Lecticans in Vascular Remodeling of the Pulmonary and Fetal Circulation

Implications for Children with End-Stage Heart Disease

van der Have, Oscar

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Implications for Children with End-Stage Heart Disease

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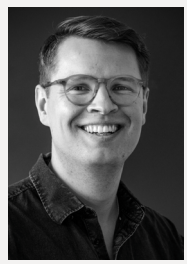

Oscar van der Have first became involved in experimental research with the Vessel Wall Biology group as a preclinical medical student and started his PhD studies upon graduating from medical school at Lund University in 2020. He is a resident in pediatric cardiology at the Pediatric Heart Center, Skåne University Hospital in Lund, Sweden.

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Lecticans in Vascular Remodeling of the Pulmonary and Fetal Circulation

Implications for Children with End-Stage Heart Disease

Oscar van der Have, MD

LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Abstract

Introduction: Elevated pulmonary vascular resistance (PVR) is observed in both physiological and pathological states. Pulmonary hypertension (PH) is a condition associated with significant morbidity and mortality in neonates and children and curative pharmacotherapy is lacking in most cases. *In utero*, a high PVR allows for blood to bypass the lungs through the open ductus arteriosus (DA). Vascular remodeling in PH development and DA closure is characterized by abundant deposition of extracellular matrix, including proteoglycans. The proteoglycan sub-family lecticans have remained largely un-studied in these settings. In children with end-stage heart disease, high PVR complicates clinical management and has been associated with poor outcome following pediatric heart transplantation (pHTx).

Methods: The accumulation, spatial distribution and temporal regulation of the two lecticans aggrecan and versican was investigated in human PH and DA specimens using a combination of synchrotron-based phase-contrast microcomputed tomography and histology, immunostaining, mRNA *in situ* hybridization and spatial proteomics. In addition, we investigated clinical practice in Sweden and internationally in children with PH listed for pHTx, through a survey- and registry-based approach.

Results: In PH, lecticans were shown to be expressed by vascular smooth muscle cells and endothelial cells and accumulated early in lesion development. Multiple different isoforms of versican were present and degraded by members of the ADAMTS protease family, and versican was found at elevated concentrations in PH patient plasma. Within the pulmonary vascular tree, aggrecan and versican preferentially accumulated at, or proximally to, sites of elevated PVR. Versican and aggrecan were furthermore shown to accumulate at high levels during the early phases of anatomical remodeling of the closing DA, with progressive degradation by ADAMTS proteases at later stages of remodeling. Surveying international practice patterns for patients with PH and end-stage heart disease revealed significant heterogeneity in hemodynamic evaluation, treatment with pulmonary vasodilators and cut-offs for precluding patients for isolated for pHTx. Within the Swedish cohort of children listed for pHTx, children with elevated PVR did not fare worse off post-pHTx but very few patients with a fixed PVRi above 6 $WU \cdot m^2$ had been listed and transplanted.

Conclusions: In summary, early lectican accumulation in pulmonary and fetal vascular remodeling appears vital and turnover of lecticans by ADAMTS proteases is an intriguing target for future pharmacological therapies. Clinical practice in children with PH and heart disease is heterogenous but does not appear to have influenced post-pHTx outcomes in Sweden.

Key words: vascular remodeling, pulmonary hypertension, ductus arteriosus, lecticans, pulmonary vascular resistance, pediatric heart transplantation

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Description: the surgically removed patent ductus arteriosus of a 6-day-old infant with transposition of the great arteries. Analyses of the vessel through four modalities are depicted: immunofluorescent stain for versican and its proteolytic fragment (top left), histological staining for glycosaminoglycans (top right) as well as structure tensor vectorial analysis (bottom right) and three-dimensional segmentation (bottom left), both performed on the synchrotron microtomography dataset of the vessel.

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To my parents

Table of Contents

List of publications **8**

Populärvetenskaplig sammanfattning **10**

Abbreviations **14**

Introduction **16**

Lecticans of the vascular wall 16

The vessel wall 16

Proteoglycans – key components of the extracellular matrix 18

Lecticans 18

Aggrecan 19

Versican 20

Proteolysis of aggrecan and versican 21

Glycosaminoglycans 22

Role in vessel wall homeostasis 23

The pulmonary and fetal circulation 24

The pulmonary circulation 24

The fetal circulation 25

Transition from fetal to neonatal circulatory physiology 27

The ductus arteriosus 27

Pediatric pulmonary hypertension 32

Pulmonary arterial hypertension 32

Pulmonary hypertension due to left heart disease 35

Diagnostics and hemodynamic evaluation 36

Treatment 39

Pulmonary hypertension and heart transplantation in children 41

Aims **44**

Material and methods **45**

Material and study design 45

Study I – III 45

Study IV 47

Study V 47

Experimental methodology 49

Synchrotron-based microtomography 49
Histology and immunostaining 51
Enzyme-linked immunosorbent assay 52
mRNA transcript analysis 53
Ethical aspects 53
Statistical analysis 54
Results 55
Study I 55
Study II 56
Study III 57
Study IV 58
Study V 59
Discussion 60
Spatiotemporal dynamics of lectican accumulation and turnover 60
Pulmonary arterial vasculopathy 60
Closure of the ductus arteriosus 62
Diagnostic and pharmacological implications 63
Pre-transplant PH in children – ascending to new heights? 64
Prospects for children with advanced heart failure in Sweden 66
Limitations 68
Concluding remarks 69
Acknowledgements 72
References 78

List of publications

This thesis is based on the following papers and manuscripts which will be referred to in the text by their Roman numerals (I-V):

I. **van der Have O**, Mead TJ, Westöö C, Peruzzi N, Mutgan AC, Norvik C, Bech M, Struglics A, Hoetzenecker K, Brunnström H, Westergren-Thorsson G, Kwapiszewska G, Apte SS, Tran-Lundmark K. *Aggrecan accumulates at sites of increased pulmonary arterial pressure in idiopathic pulmonary arterial hypertension*. Pulm Circ. 2023 Feb 21;13(1):e12200.

II. Westöö C, Mutgan AC, **van der Have O**, Mead TJ, Ahmed S, Lampei E, Koch CD, Norvik C, Aspberg A, Bech M, Peruzzi N, Brunnström H, Kwapiszewska G, Rådegran G, Apte SS, Tran-Lundmark K. *Localization, proteolytic processing, and binding partners of versican isoforms in vascular lesions of pulmonary arterial hypertension.* Manuscript in press in J Histochem Cytochem.

III. **van der Have O**, Peruzzi N, Norvik C, Nandadasa S, Shelton EL, Lampei E, Bech M, Dreier T, Tan C, Gram M, Kristiansson A, Brunnström H, Allison B, Rodriguez ER, Apte SS, Reese J, Tran-Lundmark K. *Three-dimensional morphology and spatiotemporal dynamics of lecticans assessed in the mammalian ductus arteriosus.* Manuscript.

IV. Hopper RK, **van der Have O**, Hollander SA, Dipchand AI, Perez de Sa V, Feinstein JA, Tran-Lundmark K. *International practice heterogeneity in pre-transplant management of pulmonary hypertension related to pediatric left heart disease*. Pediatr Transplant. 2023 Mar;27(2):e14461.

V. **van der Have O**, Wåhlander H, Hofbard T, Abele D, Sjöborg Alpman M, Duus Weinreich I, Böhmer J, Nilsson J, Holgersson J, Liedberg A-S, Tran-Lundmark K, Odermarsky M. *Comprehensive analysis of the first 35 years of pediatric heart transplantation in Sweden.* Manuscript.

In addition to the included papers and manuscripts, contributions were made to the following original articles, letters and reviews during the corresponding PhD period:

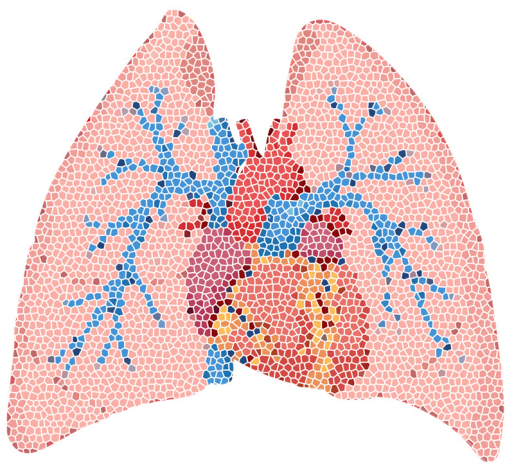
1. Hopper RK, Hansmann G, Hollander SA, Dipchand AI, **van der Have O**, Iler C, Herrington C, Rosenzweig EB, Alejos JC, Tran-Lundmark K; American Heart Association Council on Cardiopulmonary, Critical Care, Perioperative & Resuscitation; Council on Clinical Cardiology; and Council on Cardiovascular Stroke Nursing. *Clinical management and transplant considerations in pediatric pulmonary hypertension due to left heart disease: a scientific statement from the American Heart Association*. Circ Heart Fail. 2025 Jan;18(1):e000086.
2. Arévalo-Martinez M, Ede J, **van der Have O**, Ritsvall O, Zetterberg FR, Nilsson UJ, Leffler H, Holmberg J, Albinsson S. *Myocardin related transcription factor and galectin-3 drive lipid accumulation in human blood vessels*. Vascul Pharmacol. 2024 Sep;156:107383.
3. Bodmer J, Levin A, Westöö C, **van der Have O**, Peruzzi N, Tran-Lundmark K, Abman SH, Galambos C. *Intrapulmonary bronchopulmonary anastomoses in severe COVID-19-related acute respiratory failure*. Am J Respir Crit Care Med. 2022 Nov 1;206(9):1169-1170.
4. **van der Have O**, Westöö C, Ahrné F, Tian X, Ichimura K, Dreier T, Norvik C, Kumar ME, Spiekerkoetter E, Tran-Lundmark K. *Shunt-type plexiform lesions identified in the Sugen5416/hypoxia rat model of pulmonary arterial hypertension using synchrotron-based phase-contrast micro-CT*. Eur Respir J. 2022 Jun 9;59(6):2102802.
5. Westöö C, Norvik C, Peruzzi N, **van der Have O**, Lovric G, Jeremiasen I, Tran PK, Mokso R, de Jesus Perez V, Brunnström H, Bech M, Galambos C, Tran-Lundmark K. *Distinct types of plexiform lesions identified by synchrotron-based phase-contrast micro-CT*. Am J Physiol Lung Cell Mol Physiol. 2021 Jul 1;321(1):L17-L28.
6. Norvik C, Westöö C, Peruzzi N, Lovric G, **van der Have O**, Mokso R, Jeremiasen I, Brunnström H, Galambos C, Bech M, Tran-Lundmark K. *Synchrotron-based phase-contrast micro-CT as a tool for understanding pulmonary vascular pathobiology and the 3-D microanatomy of alveolar capillary dysplasia*. Am J Physiol Lung Cell Mol Physiol. 2020 Jan 1;318(1):L65-L75.

Populärvetenskaplig sammanfattning

I denna avhandling ingår fem delarbeten (I-V) som alla berör olika aspekter av ett och samma övergripande tema; *förhöjt blodtryck i lungcirkulationen hos barn*.

Först beskrivs delarbete I, II och III, vars syfte var att kartlägga närvaron, fördelningen och nedbrytningen av *lektikaner* i två tillstånd med koppling till förhöjt blodtryck i lungorna.

Kroppens organ kan liknas vid antikens stora mosaiker: likt mosaikbrickorna uppvisar *cellerna*, kroppens minsta byggstenar och funktionella enheter, variation i storlek, utseende och orientering. Alla brickor hålls samman av murbruk som tillåter de enskilda bitarna att skapa intrikata mönster, vilket vi uppfattar som mosaikkonstverk. Utan murbruket skulle det vara mycket svårt att bygga en stor, komplex mosaik. Likt mosaikbrickorna är kroppen beroende av cellens murbruk, *bindväven*, för att forma och underhålla kroppens organ. Ett murbruks grovlek, fuktighetsgrad och hållfasthet avgörs av andelarna vatten, sand och bindemedel. På liknande vis kan egenskaperna hos bindväven variera med proportionerna av dess olika beståndsdelar. Bindväven kan dessutom påverka, och påverkas av, beteendet hos de celler den omger.



Cellen – brickan i kroppens mosaik.
Illustration: Henrik Gunnarsson.

En familj av beståndsdelar i bindväven, *lektikaner*, kan liknas vid murbrukets bindemedel. Lektikaner är stora, vattenbindande, molekyler som är sammanlänkade med varandra i ett nätverk som agerar förankringspunkt för både omkringliggande celler och andra bindvävskomponenter. Det förekommer normalt en viss nyproduktion och omsättning av lektikaner, men stora förändringar i dess nybildning eller nedbrytning kan vara förenat med sjukdom. En familj av enzymer som bryter ner lektikaner i mindre beståndsdelar heter *ADAMTS*. En balans mellan intakta lektikaner och nedbrytning genom ADAMTS-enzymerna har visat sig vara viktig för att upprätthålla stabilitet och elasticitet i *kärlväggen*. Rubbningar i denna balans orsakar sjukdomar som kännetecknas av både instabilitet och förträngning i kärlväggen.

I delarbete I och II har vi studerat lektikaner i förträngda lungkärl från patienter med *pulmonell hypertension*, högt blodtryck i lungcirkulationen. Detta är ett allvarligt kroniskt tillstånd som kan uppkomma till följd av flera orsaker. Idag finns det ingen botande behandling och pulmonell hypertension leder ofta till en ökad belastning på *höger hjärthalva*, som tvingas arbeta mot ett ökat motstånd när blodtrycket i lungan stiger. Pulmonell hypertension kan även uppstå som en konsekvens av hjärtsjukdom i *vänster hjärthalva*. Är vänster hjärthalvas pumpförmåga nedsatt under lång tid kommer till slut trycket i hjärtats vänstra förmak att stiga och fortplanta sig bakåt i lungcirkulationen och skada lungkärlen, med utveckling av pulmonell hypertension som följd.

Högt blodtryck i lungcirkulationen är dock inte alltid förenat med sjukdom. Tillståndet förekommer naturligt under *fosterutvecklingen*, när blodflödet till fostrets lungor är begränsat och fostrets blod syresätts i moderkakan. Under fosterutvecklingen tillåts blod att passera förbi lungorna på sin väg ut till kroppens organ, bland annat genom ett stort öppet kärl mellan lung- och kroppspulsådern, *ductus arteriosus*. Passagen möjliggörs av att blodtrycket i lungpulsådern är högre än det i kroppspulsådern. I samband med att det nyfödda barnet tar sina första andetag, och därmed öppnar sina lungor, faller blodtrycket i lungorna markant. Strax efter förlossningen sluts ductus spontant, men kan i vissa fall förbli öppen och orsaka att för mycket blod når lungorna, då blodtrycket i kroppspulsådern nu övervinner det sjunkande blodtrycket i lungpulsådern. Denna övercirkulation av lungorna genom en *öppetstående ductus arteriosus* utgör en annan av orsakerna till utvecklingen av kronisk, sjuklig, pulmonell hypertension. I delarbete III jämförde vi lektikaners närvaro i öppetstående, och sluten, ductus arteriosus från foster och barn av olika åldrar för att bättre förstå varför ductus i vissa fall förblir öppen.

