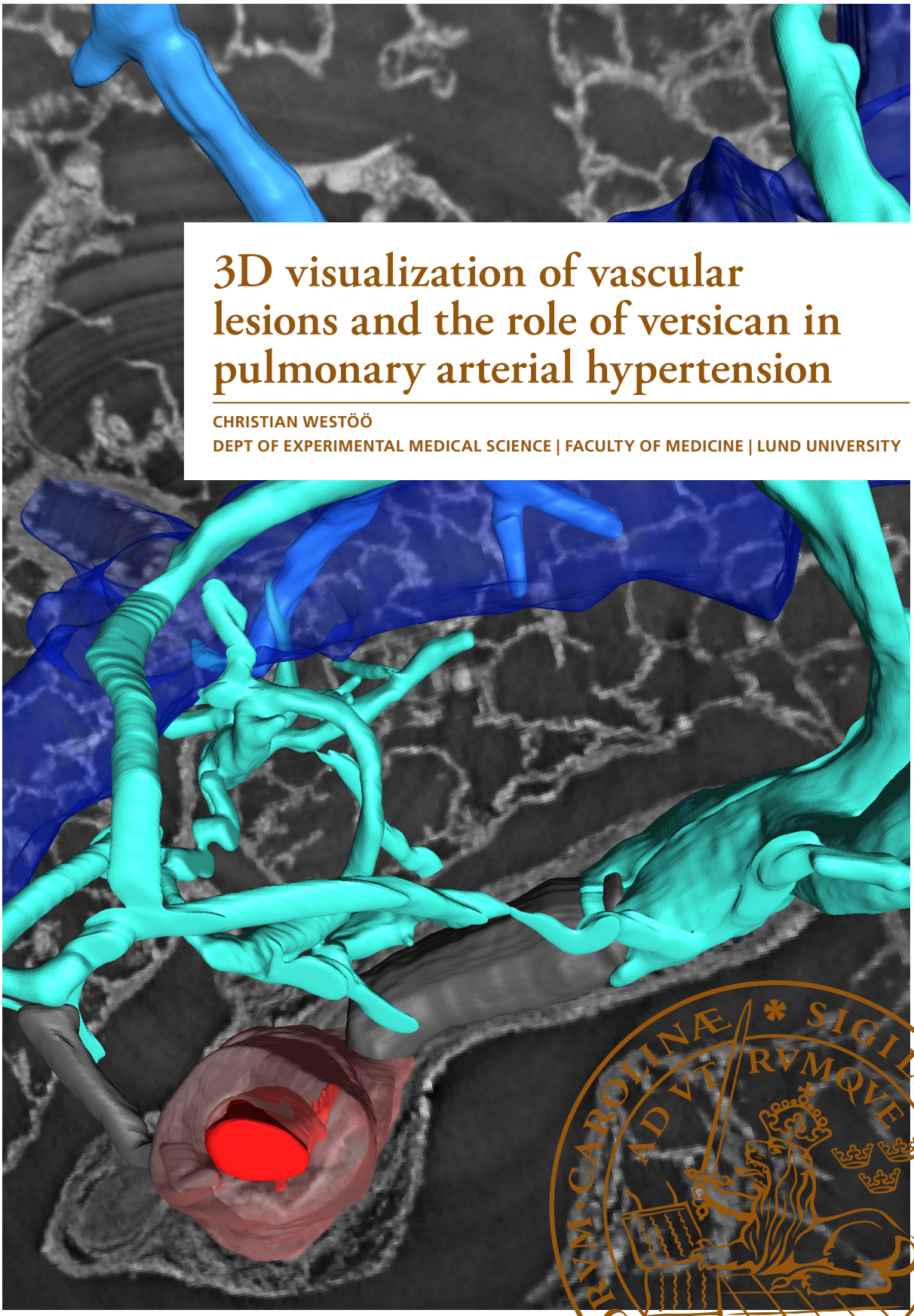

3D visualization of vascular lesions and the role of versican in pulmonary arterial hypertension

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LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Abstract

Pulmonary arterial hypertension (PAH) is a condition that leads to lethal right heart failure. Available treatments provide relief but are far from curative and the vascular remodeling in PAH has been difficult to decipher. Although pathognomonic to PAH, the plexiform lesions are difficult to define, and there is no consensus regarding their potential hemodynamic impact. Versican is an extracellular matrix proteoglycan known to accumulate in systemic vascular disease, often colocalized with hyaluronan. Previous results have shown that versican is abundant in vascular lesions of PAH. However, its functional role warrants investigation.

This PhD project have had two separate aims, of equal importance. A novel imaging technique called synchrotron-based phase-contrast micro-CT (SPμCT) was evaluated for its potential use as a tool for virtual histology of pulmonary vascular pathology. SPμCT was used in all five projects. Versican was studied in human PAH tissue by immunofluorescence and in situ hybridization, and its function in disease development was evaluated in murine models of pulmonary hypertension.

SPμCT imaging revealed the possibility of non-destructive 3D imaging of paraffin-embedded pulmonary tissue with clear contrast and high resolution. With this technique, we could demonstrate the presence of patent intrapulmonary bronchopulmonary anastomoses in a subgroup of pediatric PAH called alveolar capillary dysplasia and confirm the identity of misaligned pulmonary veins as dilated bronchial veins. SPμCT imaging of adult idiopathic PAH (IPAH) tissue enabled 3D visualization of plexiform lesions. Significant plexiform lesion heterogeneity was identified, as well as proof of shunting between the pulmonary and bronchial circulation within some lesions. These results led to a novel classification of plexiform lesions. The same technique was used for a rat model of PAH (Sugen5416/hypoxia) where for the first time an animal model has been shown to have the full spectrum of human type plexiform lesions, including shunt-type lesions.

GAG AR-α and -β containing isoforms of versican were found to be produced and deposited in lesions of PAH. Hyaluronan was found abundant in the neointima of vascular lesions, consistently colocalizing with versican. Known neoepitopes of versican, generated by enzymatic cleavage, were observed and the staining pattern indicated further enzymatic cleavage of the core protein. Serum samples from IPAH and control patients revealed significantly elevated levels of versican G3 in patient blood, making it a possible biomarker. Neither a global partial (two isoforms) or inducible complete (all isoforms) knockdown of versican affected pulmonary hypertension in mice exposed to chronic hypoxia, perhaps due to the lack of occlusive lesion formation in this model. SPμCT imaging revealed gradual formation of occlusive vascular lesions, with versican deposition, in a mouse model where exposure to house dust mite generates pulmonary hypertension. These results indicate that this model may be more suitable for studies of the role of versican in vivo.

In conclusion, SPμCT imaging and the novel classification of plexiform lesions have the potential to advance the PAH field significantly, and versican G3 is a potential biomarker for pulmonary vascular remodeling.

Key words: Pulmonary arterial hypertension, Synchrotron, Imaging, Phase-contrast, Plexiform, Versican, Biomarker

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Till mina föräldrar som gjorde allt rätt

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Article II

Christian Westöö, Christian Norvik, Niccolò Peruzzi, Oscar van der Have, Goran Lovric, Ida Jeremiasen, Phan-Kiet Tran, Rajmund Mokso, Vinicio De Jesus Perez, Hans Brunnström, Martin Bech, Csaba Galambos and Karin Tran-Lundmark. **Distinct types of plexiform lesions identified by synchrotron-based phase-contrast micro-CT.** *Am J Physiol Lung Cell Mol Physiol* 321: L17 – L28, 2021

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Oscar van der Have, **Christian Westöö**, Filip Ahrné, Xuefei Tian, Kenzo Ichimura, Till Dreier, Christian Norvik, Maya E Kumar, Edda Spiekerkoetter, Karin Tran-Lundmark. **Shunt-type plexiform lesions identified in the Sugen5416/Hypoxia rat model of pulmonary arterial hypertension using SPµCT.** *Eur Respir J 2022, 59: 2102802*, Research Letter

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Abbreviations

ACD/MPV	Alveolar capillary dysplasia with misaligned pulmonary veins
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
AR	Attachment region
ARRGQF	N-terminal neoepitope of cleaved versican
BMPR2	Bone morphogenetic protein receptor type 2
CT	Computed tomography
DPEAAE	C-terminal neoepitope of cleaved versican
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
GAG	Glycosaminoglycan
HDM	House dust mite
HPAH	Hereditary pulmonary arterial hypertension
IPAH	Idiopathic pulmonary arterial hypertension
ISH	In situ hybridization
mRNA	Messenger RNA
PAH	Pulmonary arterial hypertension
PCWP	Pulmonary capillary wedge pressure
PH	Pulmonary hypertension
PPHN	Persistent pulmonary hypertension of the newborn
PVR	Pulmonary vascular resistance
PVOD/PCH	Pulmonary veno-occlusive disease and/or pulmonary capillary hemangiomatosis
RNA	Ribonucleic acid
RVH	Right-ventricular hypertrophy

RVSP	Right-ventricular systolic pressure
SPμCT	Synchrotron-based phase-contrast micro-CT
VEGFR2	Vascular endothelial growth factor receptor 2
VSMC	Vascular smooth muscle cell
WHO	World health organization
α-SMA	Alpha smooth muscle actin
2D	Two-dimensions or two-dimensional
3D	Three-dimensions or three-dimensional

Introduction

It'll be easy-peasy-lemon-squeezy.

No, it won't. It will be difficult-difficult-lemon-difficult. That is what it will be.

– Simon Foster exchanging words with Toby Wright

Pulmonary Hypertension

Pulmonary hypertension (PH) is a severe condition of elevated blood pressure within the pulmonary circulation. It is defined as a mean pulmonary arterial pressure ≥20 mmHg (millimetres of mercury) at rest measured by right heart catheterization.[2] PH is a diverse syndrome and is divided into five subgroups based mainly on aetiology, according to the World Health Organization classification (Table 1).[2] Group 1, where the primary underlying pathology is in the pulmonary vasculature (primarily pre-capillary) is called pulmonary arterial hypertension (PAH). Group 2 PH is caused by left heart disease (left-heart failure), group 3 is associated with chronic lung disease with or without hypoxia and group 4 is secondary to chronic pulmonary thromboembolism. Group 5 is comprised of unclear or multifactorial forms of PH. Treatments are specific to each group, but in general groups 2-5 are treated by focusing on treating the underlying primary pathology which can vary greatly. The focus for this thesis is on group 1, the highly complex and progressive condition of PAH, for which there is no cure and where available therapies only delay development of right heart failure and premature death.

Table 1
Classification according to the World Symposium on Pulmonary Hypertension

Group 1	Pulmonary arterial hypertension (PAH)
Group 2	Pulmonary hypertension due to left heart disease
Group 3	Pulmonary hypertension due to lung diseases and/ or hypoxia
Group 4	Chronic thromboembolic pulmonary hypertension (CTEPH)
Group 5	Pulmonary hypertension with unclear multifactorial mechanisms

Pulmonary arterial hypertension

"To the dumb question "Why me?" the cosmos barely bothers to return the reply: why not?"

– Christopher Hitchens

Classification and aetiology

The definition of pulmonary hypertension (PH) is, as stated above, a mean pulmonary arterial pressure ≥20 mmHg. As PAH is a pre-capillary condition (with the exemption of pulmonary veno-occlusive disease, discussed briefly below) the diagnosis requires both an elevated pulmonary vascular resistance (PVR) and a pulmonary capillary wedge pressure (PCWP) ≤15 mmHg (normal range 4–12).[3] An elevated PVR signifies pre-capillary vascular remodelling and/or increased vascular tone, and a low PCWP excludes significant post-capillary hemodynamic dysfunction (such as congestive left heart disease).

Subgroups of PAH

PAH can be further classified into several subgroups which share vascular pathology, clinical presentation and to a large extent pharmacological management.[2, 4] Over 50% of cases are idiopathic (IPAH) or hereditary (HPAH)[5], with the remaining being drug-induced or associated PAH (Table 2). Pulmonary veno-occlusive disease and/or pulmonary capillary hemangiomatosis (PVOD/PCH) has been added to group 1 (PAH) due to the similar clinical presentation, although the primary pathology for these patients is not pre-capillary.[6] Furthermore, PVOD/PCH patients generally have a worse prognosis and do not tolerate pharmacological PAH treatment with pulmonary vasodilators (high risk of pulmonary oedema).[6, 7] Persistent pulmonary hypertension of the newborn (PPHN) is also a type of PAH, often caused by a delay in the transition from the fetal circulation. In its milder forms it self-terminates or can be treated by addition of oxygen. There are however severe forms that require treatment with nitric oxide

(NO) and possibly also heart-lung machine support (extracorporeal membrane oxygenation), where life however can still be saved. In some cases, children who present as PPHN suffers from a congenital malformation of the pulmonary vascular tree called alveolar capillary dysplasia with misaligned pulmonary veins (ACD/MPV) that causes severe hypoxia where mortality approaches 100%.