För att kunna genomföra delarbetet I-III har vi använt arkiverade patientprover från både svenska och amerikanska biobanker och sjukhus. Experiment på dessa *vävnadsprover* har genomförts dels med traditionella, tvådimensionella, metoder för bearbetning och infärgning av vävnad, dels med hjälp av mycket högupplöst skiktröntgen. Den sistnämnda metoden använder *synkrotronstrålning* och har försett oss med information om vävnaden i tre dimensioner. Experiment med

synkrotronstrålning kan endast utföras vid en s.k. *elektronpartikelacceleratorer* varav en, MAXIV, är belägen strax norr om universitetssjukhuset i Lund.

Elektronpartikelacceleratorn MAXIV i Lund.
I bakgrunden skymtas ”Blocket”, Lundadômen och författaren själv. Fotograf: Perry Nordeng.

I delarbete I fann vi att *aggrecan*, en lektikan som i vanliga fall återfinns i brosk, uttrycktes och ansamlades i sjuka lungkärl hos patienter med pulmonell hypertension. Ansamlingen av aggrecan tycktes i huvudsak ske tidigt i sjukdomsprocessen och i områden belastade av högt lungblodtryck. Medlemmar ur ADAMTS-familjen uppvisade även ett högre genuttryck i sjuka lungkärl. I linje med detta visade vi i delarbete II att *versican*, en annan av lektikanerna, omsattes av ADAMTS-enzymer i sjuka lungkärl hos patienter med pulmonell hypertension. Omsättningen genererade flera fragment som uppvisade olika fördelning i kärlväggen. Vi fann dessutom att ett av dessa nya fragment ansamlades i högre koncentration i blodet från patienter med pulmonell hypertension, jämfört med friska kontroller. Delarbete III visade att både aggrecan och versican ansamlades i stora mängder i kärlväggen under fosterutvecklingen, för att sedan brytas ner av ADAMTS-enzymerna strax efter födseln. Vi visade även att det sker förändringar i cellernas sammansättning och signalering, samtidigt som ansamlingen av lektikaner pågår. Ansamlingen av lektikaner i kärlväggen hos barn med öppetstående ductus arteriosus var mindre påtaglig. Gemensamt för delarbete I-III är att vi på ett bättre sätt har kunnat förstå den tredimensionella mikroskopiska anatomin m.h.a synkrotronstrålning.

Nu följer en beskrivning av delarbete IV och V, som har en direkt koppling till vård av barn som lider av både förhöjt lungblodtryck och *transplantationskrävande hjärtsvikt*.

I de fall bägge tillstånden föreligger kan högt lungblodtryck försämra möjligheterna till överlevnad efter *hjärttransplantation*. Om lungkärlen är svårt sjuka kan nämligen det nya hjärtat ta skada, då höger hjärthalva inte är van vid det höga motståndet i lungcirkulationen. Det finns dock *inga riktlinjer* som anger hur högt motståndet i lungcirkulationen hos barn kan tillåtas vara innan en hjärttransplantation bör uteslutas. Om lungkärlen anses vara för sjuka, kan ett alternativ vara en kombinerad hjärt- och lungtransplantation, ett ingrepp som tyvärr har en betydligt sämre prognos.

Resultaten i delarbete IV byggde på en enkät som skickades ut till specialistläkare som behandlar barn med bägge tillstånden, och syftade till att kartlägga likheter och skillnader i vården. Baserat på 49 svar från läkare i 16 länder kunde vi konstatera att det föreligger meningsskiljaktigheter inom flera delar av vården: tillvägagångssätt för att mäta motstånd i lungcirkulationen, indikationer för läkemedelsbehandling och längden på denna, samt huruvida ett riktvärde för maximalt lungmotstånd bör användas överhuvudtaget.

I delarbete V, en registerbaserad studie, gjordes en omfattande beskrivning av de barn under 18 år som listats för hjärttransplantation i Sverige under åren 1989–2023. Vi lade särskilt fokus på att beskriva hur det gått för *svenska barn med pulmonell hypertension som hjärttransplanterats*. Hjärttransplantation bland barn i Sverige har blivit vanligare över de senaste 35 åren, sannolikt till följd av att vården av svårt sjuka barn har förbättrats. Överlevnaden på väntelistan har förbättrats markant över tid, men små barn under ett års ålder har fortsatt sämre förutsättningar att nå hjärttransplantation än äldre barn. Pulmonell hypertension var i vår analys inte förenat med sämre överlevnad efter hjärttransplantation. Långtidsöverlevnaden efter hjärttransplantation har varit mycket bra sedan starten 1989 och idag når en stor majoritet av de hjärttransplanterade barnen i Sverige vuxen ålder.

Sammanfattningsvis konstateras att en omfattande ansamling av lektikaner tycks spela en roll tidigt i utvecklingen av pulmonell hypertension och i slutningen av ductus arteriosus. Parallellt ökar dessutom nedbrytning av lektikaner i kärlväggen. De fragment som genereras har en viktig biologisk funktion och kan vara möjliga blodmarkörer för högt lungblodtryck. Vården av barn med högt lungblodtryck och samtidig hjärtsjukdom är komplicerad och våra diagnostiska verktyg har flera felkällor som gör det svårt att formulera tydliga riktlinjer. Till följd av bristen på riktlinjer, kan vi se bevis på att vården av barn med högt lungblodtryck och hjärtsjukdom varierar på det internationella planet. Sverige har lyckats väl med att hjälpa dessa barn att nå hjärttransplantation, med goda resultat även på lång sikt. Svensk barnhjärttransplantationsverksamhet håller en hög internationell klass, trots ett modest antal patientfall årligen.

Abbreviations

IEL – internal elastic lamina

EC – endothelial cell

VSMC – vascular smooth muscle cell

ECM – extracellular matrix

PG – proteoglycan

GAG – glycosaminoglycan

CS – chondroitin sulfate

DS – dermatan sulfate

KS – keratan sulfate

HS – heparan sulfate

HA – hyaluronic acid/hyaluronan

LP – link protein

IGD – interglobular domain

MMP – matrix metalloproteinases

ADAMTS – a disintegrin-like and metalloproteinase domain with thrombospondin type 1 motifs

LV – left ventricle

RV – right ventricle

LA – left atrium

RA – right atrium

mPAP – mean pulmonary arterial pressure

PVR – pulmonary vascular resistance

WU – Wood Unit

PAWP – pulmonary arterial wedge pressure

LAP – left atrial pressure

CO – cardiac output

CI – cardiac index

PVRi – indexed pulmonary vascular resistance

Ao – aorta

MPA – main pulmonary artery

DA – ductus arteriosus

LA – ligamentum arteriosum

PDA – patent ductus arteriosus

PGE_2 – prostaglandin E_2

NSAID – non-steroidal anti-inflammatory drug

PGE_1 – prostaglandin E_1

PH – pulmonary hypertension

RHC – right heart catheterization

LHD – left heart disease

PAH – pulmonary arterial hypertension

CHD – congenital heart disease

PH-LHD – pulmonary hypertension due to left heart disease

IPAH – idiopathic pulmonary arterial hypertension

HPAH – hereditary pulmonary arterial hypertension

AVT – acute vasoreactivity testing

NO – nitric oxide

PDE5i – cyclic guanosine monophosphate-specific phosphodiesterase type 5 inhibitors

pHTx – pediatric heart transplantation

CM – cardiomyopathy

DCM – dilated cardiomyopathy

HCM – hypertrophic cardiomyopathy

RCM – restrictive cardiomyopathy

UVH – univentricular heart

MCS – mechanical circulatory support

ECMO – extracorporeal membrane oxygenation

VAD – ventricular assist device

LVAD – left ventricular assist device

RVAD – right ventricular assist device

BiVAD – biventricular assist device

HLA – human leukocyte antigen

DSA – donor specific antibody

CDC – cell-dependent cytotoxicity

AMR – antibody-mediated rejection

CAV – cardiac allograft vasculopathy

ABOi – ABO-incompatible

FLD – failed lung donor

LCPR – Lund Cardiopulmonary Registry

E – embryonic day

dGA – day of gestation

ISH – *in situ* hybridization

ISHLT – International Society for Heart and Lung Transplantation

Re-pHTx – pediatric heart re-transplantation

SRμCT – synchrotron-radiation phase-contrast microcomputed tomography

μCT – microcomputed tomography

EvG – Elastica van Gieson

Ab/PAS – Alcian blue/Periodic acid-Schiff

mRNA – messenger RNA

ELISA – enzyme-linked immunosorbent assay

qRT-PCR – quantitative real-time polymerase chain reaction

αSMA – α-smooth muscle actin

vWF – von Willebrand Factor

dTPG – diastolic transpulmonary gradient

Introduction

Lecticans of the vascular wall

The vessel wall

Structure and categorization of arteries

The vascular wall consists of three layers (Figure 1). The innermost, *tunica intima*, separates the blood flowing through the lumen from the other layers of the wall. The middle, muscularized, *tunica media* regulates blow flow through vascular tone (vasoconstriction and vasodilatation). The intima and media are separated by a prominent elastic membrane, the *internal elastic lamina* (IEL). The *tunica adventitia* is the outermost layer, mainly composed of connective tissue, but also holds the *vasa vasorum* and *nervi vascularis*.

Arteries are divided into three categories based on their size and composition (Figure 1). The high elastin fiber content in the tunica media of *elastic arteries* grants flexibility and allows for a relatively constant pressure gradient despite fluctuations in pulse pressure, as this type of arteries lie in close proximity to the heart. *Muscular arteries* have a greater smooth muscle cell content in their tunica media compared to elastic arteries, allowing them to control blood flow to tissues. Muscular arteries eventually feed into *arterioles*, which respond to the tissues need for oxygen and nutrients and play a significant role in determining the peripheral vascular resistance.

Of note, the tunica media of *pulmonary* muscular arteries and arterioles are significantly thinner than their *systemic* counterparts and have a greater luminal diameter, contributing to the lower total peripheral vascular resistance of the pulmonary circulation.

Cells of the vessel wall

In healthy arteries the tunica intima is composed of a single layer of *endothelial cells* (EC), the endothelium. ECs determine vascular permeability and play roles in inflammation, vascular tone and coagulation, given their direct contact with blood. *Vascular smooth muscle cells* (VSMC) grant the tunica media vasoreactivity and regulate luminal diameter. VSMC phenotype is altered in the setting of vascular

injury, switching from quiescent and contractile to a synthetic phenotype with higher proliferation and migratory rate. Together with *fibroblasts*, residing in the adventitia, VSMCs produce an *extracellular matrix* (ECM) which provides structural support and elasticity to the vessel wall.

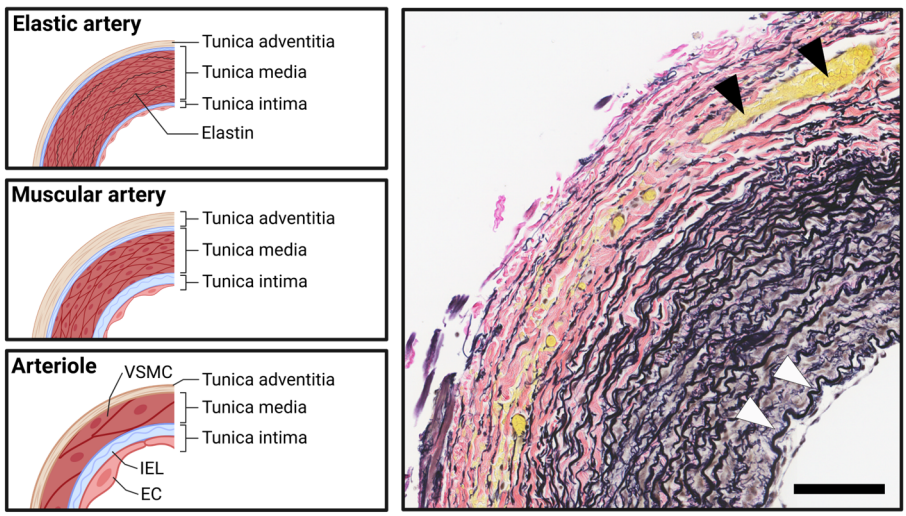


Figure 1. Composition of the arterial vessel wall.
Panels to the left outline differences in the composition of elastic arteries, muscular arteries and arterioles. The right panel shows an EvG staining of a human muscular artery. Elastic fibers stain black and collagens stain pink/red. ECM rich in PGs is found in between the layers of elastic fibers. The prominent IEL is clearly visible (white arrowheads), as is the vasa vasorum (black arrowheads). Scale bar is 100 µm. *VSMC, vascular smooth muscle cell; IEL, internal elastic lamina; EC, endothelial cell; EvG, Elastica van Gieson; ECM, extracellular matrix; PG, proteoglycan.*

Vascular extracellular matrix

The ECM is an intricate three-dimensional structural network, providing means of signaling through its interaction with surface receptors or via signaling molecules trapped in the matrix (1-3). The ECs of the vessel wall sit atop a *basement membrane*, a specialized form of ECM, composed of laminins, collagen IV, nidogens and perlecan (4). The concentric layers of VSMCs in the tunica media are separated by *elastin* filaments, creating lamellae within the wall that grant elasticity. Within the lamellae, VSMCs are embedded in a basement membrane with *proteoglycans* (PGs) believed to be important in keeping VSMCs quiescent and contractile (5). Changes in the composition of the basement membrane are associated with alterations in VSMC phenotype. Vascular *collagens*, mainly fibrillar collagens in the adventitia, provide tensile strength to the vessel. Vascular PGs will now be described in greater detail.

Proteoglycans – key components of the extracellular matrix

PGs are a diverse class of ECM molecules comprising protein cores enriched with covalently attached, negatively charged, *glycosaminoglycans* (GAGs). Based on the pattern of sulfation, GAGs associated with PGs are divided into four different types: *chondroitin sulfate (CS)*, *dermatan sulfate (DS)*, *keratan sulfate (KS)* and *heparan sulfate (HS)* (6). A fifth GAG, *hyaluronic acid/hyaluronan* (HA), is a non-sulfated GAG that interacts with and stabilizes PG complexes.

The GAG component of PGs allows for the formation of gel-like pericellular matrices through inherent negative charges within the sugar molecules, that attract cations and water to hydrate the ECM and generates an *osmotic swelling pressure* against its surroundings (3, 6) (Figure 2). Additionally, PGs act as co-receptors in signaling and GAGs harbor chemokines and growth factors, which guide migration, phenotype switching and proliferation of cells, if released (6). Although most PGs are found in the immediate pericellular or extracellular space, or are associated with the cell-surface, intracellular PGs have been described (7, 8).