Table 2
Group 1: Pulmonary arterial hypertension (PAH)

1.1	**Idiopathic**	
1.2	**Heritable**	
1.3	**Drug and toxin induced**	
1.4	**Associated with**	
	1.4.1	Connective tissue disease
	1.4.2	HIV infection
	1.4.3	Portal hypertension
	1.4.4	Congenital heart disease
	1.4.5	Schistosomiasis
1.5	**PAH long-term responders to calcium channel blockers**	
1.6	**PAH with overt features of venous/capillaries (PVOD/PCH) involvement**	
1.7	**Persistent PH of the newborn syndrome (PPHN)**	

Epidemiology, clinical presentation, diagnosis and treatment

Dr. Iannis had enjoyed a satisfactory day in which none of his patients had died or got any worse

– Louis De Bernières

Epidemiology

PAH has prevalence of 15-50 cases per million adults within Europe and the United States.[8] The incidence of PAH for women is fourfold higher than for men, but men have a higher mortality rate.[9] Because of its progressive nature, high morbidity and mortality it is essential that PAH is diagnosed early. However, due to the rarity of the diagnosis and the non-specific initial symptoms, there is often significant patient's and doctor's delay.[10]

Clinical presentation and diagnosis

Initial symptoms include fatigue, varying degrees of shortness of breath, dizziness and heart palpitations to name a few; unspecific symptoms for which there are many different causes. Furthermore, there are currently no known biomarkers that can help the physician during the initial assessment.

Once PAH is suspected, a primary screening can be done with a chest radiograph and a echocardiography (heart ultrasound, where signs of increased pulmonary pressures can be detected). To diagnose PAH a complete right-heart catheterization (insertion of a pressure sensitive catheter into the pulmonary artery, from a major vein via the right heart) is needed, which is a costly and invasive procedure that requires a specialized hospital setting.[11]

Treatment

With modern therapy in a developed health care setting (western Europe) the five-year survival rate following PAH diagnosis is 71%, and for high-risk patients 56 %.[12] This is a significant improvement, as the first comprehensive registry (1981-85) found a median survival (without specific PAH therapies) of 2.8 years and an overall five-year survival rate of 34%.[13]

Therapies that slow the progression of PAH have become available in the last two decades, in the form of different types of vasodilators that reduce the pulmonary arterial pressure and the strain on the right heart. Ongoing trials where molecular pathways are targeted appear promising, including a recent study where a drug called Sotatercept (acting to balance growth-promoting and -inhibiting pathways) have shown to significantly reduce PVR.[14] There are however so far no therapies available that convincingly have been shown to reverse the vascular pathology to the extent that lung, or heart-lung, transplantation can be avoided long term.[15] Lung transplantation is the last resort but is associated with high mortality. For IPAH patients post lung-transplantation, the three-month mortality is 23 % with a median survival of only 10 years according to a 2015 report.[16]

Pulmonary vascular anatomy

I've come down from the mountains, with an ass-full of specimens

– Stephen Maturin

Macroscopic pulmonary anatomy

The lungs are the only organ in the body that accept the full cardiac output, i.e., the same volume of blood which is pumped into the body from the left heart. As such, the pulmonary vasculature has developed to ensure high compliance and low resistance (low pressure system). Similar to the systemic, the pulmonary circulation is serially compartmentalized into arteries, capillaries and veins, with the difference being that the arteries carry de-oxygenated blood, and veins carry oxygenated blood. All vessels entering or exiting the lung do so through the pulmonary hilus. The pulmonary veins travel in septal spaces and carry oxygenated blood from the pulmonary circulation, but also a small amount of de-oxygenated blood from the bronchial circulation, as only a minority of the bronchial venous blood is believed to empty into systemic veins.[17] Another important distinction between the pulmonary and the systemic circulation is how arteries and arterioles respond to relative hypoxia. In the systemic vasculature arterioles dilate when hypoxic conditions arise, such as in a muscle that is exerted. This effect allows an increased blood flow for the increased local demand and helps to reverse the hypoxic environment (accumulation of lactic acid). In contrast, the pulmonary arterioles respond reversely i.e., they constrict. As an evolutionary response, blood flow to poorly ventilated pulmonary tissue (local hypoxic environment) is restricted, thus avoiding ventilation-perfusion mismatch for optimized oxygenation. As such, when exposed to global hypoxic conditions (such as high altitude) pulmonary arterial vessels constrict with increased PVR and pulmonary hypertension as consequence.

Vascular anatomy

The vascular wall consists of three individual layers as depicted in figure 1: tunica intima (inner most layer), tunica media and tunica adventitia (outer most layer). The intima is in most vessels composed of a single layer of endothelial cells situated on a basement membrane. The media is made up of smooth muscle cells and elastic fibres. It maintains vascular tonus, is elastic and propagates pulse waves. The adventitia is composed mainly of collagen and elastic fibres and provides tensile strength to the vessel.

The presence of elastic fibres and muscularization (vascular smooth muscle cells or VSMCs) vary with the size of pulmonary arteries. Larger pulmonary arteries have multiple elastic laminae. With smaller diameters (<1 mm) the media is seen to be clearly delineated by a single internal and external elastic lamina on either side of the media.[18] There is a gradual loss of musculature in the distal pulmonary vascular tree, where partially muscularized arteries appear adjacent to respiratory bronchioles and non-muscularized arteries accompany alveolar ducts.[18] In general, vessels $\geq$ 70 µm in diameter are muscularized in a healthy state.[19] Capillaries, situated between the arteries and veins, lack the medial layer. Pulmonary veins get muscularized as their size increase, however the thickness is estimated to be ~50% compared to pulmonary arteries of the same size.[20] They are easily identified by their thin media, and their indistinct or non-existent internal elastic lamina.[21]

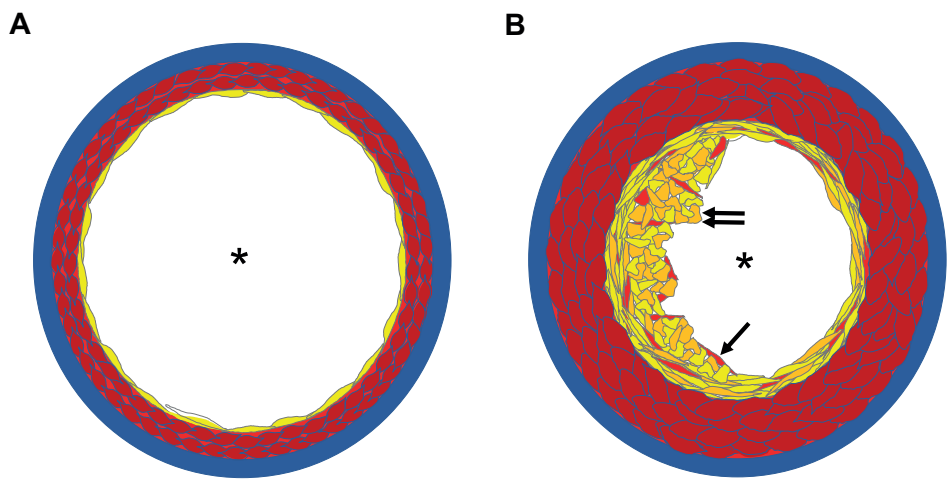

Figure 1.
Schematic and simplified illustration of a cross section of the pulmonary artery in health and disease. Blue = adventitia; red: media; yellow = intima. Asterisk marks lumen. **A**: healthy pulmonary artery. The intima is composed of a single layer of endothelial cells, and the media is composed of vascular smooth muscle cells in an orderly configuration; **B**: remodelled pulmonary artery. The intima is thickened and grows occlusive with dysfunctioning endothelial cells (orange, double arrow) as well as dysfunctioning smooth muscle cells (single arrow) within the neointima. The media is hypertrophied by both proliferation and hypertrophy of smooth muscle cells.

Pulmonary arteries and veins do not exist as bundles as they do in the systemic circulation, but are separated by the pulmonary parenchyma where gas exchange (oxygenation) occurs through pulmonary capillaries. The vessels in the pulmonary parenchyma consist of arterioles (distal arteries) and venules (<20-25 μm)[18] and capillaries (~7-17 μm).[22] The parenchyma is estimated to comprise 85% of the lung anatomic volume, with the remainder made up of vessels and airways.[23] The pulmonary arteries run alongside the airways (bronchus and bronchiole) with coincidental branching in what are known as bronchovascular bundles. The bronchovascular bundle is enveloped by a common adventitial sheath that also house connective tissue, nerves, lymphatics and branches of the bronchial circulation (discussed in detail below).[24]

In addition to the conventional dichotomous branching of the pulmonary arterial vasculature there are also so-called monopodial branches or supernumerary arteries. These are identified by their perpendicular branching angle, their comparatively smaller diameter and by the fact that they do not accompany an airway.[25]

The bronchial circulation

The bronchial circulation is part of the systemic circulation, usually originating from the aorta but also observed to originate from intercostal arteries,[26, 27] that supplies oxygenated blood to larger anatomical structures in the lung such as the airways and the tissue making up arteries and veins as well as the pleura.[27] When supplying oxygenated blood to vascular structures, it is done through a network called the vasa vasorum (from Latin: *vessels of the vessels*). In a normal, healthy, setting bronchial arteries are small with a cross-sectional measurement of 2 mm at their origin,[28] compared to the pulmonary artery which is ~24-26 mm[29], and are estimated to receive only 1%[30] of the total cardiac output (compared to the pulmonary artery which receives 100%). As described previously, the bronchial arteries are located within the bronchovascular bundle, where often plural bronchial arteries are found.[27] The bronchial arteries terminate in different ways. Firstly, through coincidental branching they give rise to capillaries that oxygenate larger structures, as described above, which then drain into bronchial veins. Furthermore they have been shown to terminate distally where they merge with nearby alveolar clusters (oxygenation of blood) forming capillary networks at the level of the preterminal bronchiole[27] and have been found to merge with pulmonary arterioles at the capillary level forming a anastomotic network that eventually drain into pulmonary veins.[24] When the bronchial artery end in a alveolar capillary network, it is often termed a bronchopulmonary artery. Pre-capillary arterial to arterial connections (pulmonary to systemic) called arterial bronchopulmonary anastomoses have also been reported to exist, which are discussed in the following section.

Arterial bronchopulmonary anastomoses

The existence of patent pre-capillary arterial bronchopulmonary anastomoses within the human lung is not fully known. In his 1969 paper on the paediatric lung (immature to 7 months old) Robertson showed that patent arterial bronchopulmonary anastomoses exist post-partum, and that they appear to increase in number within the first 2½ months. Thereafter, with increasing age, he saw clear evidence for the obliteration of these anastomoses by intimal smooth muscle cells and connective tissue. He states that "*it is apparent … that there is a dynamic relation between the pulmonary and bronchial arterial systems*" and furthermore that *"it seems probable that the majority of the arterial bronchopulmonary anastomoses normally become obliterated toward adult age"*.[31] In a 1963 paper Margaret Turner-Warwick found no evidence of pre-capillary arterial bronchopulmonary anastomoses in healthy adult lungs.[32] K. K. Pump however argued in his 1972 paper on the bronchial circulation that they do exist and that blood could flow through these anastomoses in hemodynamically altered but healthy states, such as during inspiration when pulmonary arterial pressures decrease.[27] More recently Kotoulas *et al* discussed in their review from 2014[24] that the available literature on the topic suggests that they are functionally closed but that they open during pathological conditions (excluding healthy hemodynamically altered states) such as chronic thromboembolic pulmonary hypertension (group 4 pulmonary hypertension), where they have repeatedly been shown to be patent.[32-34]

Previous studies have not found these connections in PAH to any convincing degree.[34, 35] Although recent data have indicated that there are in fact patent arterial bronchopulmonary anastomoses in PAH, their existence is still under debate as well as their potential hemodynamic impact.[36]

The extracellular matrix, proteoglycans and glycosaminoglycans

Versican is easy to study

– A statement never uttered by man

The extracellular matrix

Most cells do not exist as individual units by themselves but are connected with neighbouring cells through the extracellular matrix, or ECM. The ECM can be understood as a scaffold that is produced by the cells for the cells. It is the fundament of the tissue it is a part of, true for organs and blood vessels alike. The vessels mechanical strength, compressibility and elasticity is dependent on the state of the ECM, as is the cell-cell crosstalk that regulates differentiation, migration and proliferation: "*any deviation from this balance changes blood vessel architecture and mechanics, altering function and resulting in disease*".[37]

Examples of different types of ECM are the basement membranes underneath the endothelium, the vascular smooth muscle cell (VSMC) basement membrane and the internal and external elastic laminae of the media.[38] The healthy arterial ECM is mainly made up of the elastic elastin and to a lesser extent collagen fibres, with smaller amounts of proteoglycans and glycoproteins.[39]

Proteoglycans and glycosaminoglycans

Proteoglycans are large molecules, produced by a variety of cells including VSMCs, that consist of a core protein with varying amounts of negatively charged polysaccharide side chains (glycosaminoglycans or GAGs) covalently attached.

Based on underlying chemical structures, GAGs are divided into five classes or types: chondroitin sulphate, dermatan sulphate, heparan sulphate, keratan sulphate and hyaluronic acid.

The negative charge attracts positively charged molecules which affect cellular communication and migration and can produce a gel-like structure by the attraction of cations such as Na^+ (hydrostatic pressure). Proteoglycans are by their nature chemically active. They bind growth factors and cytokines and can modulate their activity, regulate the actions of secreted proteases as well as affect cellular function, to name a few functions.[40]

Versican

Versican is a chondroitin sulphate proteoglycan that has been shown to be of importance to tissues in both health and disease. The versican core protein consists of an N-terminal G1 domain and a C-terminal G3 domain, with glycosaminoglycan (GAG) attachment regions (AR) between the two: GAG AR-α and GAG AR-β, see figure 2.[41, 42] Fifteen exons encode the versican core protein. Exon 7 encodes GAG AR-α and exon 8 GAG AR-β. Through alternate splicing different isoforms are generated, with the four most common being isoforms V0 – V3. Most of what is known regarding the properties of versican has been attained through studies of V0 and V1. Isoform V0 is the largest, containing both GAG AR-α and GAG AR-β, and is found during embryonic development, and to a lesser extent in adult tissue.[43] V1, expressing GAG AR-β but not -α, is the most abundant in adult tissue, and has been shown to inhibit apoptosis and promote proliferation during repair and remodelling in disease, injury and inflammation.[44-46] Isoform V2, expressing GAG AR-α but not -β is mainly found in the central nervous system, and has been demonstrated to have anti-proliferative properties.[47, 48] V3 does not contain any AR, and has not been associated with disease to any significant extent, though few studies have been successful in detecting isoform V3 in tissue or cells.[44] mRNA expression of V3 has however been studied, and the data indicate that isoform V3 have antiproliferative properties.[46, 49] Accumulation of the versican core protein has been found in systemic vascular lesions,[37] and has by our group been found to be abundant in lesions of PAH.[50] Furthermore, a whole exome sequencing performed on 12 unrelated IPAH patients in 2014 by de Jesus Perez and colleagues identified nine genes that were modified/ mutated. The versican gene VCAN was found to be one of the genes most highly affected.[51]

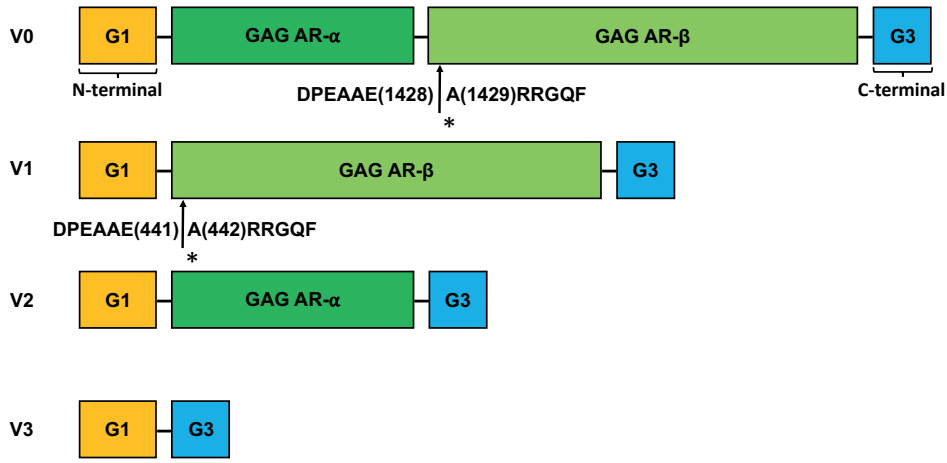

Figure 2.
Schematic illustration of versican, showing the four most common isoforms. The versican molecule consist of an N-terminal G1 domain and a C-terminal G3 domain, with glycosaminoglycan (GAG) attachment regions (AR) between the two: GAG AR-α and GAG AR-β. Isoform V0 contains both GAG AR-α and GAG AR-β, V1 only GAG AR-β and V2 only GAG AR-α. Isoform V3 lacks glycosaminoglycan attachment regions. Asterisks mark the cleavage site in the GAG AR-β domain for ADAMTS enzymes that generates fragments with neoepitopes DPEAAE and ARRGQF at site Glu1428-Ala1429 in isoform V0 and Glu441-Ala442 in isoform V1.

In the systemic circulation versican is known to affect cell adhesion, migration and proliferation. Its functional role in the progression of lesions of PAH is however still largely unknown.

Newly synthesised versican is either deposited in the ECM or rapidly degraded by enzymatic activity. A number of different proteases degrade versican, and cleavage by certain ADAMTS-enzymes (a disintegrin and metalloproteinase with thrombospondin motifs) generates an amino-terminal fragment with the neoepitope sequence DPEAAE by cleavage in the AR-β of isoforms V0 and V1 (figure 2).[52] The fragment, also known as Versikine when generated from isoform V1, has been shown to be biologically active in settings such as mouse embryonic development[53], and likely has modulating roles in colorectal cancer[54] and in myeloma.[55] Furthermore, recent work by Islam and colleagues demonstrated significantly faster wound healing in a mouse model with ADAMTS-cleavage resistant versican, indicating proliferative effects from versican accumulation.[56] Turnover of versican by ADAMTS enzymes had not previously been studied in lesions of PAH.

Versican is known to interact with hyaluronan (hyaluronic acid) via its N-terminal domain. Hyaluronan is a glycosaminoglycan (GAG), and is unique among GAGs as it is not attached to a protein core. Furthermore, it is very large in comparison to other GAGs (approximate molecular weight 10^5-10^7 compared to chondroitin sulphates 10-50 x 10^3).[57] Hyaluronan plays important roles in the ECM: it is for example one of the first ECM molecules produced by plated cells.[58] It is found in

most tissues and organs, with the highest concentrations generally found in connective tissues. The lowest concentrations of hyaluronan are found in the blood.[57] Through its large size, and through its intrinsic properties, it can bind large amounts of water as well as having hydrophobic patches where it can crosslink other hyaluronan molecules or lipid structures.[59] In an extensive affinity-histochemical analysis of the rat, only minor amounts of hyaluronan were identified in healthy arteries.[60] The author have found no evidence that this pattern would be different for healthy human arteries, systemic or pulmonary.

Versican and hyaluronan form pericellular matrices. These have been demonstrated to be central for proliferation of VSMCs and have been found in re-stenotic vascular lesions of the systemic circulation.[61] In IPAH, hyaluronan has been identified in pulmonary vascular lesions, and elevated levels have been found in patient plasma.[62] Interestingly, elevated hyaluronan deposition by fibroblasts in the ADAMTS-cleavage resistant versican mouse model, mentioned above, have been identified.[56] It is however unknown what relationship versican and hyaluronan have in the different vascular lesions of PAH, as it had not previously been studied.

Pathogenesis of PAH and plexiform lesions

If the radiology resident and the [medical student] both see a lesion on the chest X-ray, there can be no lesion there

– Law XII, House of God

Pulmonary vascular remodelling

Pulmonary arterial remodelling is pathognomonic for PAH. Although complex, the remodelling can generally be summarized into intimal and medial changes in distal pulmonary arteries and arterioles. The intima is found to grow from a single endothelial layer to a thick neointima, rich in ECM produced by both endothelial and VSMCs, that compromises the vascular lumen (figure 1B). The media hypertrophies by both proliferation and hypertrophy of VSMCs, along with increased deposition of ECM molecules and proteins. Smaller, previously non-muscularized arteries get muscularized. Although the adventitia can grow thicker, on average it is not significantly increased in PAH compared to control patients.[63] Taken together, these changes are referred to as vascular lesions. These lesions reduce the cross-sectional lumen area of the pulmonary vasculature and increase the PVR. The vascular changes are incompletely understood, and there are no available treatments that can reverse the vascular remodelling.

Historical perspective on vascular lesions of PAH

In a hallmark paper by Heath and Edwards[64] in 1958, the lesions of pulmonary hypertension were described and graded on a scale from one to six, with grade six being the most remodelled. Grade 1 is defined by medial hypertrophy and grade 2 by initial cellular intimal proliferation. Grade 3 with increasing intimal remodelling and intimal occlusion. Grade 4 was described as progressive generalized dilatation lesions of different types, where the remodelling shifts from hypertrophy to dilatation. Included in grade 4 is the hallmark lesion of PAH, the plexiform lesion,

see figure 3 for representative examples. Although difficult to define, a generally accepted definition by Wagenvoort from 1994 states that the plexiform lesion consists of a plexus of small vessels within a focal dilatation of the pulmonary arterial branch (plexiform lesions will be discussed in more detail below).[65] With grades 5 and 6, the authors described a chronic appearance with fibrosis (scarring) and rigidity. Grade 6 was defined as necrotizing arteritis. They only found these lesions in one patient and made a point of stating that this lesion type is highly uncommon in PH. In summary, the vascular pathology was described to start with progressive medial hypertrophy followed by cellular intimal reactions/occlusive lesions which later becomes fibroelastic. Dilatation lesions, as a result of atrophy, were then described to follow.

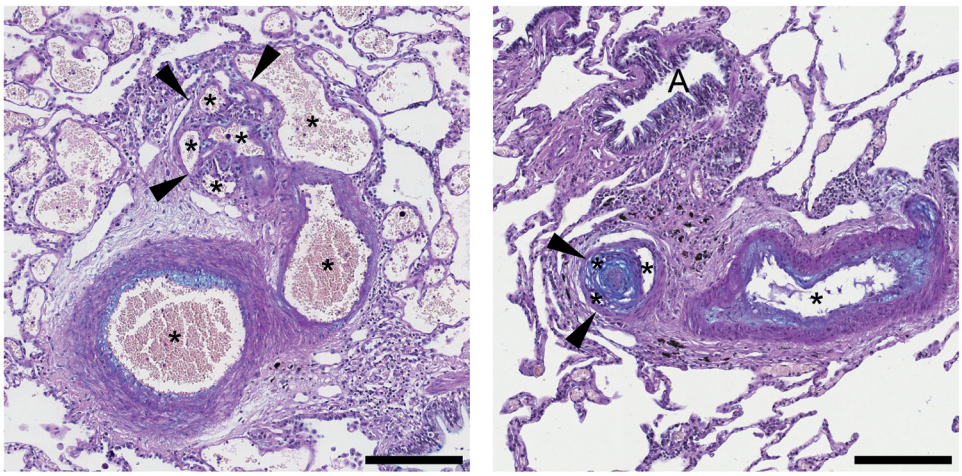

Figure 3.
Representative histological images of plexiform lesions. Both are Ab-PAS stainings, where proteoglycans are seen in blue. Asterisk marks vascular lumen, arrows indicate the plexiform lesions. In the right hand image the airway is annotated with an A. Scale bars = 200μm.

In essence, Heath and Edwards presented a proliferative and a subsequent degenerative evolution of lesions. C.A. Wagenvoort built on this work with his paper from 1973 where he modified the classification of the lesions of pulmonary vascular disease.[66] He did this in an effort to aid clinicians and pathologists in their clinical work (for reference it is important to note that eight years later he published a revision of his arguments stating that diagnosis cannot be based largely or solely on lesion grade due to the complexity of the disease).[67] Furthermore, in 1986 he argues (responding to a simplified grading system suggested by W. C. Roberts) in the American Journal of Cardiology that due to the "*variety of pulmonary vascular changes any classification in the sense of grading has become of limited value and may be misleading"* and ending his argumentation with that what should be

evaluated is the *"entire morphological picture".*[68] These publications and commentaries are presented to demonstrate for the reader that there has been a long-standing debate regarding the nature of pulmonary vascular lesions. Furthermore, it demonstrates that an accepted pathological model explaining severe PAH was missing, a theory that would explain the creation of the lesions (intimal and plexiform). There were differing theories, centred primarily upon cause and effect: either that there exists a primary pathology causing lesion formation and that the lesions in turn increase the pressure or, on the other hand, that an altered hemodynamical state occurs first, stressing the pulmonary vasculature, with lesion formation as a result.

Theories on the development of PAH

One such theory is the chronic hypoxia theory, based on research done by Reid and Wagenvoort that was focused to a large extent on experiments with rats.[69, 70] As explained in section *Pulmonary vascular anatomy* on page 18, pulmonary arteries respond with vasoconstriction to hypoxic conditions. Rats exposed to hypoxia (see *Animal models* on page 34 below) respond with medial hypertrophy and increased vasoconstriction leading to pulmonary hypertension. This form of experimental pulmonary hypertension has been used extensively and can successfully be treated with a range of drugs.[71-73] However, human PAH has so far been shown to respond poorly to many types of drugs which gave promising effects on the remodelling seen with animal models of chronic hypoxia, and it is now accepted that only a small minority of patients present with a significant reversibility of disease with oxygen and/or nitric oxide at the time of diagnosis.[74] Another crucial consideration of the hypoxia theory is that the model does not generate human-like intimal or plexiform lesions, with the latter being the histopathological hallmark of PAH.

In 2008 Rai and colleagues published an article entitled "*The cancer Paradigm of Severe Pulmonary Arterial Hypertension".*[75] They proposed a new explanatory model of PAH, one that would also explain the occurrence of plexiform lesions (which the authors dubbed "complex vascular lesions"). Their interpretation of the then published research was that a cancer-like phenotype of injured endothelial cells is the root cause of PAH and that a "*neoplastic, angioproliferative disorder likely more accurately reflects the clinical reality ... of patients with severe PAH than a vasomotor tone (vasoconstriction and impaired vasodilation)-centred model".* Furthermore, the authors hypothesized that the appearance of plexiform lesions in specific locations within the pulmonary arterial vascular tree could be *"akin to the process of widespread metastases resulting in innumerable micronodules in a characteristic distribution (the seed and soil hypothesis)".*[75] To this authors knowledge however, no corroborative evidence of this metastasis hypothesis of plexiform lesions has been published. However, what is accepted in the field is that dysregulated vascular cell growth is integrally linked to the development of

plexiform lesions, where angioproliferative markers have been found to be upregulated.[76, 77] Experimental PAH models using the receptor tyrosine kinase inhibitor SU5416 and hypoxia also provide data in support of a proliferative hypothesis. With SU5416, vascular endothelial growth factor receptor 2 is blocked which leads to widespread pulmonary vascular endothelial cell apoptosis with the result that apoptosis-resistant hyper-proliferative cells are selected. These in turn cause obliterative lesions in distal arterioles and an increase of PVR.[78] Hence, there are clear data in support of a dysregulated proliferative component within lesions of PAH. However, there are interesting contradictory data to this hypothesis which are discussed in a 2017 review article by Chaudhary and colleagues with the subtitle "*Have we put the cart before the horse*?"[79] Among other results, they discuss the findings of Abe et al demonstrating that when blood flow was reduced through arterial banding, rats exposed to SU5416 and hypoxia did not develop vascular lesions.[80] This phenomenon has also been observed in a case report, where regression of plexiform lesions were identified after single lung transplantation in the non-transplanted lung.[81] This would indicate that hemodynamic abnormalities are the trigger and not the other way around.