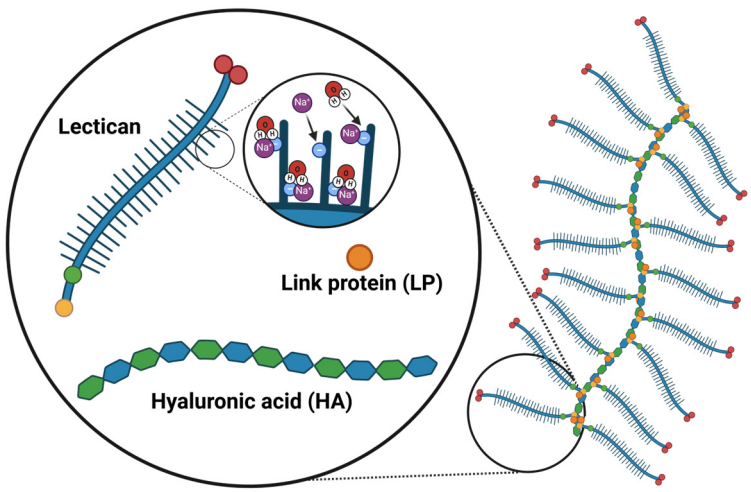


Figure 2. The ternary complex formed by HA, LP and lecticans hydrates the ECM
Lecticans form massive supramolecular complexes together with HA and LP. Inherent negative charges within the GAG side chains grant lecticans their hydrophilic properties. *LP, link protein; HA, hyaluronic acid/hyaluronan; ECM, extracellular matrix.*

Lecticans

Lecticans are a distinct group of extracellular, CS-rich, PGs. Four members of the lectican family are widely acknowledged: *aggrecan*, *versican*, *brevican* and *neurocan*. All lecticans share highly conserved G1 and G3 globular core protein domains at their N- and C-termini, respectively. The term “lectican” is derived from

the common C-type lectin domain within the G3 globular protein (9), whereas accounting for the HA-binding property of the G1 domain prompted the alternative name “hyalectans”. The ability of lecticans to bind HA, requiring the presence of *link protein* (LP), at their N-terminus enables the formation of large supramolecular complexes which adds to their *hydrophilic* capabilities (Figure 2).

The presence of brevican and neurocan is restricted to the central nervous system, where they have roles in neuronal tissue development and repair (6, 9). Hereinafter, only aggrecan and versican will be considered relevant for studies of lecticans in the cardiopulmonary system.

Aggrecan

Structure

The first PG was identified in human cartilage in 1978 by Dr. Helen Muir and was later named aggrecan (10-12) (Figure 3). Suggested by its name, aggrecan forms large aggregates, >200 MDa, together with HA and LP (6). It is the main PG of articular cartilage, where its biomechanical properties contributes to the load-bearing function of articular cartilage (13-15). The core protein of aggrecan contains an unique globular domain, G2, amounting its total protein mass to ~250 kDa (14). Between the G1 and G2 domains lies the *interglobular domain* (IGD), which is often the site for proteolytic cleavage (14), discussed later.

Aggrecan hosts a large number of CS and KS chains, which account for ~90% of its overall molecular weight of ~2500 kDa (16). The CS attachment domain contains ~100 CS chains and is located between the KS domain and the G3 core protein (14, 17). The KS domain is anchored to the G2 globular domain at its N-terminal end and has up to 60 KS attachment sites in humans (14, 16, 17).

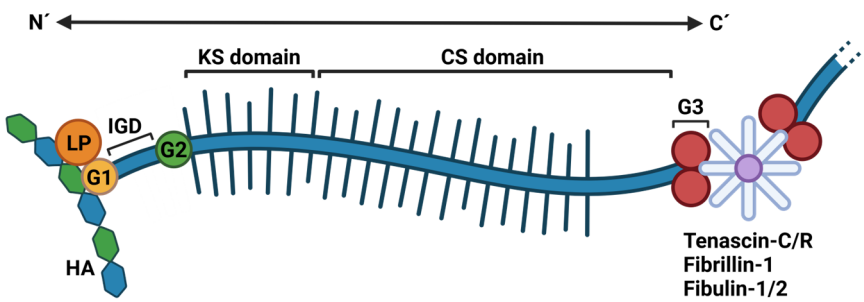


Figure 3. Structure and interactions of aggrecan
HA, hyaluronic acid/hyaluronan; LP, link protein; IGD, interglobular domain KS, keratan sulfate; CS, chondroitin sulfate

Interactions and binding partners

The G1 domain and LP are highly homologous in their structure, both consisting of an immunoglobin-like repeat (A subdomain) and two PG tandem repeats (B and B´ subdomains), where the latter two subdomains mediate the interaction with HA (16, 18) (Figure 3). The A subdomains of G1 and LP interact with each other to stabilize the ternary complex (19). The G2 domain of aggrecan is homologous to the PG tandem repeat, however, lacks interactions with HA or LP, and has not been shown to have any other significant ECM interaction to date (16, 20).

At the C-terminal end, the G3 domain connects aggrecan to other components of the ECM (16). The domain consists of two epidermal growth factor-like repeats, the C-type lectin subdomain and a complement regulatory protein domain, where the most prominent interactions are seen between the C-type lectin subdomain and *tenascins*, *fibulins* and *fibrillin-1* (16, 20) (Figure 3). Interestingly, these multimeric proteins have been shown to cross-link multiple aggrecan-LP-HA complexes (21), further stabilizing the ECM (Figure 3).

Versican

Structure and isoforms

Versican lacks the G2 and IGD domains of aggrecan but hosts two CS attachment regions, αGAG and βGAG, which through alternative splicing of *VCAN* exon 7 and 8 can generate a total of five different *isoforms* of versican (V0-V4) (22) (Figure 4). αGAG and βGAG has one and eight attachment sites for CS, respectively (17). Thus, the different isoforms of versican only confer a fraction of the hydrophilic properties of aggrecan.

The full length, V0, isoform of versican contains both the αGAG and βGAG attachment regions and is ubiquitously expressed throughout embryonic development (23, 24). V1 expresses the βGAG attachment region and is the most common isoform found in adult tissues (22, 25), whereas isoform V2 is found exclusively in the central nervous system and only contains the αGAG attachment region (26, 27). Lacking both GAG attachment regions, isoform V3 has not been associated with disease, however, was found to influence VSMC phenotype *in vitro* (28) and to improve elastin formation following neointimal injury (29). Little is known about isoform V4, only containing the N-terminal end of βGAG, aside from its upregulation in human breast cancer (30).

Interactions and binding partners

Given a high degree of homology between the common core protein domains of versican and aggrecan, their N- and C-terminal interactions are almost identical, albeit with small differences in ligand affinity and subdomain interactions (17). Like

aggrecan, versican forms large complexes with HA and LP via interaction with the B-B´subdomains of G1, however this paired domain also mediates the interaction with LP (19). Interacting partners at the C-terminal end of versican are similar to those of aggrecan (31) (Figure 4).

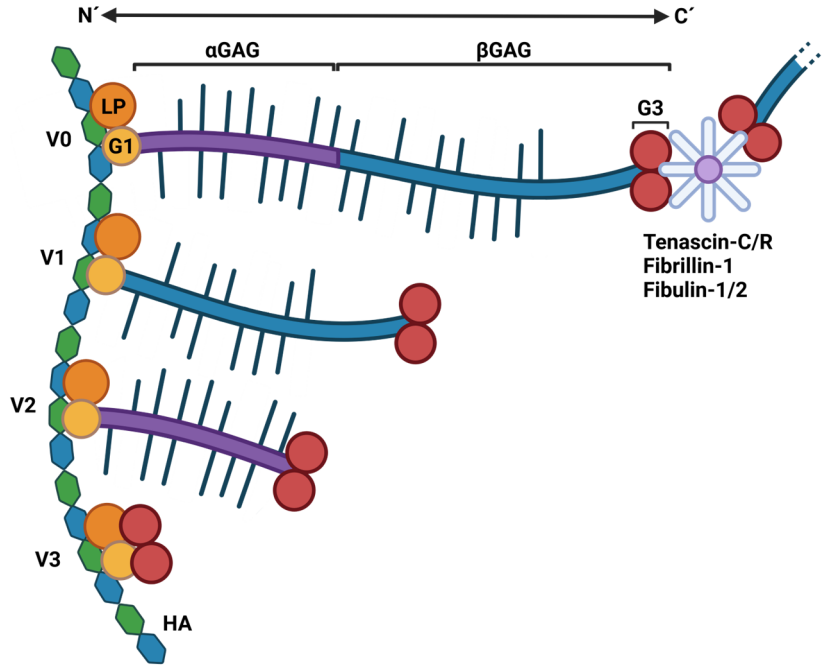


Figure 4. Structure, isoforms and interactions of the classic versican isoforms, V0-V3
HA, hyaluronic acid/hyaluronan; LP, link protein

Proteolysis of aggrecan and versican

The irreversible, post-translational, *proteolysis* of aggrecan and versican core proteins have several implications, including the release of bound morphogens/growth factors, generation of biologically active fragments and loss of ECM integrity (32). Several families of proteases, including *matrix metalloproteinases* (MMPs), have lecticans as their substrates. Versican and aggrecan proteolysis mediated by members from the ADAMTS (*a disintegrin-like and metalloproteinase domain with thrombospondin type 1 motifs*) family is important in the orchestration of cardiac development (20, 33). Additionally, dysregulated activity of ADAMTS proteases has been ascribed an emerging role in regulating vascular patency (34-37) and in cardiopulmonary disease (37-39).

The ADAMTS protease family

Since the discovery of ADAMTS1 in 1997 (40), a total of 19 ADAMTS proteases have been described (41). Structurally and genetically paired, ADAMTS proteases appear to act in a coordinated, and sometimes overlapping, manner as illustrated by the similar clinical presentation of genetic mutations within certain pairs (42).

Much of what is known about cleavage of aggrecan by ADAMTS proteases stems from research on cartilage breakdown in joint injury (43). The principal site of ADAMTS-mediated cleavage in aggrecan lies within the IGD at the Glu^{373}-Ala^{374} peptide bond, generating a C-terminal fragment that carries a vast majority of the GAG chains. The N-terminal sequence of the neoepitope is NITEGE (14). Cleavage at this site is mediated by ADAMTS4 and ADAMTS5, but also MMP-8, where the activity of ADAMTS5 has been proven much greater than ADAMTS4 under physiological conditions (44).

In versican, the most well-studied site of ADAMTS-mediated cleavage lies at the N-terminus of the βGAG (Glu^{441}-Ala^{442}) domain in versican V0 and V1 (45). The fragment, with the C-terminal sequence DPEAAE, was named *versikine.* Versikine was shown to have an independent biological function at several stages of embryogenesis (33) and was ascribed a role in atherosclerosis development (36). The principal versicanases are ADAMTS1, ADAMTS4, ADAMTS5, ADAMTS9, ADAMTS15 and ADAMTS20.

As suggested by recent work utilizing a novel proteomics approach (46), multiple additional ADAMTS cleavage sites likely remain to be uncovered within the core proteins of versican and aggrecan (32).

Glycosaminoglycans

Chondroitin sulfate and keratan sulfate

Both CS and KS are unbranched polysaccharides consisting of repeated disaccharide structures, modified with sulfate residues at differing positions (Figure 5). CS polymerization starts after the synthesis of a linker region, that interacts with the CS-bearing domains of lecticans and is undertaken by numerous synthetases and transferases in the *Golgi apparatus* (47). KS does not contain a linker region but is instead attached to the core protein as N-linked (KS type I), *O*-linked (KS type II), and 2-*O*-mannose-linked (KS type III) carbohydrates (17).

Hyaluronic acid/hyaluronan

HA is synthesized as a free polysaccharide at the plasma membrane and is directly secreted into the ECM, thus avoiding sulfation and other modifications in the Golgi apparatus (48) (Figure 5). It ranges in size from 500 kDa to 10 MDa, thus often exceeding the size of other GAGs (47). Aside from its interactions with lecticans

and its hydrophilic properties, HA contributes to cell signaling via binding to cell surface receptors, and can be either pro- or anti-inflammatory depending on its molecular weight (49).

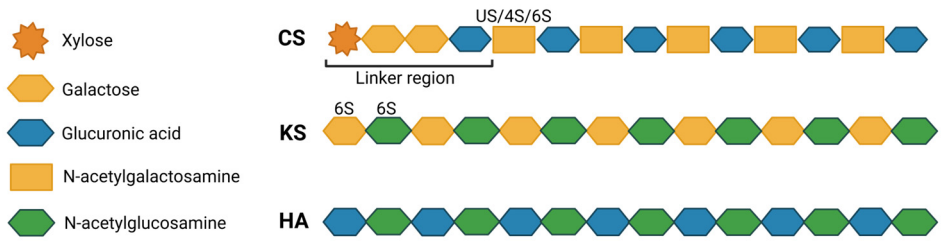


Figure 5. Composition of GAGs associated with lecticans
The N-acetylgalactosamine of CS can be unsulfated or sulfated at the 4-*O* and/or 6-*O* position. The N-acetylglucosamine and galactose of KS can both be sulfated at their 6-*O* positions, whereas HA is unsulfated upon secretion into the ECM. Adopted from (48). *CS, chondroitin sulfate; KS, keratan sulfate; HA, hyaluronic acid/hyaluronan; US, unsulfated; 4S, sulfated at the 4-O position; 6S, sulfated at the 6-O position; GAG, glycosaminoglycan.*

Role in vessel wall homeostasis

Small aggregates of GAGs and lecticans surround VSMCs of the tunica media of elastic arteries, and are important in pressurizing the intralamellar space, which maintains a baseline tension in the circumferential elastic filaments of the artery (50). Elastin and collagen provide a counter-pressure against the osmotic swelling pressure of GAGs and lecticans. This biomechanical equilibrium contributes to vascular patency and facilitates *mechanosensing*, as VSCMs form elastin-contractile units through focal adhesions with elastic filaments within the tunica media (51). If the delicate balance is disturbed, the result is loss of structural integrity and dysfunctional mechanosensing. In the setting of GAG and lectican pooling, as observed in dissection of the thoracic aorta (39), disturbed cell-ECM interactions and delamination of the arterial wall were suggested to be consequences of increased intralamellar pressure (52). Members of the *ADAMTS* family were found downregulated in areas with aortic dissection (39), suggesting that a baseline expression of *ADAMTS* proteases contributes to vascular homeostasis. Differences in the ADAMTS-mediated proteolysis of aggrecan and versican was shown to cause postnatal closure of the umbilical artery, but not vein, preventing excessive postnatal bleeding across mammalian species (34).

The pulmonary and fetal circulation

The pulmonary circulation

Anatomy

The adult human circulatory system consists of two serially connected circuits, the *systemic* and *pulmonary circulation,* where blood is impelled forward by the *left ventricle* (LV) and *right ventricle* (RV). The trachea, pulmonary vessels and pulmonary parenchyma are provided with nourished and oxygenized blood through a segment of the systemic circulation, termed the *bronchial circulation.*

The LV and RV generate force to drive blood forward through the *arteries* of the vascular system, so that functional exchange can occur throughout the *capillary network.* The *venous system* returns blood to the opposing side of the ventricle of origin, to the *left atrium* (LA) and *right atrium* (RA) of the heart. Bronchial veins from larger airways and the hilum drain into the RA via the azygos system, whereas intrapulmonary bronchial arteries connect to the pulmonary venous system that drain into the LA.

Hemodynamics

The *mean arterial pressure* and *mean pulmonary arterial pressure* (mPAP) required to maintain homeostasis is different for the systemic (<100 mmHg) (53) and pulmonary (<20 mmHg) (54) circulation, due to the difference in total peripheral vascular resistance between the two circuits.

In the adult circulation, the inherent *systemic vascular resistance* is ten-fold greater than the *pulmonary vascular resistance* (PVR) due to the increased muscularization of systemic arteries, and that the systemic capillary network supplies a greater number of end-organs (55). PVR is approximated using the hydraulic equivalent of Ohm´s law (56):

$$[\mathrm{PVR}] = \frac{\text{transpulmonary gradient}}{\text{pulmonary blood flow}} = \frac{(\mathrm{mPAP} - \mathrm{PAWP})}{\mathrm{CO}} = \mathrm{mmHg}^{-1} \cdot \mathrm{min/L} =$$

$$= 1 \textit{ Wood Unit (WU)}$$

The *pulmonary arterial wedge pressure* (PAWP) is an estimation of the *left atrial pressure* (LAP), where the difference between mPAP and PAWP reflects the drop in driving pressure that occurs over the pulmonary circuit. Although *cardiac output* (CO) is an important determinant of vascular resistance, it is presumed to be relatively constant between the LV and RV. The transpulmonary gradient is therefore the main reason underlying the difference in vascular resistance between the adult systemic and pulmonary circulation.