Plexiform lesions

Much of these above referenced studies, reviews and discussions have been centred on plexiform lesions. It has been the focus of much PAH research since it is the lesion type that is specific to PAH, group 1 pulmonary hypertension, and since it is associated with a high disease burden.[82] If this lesion can be understood, then perhaps the pathophysiology of PAH could be understood. However, a consensus needs to be established as there is obvious uncertainty in the field regarding which lesions are plexiform. That the terms plexiform and plexogenic (plexiform-like) are used interchangeably is a clear sign of this. Furthermore, as argued above, there is still no accepted pathophysiological model for plexiform lesions: are they a *cause for* disease or are they a *marker of* disease? Another point of confusion in the field is a paper from 2016 by Galambos et al, based on computer assisted three-dimensional visualization, that indicate that plexiform lesions might not arise within the pulmonary vascular bed at all, but that they might originate from the bronchial microcirculation in anastomotic connections.[36] As Chaudhary and colleagues point out: *If correct, this would have profound implications … if plexiform lesions do not actually occur within the pulmonary bed, then how can they contribute directly to the increased pulmonary vascular resistance*?"[79]

Why has there been so much confusion regarding this central lesion that has been known and defined for decades? One hint to what could be a cause for this confusion lies in how it has been defined: "*a plexus of small vessels within a focal dilatation of the pulmonary arterial branch*".[65] This is ambiguous, but this ambiguity is perhaps the logical consequence when defining a three-dimensional structure that

have only been studied in two dimensions. This ambiguity or uncertainness of what a plexiform lesion is, or where it is located, can be seen in the different schematic illustrations of plexiform lesions found in publications, shown in figure 4.

There have been several previous attempts by researchers to answer these questions by trying to visualize and study plexiform lesions in three-dimensions.[76, 83, 84] However, due to technical limitations only small tissue volumes could be analysed with relatively low resolution. Through serial sectioning with computer assisted reconstruction, Yaginuma et al[83] found plexiform lesions in supernumerary arteries while Cool et al[84] found them distal to dichotomous bifurcations, sometimes ending in aneurysm-like lesions. Jonigk et al[76] used a slightly different approach by imaging/ scanning 30 µm thick tissue slides with fluorescent stainings, and reported the lesions to be adjacent to the pulmonary artery.

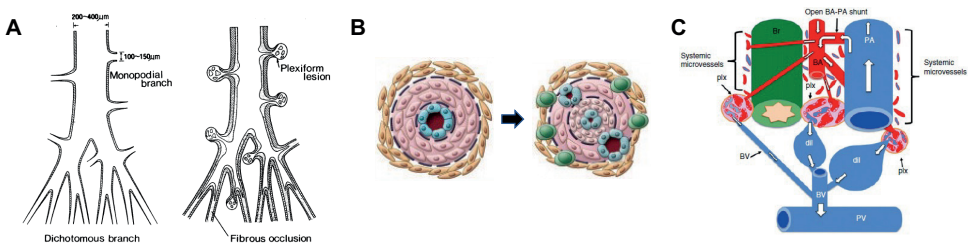

Figure 4.
Examples from published litteratures on schematic depictions of plexiform lesions. **A:** Plexiform lesions were identified primarily in supernumerary arteries (monopodial branches); **B:** occlusive neointima formation leading to endothelial channel formation; **C:** Plexiform lesions develop at sites where systemic microvessels originally reside. References: A: *Reproduced with permission by BMJ. Yaginuma et al, Distribution of arterial lesions and collateral pathways in the pulmonary hypertension of congenital heart disease: a computer aided reconstruction study, Thorax 1990;45:586-590*; B: *Freely available content, Rabinovitch, Molecular pathogenesis of pulmonary arterial hypertension, J Clin Invest. 2012; 122(12):4306-4313*; C: *Adapted with permission of the American Thoracic Society. Copyright © 2022 American Thoracic Society. All rights reserved. Intrapulmonary Bronchopulmonary Anastomoses and Plexiform Lesions in Idiopathic Pulmonary Arterial Hypertension,Galambos, et al, Am J Respir Crit Care Med. 2016 Mar 1;193(5):574-6. The American Journal of Respiratory and Critical Care Medicine is an official journal of the American Thoracic Society. Readers are encouraged to read the entire article for the correct context at [https://doi.org/10.1164/rccm.201507-1508LE]. The authors, editors, and The American Thoracic Society are not responsible for errors or omissions in adaptations.*

The authors had differing results, but perhaps that is telling. Perhaps the plexiform lesion is not homogenous in nature but heterogenous, which could help explain the different findings of not only these three-dimensional reconstructions, but of previous studies as well. Is the plexiform lesion a result of elevated pulmonary pressures or a response to it, is it located in the pulmonary circulation or is it as Galambos and colleagues have suggested in the bronchial microcirculation? Are they hemodynamically important?

Given that current research modalities have so far been unable to answer these questions, perhaps new techniques are required to solve the riddle of the plexiform lesion.

Methods used for projects included in this thesis

I am the camera man

– Solomon Vandy

Histology and immunostaining

Histology and the preparation of tissues

Histology is the study of microanatomy through a microscope, and is fundamental within biological pre-clinical research. Organs, tissues and cells can be visualized and their structure and or function can be assessed. When tissues of disease are studied, the term histopathology is often used.

With few exceptions (most notably live cells in culture) all biological tissue needs to be fixated and often embedded before they can be viewed through the microscope. Fixation is performed to maintain and preserve the structure of tissues and cells, embedding to allow for subsequent sectioning of the tissue. The general preparatory steps are presented below for how human pulmonary tissue is preserved and prepared for histological studies. The process applies for mouse and rat tissue as well, although with their smaller sizes individual lobes of the lung can be embedded intact.

Pulmonary tissue is obtained from a donor, either during transplantation or post mortem during autopsy. For tissue to be retrieved and used for science, an ethical permit has to have been attained. Due to the size of the lobes of the lung, smaller sections or parts are cut, 1-2 cm x 1-2 cm with a depth of a couple of mm. The tissue is placed in plastic cassettes and submerged in formalin or another form of fixative for 24-48 hours. The fixated tissue is then dehydrated by submerging it into increasing concentrations of alcohol, starting at 70% and ending at 99,5 or 100%. The completely dehydrated tissues are then submerged in a clearing agent such as xylene, making the tissue transparent. Furthermore, clearing agents such as xylene are highly miscible with paraffin, which alcohol is not. After this, the tissue cassettes

are placed in a container with warm liquid paraffin. The tissue is then removed from the plastic cassettes and moved to a small, temporary, metal mold and warm liquid paraffin is then poured into the mold completely submerging the tissue again. The mold with the tissue is then cooled. After this, the embedded tissue block can be stored in room temperature for years, for example in a bio-bank. Fixed paraffin-embedded tissues are completely safe to handle, as no living entity (cells, bacteria, viruses, parasites) survive the process.

To study tissue that has been embedded and preserved, small sections have to be cut. These are cut from the tissue block using a microtome that has a precise mechanical advance. Normally, sections are cut to a thickness of between 3 and 5 micrometres (1 micrometre = 0.001 millimetre). These thin sections are then placed on glass slides where they adhere firmly. As a final step, the slides are dried (baked) to remove any remaining water.

The tissue is thereafter ready for histological examination, although before any staining the tissues need to be deparaffinized and rehydrated. Other than general anatomy, there is little else however that can be assessed on an unstained tissue section. Standard stains such as haematoxylin, eosin or alcian blue can be used, highlighting general cellular and extracellular aspects of the tissue. However, when the researcher wants to actively look for specific cells or proteins (or proteoglycans) either immunohistochemical or immunofluorescence stainings have to be performed.

Immunohistochemistry, immunofluorescence and inherent limitations

Both immunohistochemistry and immunofluorescence use antibodies to stain for specific antigens (proteins) presumed or known to be present in the paraffin embedded tissue. The main difference between the methods is how a colour signal of the antigen is obtained. Once a primary antibody has bound to the target antigen, a secondary antibody is applied which binds to the primary antibody. With immunohistochemistry (or chromogenic immunohistochemistry), the secondary antibody is conjugated to an enzyme, such as peroxidase, that can catalyse a colour reaction. With immunofluorescence, the secondary antibody (or a primary antibody) is tagged to a fluorophore that only emits light of specific wavelengths. The benefits with fluorescence are that multiple antigens can be stained at the same time (most commonly up to three separate signals with the addition of a certain nuclear signal). The downside of this method is the short shelf life of the stained tissue: the signal is only usable for a maximum of a few weeks. It is also generally more expensive. Immunohistochemical stains however have a very long shelf life, with the results visible for months, perhaps years, and is relatively cheap. Multiple antigens can be stained, although in its most used form only one antigen is targeted. See figure 5 for representative images of immunohistochemistry and immunofluorescence

respectively. Once a tissue section has been stained, for most intents and purposes it cannot be stained again.

Although these methods are one of the fundamental instruments in pre-clinical science, they have inherent drawbacks. The most notable disadvantages are that once stained the tissue is spent, but also that the tissue can only be studied in two dimensions. This does not necessarily constitute a negative if the region of interest is best presented in a flat plane. However, if three-dimensional structures are to be studied, such as plexiform lesions, the analysis of these will be limited. While studying plexiform lesions, researchers have tried to overcome this limitation by serial sectioning and using computer power to create a virtual three-dimensional volume of the tissue, as stated previously on page 30.

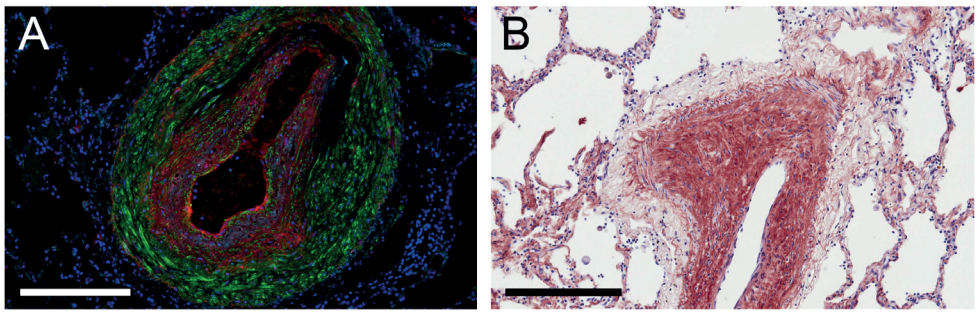

Figure 5.
Two remoddeled pulmonary arteries, from IPAH tissue, of similar size with neointima and media hypertrophy stained using different methods. **A:** Immunofluoresence. Versican = red, Hyaluronan = turquoise, alpha smooth muscle actin = green, DAPI nuclear stain = blue; **B**: Immunohistochemistry. Versican = red, DAPI nuclear stain = blue. Scale bars = 200μm.

With the intent of evaluating if new technology could improve the visual analysis of microscopic tissue, the author and colleagues have evaluated and utilized synchrotron radiation imaging, a resource which to our knowledge had not previously been used within the PH field. This technique is presented under the heading "*Synchrotron radiation micro-CT and the applications for soft tissue imaging"* below on page 37 and is used in all papers included in this thesis.

RNA in situ hybridization

RNA In situ hybridization (RNA ISH) is a method used to identify RNA in a tissue section. It shows where, and in which cells, a specific RNA transcript is produced and thus which genes undergo transcription. The major advantage of this technique is that it enables the researcher to determine how gene expression relates to protein production through combined staining with immunohistochemistry and/ or

immunofluorescence. It is a relatively expensive method however, and it requires fresh tissue (< three months after fixation and embedding).

Animal models

General background on rats and mice in research

Animal models are used to mimic human disease states, with the goal of better understanding disease mechanisms and for tests of experimental treatments. Of the many species used for this purpose in science, mice and rats are the most common. Because of their genetic homologies and similar physiologies, these animals present good models for studying biological processes that have been conserved through evolution.[85, 86] They are different species, each with their advantages and disadvantages as models for experimental science. Mice are smaller, easier and cheaper to maintain and breed, and have incredible resilience to genetic alterations. Rats on the other hand are larger with a physiology more closely resembling humans, with the relative downside that handling and breeding is more complicating and more expensive. Furthermore, for the specific interests of this thesis work, a bronchial circulation exists in rats, but its functional existence in mice is to the author's knowledge still not fully elucidated.[87, 88]

Genetic modifications

As mentioned in the previous paragraph, mice used for research have incredible resilience to genetic alterations. Two different types of genetically modified mice have been used in this thesis work: both modifications have been implemented to alter the production of versican.