Importantly, a variation in CO is observed in children and ranges from <0.5 L/min in neonates to ~4-5 L/min in adolescents (57). This generates significant variability in the estimation of PVR, based on the assumption that the transpulmonary gradient does not change with body size and motivates normalization of PVR against body surface area in pre-pubescent children. As a result, *cardiac index* (CI) is used instead of CO, for the estimation of an *indexed pulmonary vascular resistance* (PVRi) in this population (56):

$$[\mathrm{PVRi}] = \frac{\text{transpulmonary gradient}}{\text{pulmonary blood blod / body surface area}} = \frac{(\mathrm{mPAP} - \mathrm{PAWP})}{\mathrm{CI}} =$$

$$= \mathrm{mmHg}^{-1} \cdot \mathrm{min/L} \cdot \mathrm{m}^2 = 1 \; \textit{indexed Wood Unit (WU} \cdot \textit{m}^2\textit{)}$$

Levels of mPAP and PAWP appear to be relatively stable at rest, independent of both age and sex (54). Pathology of the left heart, pulmonary vasculature and parenchyma are all associated with elevations in PAWP and mPAP (58), however fluctuations in pulmonary hemodynamics are not exclusively associated with disease.

During exercise an increased variability in both mPAP and PAWP appears to be dependent on age and the degree of exercise, as mPAP levels >30 mmHg were reported in 47% of individuals ≥50 years during mild exercise (54). In this setting of increased CO, the recruitment of dormant arteriovenous *intrapulmonary shunts* limits the rise in local mPAP, protecting the pulmonary vascular bed from hypertensive injury (59). Shunting from the pulmonary to the bronchial circulation has also been demonstrated in the setting of developmental lung disease in neonates (60, 61). Whether physiological arteriovenous shunting also involves *broncho-pulmonary anastomoses*, or not, is unknown.

In fetal circulatory physiology, a high PVR ensures that blood can be shunted past the pulmonary circulation, allowing for efficient distribution of oxygenated blood.

The fetal circulation

Anatomy

Throughout intrauterine development, gas exchange occurs at the level of the *placenta* instead of in the constricted, high resistance, pulmonary vascular bed (Figure 6). Oxygenated blood (~70-80% saturation) from the placenta reaches the fetus via the single *umbilical vein*, where it mixes with blood from the portal vein. Approximately 50% of this total volume is shunted past the liver via the *ductus venosus* to directly reach the *inferior vena cava* and the RA (62). A high volume of venous return to the RA causes a right-to-left gradient across the *foramen ovale*,

which connects the RA and LA. Within the RA, the Eustachian valve preferentially diverges oxygenated blood from the ductus venosus into the LA (63). From that point, it is propagated into the ascending *aorta* (Ao) to supply the upper body and brain with relatively well-oxygenated blood (~65% saturation) (64).

The remainder of the venous return, mainly deoxygenated blood from the *superior vena cava,* flows from the RA into the RV and is ejected into the *main pulmonary artery* (MPA). However, only 10-25% of the RV CO reaches the pulmonary vascular bed (65). The majority of this deoxygenated blood (~35% saturation) is diverted through the *ductus arteriosus* (DA) into the descending Ao due to the high resistance presented by the pulmonary vascular bed (63). Aside from perfusing the abdomen and lower body, this portion of blood reaches the placenta via the two *umbilical arteries* (Figure 6). The placenta possesses a high-flow, low-resistance, low-velocity vascular bed, similar to the adult pulmonary circulation, which allows for efficient gas and nutrient exchange with maternal blood.

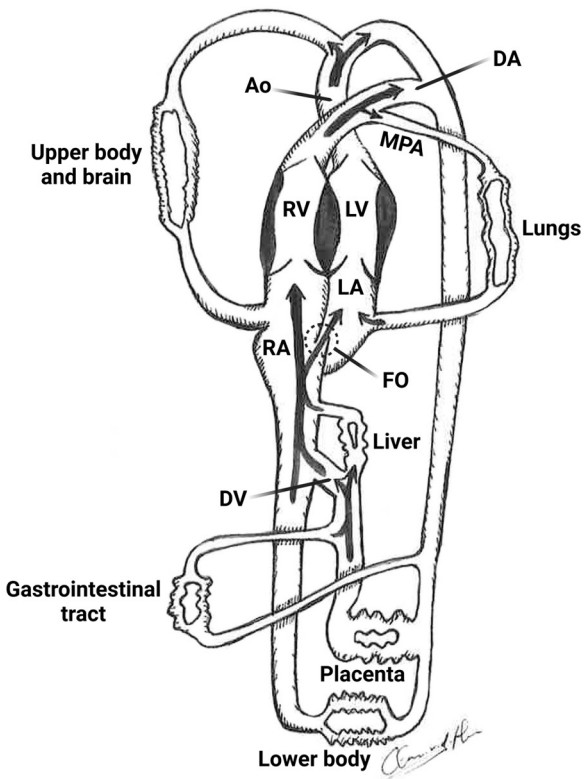


Figure 6. Anatomy and direction of blood flow in the fetal circulation
Ao, aorta; DA, ductus arteriosus; MPA, main pulmonary artery; LV, left ventricle; RV, right ventricle, LA, left atrium; RA, right atrium; FO, foramen ovale; DV, ductus venosus

Transition from fetal to neonatal circulatory physiology

The transition from fetal to neonatal circulatory physiology is a transient event which encompasses a series of hemodynamic and anatomical alterations that allow for adaptation to life outside the womb (63). It begins as the newborn inflates its lungs with air, opening the pulmonary vascular bed. This rapid drop in PVR increases pulmonary blood flow, and thus venous return to the LA. The resulting increase in LAP reverses the shunt over the atrial septum and closes the foramen ovale.

Simultaneously, as the umbilical cord is clamped and tied of, the connection to the placental vascular bed is broken. Decreased blood flow through the ductus venosus is believed to predispose its closure within a few days after birth (66), however the mechanisms involved are incompletely described (67).

Relative hypoxia in fetal blood, as well as circulating prostanoids, maintains DA patency during the intrauterine period. Increased oxygenation and degradation of placental prostanoids predisposes rapid functional closure of the DA, most often within three days of birth, as determined by echocardiography (68, 69). Anatomical closure of the DA is completed after 3-6 months, following fibroelastic vascular remodeling and collagen deposition to form the *ligamentum arteriosum* (LA) (51).

Inability to close any of the three fetal shunts causes persistent postnatal shunting in the neonate, often in a reverse direction to the intrauterine shunt as the transition to neonatal physiology has altered the hemodynamic equation.

Inability to close the DA is associated with the development of chronically elevated PVR, as left-to-right shunting over the DA exposes the pulmonary vascular bed to systemic arterial pressures. Vascular remodeling underlying functional and anatomical DA closure will be the focus of the next section.

The ductus arteriosus

An enigmatic structure, the DA has been the subject of investigation for centuries and the complete mechanisms underlying the closure of this muscular artery have yet to be fully understood (70). Failure to close the DA, *patent ductus arteriosus* (PDA), is observed in both preterm and term infants with a strong positive correlation to lower gestational age and weight at birth (Figure 7) (68). PDA is a major cause of mortality and morbidity in preterm infants (71, 72), however, maintaining DA patency is lifesaving in the setting of DA-dependent congenital heart defects in the same population. Modulation of DA patency is therefore an important cornerstone of neonatal intensive care. Rapid physiological closure of the DA can be attributed to two synergistic mechanisms: VSMC vasoconstriction (*functional closure*) and intrauterine formation of *intimal cushions* (*anatomic closure*).

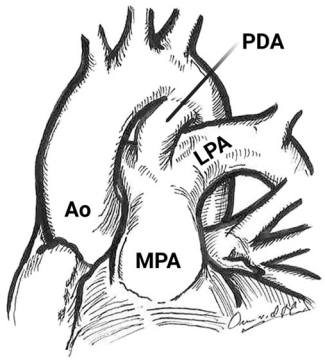


Figure 7. Anterior view of the PDA and great vessels
Ao, aorta; MPA, main pulmonary artery; LPA, left pulmonary artery; PDA, patent ductus arteriosus

Functional closure

Increased postnatal pulmonary blood flow causes an elevation of PaO_2 in neonatal blood, resulting in DA vasoconstriction through several pathways (73). Prostanoids that contribute to intrauterine vasodilation, especially prostaglandin E_2 (PGE_2), are reduced after birth as the placental source is removed and PGE_2 is metabolized in the lung (73, 74). Increased PaO_2 furthermore causes decreased PGE_2 sensitivity in the DA, thus further inhibiting vasodilation (75). Unfortunately, the DA can reopen if the neonate is under hypoxic stress or experiences a diminished response to PGE_2 withdrawal (76).

Anatomical closure

In small mammals, such as rodents or rabbits, vasoconstriction alone is sufficient to achieve complete postnatal functional closure of the DA, whereas the greater diameter of the DA in large mammals appears to require a second mechanism for efficient DA obstruction (77-79).

Intimal cushions are GAG-rich, intimal swellings that protrude into the lumen (Figure 8) and enable finalization of DA closure in humans and other large mammals, such as dogs (80), pigs and lambs (77). Formation of intimal cushions in humans begins during the second trimester with the fragmentation of the DA IEL and transmigration of VSMCs from the tunica media into the *subendothelial region* (81, 82). Interestingly, competitive interaction of CS with a 67 kDA elastin-binding protein was shown to impair elastin assembly in the lamb DA (83, 84). Deposition of HA, induced by PGE_2-signaling through its EP4 receptor (74, 85, 86), is believed to be a key event mediating VSMC transmigration and subsequent transdifferentiation to a secretory phenotype (81). A complex of EC-derived versican and fibulin-1 was shown to be important for DA VSMC migration in intimal cushion formation (87). Four morphological maturation stages of the DA,

independent of infant age, were described by Gittenberger-de Groot *et al* in 1980 (82) and remains relevant to date (51, 78, 88, 89) (Figure 9).

Given the role of ADAMTS versicanases and aggrecanases in rapid umbilical artery postnatal closure (34), lecticans and their turnover were highlighted as a potentially interesting target for future studies of DA closure. In line with this, *ADAMTS9* was consequently shown to be upregulated across several studies comparing the DA and Ao transcriptome (90). In the PDA, lack of IEL fragmentation and underdeveloped intimal cushions are observed (91). Following successful functional DA closure, apoptosis of medial VSMCs is believed to contribute to the irreversible closure of the DA (92). Over the weeks and months following birth, obliterative remodeling of the DA continues, with degeneration of VSMCs, collagen deposition and fibrotic turnover with focal calcification (93). The LA is the remnant of this process.

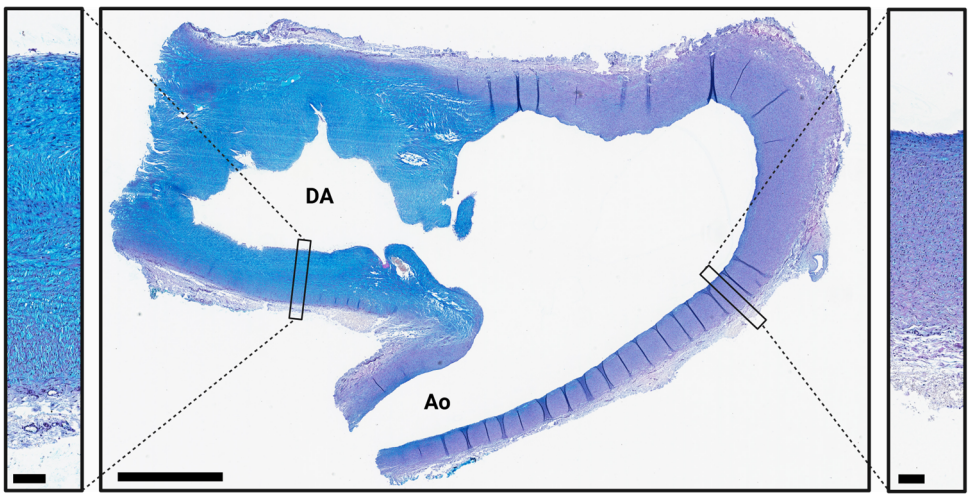


Figure 8. The ductal and aortic vessel wall
The wall of the Ao, the largest elastic artery in our body, is shown to the right. Few GAGs are accumulated in the healthy aortic wall. The wall of the DA, the largest muscular artery in our body, with well-developed intimal cushions is shown to the left. Note the mucoid lakes, large pools of GAGs present in the closing DA, visualized through Ab/PAS staining (GAGs stain light blue). Scale bar for the central panel is 2 mm, and 200 µm for the magnified panels. *DA, ductus arteriosus; Ao, aorta; GAG, glycosaminoglycan; Ab/PAS, Alcian blue/Periodic acid-Schiff.*

Manipulation of ductus arteriosus patency

Although some PDAs undergo spontaneous closure, especially in preterm neonates that reach term age outside of the womb, pharmacological or surgical intervention against the PDA is necessary in some cases.

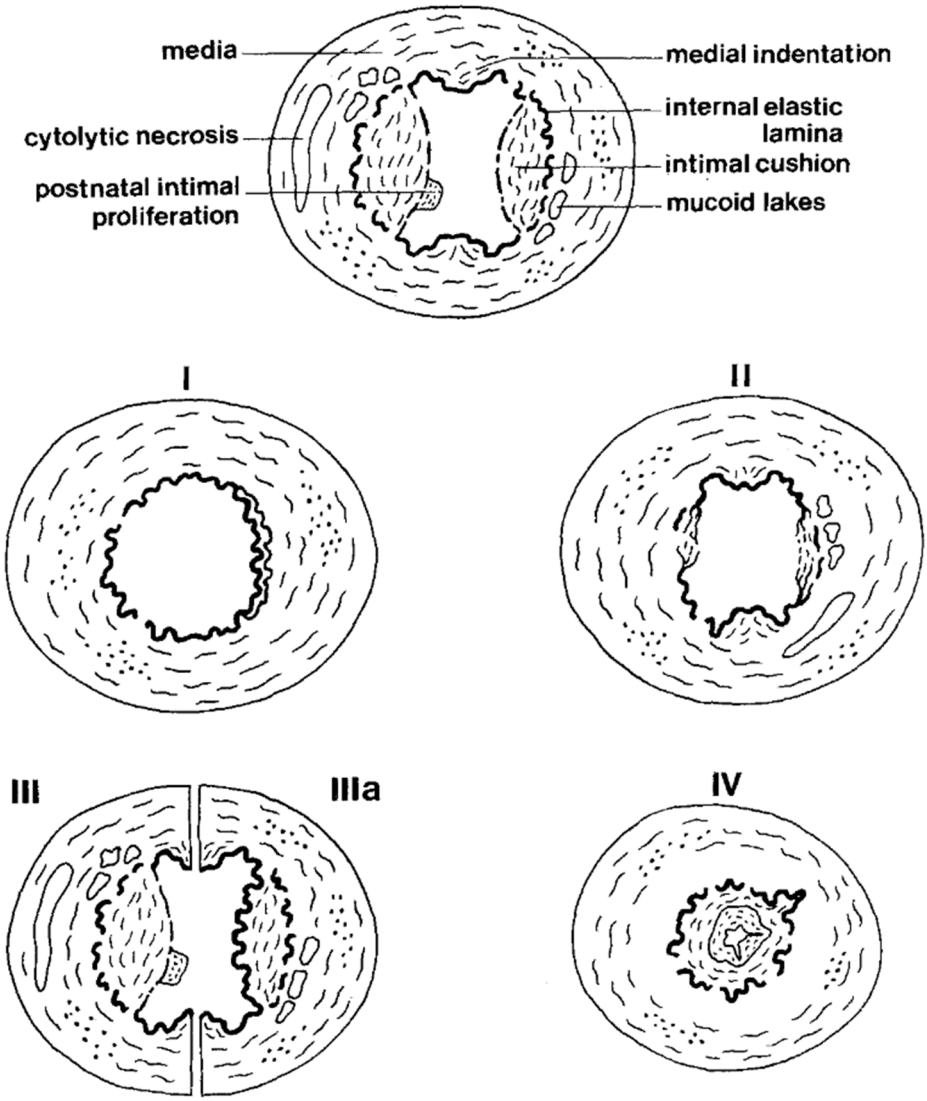


Figure 9. Morphological maturation stages of the DA

In stage I, the DA resembles a large muscular artery with a single IEL that has multiple, small, interruptions. Intimal cushion formation begins during stage II and progresses until stage III which is commonly observed in all term infants: cytolytic necrosis and mucoid lakes are signs of imminent DA closure. Stage IIIa represents the PDA, which lacks many features of well-developed intimal cushions. In stage IV, intimal cushions fuse and postnatal intimal proliferation fills the remaining lumen. Reprinted from Gittenberger-de Groot AC *et al*, Journal of Pediatrics 1980, with permission (82). *IEL, internal elastic lamina; DA, ductus arteriosus; PDA, patent ductus arteriosus.*

Cyclooxygenase inhibitors are non-steroidal anti-inflammatory drugs (NSAID) which block prostaglandin synthesis and can be used to enforce vasoconstriction of the DA (94). However, therapy-resistant PDAs are observed in close to 30% of preterm infants (95). Furthermore, treatment with NSAID is associated with serious complications, including renal failure, necrotizing enterocolitis and platelet abnormalities (96). Indomethacin (intravenous) and ibuprofen (oral) are the most common drugs used, where ibuprofen is considered to have fewer side effects (97). Acetaminophen, with less effect in immature vessels (98), has been proposed as an alternative in patients with contraindications to NSAID treatment (95, 97). Surgical ligation or percutaneous closure of the PDA may be necessary if pharmacotherapy is insufficient or contraindicated.

Currently, a PDA can only be kept open with continuous intravenous infusion of PGE_1, although treatment is associated with peripheral vasodilation, apnea and fever (99).

Despite suboptimal efficacy, and the multiple complications observed with current pharmacological therapies, few efforts have been made in the last decades to develop new drugs to manipulate DA patency (97). A greater understanding of the mechanisms and modulators underlying intimal cushion formation in humans could uncover new targets for PDA therapy, to avoid development of *pulmonary hypertension* and other feared complications in neonates.

Pediatric pulmonary hypertension

Pulmonary hypertension (PH) is a heterogenous condition with an estimated global prevalence of ~1% (100, 101). No pharmacological therapy halts PH progression, which eventually causes end-stage RV failure and death.

Common symptoms of PH include fatigue, exertional dyspnea, pre-syncope and syncope and are related to cardiovascular, rather than ventilatory or muscular, limitations (102). Failure-to-thrive is a non-specific sign in children that should raise clinical suspicion of PH (103). Abdominal distension and ankle edema develop as signs of RV failure but are rarely observed in children. PH is defined by the elevation of mPAP >20 mmHg at rest, determined via right heart catheterization (RHC) (54, 104). It is, however, essential that results of hemodynamic testing are interpreted in its clinical setting to properly phenotype the disease. Correct clinical phenotyping is vital for risk stratification, determining prognosis and optimizing treatment of PH (105-107).

Classification

The World Symposium on Pulmonary Hypertension guidelines pools etiologies of PH into rare vascular disorders (PH group 1 and 4, Table 1) and PH associated with more common cardiopulmonary disorders such as *left heart disease* (LHD) (PH group 2) and lung disease and/or hypoxia (PH group 3) (100, 104). PH group 5 gathers unclear or multifactorial causes of pulmonary vascular disease (100, 104) (Table 1).

PH from all groups are observed in children and neonates, although *pulmonary arterial hypertension* (PAH) (PH group 1) is a more predominant cause of disease than in adults (103, 108). *Congenital heart disease* (CHD) is the dominant etiology of neonatal and pediatric PH in both groups 1 and 2 (109). *PH associated with LHD* (PH-LHD) (PH group 2) presents unique challenges in the diagnostics, clinical management and treatment of PH in the pediatric population (103, 110, 111), especially in the setting of *end-stage heart failure* (112).

Pulmonary arterial hypertension

PH group 1, PAH, involves vasoconstriction and obliterative remodeling of arterioles and small pulmonary arteries (113, 114) and conveys a poor prognosis (101), especially in children (115, 116). *Idiopathic PAH* (IPAH), *hereditary PAH* (HPAH) and *PAH associated with CHD* constitute some of the main causes of PH in children (Table 1) (117-119).

Table 1. Clinical classification of PH.
Clinical classification of PH, based on the 7th World Symposium on Pulmonary Hypertension, with PH group 1 and 2 subtypes outlined. The clinical sub-classification of CHD-associated PH in neonates and children (A-E under 1.4.4), as suggested by the pediatric task force of the 7th World Symposium on Pulmonary Hypertension, is shown. Adopted from (104) and (111). *PH, pulmonary hypertension; PAH, pulmonary arterial hypertension; HIV, human immunodeficiency viruses; CHD, congenital heart disease; ASD, atrial septal defect; PVOD, pulmonary veno-occlusive disease; PCH, pulmonary capillary hemangiomatosis.*

Group 1: PAH
1.1 Idiopathic
1.1.1 Long-term responders to calcium channel blockers
1.2 Heritable
1.3 Associated with drugs and toxins
1.4 Associated with:
1.4.1 connective tissue disease
1.4.2 HIV infection
1.4.3 portal hypertension
1.4.4 CHD
A. Eisenmenger syndrome
B. left-to-right shunt, correctable (1) or not correctable (2)
C. coincidental shunts, including all isolated ASDs in childhood
D. corrected CHD
E. without prolonged initial shunt, e.g. neonatally corrected transposition
1.4.5 schistosomiasis
1.5 PAH with features of venous/capillary (PVOD/PCH) involvement
1.6 Persistent PH of the newborn
Group 2: PH associated with left heart disease
2.1 Heart failure
2.1.1 with preserved ejection fraction
2.1.2 with reduced, or mildly reduced, ejection fraction
2.1.3 cardiomyopathies with specific etiologies
2.2 Valvular heart disease
2.2.1 aortic valve disease
2.2.2 mitral valve disease
2.2.3 mixed valvular disease
2.3 Congenital/acquired cardiovascular conditions leading to post-capillary PH
Group 3: PH associated with lung diseases and/or hypoxia
Group 4: PH associated with pulmonary artery obstructions
Group 5: PH with unclear and/or multifactorial mechanisms

Hemodynamic definition

In addition to elevated mPAP >20 mmHg, *pre-capillary PH* observed in pediatric PAH entails an increase in PVR ≥3 WU·m^2, and a PAWP ≤15 mmHg (100, 104), thus lacking elevated LAP.

Pathobiology

Although there are patients presenting with pure HPAH, IPAH or PAH related to systemic-to-pulmonary shunts in CHD, the complex interaction between

hemodynamics, pathogenic variants (120) and vascular, as well as parenchymal, remodeling in disease development is increasingly being recognized (104, 113).

In PAH, vasoconstriction of pulmonary arterioles is favored due to pulmonary EC dysfunction, resulting in an imbalance of vasoactive substances (113). Such EC injury is furthermore believed to be important for the initiation of obstructive vascular remodeling in distal muscularized pulmonary arteries (70-500 µm in diameter) (113). In the setting of systemic-to-pulmonary shunting in CHD, it is believed that altered shear stress due to increased pulmonary blood flow may trigger EC dysfunction (117). A pre-tricuspid shunt, such as an *atrial septal defect*, causes an increase in pulmonary blood flow but does not convey systemic pressure into the pulmonary circulation. A post-tricuspid shunt, such as a *ventricular septal defect*, is therefore believed to cause a greater amount of damage to the pulmonary vascular bed, as it directs both high flow and systemic pressure towards the pulmonary circulation (121). Loss-of-function in bone morphogenetic protein receptor 2, found in 70-80% of patients with HPAH, generates a similar EC dysfunction. However, only 20% of heterozygous mutation-carriers present with PAH, with an increased penetrance of the genotype amongst females. This indicates that an additional injury or trigger, unknown to date, is required to initiate the development of PAH.

The arteriopathy of PAH was first described in detail, and graded, in 1958 by Heath and Edwards (122) based on the intimal reaction and state of the media of small arteries and arterioles (Table 2).

Table 2. The Heath and Edwards classification of pulmonary arterial vascular remodeling.
Note that the histopathological classification is based on PAH associated with CHD ("*large ventricular septal defects and functionally related diseases*"). Reprinted from Heath D, Edwards JE, Circulation 1958, with permission (122).

TABLE 1.—*Basis of Grades of Hypertensive Pulmonary Vascular Disease Found in Association with Large Ventricular Septal Defects and Functionally Related Diseases*

	Grade of hypertensive pulmonary vascular disease					
	1	2	3	4	5	6
Type of intimal reaction	←—None—→	←———	———	—Cellular—	———	———→
			←———	— Fibrous and fibroelastic—	———	———→
				←——— "Plexiform lesion" ———		———→
State of media of arteries and arterioles	←———	———	——— Hypertrophied ———		———	———→
			←———	— Some generalized dilatation ———		———→
				←——— Local "dilatation lesions" ———		———→
					←——— P H * ———	———→
						←—N A †—→

* Pulmonary hemosiderosis associated with distended, thin-walled, arterial vessels throughout the lung.
† Necrotizing arteritis.

From this classification, three main types of vascular changes are identified: *hypertrophy of the muscularized tunica media*, *cellular intimal reactions/occlusive lesions* and *plexiform lesions* (Figure 10). The latter is the pathognomonic lesion for advanced PAH, where certain sub-types have been proposed to contain pressure-relieving shunts to the bronchial circulation (61, 123, 124). PAH vascular lesion progression follow a stepwise development, although "early" lesion types can be observed also in more advanced histopathological stages of the disease (122).

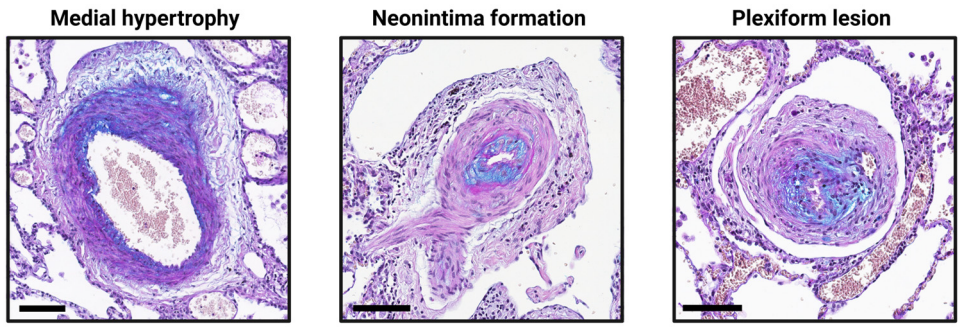


Figure 10. Pulmonary arteriopathy in PAH
The three main lesion types in PAH. Note the accumulation of GAGs, visualized through Ab/PAS staining (GAGs stain light blue), throughout all lesion types. Scale bars are 100 µm. *PAH, pulmonary arterial hypertension; GAG, glycosaminoglycan; Ab/PAS, Alcian blue/Periodic acid-Schiff.*

EC dysfunction generates a pro-inflammatory and apoptosis-resistant EC phenotype, which in combination with excessive ECM deposition and turnover, permits migration and proliferation of VSMCs and adventital fibroblasts (113). Growth factors, such as fibroblast growth factor 2, harbored within PGs, are released upon proteolytic cleavage and were shown to increase pulmonary arterial VSMC proliferation and contribute to PAH disease progression (125). Furthermore, previous work from our group has shown that increased expression and deposition of versican in IPAH vascular lesions is mediated by VSMCs in response to hypoxia (126). The presence of aggrecan and different isoforms of versican in PAH vascular remodeling, as well as their interacting partners and associated proteases, remained to be determined at the time of initiation of this thesis project.

Pulmonary hypertension due to left heart disease

Constituting 65-80% of cases across all age groups, PH-LHD (PH group 2) is arguably the most common form of PH (127). Patients with PH-LHD are, however, often under-represented in international registries as they often are managed by heart failure/transplant teams, rather than PH teams.

Hemodynamic definition

All etiologies of PH-LHD (Table 1) converge at the elevation of LAP (Figure 11). The resulting pulmonary congestion causes isolated *post-capillary PH*, displaying PWAP >15 mmHg and PVR <3 WU·m^2 in children, aside from elevated mPAP >20 mmHg (100, 104). With persisting post-capillary PH, secondary pulmonary arterial remodeling predisposes development of *combined post- and pre-capillary PH*, with elevation of PVR to ≥3 WU·m^2 (112).

Pathobiology

The development of PH-LHD is multifactorial and incompletely understood. In LHD with acute onset, the sudden increase in pulmonary venous pressure causes pulmonary edema (112). Long-standing pulmonary congestion causes thickening of the alveolar-capillary membranes, but also transmits the pressure to the arterial side, resulting in EC damage and excess vasoconstriction (113). Pulmonary artery medial hypertrophy, and in rare cases neointima formation, are observed. Plexiform lesions are usually not observed in PH-LHD.

Reflex-like vasoconstriction of precapillary pulmonary arteries, elevating the transpulmonary gradient and thus PVR, is believed to be mediated via the *Hermo-Weiler reflex* (128) (extension of the Kitaev reflex) in the setting of LHD. Mechanoreceptors in the LA have been suggested to be responsible for this effect, preventing hypertensive damage to the pulmonary capillaries, although the underlying mechanism remains unclear (129). The Hermo-Weiler reflex could be the explanation for "out-of-proportion" PH observed in mitral stenosis (128) and explains why the severity of post-capillary PH is unrelated to the grade of aortic valve stenosis (130). More important, in children with mitral stenosis and pulmonary vascular remodeling, elevation of mPAP was often reversed after surgical intervention (131).

Diagnostics and hemodynamic evaluation

Due to its relative rarity and non-specific symptoms, there is a risk for both patients and doctors delay in the diagnosis of PH. In the setting of predisposing conditions, such as CHD or LHD, practitioners should have an increased awareness of the risk for PH development.