One mouse strain was engineered to express only two out of the four common isoforms of versican by lacking isoforms V0 and V2 (see page 23 for information regarding the isoforms of versican). An ER-retention signal (retaining proteins in the endoplasmic reticulum, thus preventing any export from the cell) as well as a translational (the cellular process of building proteins) stop codon was inserted in exon 7 of the versican gene. This prevented versican isoforms containing GAG AR-α to be produced and exported, resulting in a global knockout of isoforms V0 and V2.[48]

The other mouse strain used were engineered to utilize the Cre-Lox recombination system that allows for *inducible* genomic alterations[89], and for our purposes a complete versican knockdown. An inducible knockdown was needed due to versican being essential for the development of the heart (a complete global

knockdown or knockout is lethal for the developing foetus).[90] In short, the system uses an enzyme called Cre recombinase that can alter the genome through strategic editing at loxP sites, the latter determining which gene or exon that is targeted (activated, repressed, removed, altered). The enzyme Cre recombinase can be designed to be activated by certain stimuli, in this case through exposure to tamoxifen (chemical signal). When mice homozygous for versican with loxP sites and tamoxifen-inducible Cre recombinase are exposed to tamoxifen, a global knockdown of all isoforms of versican is attained.

Ethical considerations

In the context of animal models in science, scientists try to maintain the principles of the three r's: replacement, refinement and reduction. In essence, we aim to replace animals for other models whenever it can be done, to refine our methods to get the most accurate results and finally to try and use as few animals as possible while maintaining the integrity of the experiment. Furthermore, before any animal trials can be attempted, ethical permits must be granted.

Animal models of chronic hypoxia

For this thesis work, the author has utilized both mice and rat models in the attempt to answer fundamental pathological questions of pulmonary hypertension. As outlined in the section *Pulmonary vascular anatomy*, page 18, the pulmonary vascular response to hypoxic conditions is vasoconstriction. This response is conserved through evolution and is observed nearly universally among mammals, including mice and rats.[91] The response brings balance between ventilation and perfusion by directing blood from poorly ventilated segments to well ventilated segments. If, however, all airways are hypoxic a global pulmonary vasoconstriction happens which increases the pulmonary vascular resistance (PVR) inducing pulmonary arterial hypertension. The discovery, connecting hypoxia with pulmonary hypertension, was first published by Euler and Liljestrand in 1946 and has since been used as a model for the condition.[92] In short, animals are kept at hypoxic conditions of approximately 10% oxygen (the equivalent of ~5500 m altitude) for three to four weeks. Pulmonary hypertension is verified by in vivo right-heart catheterization by insertion of a pressure sensitive catheter via the internal jugular vein. Animals are anesthetized during the procedure, and during subsequent sacrifice.

The resulting morphological changes in response to chronic hypoxia are very similar across species although the magnitude does depend on the species, sex and developmental stage.[93, 94] The remodelling seen includes muscularization of previously non-muscularized arterial vessels (distal migration and proliferation of VSMCs and/ or differentiation of pericytes), medial thickening (accumulation and

hypertrophy of VSMCs and deposition of extracellular matrix proteins) of muscular and elastic vessels and right ventricular hypertrophy.[95, 96] The degree of morphological changes are found to be more pronounced in rats compared to mice.[96] Endothelial cell proliferation or other types of lumen occlusive lesions are however not found following chronic hypoxia in either species. Both models (species) have their strengths and weaknesses. Below are summaries of the two models used for this thesis work.

Murine chronic hypoxia model

Although the vascular remodelling in general is not as pronounced in mice as in rats, the possibility of genetic modifications that the mouse model allows for is a great resource. With this model, we aimed to study the effects of versican on the development of pulmonary hypertension. Two separate genetically modified mice were used, as outlined under *Genetic modifications* on page 34. One model was deficient in versican isoforms V0 and V2 while the other model was deficient in all isoforms. The main strength is that mice allow for this type of genetic alteration: the possibility to evaluate what effect a certain gene or protein has on disease progression. The main disadvantage of this model is that it does not reproduce human like vascular occlusive lesions. Although medial hypertrophy can to some extent affect lumen diameter, it is mainly the development of neointima that causes lumen occlusion. Another weakness of this model is that it is a representation of a vasoconstrictive phenotype of pulmonary hypertension (not a PAH specific model).

Sugen5416/Hypoxia model

Sugen5416 is a receptor tyrosine kinase inhibitor which blocks vascular endothelial growth factor receptor 2 (VEGFR2). When it is administered in conjunction with hypoxia in rats, human like intimal lesions develop albeit without the presence of plexiform lesions.[78] Although an exaggerated pulmonary hypertension is seen in mice exposed to the same regiment, intimal lesions do not develop.[97] As such, this has been the dominant rat model of pulmonary hypertension for close to two decades. The rationale is that the inhibition of VEGFR2 with hypoxia causes endothelial cell death, which selects for apoptosis resistant cells with a pathological proliferation as described on page 29. This is a two-hit model as administration of Sugen5416 alone does not cause this reaction. The major drawback has been that it does not generate plexiform lesions. Within the last decade it has been discovered however that if time until sacrifice, post hypoxia, is extended for many weeks plexiform lesions are created.[98]

Synchrotron radiation micro-CT and the applications for soft tissue imaging

Computed tomography

The major limitation with histology is that it limits the researcher to a two-dimensional analysis. There are within clinical medicine and other fields of research techniques that allow for three-dimensional (3D) analysis, such as computed tomography (CT). Through the use of an X-ray tube with detectors, placed in a rotating gantry, a three-dimensional view of the organ or tissue of interest is generated by tomographic reconstruction. The technique utilizes the fact that X-rays are attenuated when passing through a medium (tissue). Different tissues have different attenuation coefficients, which in turn generates contrast. Soft tissues such as the liver, spleen and lungs however have low and homogenous attenuation, with poor contrast as a result. To overcome this, a contrast agent is often given into the patients' blood stream. The main advantage of a CT-scan compared to a standard X-ray scan is that a 3D image is generated: an area or organ of interest is not hidden, concealed or otherwise affected by superimposed or nonconcurrent tissue and its 3D shape can be fully visualized. However, due to how CT-scanners are designed, they can only visualize macroscopic structures (> millimetre in diameter), and that at a resolution that is insufficient for many pre-clinical researchers.[99] There are micro-CT scanners that can distinguish structures at a micrometre level, which have been used for non-destructive (compared with standard histological studies) 3D imaging of pulmonary tissue for the last decade.[100, 101] Generating sufficient contrast from soft tissue has continued to pose a problem however, which is why attempts have been made of using contrast-enhancing agents that provide usable data; this however exempts tissue already embedded and stored in biobanks.[102, 103] In a review article on micro-CT imaging of the lungs and pulmonary vascular system by Ritman in 2005 this problem is recognized, and he proposes that the use of phase-shift could be the solution. The technology needed was however not available at the time.[104]

Synchrotron radiation and the use of phase contrast

X-rays passing through a medium are not only attenuated but undergo a phase-shift as well (figure 6), and this phenomenon can be used for phase-contrast imaging. The X-rays need to be of certain quality however, which are typically only available at synchrotron sources.

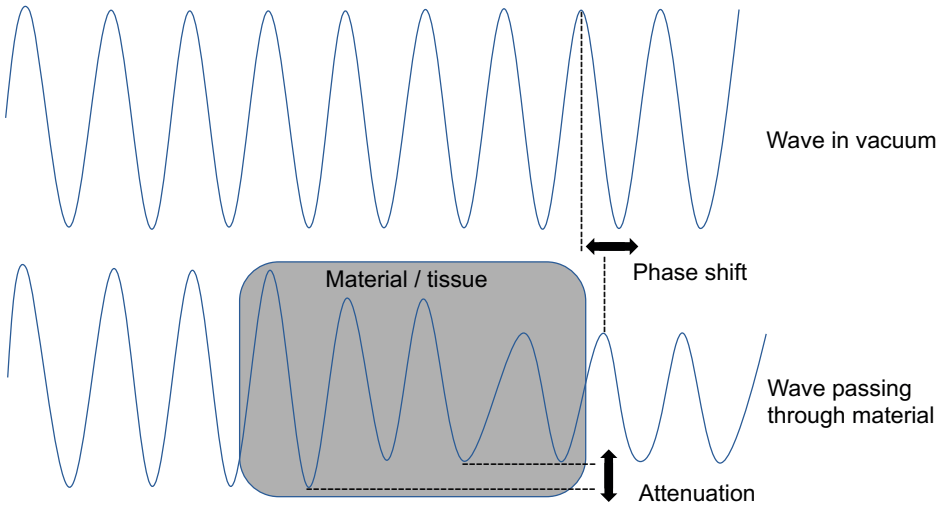

Figure 6.
Comparison of an X-ray traveling in a vacuum and through a material. In the latter, the waves intensity is attenuated and it undergoes a phase shift.

A synchrotron source is by and large a particle accelerator that generates high energy X-rays used for scientific purposes.[105] Electrons are kept circulating at relativistic speeds in a storage ring, with powerful bending magnets strategically placed to keep the electrons in a closed orbit. The electrons are then manipulated in such a way that X-rays are produced that have very high brilliance and high coherence. The X-ray emission, or beam, is then captured at a beamline, which is the experimental end station that conditions the beam according to the particular experiment or imaging that are to be performed there (Figure 7).

With the construction of MAX IV, which is a new synchrotron facility in Lund, Sweden, meetings were held in order to promote international scientific communication and to exchange ideas. At those meetings, physicists from Lund University and well as from the Swiss Light Source synchrotron at Paul Scherrer Institute in Villigen, Swizerland, presented data where they had successfully imaged mouse lung at the alveolar scale, with high resolution and clear contrast, non-destructively in 3D.[106, 107] Those meetings became the start of fruitful collaborations between our group and radiation physicists with the aim of imaging pulmonary vascular remodelling, in human tissue and animal models, in 3D.

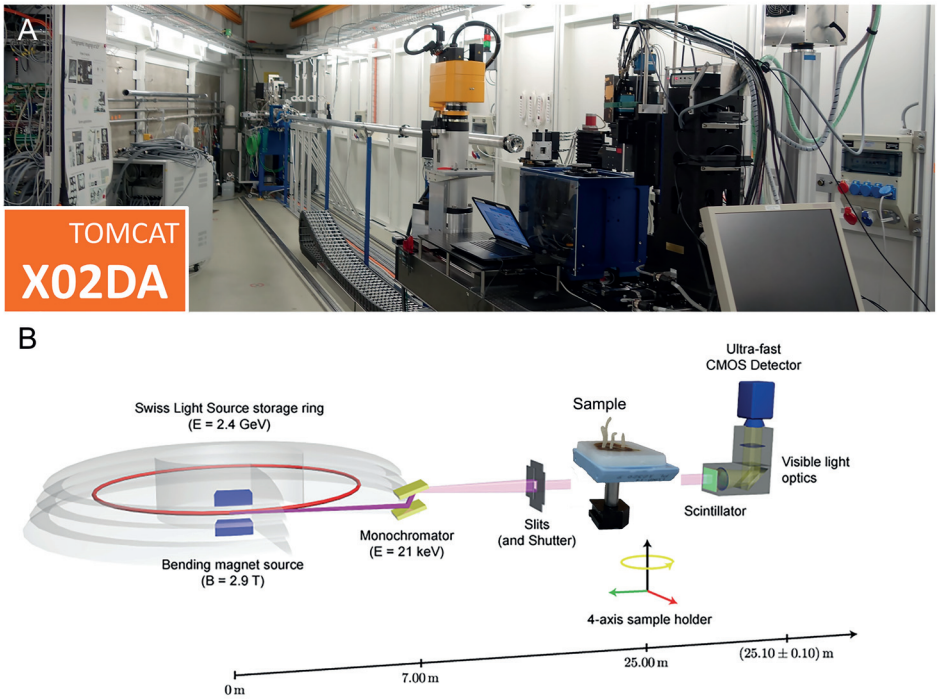

Figure 7.
TOMCAT beamline at the Swiss Light Source, Paul Scherrer Institute, Villigen, Swizerland
A: Image of the experimental hutch at the TOMCAT beamline; **B**: Schematic illustration of the synchrotron and beamline. The sample is placed on a rotating sample holder. In the schematic a photography of a paraffin-embedded pulmonary tissue sample is shown. On the sample, small shapes of wax were placed in order to keep an area of interest central through sample rotation during image acquisition.

Aims of the thesis

The initial title of this PhD thesis was *The role of versican in pulmonary arterial hypertension.* With current PAH therapy having unsatisfactory effects, being focused primarily on vasodilation, pre-clinical research in general is focused on understanding the vascular remodelling process. As versican has been found abundant in the ECM of vascular lesions, including plexiform lesions, its functional role in this setting warranted investigation. With the possibility of exploring a novel imaging technique allowing for potential 3D visualisation of paraffin-embedded pulmonary tissue the focus of the thesis was slightly altered.

As such, the thesis title was changed to *3D visualization of vascular lesions and the role of versican in pulmonary arterial hypertension*. The aim was now two-fold, in no particular order of importance. On the one hand to evaluate if this novel imaging technique could be useful in the study of pulmonary tissue and possibly aid in the understanding of pulmonary vascular lesions. On the other hand to further investigate versican in human vascular lesions of PAH, and to evaluate the effect versican has on murine experimental pulmonary hypertension.