RHC remains the gold standard in PH diagnostics (104), although alternative invasive and non-invasive diagnostic tools exist. In small (<2-5 kg), critically ill, or vulnerable children RHC is not always feasible (111), why medication in some cases is initiated based on secondary signs of PH (109). Such signs include, but are not limited to, RV hypertrophy, enlargement of the RA, significant tricuspid insufficiency and jet velocity, septal flattening and increased velocity in the MPA. These signs may be detected via non-invasive approaches such as

echocardiography, electrocardiogram, radiography/computed tomography of the chest and ventilation-perfusion scintigraphy and are vital in determining the sub-type of PH. Both known and *de novo* genetic variants associated with PAH are enriched in children and neonates, and genetic testing with either exome or genome sequencing is recommended for several sub-types of PAH in both children and adults (132).

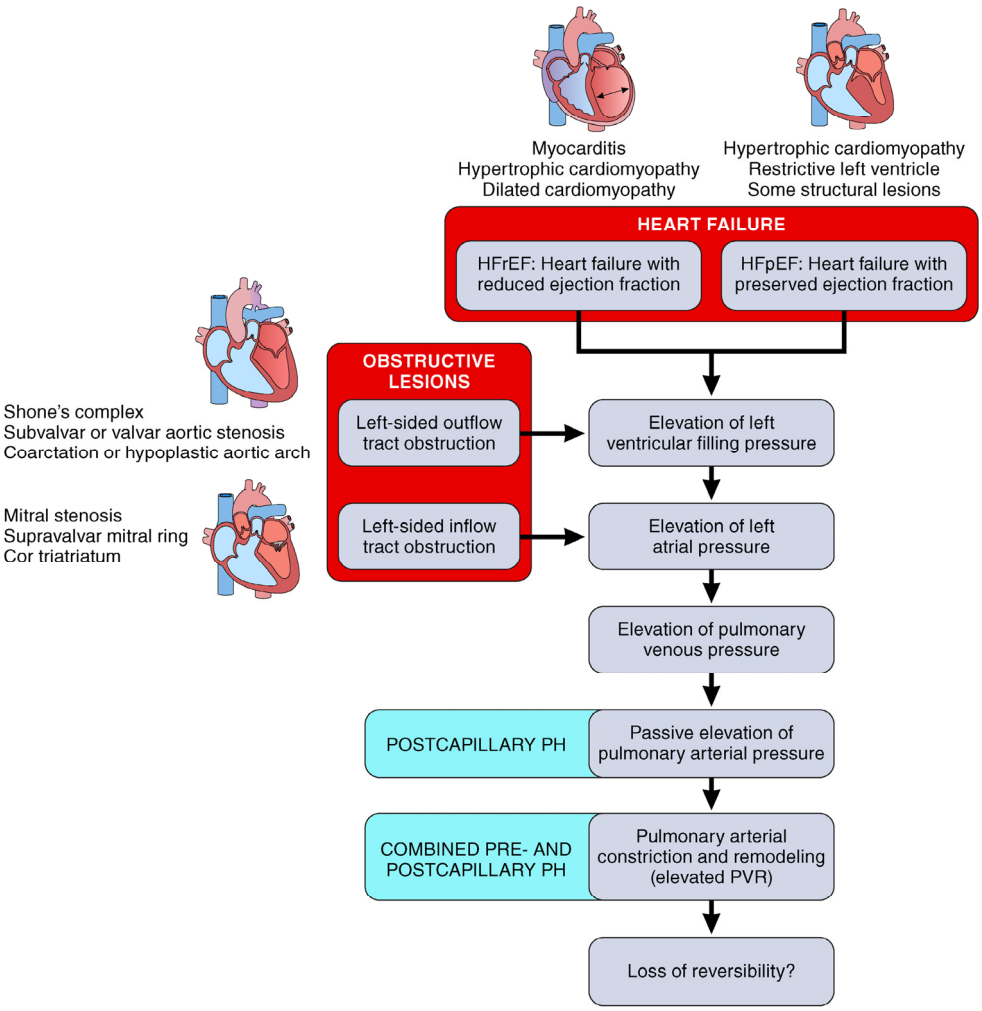


Figure 11. Mechanisms of PH-LHD development in children and adolescents.

Reprinted with permission from Circ Heart Fail. 2025;18:e000086 doi: 10.1161/HHF.0000000000000086 ©2025 American Heart Association, Inc. *HFrEF, heart failure with reduced ejection fraction; HFpEF, heart failure with preserved ejection fraction; PH, pulmonary hypertension; PVR, pulmonary vascular resistance.*

Right-heart catheterization

Through the introduction of a *Swan-Ganz catheter* via the jugular or femoral vein, the RA and RV pressure can be measured. Advancing the catheter further allows for measurement of mPAP in the MPA. PAWP is determined through the inflation of a balloon in a distal pulmonary arterial branch, temporarily obstructing the vessel to measure the post-stenotic pressure.

To calculate PVR and PVRi, CO is either calculated via the *Fick principle* or measured through *thermodilution*. The Fick principle states that if oxygen consumption and the arteriovenous oxygen difference is known, CO can be calculated, but assumes constant levels of hemoglobin and that no oxygen is consumed by the pulmonary tissue (133). The oxygen consumption can either be assumed (indirect Fick method) or precisely determined (direct Fick method) through a labor-intensive process seldomly applied in children. Thermodilution determines CO based on the temperature change of an injected cold saline solution.

Although the direct Fick method and thermodilution measurements display good correlation in children (57), disagreement between these two methods and the indirect Fick method is significant (134). The indirect Fick method was shown to overestimate CI by ~20% compared to thermodilution in small children (135), which inevitably affects reliability of calculated PVRi. Currently, the preferred method for determining CO in children with PH group 1 and 2 is a subject of debate.

Several other factors influence the reliability of calculated PVRi. Children are often under sedation or general anesthesia during RHC, increasing both the procedural risk and the potential confounders in hemodynamic measurements. Additionally, estimations of CO are sensitive to anatomical defects and shunts, and is less reliable if CO is low (134).

Table 3. Criteria for positive AVT

AVT, acute vasoreactivity testing; REVEAL, Registry to Evaluate Early and Long-term PAH disease management; mPAP, mean pulmonary arterial pressure; CI, cardiac index; PVR, pulmonary vascular resistance; SVR, systemic vascular resistance; CO, cardiac output.

REVEAL pediatric critera by Barst et al. (136)	
I.	A decrease in mPAP of ≥20%,
II.	and an unchanged, increased, or <10% decrease in CI,
III.	and a decreased or unchanged PVR/SVR ratio.
Adult criteria by Sitbon et al. (137)	
I.	Reduction of mPAP of ≥10 mmHg,
II.	to reach an absolute mPAP ≤40 mmHg,
III.	and an increased or unchanged CO.

Acute vasoreactivity testing (AVT) is performed during RHC to investigate responsiveness to vasodilator therapy. A positive AVT in children with PH group 1 is most appropriately determined with the *Sitbon* (138) or *modified Barst* (136)

criteria (Table 3), whereas there are no widely accepted definitions of positive AVT in pediatric PH group 2. Additionally, indications for AVT are not standardized in children and adherence to the Barst and Sitbon criteria seems poor (138). AVT is used in risk stratification prior to cardiac surgery and to identify long-term responders to calcium-channel blockers (115, 137) (see Table 1, line 1.1.1). The most common vasodilator used for AVT in children is inhaled *nitric oxide* (NO) with or without the addition of oxygen (138). In children with PH-LHD, AVT carries with it the risk of pulmonary edema, although consensus on relative and absolute contraindications and standardized protocols are lacking (112).

Treatment

Therapies for pediatric PH are based on expert opinions, extrapolation of data from adult clinical trials or small pediatric cohorts, which poses a challenge for practitioners globally (139). In children, the most efficient way to prevent and potentially reverse pulmonary vascular remodeling is the correct diagnosis, as well as medical, interventional and surgical treatment, of underlying CHD and acquired heart disease. The armamentarium against PH mainly comprises medical therapy, although interventional and surgical elements can be involved at later stages if an *atrial septostomy* or *Potts shunt* is needed to relieve the RV (111). In rare cases, children with PH require a *lung transplantation*. Medical therapies for pediatric PH will be outlined in this section.

Pulmonary vasodilator therapy

To reduce mPAP, vasodilation of pulmonary arterioles can be induced through the modification of three pathways affecting VSMC contractility (Figure 12). Medications can be inhaled, taken orally or administered via subcutaneous or intravenous injection/infusion. Importantly, pulmonary vasodilators have not been shown to modify or reverse vascular remodeling, why they are mainly considered a symptomatic treatment that protects the RV.

The endothelin pathway is activated through interaction of endothelin-1 with endothelin receptor A and B on VSMCs, inducing vasoconstriction. Endothelin receptor antagonists macitentan and bosentan induce vasodilation through dual antagonism of endothelin receptor A and B, whereas ambrisentan is a selective antagonist for endothelin receptor A (140).

Activation of soluble guanylate cyclase by NO leads to an increase in the potent VSMC vasodilator cyclic guanosine monophosphate. In addition to supplementing the *nitric oxide pathway* with exogenous NO, the pathway can be stimulated via enhancement of soluble guanylate cyclase activity with riociguat. Treatment with *cyclic guanosine monophosphate-specific phosphodiesterase type 5 inhibitors*

(PDE5i), most often sildenafil or tadalafil, is the most common approach to achieve pulmonary vasodilation in Swedish children (109).

The prostacyclin pathway mediates vasodilation through the increase of cyclic adenosine monophosphate, deriving from the interaction of EC-derived prostacyclin with prostaglandin receptors. The prostacyclin analogue epoprostenol is given as a continuous intravenous infusion due to its short half-life (3-5 min) and requires careful monitoring. Similar effects are achieved through iloprost for inhalation, and treprostinil for subcutaneous or intravenous infusion. Selexipag (oral administration) has a similar mechanism but is a non-prostanoid receptor agonist.

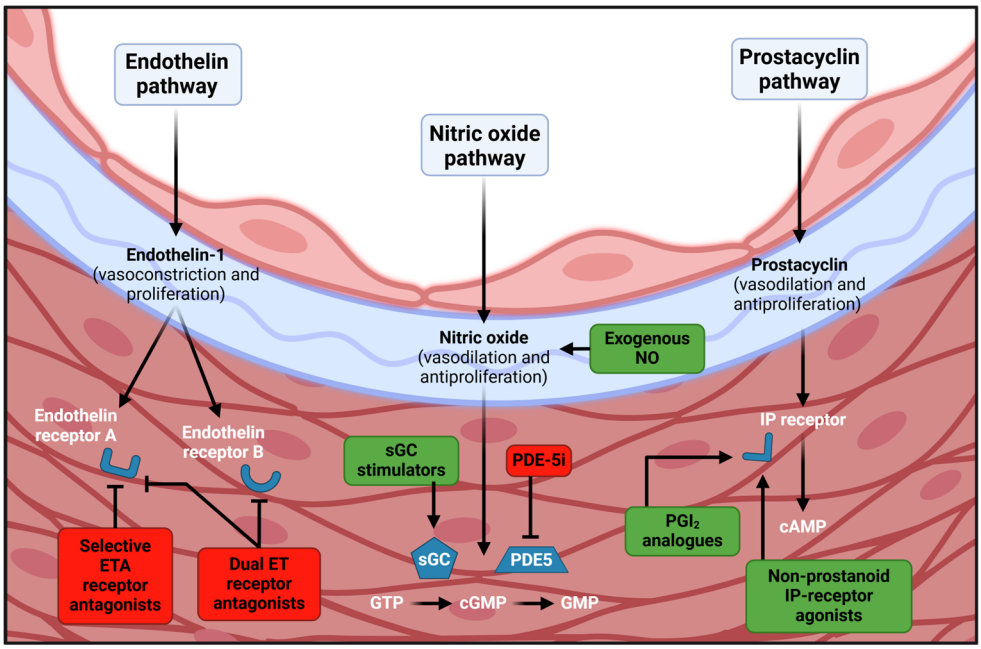


Figure 12. Pathways and drugs modulating vasoreactivity of pulmonary arteries
ETA, endothelin receptor A; ET, endothelin receptor; sGC, soluable guanylate cyclase; NO, nitric oxide; PDE-5i, cGMP-specific phosphodiesterase type 5 inhibitors; PDE-5, cGMP-specific phosphodiesterase type 5; GTP, guanosine triphosphate; cGMP, cyclic guanosine monophosphate; GMP, guanosine monophosphate; PGI_2, prostacyclin I_2; IP, prostacyclin I_2 receptor; cAMP, cyclic adenosine monophosphate.

Oxygen and nitric oxide

Potent vasodilators, albeit difficult to administer outside of the hospital setting, oxygen and inhaled NO are often used in the acute or post-operative phase of PH treatment in children managed at the intensive care unit.

Calcium channel blockers

Calcium channel blockers act on ion channels in VSMCs, modulating the influx of Ca^{2+}, to reduce vasoconstriction and are mainly used for the treatment of systemic hypertension. In subsets of both adult and pediatric IPAH/HPAH patients, with positive AVT, calcium channel blocker treatment has been associated with significantly improved long-term survival (111, 138).

Sotatercept

The pharmacodynamics of sotatercept differ from those of the other PAH-specific drugs, as it aims to restore the balance between pro- and anti-proliferative bone morphogenetic protein pathways through its function as a ligand trap. It was recently approved for use in adult PAH in the US, and clinical trials in children are ongoing (111).

Pulmonary hypertension and heart transplantation in children

Severe PH with fixed, unresponsive, PVRi is associated with acute RV failure and increased early mortality after *pediatric heart transplantation* (pHTx), as the un-adapted RV of the donor heart is forced to work against a pulmonary circuit with high resistance (141). High PVRi can accordingly be considered an absolute or relative contraindication for isolated pHTx (142-144), depending on overall clinical status and the level of PVRi elevation. Selected patients may be eligible for combined heart-lung transplantation instead. Given the inferior long-term outcomes of combined heart-lung transplantation (145, 146), optimal management and monitoring of PH is required to allow for isolated pHTx if deemed feasible (112). Furthermore, pediatric PH-LHD was shown to normalize post-pHTx in most cases (147). Pediatric patients with end-stage heart disease and PH is a growing population, as improved diagnostics and management of heart disease have led to improved chance of survival (110).

The interpretation of pulmonary hemodynamics in the presence of heart failure is complex, especially since measurements obtained during RHC not always are relevant or true to the dynamic physiology of the growing child (112). Hemodynamic cutoffs to guide the timing and interpretation of AVT exist for adults (148), but not in the pediatric population. The literature investigating optimal PVRi cutoffs in children listed for pHTx is ambiguous and non-conclusive (149-155). Due to the lack of evidence, consensus guidelines and hemodynamic cutoffs to preclude children with PH and end-stage heart disease from isolated pHTx are lacking.

The following section will, in short terms, introduce the concept of pHTx for end-stage heart disease. Additionally, it will give an overview of pediatric-specific aspects of listing as well as pre- and post-pHTx management, in Sweden.

Etiologies of heart failure

End-stage heart disease necessitating pHTx is most commonly caused by CHD or *cardiomyopathies* (CM) (156). There are multiple phenotypes of CM (157): *dilated CM* (DCM) is the most common form and presents with congestive heart failure due to dilatation of the LV and forward (systolic) failure. *Hypertrophic CM* (HCM) causes increased LV wall thickness, independent of increased loading, and may debut with arrhythmia, congestive heart failure and sudden cardiac death. Patients with HCM often have normal or supranormal systolic function, paired with diastolic dysfunction. *Restrictive CM* (RCM) is the rarest form of CM and encompasses diastolic dysfunction of the LV and/or RV with atrial enlargement and the risk of arrythmia and thromboembolism.