Specific aims included:

Paper I

To investigate whether synchrotron-based phase-contrast micro-CT could be used to study paraffin-embedded pulmonary tissue and to image the vascular pathology of alveolar capillary dysplasia with misalignment of pulmonary veins.

Paper II

To use synchrotron imaging to characterize the plexiform lesions in PAH

Paper III (research letter)

To use synchrotron imaging to determine if the prolonged Sugen5416/hypoxia rat model generates human-like plexiform lesions

Paper IV

To further characterize versican accumulation and turnover in vascular lesions of PAH

Paper V

To investigate what functional effect versican has on the development of pulmonary hypertension using the murine hypoxia model

Results and discussion

Paper I

Synchrotron-based phase-contrast micro-CT as a tool for understanding pulmonary vascular pathobiology and the 3-D microanatomy of alveolar capillary dysplasia

Here, the aim was to evaluate whether synchrotron-based phase-contrast micro-CT (SPμCT) could be a useful tool for structural assessment of paraffin-embedded pulmonary tissue. The tissue in question was from a rare form of pulmonary hypertension called alveolar capillary dysplasia with misaligned pulmonary veins (ACD/MPV) along with healthy control tissues.

From the section *Pathogenesis of PAH and plexiform lesions* on page 26 the reader will have understood that standard histological techniques have not been able to answer all questions raised by researchers regarding microanatomy in general and vascular anatomy in particular. Smaller vessels do not traverse tissues in a straight line, and in most cases only a very limited part of a vascular lesion can be studied in a 3-5 micrometre-thin two-dimensional tissue section. The author and the research group to which he belongs have encountered this problem, the same way as most researchers in this field have. When Lovric and colleagues showed that they could successfully image murine lung with good contrast, an opportunity presented itself.[107] Could human lung be imaged in the same way? One possible problem was evident however: they had imaged non-paraffin-embedded pulmonary tissue, whereas practically all tissue stored for research is paraffin-embedded. Would the paraffin cause any interference or affect contrast?

We had already established a research collaboration with a paediatric pulmonary pathologist, Professor Csaba Galambos, who studies pulmonary vascular disease with focus on intrapulmonary shunting. He had at the time recently published results, based on computer-assisted reconstruction of serial tissue sections, which suggested involvement of the bronchial circulation within lesions of PAH (see Figure 4C).[36] Professor Galambos had also used this technique to decipher a histological mischaracterization of ACD/MPV.[108] He argued that the misaligned pulmonary veins were in fact not pulmonary veins, but dilated bronchial veins. As a tool to study this, he had injected a green tissue marking dye, used routinely by

pathologists, into the pulmonary artery to help with histological assessment of possible intrapulmonary shunts.

This project was a joint venture where our group collaborated with the physicists and engineers at the TOMCAT beamline of the Swiss Light Source (where the initial imaging of the mouse lung had been performed), radiation physicists from Lund University and pathologists (adult and paediatric). Our aim was to investigate if this technique could image paraffin-embedded pulmonary tissue of health and disease, and if possible shed further light on the anatomical questions raised regarding the syndrome ACD/MPV.

SPμCT imaging of paraffin-embedded tissue worked even better than expected. It was possible to study pulmonary histology in 3D, without any interference from the paraffin (figure 8). Furthermore, the tissue proved unaffected by the irradiation. Subsequent sectioning and histochemical staining of the irradiated tissue was unaltered when compared to non-irradiated tissue. The 3D structures of vascular lesions or areas of interest could be matched with histology and immunohistochemistry. Secondly, we could add valuable information on ACD/MPV. In the limited amount of tissue studied, we found numerous arterial anastomoses between the pulmonary arteries and the bronchial circulation, thus proving their existence in the disease. We could also trace the so called misaligned pulmonary veins and demonstrate that they are in fact dilated bronchial veins, as previously suggested.[108]

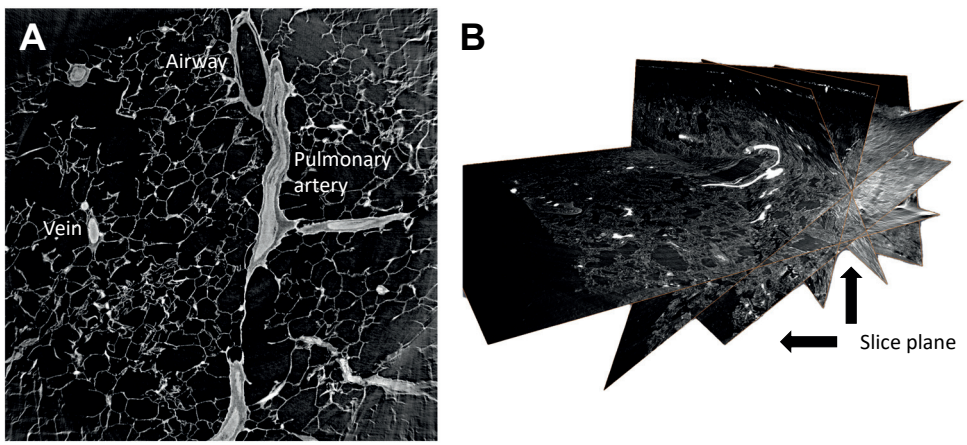

Figure 8.
Histology by SPμCT. **A**: Representative image from healthy lung showing the resolution and contrast. Airway, pulmonary artery, vein and parenchyma clearly visible; **B**: Example of how virtual sections can be used to vizualise the course of a vessel or structure of a lesion in 3D.

Finally, we discovered by chance that the green dye, used routinely by pathologists, is highly attenuating. The high attenuation is seen as white in the CT volumes and this allowed for automatic segmentation of the dye filled vessels and 3D rendering

of the complete vascular tree (within the imaged volume) by image analysis software, without affecting the visualization of surrounding tissue to any degree. The vasculature could be rendered without the dye as well, although this was then a semi-manual effort.

Comparison of the digital 3D images of the tissue with standard histology proved that image/data capture and processing, including the generation of contrast through phase-shift algorithms, gave an accurate representation of reality. In other words, the imaging showed the tissue as it is, without confounding artifacts or distortions.

That arterial anastomoses between the pulmonary circulation and the bronchial circulation were found and could be visualized was exciting and opened new possibilities. It added valuable information to the debate on the existence of intrapulmonary shunts in PAH (their existence in adult PAH had been suggested, but was not yet proven) and it could also explain some on the hypoxia suffered by the affected patients. Previously, only the dysplastic alveolar capillaries had been understood to cause the hypoxia. With open anastomoses between the circulations, deoxygenated blood will be shunted from right to left due to the supra-systemic pulmonary arterial pressure before it has been oxygenated. To what extent this contributes to the overall hypoxia we could not estimate however. That we could identify the misaligned pulmonary veins as dilated bronchial veins is a strength of this technique. It had been suggested previously, but we could now prove it definitively. The reason for the anatomical mischaracterisation possibly lies in the fact that bronchial veins should not be visible to this degree due to the limited flow in the bronchial circulation in a healthy state (see section *The bronchial circulation* on page 20). As such, the veins have probably been assumed pulmonary. However, with the knowledge that blood is shunted from pulmonary arteries to the bronchial microcirculation, it follows that a larger volume of blood must be carried by the bronchial veins, causing them to distend and giving the disease this characteristic histological pattern.

Paper II

Distinct types of plexiform lesions identified by synchrotron-based phase-contrast micro-CT

With the potential of virtual histology granted by SPμCT, our aim with this project was to use this technique to visualize plexiform lesions in 3D. As the reader will have understood from the section *Pathogenesis of PAH and plexiform lesions* on page 26, plexiform lesions are incompletely understood. There is uncertainty, or a

differing of opinion, regarding their nature (which lesions are plexiform), and their positioning within the pulmonary vascular tree vis á vis the bronchial (systemic) circulation.

For this paper, paraffin-embedded tissue from 14 patients with PAH was collected. All patients had clinical characteristics and hemodynamic profiles consistent with PAH. 13 of these were diagnosed with IPAH (idiopathic) and one patient with a known BMPR2 mutation (a type of familial PAH with a penetrance of ~20%).[109, 110] Healthy pulmonary tissue was used as control. The reason for including tissue from the BMPR2 mutation carrier was that the pulmonary arteries in this tissue had been injected with the green dye found to be radiopaque in paper I. Previous publications had also shown that BMPR2 carriers had no discernable differences in vascular lesion density or morphology compared to IPAH.[76, 111]

For this study, any vascular structure, connected to a pulmonary artery, with at least two channels for blood to pass was identified as plexiform. Primary identification of plexiform lesions was based solely on the IPAH tissue; dye-injected BMPR2 tissue was used for visualization of selected lesion types. Three patients were excluded from the IPAH cohort because of comorbidities or otherwise confounding historical clinical data.

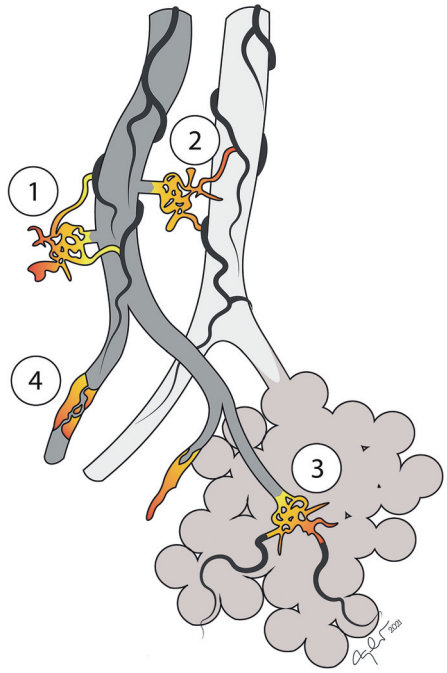

Figure 9.
Four types of plexiform lesions.
The following definitions of the types are from the published article.[1] Type 1: in monopodial branches, often with a connection to the vasa vasorum. Type 2: between pulmonary arteries and airways as a tortuous transformation of intrapulmonary bronchopulmonary anastomoses (IBA), connecting the pulmonary artery with peribronchiolar vessels. Type 3: at unexpected abrupt ends of distal pulmonary arteries/arterioles … as spherical structures that terminated the vessel. Type 4: completely occluded pulmonary arteries with recanalization, or incomplete blockage (any occluded pulmonary artery with at least two lumens were identified as type 4). Figure originally published in: van der Have O, Westöö C, Ahrné F, et al. Shunt-type plexiform lesions identified in the Sugen5416/hypoxia rat model of pulmonary arterial hypertension using synchrotron-based phase- contrast micro-CT. Reproduced with permission of the ERS. Eur Respir J 2022; 59: 2102802 [DOI: 10.1183/13993003.02802-2021].

In total, 108 plexiform lesions were identified in the IPAH tissue that all fit the definition. None were observed in control tissue. Although the lesions demonstrated heterogeneity, specific characteristics and similarities became apparent during the analysis. We found that all identified lesions could be subdivided into four specific subgroups or types, defined and visualized in figure 9.

With these results we could show that previous publications where plexiform lesions were found either at different localizations, bifurcations or in supernumerary arteries to name two examples, were not contradictory but that both were correct (figure 10). Plexiform lesions are heterogenous. We could also show that some plexiform lesions do act as shunts or anastomoses between the pulmonary and systemic circulations and that these connections appear common, thus definitively answering that debated question as well.

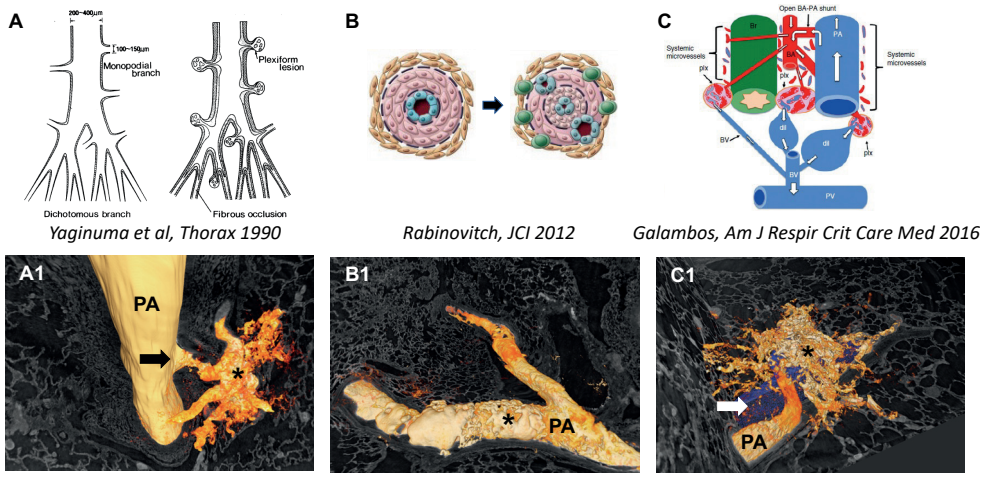

Figure 10.