CHD is a heterogenous group of congenital malformations of the heart and great vessels, where *univentricular heart* (UVH) encompasses especially severe combinations of defects that predispose anatomical or functional *single ventricle physiology.* Palliative cardiac surgery in multiple stages can be performed to bridge infants to older age, although mortality and morbidity is significant and pHTx remains a therapeutic option in this population (158, 159). Aside from increased surgical complexity (160), patients with CHD often present with multiple risk factors that increase risk of adverse outcomes in the early post-pHTx period, including renal failure, infection and significant immunization (161).

Listing and pediatric heart transplantation organization in Sweden

Since the adoption of brain death criteria in 1989, Swedish pHTx practice has been centralized to Queen Silvia Children´s Hospital in Gothenburg and Skåne University Hospital in Lund, together providing for ~10.5 million inhabitants. Sweden is a part of Scandiatransplant, an organ allocation organization which coordinates all listing and solid organ exchange for both children and adults in the Nordic countries (Norway, Denmark, Finland, Iceland and Sweden) and Estonia (162). Scandiatransplant maintains a common wait list for all patients <18 years of age listed for pHTx, aiming to optimize organ utilization in the region.

Mechanical circulatory support

Children with end-stage heart disease may require *mechanical circulatory support* (MCS) on short- or long-term, either as a bridge to recovery or pHTx, or in very rare cases as destination therapy. *Extracorporeal membrane oxygenation* (ECMO) is used as short-term MCS in the setting of acute cardiac decompensation. The use of pre-pHTx ECMO is associated with thromboembolic events, bleeding and infection, and with impaired long-term outcomes post-pHTx (163).

Surgical implantation of a *ventricular assist device* (VAD) for longer-term MCS has been associated with non-inferior post-pHTx long-term outcomes and improved

wait list survival (164). VADs for both the left (LVAD) and right (RVAD) ventricle can be used, as well as a biventricular (BiVAD) support.

Immunological considerations

After the introduction of several new immunosuppressants in the 1980's, the initial problems with graft rejection were overcome (165, 166). Today, a combination of drugs targeting different parts of the immune system is used as peri-operative induction and long-term maintenance immunosuppressive treatment to minimize *acute* and *chronic rejection* of the transplanted heart.

For induction, the most common agents in children are *anti-thymocyte globulin* or *interleukin-2 receptor antagonists* together with *corticosteroids*. Next, a combination of *calcineurin inhibitors*, *anti-proliferative agents* and *proliferation signal inhibitors* are used to combat rejection (167), however long-term use is associated with nephrotoxicity, gastrointestinal side effects and lymphoproliferative malignancies (156).

Due to the high frequency of prior surgery, transfusion and MCS in pHTx candidates, a significant portion of recipients have developed anti-*human leukocyte antigen* (HLA) antibodies against the antigens of the proposed donor (*donor-specific antibodies*, DSA) at pHTx (168). This is considered especially critical in the presence of a positive *cell-dependent cytotoxicity* (CDC) crossmatch. Listing with the requirement of a negative CDC crossmatch confers impaired survival due to longer wait list duration (169), why protocols for peri-operative antibody removal and augmented immunosuppression have been developed to allow for pHTx against positive CDC crossmatch (170-172). There are still concerns that recipients who develop *de novo*, or have *preformed*, DSA are at higher risk of *antibody-mediated rejection* (AMR) and *chronic allograft vasculopathy* (CAV) (168). Certain sub-types of DSA, and as well as persistent DSA post-pHTx, are considered particularly unfavorable (173).

The immaturity of the infantile immune system has allowed for the development of *ABO-incompatible* (ABOi) pHTx with non-inferior outcomes demonstrated at the long term (174, 175), shortening wait list duration in this population with a limited donor pool.

Aims

Study I

Study I aimed to determine the expression and presence of aggrecan and ADAMTS proteases in vascular lesions of advanced human IPAH and to describe the spatial distribution of aggrecan-rich lesions in the pulmonary vascular tree.

Study II

The aim of Study II was to investigate the two- and three-dimensional distribution of the different isoforms of versican, their interacting partners as well as fragments generated by ADAMTS-mediated proteolysis, in vascular lesions of human IPAH.

Study III

In Study III we aimed to assess versican and aggrecan dynamics across the fetal and postpartum maturation of the DA and PDA, as well as to determine DA three-dimensional morphology, in humans and other mammals.

Study IV

The aim of Study IV was to survey international practice patterns amongst physicians in the pre-pHTx management of PH in children with end-stage LHD.

Study V

Study V aimed to describe the outcomes of the first 35 years of pHTx practice in Sweden, with specific emphasis on recipients with elevated PVRi, MCS, single-ventricle physiology and elevated immunological risk, as well as those that underwent ABOi pHTx.

Material and methods

Material and study design

Study I – III

Human tissue and plasma specimens

Study I-III were experimental studies using fixed, paraffin-embedded tissue acquired from clinical pathology biobanks at Skåne University Hospital (Lund, Sweden), Vanderbilt University Medical Center (Nashville, TN, USA) and the Cleveland Clinic (Cleveland, OH, USA).

Tissue blocks for Study I and Study II were from the pathology biobank at Skåne University Hospital. Pulmonary tissue was collected from the explanted lungs of transplant recipients (n=11), patients with late-stage IPAH. Control specimens were collected from donor lungs not utilized for transplantation (FLD) (n=5). For Study II, only ten of the IPAH specimens and three of the FLD controls were used, based on the availability of sections with adequate quality.

In Study I, RNA was collected from laser capture micro-dissected small pulmonary arteries from a separate cohort of IPAH patients (n=13) and downsized donor lung controls (n=8). These cohorts were from the Medical University of Graz (Graz, Austria).

For Study II, plasma samples were acquired from the Lund Cardio-Pulmonary Registry (LCPR) biobank. Non-fasting venous blood samples were collected from IPAH and HPAH patients' introducers during routine diagnostic RHC. Samples were collected from patients both with (Cohort I, n=10) and without (Cohort II, n=10) PAH-specific therapy. Peripheral venous samples were collected from healthy subjects (n=10) and used as controls for both groups. Both Cohort I and II were retrospectively, actively, selected to match the hemodynamic parameters of the IPAH tissue cohort.

PAH was defined according to prevailing guidelines at the time of diagnosis (58). Patients underwent an extensive evaluation at a PH center, excluding other causes of the disease. Patients with IPAH lacked any other known cause of PAH. HPAH patients had a known genetic abnormality associated with PAH. Advanced vascular

remodeling was defined as Heath and Edwards histopathological grade >3 (122) (Table 2).

DA specimens for Study III were obtained from corrective surgeries for CHD (n=25), during which part of the DA was removed due to the nature of the surgical procedure. Control specimens were collected from organ donor surgeries in children (n=2), autopsies of stillborn fetuses (n=2) and an autopsy of a victim of sudden infant death syndrome (n=1). Blocks were pooled from the clinical biobanks at Skåne University Hospital, Vanderbilt University Medical Center and the Cleveland Clinic. Three DA specimens from corrective surgeries were excluded due to damage obtained during tissue sampling and/or processing.

In Study III, the fetal period temporal windows were defined as follows: late preterm (34 0/7–36 6/7), early term (37 0/7–38 6/7) full term (39 0/7– 40 6/7). Determination of DA morphological maturation stage was based on the description by Gittenberger-de Groot *et al* (82, 91) (Figure 9). All DA specimens were reviewed by expert cardiac pathologists to determine which segments were from the central (ductal) part of the vessel or the proximal or distal (great artery) end.

Clinical data

Medical records at the center of origin were reviewed to obtain relevant clinical and demographic variables for patients contributing tissue to the studies. Patient characteristics for the RNA cohorts in Study I were previously reported (176). For Study II, clinical and demographic variables for the plasma cohorts were obtained from the LCPR.

Animal tissue

Fixed, paraffin-embedded DAs from mice, rabbits and lambs were collected for Study III. Tissue was sampled from animals used as controls in other studies and were generously provided by our collaborators.

Torsos from embryonic day (E)18.5 C57BL/6 control mice (n=6) (full term E19-E21) were harvested from timed pregnancies.

Heart-lung blocks, including the great vessels emerging from the heart, were dissected from E29 New Zeeland White control rabbit pups (full term E31-E32) at 0-2 hours (n=5), 24 hours (n=1) and 72 hours (n=3) postnatally.

The DA from E105 (n=2), E108 (n=2) and E118 (n=1) lamb fetuses (148 days gestation (dGA) was full term) was carefully dissected. In a similar manner, the DA from lambs born at dGA127 (euthanized 8 hours after birth) and dGA138 (euthanized 24 hours after birth) was obtained.

Tissue processing and sectioning

Fixation, dehydration, clearing and paraffin-embedding of tissue specimens followed clinical protocols at the pathology department or institute of origin. Tissue sections, 4-5 µm each, were taken for subsequent histology, immunostaining, RNA *in situ* hybridization (ISH) and spatial proteomics.

Study IV

Study IV was a quantitative and qualitative survey study of international practice patterns in pre-pHTx management of PH in children, <18 years of age, with end-stage heart disease. A questionnaire with a combination of categorical answers, Likert scales and free text responses was constructed (see Supplemental material for Study IV). Data was collected and stored using REDCap (Research Electronic Data Capture, Vanderbilt University, Nashville, TN, USA). The questionnaire was administered via email to physicians who cared for children with heart failure requiring pHTx and/or children with PH, and were members of the International Society for Heart Lung Transplant (ISHLT) Pediatric Council, the Pediatric Pulmonary Hypertension Network, The Pediatric Task Force of the Pulmonary Vascular Research Institute, The European Pediatric Pulmonary Vascular Disease Network, and the Working Group for Pulmonary Hypertension, Heart Failure and Transplantation of the Association for European Pediatric and Congenital Cardiology. Self-selection for survey inclusion criteria included experience in the care of children with LHD requiring pHTx.

Study V

This was a nationwide, multicenter, retrospective observational cohort study resulting from a collaboration between the pediatric heart centers at Sahlgrenska University Hospital (Gothenburg, Sweden) and Skåne University Hospital (Lund, Sweden). All children <18 years residing in Sweden that were listed for pHTx, including simultaneous listing for other organs, between January 1st 1989 and December 31st 2023 were included (Figure 13). Children listed for combined heart-lung transplantation were excluded, as they were considered to have significantly different clinical courses. Data at wait list entry, as well as recipient and donor characteristics at both listing and pHTx, were retrospectively collected from the Scandiatransplant (Aarhus University Hospital, Aarhus, Denmark) registry. Medical records were reviewed as needed.

Patients were grouped into two eras of pHTx, based on the availability of detailed immunological data: January 1st 1989 – December 31st 2008 (ERA I, 20 years, no detailed immunological data available), January 1st 2009 – December 31st 2023 (ERA II, 15 years, detailed immunological data available). Immunological variables

were obtained from HLA Fusion (One Lamba/Thermo Fisher Scientific), ProSang (Omda AS) and Scandiatransplant.

One day was considered the minimum duration for both wait list time and post-pHTx survival. The total number of days on the waiting list was accounted for. Those that deteriorated and died shortly after delisting were considered as deceased in wait list mortality analysis. The censor date, and thus end of follow-up, was December 31st 2023. Children withdrawn from the list due to improvement or deterioration, that were later re-listed and transplanted, were included once in wait list and post-transplant analyses (only accounting for the listing occasion preceding pHTx or death). Patients listed for pediatric heart re-transplantation (re-pHTx) were excluded from the main analyses.

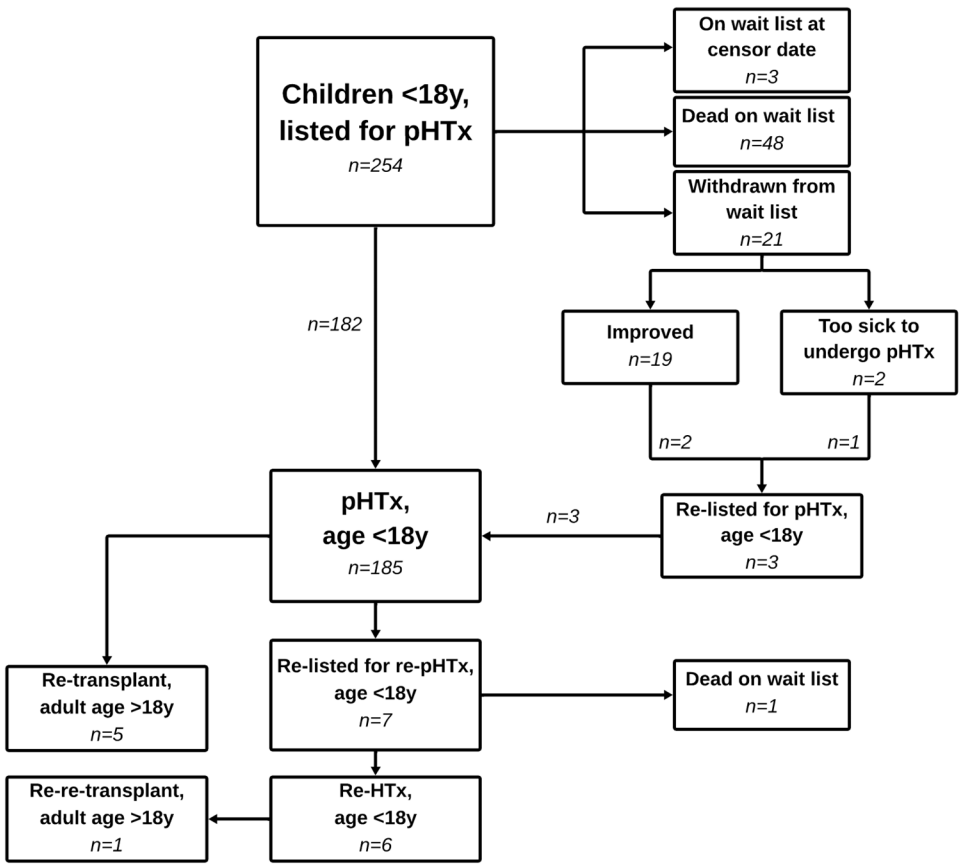


Figure 13. Flow chart of children listed for pHTx in Sweden, 1989-2023.
pHTx, pediatric heart transplantation; re-pHTx, pediatric heart re-transplantation

Experimental methodology

In the following pages the methods utilized for experimental Study I-III are described. To optimize data extraction from specimens, and to preserve the three-dimensional morphological structure even after tissue sectioning, a workflow utilizing non-destructive synchrotron-radiation phase-contrast microcomputed tomography (SRμCT) was developed by our group (60) (Figure 14). All SRμCT experiments for Study I-III were performed at the X02DA TOMCAT beamline at the Swiss Light Source (Villigen, Switzerland). Laboratory microtomography (μCT) of DA specimens were performed to control for artefacts prior to SRμCT imaging in Study III, the setup of which is outlined in (177).