SPµCT imaged 3D rendered plexiform lesions (**A1**, **B1**, **C1**) compared to the previously published schematic drawings (**A**, **B**, **C**) of plexiform lesions shown previously in Figure 4. Vascular dye is seen in yellow/orange. Asterisk marks plexiform lesion, PA indikates pulmonary artery. **A** and **A1**: Type 1 plexiform lesion in monopodial branch/ supernumerary artery (black arrow); **B** and **B1**: Type 4 plexiform lesion with endothelial channels within the original lumen; **C** and **C1**: Type 2 plexiform lesion between the pulmonary and bronchial circulation as a tortuous transformation of an intrapulmonary bronchopulmonary anastomose. Airway in blue is indicated by the white arrow. *See Figure 4 legend, page 30, for references and permissions regarding A, B, C.*

The results furthermore gave indications on the hemodynamic effect these lesions might have. Types 1 and 2 potentially relieve pressure as the pulmonary arteries were invariably occluded distally. Types 1 through 3 were in addition often surrounded by thin-walled vessels that were seen to connect to peri-bronchial vessels, to the vasa vasorum and to pulmonary veins. Collaterals bypassing type 4 lesions were often observed. In summary, we found ample evidence of shunt-like vessels originating from plexiform lesions. Based on these results, we inferred that neointimal thickening, type 4 lesions and complete vascular occlusions increase the pulmonary vascular resistance which in turn generate type 1 and 2 lesions proximally / upstream.

The nature of type 3 lesions is still unclear. The type was unevenly distributed in the cohort, with almost half of total lesions identified in one patient, thus making

well founded argumentation on its nature or role difficult. However, its placement in the vascular tree is interesting. In a paper from 1972, K. K. Pump found what he believed to be anastomoses of pre-capillary bronchopulmonary and pulmonary arterial vessels, a location which would fit with our results.[27] Another possibility is that they originate from remodeled capillary networks, as it has been demonstrated that small pulmonary arteries can abruptly end into capillary networks.[18] In the paper we argue that for further study of type 3 lesions, a larger cohort, preferably dye injected, is needed along with imaging at a higher magnification.

A key challenge with this project was how to identify a lesion as plexiform. All previous definitions or descriptions have been based on 2D histology, and we knew from our preliminary 3D data that the appearance of a plexiform lesion changes considerably when the viewing angle is changed. We chose a broad definition that would invariably include lesions previously defined as angiomatoid, cavernous as well as obstructive to name a few. However, as identification and separation of these lesions from each other have been difficult, as made apparent by published literature, this was not considered negative. Perhaps a broad definition that is easy to implement is what is needed for further studies of these lesions.

A major limitation of this study was the small cohort of 11 patients, and future studies are needed to corroborate these findings and this classification. Another limitation is that we could only infer, and not substantiate, the temporal aspects of lesion formation, as well as their hemodynamical impact. Due to the nature of the disease, it is near to impossible to collect human biopsies over time. As such, an animal model that reliably produce human-like plexiform lesions would be needed.

Paper III (research letter)

Shunt-type plexiform lesions identified in the Sugen5416/hypoxia rat model of pulmonary arterial hypertension using synchrotron-based phase-contrast micro-CT

The Sugen5416/hypoxia rat model is a well-documented model of pulmonary arterial hypertension. When time until sacrifice, following hypoxia, has been extended it has been shown to produce plexiform lesions, both within the pulmonary artery and outside the vessel lumen as aneurysms.[98, 112] Recently, a publication on this model showed that the extra luminary lesions form in supernumerary arteries and furthermore that the lesions observed in this model do not communicate with the bronchial circulation.[113] 2D histology was used for the analysis. The aneurysm-like plexiform lesions described from this model appeared similar to what we had described in human tissue (Paper II). Given this, and the proven potential of SPμCT,

our aim was to use our imaging protocol to further characterize the plexiform lesions in this animal model.

The project was a collaboration with colleagues at Stanford University, USA, who had the animal model established in their lab. Radiopaque green dye was used to better visualize the pulmonary circulation. Imaging was performed using the same protocol as in papers I and II.

Results showed that this model creates vascular pathology similar to human PAH. We identified all four types of plexiform lesions previously described in human PAH (paper II). We could demonstrate that there were patent arterial anastomoses between the pulmonary and bronchial circulation in type 1 and 2 lesions. Vascular occlusions, including type 4 lesions, were also observed. In general, the vascular occlusive pathologies were observed more distal in the vascular tree compared to human PAH.

In summary, we could demonstrate that the prolonged Sugen5416/hypoxia model produces vascular pathology, including shunt-type plexiform lesions, similar to both idiopathic and familial PAH. To our knowledge, this is the first time in this field an animal model has shown to do so. These results further highlight the strength of SPμCT imaging, as the technique reveals results otherwise hidden with standard histological methods.

Given this knowledge, there are potential great benefits to be gained from this animal model. Now, the temporal aspects of lesion formation of PAH can be studied as well as the hemodynamic roles of the different lesion types. With this model, the vascular pathology of PAH could perhaps be understood, which could bring us closer to developing new treatments for this disease.

Paper IV

Positioning, enzymatic processing and binding partners of versican in vascular lesions of pulmonary arterial hypertension

We have previously found that versican accumulates in lesions of PAH, in hypertrophied media, neointima and in plexiform lesions.[114] Versican is a large and complex proteoglycan, which can exhibit different functions and exert different effects depending on which isoform is present and to which other proteins or molecules it binds. Versican is also known to be degraded by ADAMTS-enzymes, and products of degradation are known to be biologically active. The aim of this project was to further investigate the positioning and role of versican in remodeled vessels of PAH, by identifying specific isoforms, products of enzymatic digestion and binding partners.

Tissue from ten IPAH patients were used. Paraffin-blocks were imaged by SPμCT and subsequently sectioned and analysed by immunohistochemistry and in situ hybridization. Patient blood was collected from a biobank and enzyme-linked immunosorbent assay (ELISA) was used to measure plasma concentration of versican G3.

RNA in situ hybridization showed that versican is produced locally within the hypertrophied media, neointima and in plexiform lesions: both GAG AR-α and -β were produced and deposited. From the expression pattern, we deduced that both isoforms V1 and V0 were present. Double-staining for different parts of the versican core protein found the G3-domain located closer to the endothelium compared with G1-domain, indicating cleavage of the core protein. Presence of the Neoepitopes DPEAAE and ARRGQF (see figure 2, page 24) were identified, confirming ADAMTS-mediated cleavage. Interestingly, DPEAAE consistently stained the neointima and endothelium, while ARRGQF invariably gave a circular staining pattern close to the media: separation of cleavage products was apparent. Abundant hyaluronan was found in the neointima of all examined lesions, strongly colocalizing with DPEAAE. Furthermore, hyaluronan consistently colocalized with versican G3, indicating presence of non-cleaved versican core protein (hyaluronan binds the G1-domain). Tenascin-C (TN-C) is a glycoprotein and a high-affinity ligand of the versican G3 domain that has previously been found elevated in serum of PAH patients.[115, 116] In this study, we found that it co-localized with versican G3 consistently in the neointima and stained the endothelium of some, but not all, lesions. Interestingly, TN-C consistently stained the endothelium of lesion associated thin-walled vessels and collaterals.

Further cleavage of versican was indicated by the distinct separation of the G3-domain (neointima and endothelium) and neoepitope ARRGQF (media adjacent). With this finding, we hypothesized that proteolytic fragments of the C-terminal end with the G3-domain might be released into the circulation. Plasma samples were collected from the Lund Cardio Pulmonary Registry, from a cohort chosen to resemble the existing tissue cohort as far as possible regarding clinical data. ELISA was run in triplicates on patient and control plasma and revealed significantly increased levels of Versican G3 in patient samples.

Results showed that both versican and hyaluronan are abundant in PAH lesions, and it is reasonable to assume that they are important for lesion development. There is no existing drug that targets versican. There is however an available drug that inhibits the synthesis of hyaluronan.[117] If hyaluronan is shown to accumulate in the same fashion in animal models of PAH, perhaps its role in lesion formation could be deduced from its inhibition. As previously mentioned, TN-C has been found elevated in patient plasma. Our results showed that lesion-associated thin-walled vessels consistently stained positive for TN-C. It is possible then that when elevated levels of TN-C are detected, a manifest vasculopathy with collateral vessels have

already been created. We found elevated levels of versican G3 in patient plasma. Perhaps elevated levels of G3 could be an earlier indicator of vascular remodeling?

We sampled plasma from a cohort matching our existing tissue cohort to the extent that it was possible. However, clinical data show that the plasma cohort was markedly healthier with greater exertional endurance, lower WHO functional class and lower PVR to name a few examples. As such, we believe this strengthens our findings regarding versican G3 as a potential novel biomarker for PAH. For validation, a larger cohort is needed as well as samples from other diseases with similar clinical presentations such as heart failure and idiopathic pulmonary fibrosis.

Paper V

Versican-deficiency does not affect murine hypoxia-induced pulmonary hypertension but versican accumulates in neointima following house dust mite exposure

We have previously demonstrated the extensive and abundant deposition and accumulation of versican in lesions of PAH. From previous literature, it has been shown that versican is central for VSMC proliferation in vitro.[61] However, we have not been able to demonstrate or otherwise clearly infer based on experimental evidence what role versican holds in the development of PAH. As such, the aim with this project was to experimentally examine what effects versican have on the development of PAH, using the murine model of chronic hypoxia.

In **Paper IV,** production and deposition of both GAG AR-α and -β containing isoforms was found in vascular lesions. In a whole exome sequencing of 12 IPAH patients, de Jesus Perez and colleagues found that the gene coding for versican (VCAN) was among the genes with the highest frequency of single nucleotide variations (mutations).[51] Out of these, six were located in the two exons coding for GAG attachment regions; two in exon 7 (coding for GAG AR-α) and four in exon 8 (coding for GAG AR-β). Detrimental effects of these mutations would affect production and/ or function of full-length versican (isoform V0), and either isoforms V2 or V1 depending on the exonal location. To examine the functional role of full-length versican (V0) in disease development, we used an exon 7 targeted mutant mouse strain deficient in GAG AR-α containing isoforms V0 and V2 ($V0/V2^{-/-}$).[48, 118] Following 4 weeks of chronic hypoxia exposure, mice developed pulmonary hypertension with significantly increased right-ventricular systolic pressures (RVSP) and right-ventricular hypertrophy (RVH), compared to normoxic controls. Muscularization of normally non-muscularized distal arteries (10-50 um) is the main vascular change leading to increased PVR in murine hypoxia-induced PH.

Here, coverage of positive alpha smooth muscle actin (α-SMA) signal in distal arteries was quantified to estimate the degree of distal arterial muscularization. We hypothesized that a lack of full-length versican (V0) would ameliorate PH by reducing distal migration and proliferation of VSMCs. However, no differences in distal arterial muscularization, RVSP or degree of RVH between V0/V2$^{-/-}$ and controls were observed. Positive signal for versican G3 was preserved in V0/V2$^{-/-}$ lungs, which most likely represents versican V1 as translation of exon 8 was uncompromised. Staining pattern for versican G3 was comparable to that in normoxic control lungs, and was unaffected following hypoxia. As such, we hypothesized that a knockdown of versican isoform V1 expression, in addition to V0, would result in altered VSMC migration and proliferation and thereby hinder distal arterial muscularization.

Due to the fact that versican is essential for embryonal development, as discussed on page 34, constitutive global knockout of all isoforms is not viable. In a consecutive trial, we utilized a tamoxifen-inducible Cre-loxP system targeting exon 4 which results in global knockdown of all versican isoforms (V0-3$^{-/-}$).[119] Knockdown was induced two weeks prior to experimental start point and pulmonary hypertension was induced by 4 weeks of chronic hypoxia exposure. Successful exon 4 deletion was verified using PCR (polymerase chain reaction). However, again, no differences in staining pattern for α-SMA or degree of distal arterial muscularization, RVSP or degree of RVH were observed for knockdown mice, compared to controls. And again, we found no discernible differences in α-SMA coverage or for versican G3 in V0-3$^{-/-}$ lungs compared to controls.

In conclusion, deficiency in both V0 and V1 did not affect distal arterial muscularization and development of pulmonary hypertension in mice following 4 weeks of chronic hypoxia exposure. Preserved positive signal for versican G3 in V0-3$^{-/-}$ lungs was expected considering that versican turnover is a slow process and therefore likely represents versican deposited prior to knockdown. Also, although exon 4 deletion was confirmed in all animals that received tamoxifen, cell to cell variations in Cre recombinase activity most likely result in preserved versican production in some cells. Hence, this partially preserved translation of versican isoforms in V0-3$^{-/-}$ mice and uncompromised translation of versican isoform V1 in V0/V2$^{-/-}$ mice could be sufficient for continued VSMC migration and explain the absent hypothesized effect on distal arterial muscularization, RVSP and RVH. It could also be that versican has no functional role in the non-occlusive vascular changes (distal arterial muscularization) induced in the murine chronic hypoxia model, which is supported by the fact that no significant accumulation of versican was observed in/around muscularized distal arteries. We have previously shown that versican accumulates in vascular lesions of PAH,[114] and in project IV that both GAG AR-α and -β containing isoforms are produced and deposited. Therefore, we argue that it is rather in settings of lumen-occlusive pulmonary vascular remodeling characterized by resident VSMC proliferation, hypertrophy and ECM expansion, as

seen in human PAH (demonstrated in **paper IV**), where versican might play a crucial role. If true, this further adds to previous concerns raised in the literature regarding the human translatability of the murine chronic hypoxia model of PH, especially when structural changes in ECM are investigated.

Endeavors to induce more human-like pulmonary vascular remodeling in mice to improve translatability have been many and with varying degrees of success. In a recent publication Steffes et al showed the house dust mite model (HDM) of asthmatic disease as a promising murine model of PH.[120] Mice developed severe lumen-occlusive vascular remodeling with medial thickening and neointima formation leading to increased RVSP and RVH. Here, SPμCT imaging of paraffin-embedded lung tissue from mice exposed to HDM (control, 4-, 5- and 6-week exposure) showed a gradual (temporal) occlusion of the pulmonary arteries. Immunofluorescence of lungs from mice exposed for six weeks showed widespread severe remodeling of pulmonary arteries with medial thickening, neointima formation and complete occlusions. Stainings for versican G3 revealed significant accumulation in hypertrophied media and neointima, similar to previous observations in human PAH presented in **paper IV**. However, whether this accumulation of versican plays a functional role in lumen-occlusive vascular remodeling remains unanswered and a next step would be to examine this in the HDM model of pulmonary hypertension.

Conclusions and future perspectives

With this thesis I have studied novel imaging techniques of the pulmonary microanatomy and further explored the role of versican in lesions of PAH. From the results that I have obtained, there are a number of conclusions to be drawn that will be discussed below. The order in which I have discussed these should not infer inherent value of one result or conclusion over another, as I leave the judgment of scientific significance to the individual researchers' volition.

SPμCT imaging has been found to be an excellent resource for studies of pulmonary microanatomy. The technique has revealed information previously hidden from researchers, which is exemplified by the discovery of the heterogenous nature of the plexiform lesion, and in particular that these lesions could be visualized in the Sugen5416 hypoxia rat model (Paper III). In the latter example, previous researchers (due to technical limitations) had not been able to identify shunt-type plexiform lesions and thus concluded that their results, in accordance with earlier results and/ or publications, showed that plexiform lesions do not communicate with the bronchial circulation. This example attests to the potential of SPμCT. The main limitation with SPμCT is the relative scarcity of synchrotrons and optimized beamlines. Furthermore, beamtime (getting allocated imaging time) have to be applied for (similar to a research grant) and comes with travel expenses. However, individual scans are quick (order of minutes) and an amazing amount of data can be obtained from a single beamtime. The technique has many potentials, and results from this thesis work will hopefully lead other researchers to use SPμCT as well. Perhaps future advancements in imaging will allow for laboratory-based micro-CT scanners to be improved to such a degree that they could be a viable alternative.

With this thesis the plexiform lesion has been found to be heterogenous, and this is an important finding. In all likelihood, the different types of plexiform lesions have differing hemodynamic impacts, and this highlights the need for a degree of specificity when the lesion is discussed or researched. For example, a type 4 lesion is in its nature different from a type 2 lesion: different in positioning, vascular connectivity and are in all likelihood caused by differing pathologic processes. Hopefully the scientific community will adopt this subgrouping of plexiform lesions, since a commonly held and applied definition benefits research.

We did not identify any functional effect of versican in the knockout and knockdown murine models of pulmonary hypertension. It is possible that these

results reflect reality, as previously argued, however we still believe that versican has a deleterious effect in PAH. We have concluded that the inconclusive results are most likely the consequence of the study design: the murine model of chronic hypoxia doesn't accurately represent human PAH and as such should be used with caution, especially when the study focuses on the ECM. That this model has its limitations is not novel, it has been discussed previously in both review and research articles, and the results presented in this thesis reinforces this. The prolonged sugen5416/hypoxia is in my opinion a better candidate, and future studies will determine if the HDM mouse model can be of scientific use for PAH studies as well.

We did detect significant turnover of versican in lesions of PAH, a phenomenon that warrants further examination. This turnover led us to measure patient plasma for a versican G3 containing fragment, which we found to be significantly increased. This is a potential biomarker, but it needs further validation. Firstly, the analysis needs to be replicated in a larger cohort. Secondly, the analysis needs to be performed on plasma from other pulmonary and/ or vascular diseases as well. As of now, we cannot know it elevated versican G3 levels are specific or not for pulmonary vascular remodelling. If, however, the analysis would prove unable to differentiate between different pulmonary diseases, or diseases generating vascular remodelling in general, it could still prove clinically useful. As stated in *clinical presentation and diagnosis* on page 16, PAH is invariably linked with significant patient and doctors' delay simply due to the diffuse nature of the symptoms, and a biomarker could significantly reduce the doctors' delay. A positive titre would strongly indicate vascular pathology, and since the typical patient is a young woman one of the first diseases a physician would want to exclude would be lung related, as systemic arteriopathy are usually linked with older age groups.

In summary, I believe that this thesis work has added valuable results to the PAH research field. I hope that these findings will be the stepping stones for future researchers aiming, like I have done, to untangle the mystery that is PAH. Lastly it is my sincere hope that this thesis work will, somewhere down the line, result in real clinical implications for the individual patient.

Populärvetenskaplig sammanfattning

Pulmonell hypertension är ett tillstånd med förhöjt blodtryck i lungkärlen. Kroppen har två separata blodomlopp som utgår från var sin del av hjärtat: arteriella systemkärl som försörjer kroppen med syrerikt blod utgår från vänsterhjärtat, och från högerhjärtat utgår arteriella lungkärl med syrefattigt blod som skall syresättas i lungorna. Det finns flera orsaker till pulmonell hypertension, varför syndromet är indelat i fem olika grupper. Generellt sett så behandlar man syndromet genom att angripa grundorsaken till sjukdomen, som kan vara vänstersidig hjärtsvikt eller specifika koagulationsrubbningar. För undergrupp 1, som kallas för pulmonell arteriell hypertension (PAH), sitter huvudproblemet i artärerna i lungan och det är mycket svårbehandlat. I dagsläget finns ingen botande behandling, och de drabbade patienterna har ett stort lidande och sjukdomen leder till en alltför tidig död. Varför blodtrycket stiger i lungkärlen vid så kallad idiopatisk PAH är okänt. Vad som är känt är att lungkärlen förändras på ett karakteristiskt vis samt att det förhöjda trycket leder till svikt av högerhjärtat då det måste pumpa mot allt högre motstånd (resistans). De karaktäristiska kärlförändringarna (lesioner) består av en sjuklig tillväxt av den innersta delen av artären (intima) samt den muskulära delen (media); dessa resulterar i ett kärlets volym blir mindre vilket genererar ett högre tryck. En specifik kärlförändring vid namn plexiform lesion uppstår efter en tids sjukdom, och denna typ av kärlskada återfinns enbart inom PAH. Trots att denna kärlskada är välkänd och dokumenterad sedan årtionden finns det flera oklarheter kring den plexiforma lesionen: bl.a. vart i kärlträdet den återfinns, om den är en orsak eller konsekvens av sjukdom samt om den kommunicerar med systemkärl eller ej. Den forskargrupp jag tillhör har tidigare visat att en viss typ av protein (proteoglykan) vid namn versican ansamlas i PAH associerade kärlskador, inklusive plexiforma lesioner.

Denna avhandling har haft två separata fokus, utan inbördes rangordning. Dels har en ny typ av avbildningsmetod (synkrotronstrålning) för lungvävnad utvärderats och använts för att avbilda kärlförändringar. Fokus nummer två för denna avhandling har varit proteoglykanen versican. Dess plats i mänskliga lungkärlsförändringar har analyserats och djurmodeller har använts för att undersöka hur versican påverkar sjukdomsförloppet (tryckstegring).

I **artikel I** utvärderades om synkrotronstrålning kunde användas som ett komplement till vanlig histologi för vävnadsstudier, genom att avbilda en undergrupp av PH kallad alveolär kapillär dysplasi (ACD). Vi kunde visa att denna metod på ett icke-destruktivt sätt kunde avbilda inbäddad lungvävnad, och generera tredimensionella data. Vävnaden påverkades inte negativt av strålningen utan kunde analyseras efteråt med konventionella histologiska metoder. Vidare kunde vi i denna artikel visa att patienter med ACD har kärlkopplingar mellan lung- och systemkärl vilket försvårar deras möjlighet att syresätta sig. Vi visade också att en viss typ av vener som man tidigare trott var avvikande lungvener faktiskt är vidgade bronkialvener, troligtvis vidgade på grund av kärlkopplingarna som ger ett ökat flöde.

I **artikel II** använde vi synkrotronstrålning för att undersöka och försöka förstå plexiforma lesioner. Inbäddad lungvävnad från flera patienter med PAH avbildades samt vävnad som hade injicerats med en typ av markeringsbläck som är vanligt förekommande hos patologer. Resultaten visade att plexiforma lesioner är en heterogen lesion, som kunde delas in i fyra distinkta subgrupper baserat på deras placering i kärlträdet och vad de hade för kärlkopplingar. Vi visade att två typer ofta kommunicerar med systemkärl och verkar agera som shuntar. Baserat på våra resultat lade vi fram hypotesen att vissa typer av kärlskador inklusive en typ av plexiform lesion troligtvis ligger till grund för sjukdomsförsämring, samt att två typer av plexiforma lesioner troligtvis minskar sjukdomsbördan.

I **artikel III** undersökte vi om vi med hjälp av synkrotronstrålning kunde avbilda och visualisera de fyra typerna av plexiforma lesioner i en råttmodell av PAH. Ett hinder inom PAH forskning har varit att ingen djurmodell på ett adekvat sett frambringar mänskliga kärlskador och plexiforma lesioner. Dock så har forskare relativt nyligen visat att denna modell kan generera plexiforma lesioner, men menat att dessa inte har någon koppling till systemkärl. Vi upplevde att de plexiforma lesioner de dokumenterat hade likheter med vår indelning från artikel II, varför vi ville undersöka denna modell vidare. Detta projekt var ett samarbete med forskare på Stanford University, USA. Vi kunde visa att denna råttmodell genererar alla de fyra typerna av plexiforma lesioner som har identifierats i mänsklig vävnad, samt att två typer på samma sätt som i människa kommunicerar med systemkärl. Detta är mycket lovande, då man med hjälp av denna djurmodell kan förstå vad som genererar kärlskadorna, och i vilken ordning de uppkommer.

I **artikel IV** undersöktes versican i mänskliga PAH-relaterade lungkärlsskador med hjälp av konventionella histologiska metoder i kombination med synkrotronstrålning. Vi kunde visa att versican produceras och deponeras i

kärlskadorna, och att versican genomgår betydande enzymatisk modulering. Vidare binder versican genomgående till molekylen hyaluronan samt till proteinet tenascin C. Baserat på var versican återfanns, och på grund av den enzymatiska moduleringen, eftersöktes om ett specifikt fragment kunde detekteras i patientblod. Resultaten visade att detta fragment finns i betydligt högre nivåer i patientblod jämfört med friska kontroller. Detta skulle kunna vara en biomarkör för tillståndet.

I **artikel V** undersöktes versicans roll i sjukdomsutveckling i en musmodell, där sjukdom orsakas genom långvarig exponering för hypoxi (lågt syretryck). Denna musmodell får högt tryck på grund av ökad muskelförtjockning i kärlen. Två genmodifierade musstammar användes. Dels en som saknade två av de totalt fyra normalt förekommande varianterna av versican, samt en modell som saknade alla fyra varianter. Ingen skillnad i lungkärlstryck kunde dock uppmätas när modellerna jämfördes med kontrollmöss. Slutsatsen från detta arbete är att brist på versican inte begränsar muskelförtjockning i lungkärl hos möss, samt att en djurmodell där kärlförträngande förändringar bildas bör övervägas vid framtida studier av detta slag.

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Uwe Rauch. I have found that if you don't know the answer to a science related question, the answer isn't worth knowing. I would come to you with a question, perhaps something that I've just read or wanted to learn more about, and you would reply "yes, maybe" or something to that effect in your low tempered kindly manner. An hour later or so you would knock on my door and show me two papers you have previously published on that specific topic, as well as results from the mouse model you created long ago to answer the question. Amazing. You are a world of knowledge and an institution. Thank you for helping to guide me, in your own way, through this process.

To the man with Star Wars figurines in his office, with the world's highest concentration of fingerprints per square inch on his computer screen and with the constant smile on his face, thank you **Vinicio**. You welcomed me in to your lab at Stanford before I started my PhD, when I was only a bumbling fool of a medical student. You and **Mark** gave me the best of experiences, and both of you were (and are) excellent teachers. And, since I still carry the memory, I reiterate that I was not the one responsible for the incubator infection debacle of 2014.

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Christian Norvik. I did not think either of us would ever finish this. We really did not know what we were getting into when we started at the lab, nine years ago. The blind leading the blind. We continued, without fault, to insist on learning by *doing* (instead of the perhaps more sensible approach where one studies and plans ahead). We did it all, for better and for worse. Confused in the lab, check. Confused in the in-vitro room, check. Confused notes in the lab book (which version?), check. PH-meter? Complete confusion and check. Animal house: pun intended, twice. We did learn some valuable lessons along the way though, I am sure! However, for the life of me I cannot at the moment remember them. Perhaps with time it will become clear. No, all joking aside I have come out at the other side a much cleverer man and I owe so much of that to you. Thank you for being my friend and colleague, and for putting up with me though all these years. Lemon.

Niklas, Mattias, Martin. Nuke, double vein and chungus. Gaone. PhDone. Golf!

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On a different note, I will from now on require that you all address me as Dr. Dr. whenever you want my attention. Good bye.

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