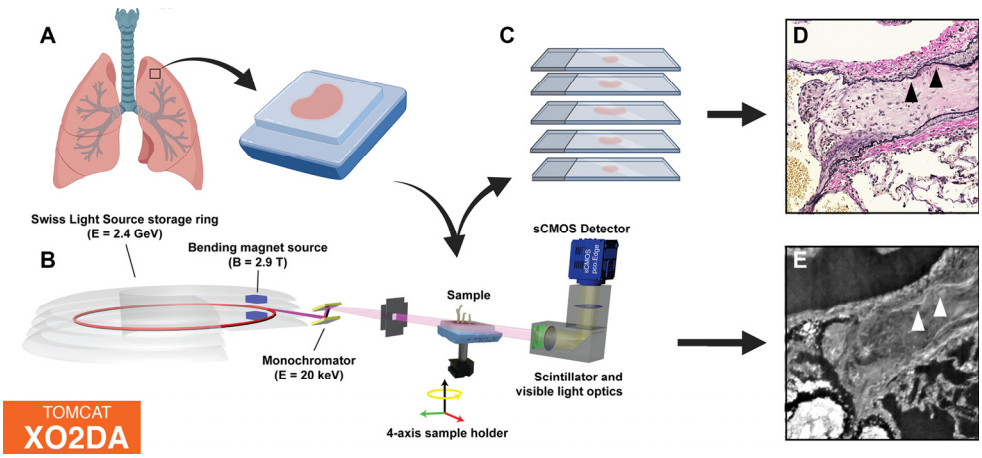


Figure 14. Schematic workflow of the experimental setup for Study I-III.
Fixed and paraffin-embedded tissue specimens (A) underwent SRμCT at the TOMCAT beamline at the Swiss Light Source (B). A 2.9 T bending magnet inserted on the 2.4 GeV storage ring produced synchrotron radiation which was led through a monochromator and tuned to 20 keV. The sample holder allowed for 180° rotation, enabling tomographic image acquisition. A detector system, comprising a scintillator and an optical microscope coupled to a sCMOS detector, was used to collect images. Areas of interest were indicted with wax markers. When SRμCT was performed, tissue sections were taken (C) and stained for markers of interest (D, EvG staining, elastic fibers stain black and collagens stain pink/red. Black arrowheads indicate the IEL). Comparison to SRμCT datasets (E, white arrowheads indicate the IEL), allowed for integration of histology into the three-dimensional space. Reprinted from van der Have O *et al*, Pulmonary Circulation 2023, with permission (178). *B, magnetic field; E, energy; GeV, giga-electronvolt; keV, kilo-electronvolt; sCMOS, scientific Complementary Metal-Oxide-Semiconductor; T, Tesla; SRμCT, synchrotron-radiation phase-contrast micro-computed tomography; EvG, Elastica van Gieson; IEL, internal elastic lamina.*

Synchrotron-based microtomography

A synchrotron light source produces synchrotron radiation, x-rays, through the acceleration of electrons to relativistic speeds (Figure 14) (179). High-brilliance, tunable, radiation is generated which allows for use of both *attenuation* and *phase-*

shift to generate contrast from the detected signal (Figure 15). In clinical practice, a radiograph or computed tomography utilizes x-ray imaging that is based on variations in attenuation. Attenuation of the signal is dependent on the density and thickness of the imaged material, as well as the energy and intensity of the incoming radiation (Figure 15). The interaction of radiation with material will furthermore cause a phase-shift of the x-rays, a phenomenon that is more prominent than differences in attenuation for homogeneous biological tissue (Figure 15).

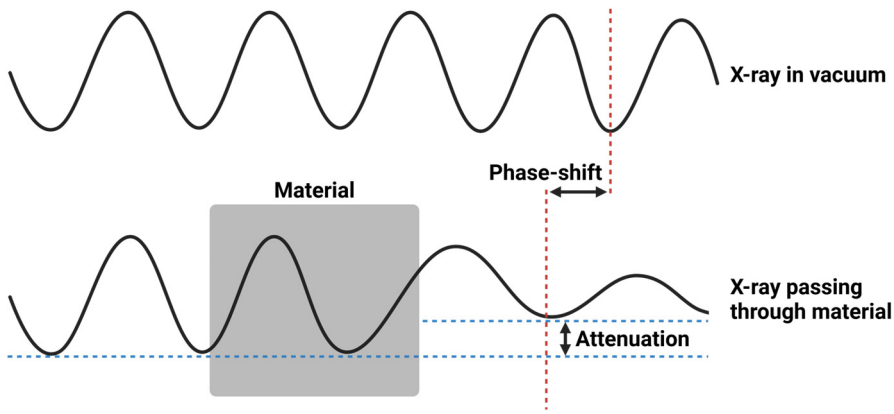


Figure 15. Attenuation and phase-contrast
Phase-shift reflects the difference in wavelength of the x-ray after passing through a material, whereas differences in attenuation correspond to the loss of amplitude, or intensity, of the x-ray. Both principles are used for SRμCT imaging. Adopted from (180). *SRμCT, synchrotron-radiation phase-contrast microcomputed tomography.*

Image acquisition and reconstruction

The field of view for SRμCT imaging depended on the magnification (4x magnification: 4.16 mm x 4.16 mm x 3.51 mm. 20x magnification: 0.83 mm x 0.83 mm x 0.7 mm). 4x imaging was used for Study I and II, whereas both magnifications were used for Study III. Additionally, stitching 2x2 4x datasets yielded a greater field of view of (~7.13 mm x ~7.13 mm x ~3.51 mm), used to capture entire DA specimens for Study III. Pixel sizes were 1.625 μm (4x magnification) and 0.325 μm (20x magnification), respectively. Microtomography reconstructions were performed using the Gridrec (181) algorithm after applying Paganin phase-retrieval (182), outlined in detail in previous work by our group (60).

Visualization, segmentation and structure tensor analysis

The FIJI (ImageJ by National Institutes of Health, Bethesda, MD, USA) and Amira (Thermo Fisher Scientific, Waltham, MA, USA) software were used for visualization, manual and semi-automated segmentation of vascular structures in SRμCT datasets. All segmentation was based on grayscale thresholding, with

varying pre-processing, background subtraction and/or enhancement to promote optimal contrast. Information about the fiber directionality of adjacent pixels was extracted by performing structure tensor analysis on DA SRμCT datasets in Study III. A script in Python, using the implementation by Jeppesen *et al* (183), was applied to generate vector-based information that was combined with segmentation data in the Amira software.

Histology and immunostaining

Histology

Stainings for histological evaluation of tissue specimens were used throughout Study I-III, all performed according to standard protocols the Department of Pathology, Skåne University Hospital, Lund. Elastica van Gieson (EvG), also known as Verhoeff´s stain, combines a staining for elastin (black) with a counterstain for collagen fibers (red/pink), whereas other tissue elements are stained yellow. Alcian blue/Periodic acid-Schiff (Ab/PAS) staining visualizes acidic polysaccharides, such as GAGs (light blue) and neutral mucins (purple/magenta).

Immunohistochemistry and immunofluorescence

Both immunohistochemistry and immunofluorescence were used to identify protein epitopes in tissue via primary and/or secondary antibodies (Figure 16). Antibodies can be directly conjugated to enzymes or fluorophores (primary conjugated antibodies) or use the principle of a secondary conjugated antibody against the F_c region of a primary antibody. Both immunohistochemistry and immunofluorescence were used to detect multiple epitopes, including lectican core proteins, in Study I-III.

Through the addition of a substrate, in the setting of an enzyme-conjugated antibody for immunohistochemical staining, consumption by the enzyme generates a signal that can be assessed in a microscope (Figure 16). Similarly, through the excitation of a fluorophore-conjugated antibody in an immunofluorescent staining, the emission of light at a specific wavelength can be detected in a fluorescence microscope (Figure 16). Stainings that use secondary antibodies amplify the epitope signal, as multiple secondary antibodies may bind to a single primary antibody. An important difference between the two methods is that immunohistochemistry enables additional amplification, as substrate consumption by the enzyme is dependent on the length of their exposure to each other. Immunofluorescent staining for multiple epitopes can be performed by using multiple fluorophore-conjugated primary antibodies emitting light of distinct wavelengths. Another method to visualize multiple epitopes is the combination of primary and secondary antibodies produced in different animals.

For most immunohistochemical and immunofluorescent stainings, pre-treatment with heat-induced epitope retrieval is required to expose the epitope. For lecticans, additional processing is sometimes needed as core proteins may be "hidden" beneath CS and KS side chains. Treatment with Chondroitinase ABC from *P.vulgaris* was used in some experiments to remove CS chains prior to incubation with primary antibodies.

Spatial proteomics through cyclic immunofluorescence

To enable identification of an even greater number of epitopes in a single tissue section, with preserved spatial information, repeated immunofluorescent stainings interlaced with image acquisition and antibody removal can be performed (Figure 16). This method is sometimes referred to as "spatial proteomics", however the method uses a pre-selected panel of antibodies and should not be equated to an un-biased proteomic analysis. For Study III, we performed cyclic immunofluorescence experiments on the MACSima™ Platform (Miltenyi Biotec, Cologne, Germany) with analysis performed undertaken in the MACS® iQ View software (Miltenyi Biotec, Cologne, Germany).

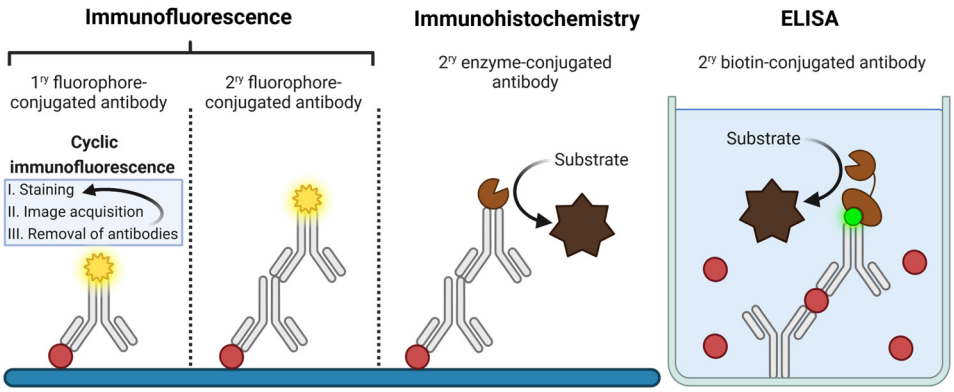


Figure 16. Principles for antibody-based experiments
ELISA, enzyme-linked immunosorbent assay.

Enzyme-linked immunosorbent assay

To quantify circulating versican core protein in plasma for Study II, a bi-antibody sandwich enzyme-linked immunosorbent assay (ELISA) was performed (PG-350 ELISA kit, Cusabio Technology) (Figure 16). The epitope in plasma samples was fixed via an immobilized primary antibody in the well. A secondary, biotin-conjugated, antibody was added. Streptavidin, with affinity to biotin, was added. Streptavidin then consumed the added substrate to generate a signal which could be

quantified. Samples from IPAH/HPAH patients and controls, as well as the standard, were analysed in triplicates or duplicates for ELISA experiments.

mRNA transcript analysis

In situ hybridization

RNA ISH was performed in Study I-III to detect messenger RNA (mRNA) transcripts of *ACAN, VCAN* exon 7 and exon 8*,* and *ADAMTS* proteases in tissue sections. This method allowed for subsequent immunofluorescent double- or triple staining which was performed in some cases. Labeled oligonucleotides from RNAScope (Advanced Cell Diagnostics) enabled both fluorescent and enzymatic detection of the transcripts of interest, the methodology of which was described in detail previously (184).

Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to quantify *ACAN* and *ADAMTS* mRNA expressions in laser-dissected pulmonary arteries in Study I. Following the conversion of mRNA to complementary DNA with a reverse transcriptase, the rapid temperature changes in a thermal cycler allowed for the elongation of certain transcripts using a pair of primers, deoxyribonucleotide triphosphates and a Taq DNA polymerase with a fluorescent tag (allowing for monitoring in real-time).

Ethical aspects

For Study I-III, all individuals contributing tissue, plasma and/or clinical data were deidentified. Studies were approved by the Swedish Ethical Review Authority (Dnr: 2017/597 and 2019-01769), with additional permit granted for the use of plasma samples for Study II (2010/114, 2011/368, 2015/270). For Study III, ethical permit was granted by the Institutional Animal Care and Use Committee (IACUC protocol nos. 18–1996 and 18–2045), the Swedish Animal Ethics Committee in Malmö/Lund, Sweden (Dnr 5.8 18-00919/2024) and the Hudson Institute Animal Ethics Committee (MMCA 2017/38, MMCA21/10, MMCA24/01, MMCA 24/07).

Participation in Study IV was anonymous and voluntary, and the purpose of the study was outlined in text as part of the questionnaire introduction. Implied consent was assumed through completion of the questionnaire.

Study V was approved by the Swedish Ethical Review Authority (Dnr 2020-02140, Dnr 2023-05036-02 and Dnr 2024-05951-02) and conducted in accordance with the

ISHLT ethical statement. The requirement of informed consent was waived due to the retrospective nature of the study.

Statistical analysis

Normality distribution was assessed with histograms, normality tests (Kolmogorov-Smirnov and Shapiro-Wilk), Q-Q plots and skewness. Categorical variables were described using frequencies and percentages, whereas mean and standard deviation or medians and interquartile range was used for continuous variables. Continuous variables were compared by independent samples t test or Mann-Whitney test (2 groups) and one-way ANOVA or Kruskal-Wallis test (>2 groups). Categorical variables were compared by Fishers exact test (2 groups) or Pearson chi^2 test (>2 groups).

For Study V, survival rates for all-cause mortality were estimated using the Kaplan-Meier method, and the log-rank test was used to compare differences between groups. The Cox proportional hazard model was used with and without stepwise multivariable adjustment to estimate hazard ratio and 95% confidence interval for the association between era of transplantation and wait list mortality or post-pHTx survival, with ERA I as the reference time period. Variables were selected *a priori* based on clinical experience. Mortality on the waiting list was adjusted for age, sex, blood group and diagnosis. Postoperative survival was adjusted for recipient and donor sex, recipient and donor age groups, recipient and donor weight mismatch ratio at transplant, diagnosis, urgency, preoperative kidney function (estimated or measured glomerular filtration rate), cold ischemia time, cytomegalovirus mismatch, ABOi pHTx and pre-transplant treatment with VAD or ECMO. Simple linear regression analysis was performed to investigate the relationship between pre- or post-AVT and post-pHTx survival time.

Unless otherwise stated, a 2-sided p-value <0.05 was considered statistically significant. Statistical analyses relied on the Stata software (StataSE, version 17.0, StataCorp) and GraphPad Prism (GraphPad Software, versions 8.1.1 through 10.4.1).

Results

Study I

Immunohistochemical and immunofluorescent stainings for an epitope within the G1-IGD-G2 core protein complex of aggrecan revealed accumulation in all three major PAH lesion types. No aggrecan was observed in healthy pulmonary arterioles of FLD controls, or within the pulmonary parenchyma and pulmonary veins of IPAH patients. Quantifying whole section stainings for aggrecan revealed a significantly greater accumulation of total positive pixels in IPAH patients, compared to FLD controls.

Qualitative assessment of EvG, Ab/PAS and immunohistochemical staining demonstrated significant heterogeneity in PAH lesion ECM composition, reflecting different stages of lesion development. GAGs and aggrecan were mainly present in early lesions with provisional, cellular, matrices whereas collagen deposition and fibroelastic remodeling occurred with time in the absence of aggrecan.

RNA ISH, with and without additional immunofluorescent staining for cell markers, indicated expression of *ACAN* within all three lesion types of PAH. Cells positive for α−smooth muscle actin (αSMA), indicating VSMCs, expressed *ACAN* in pulmonary arterioles with medial hypertrophy. Cells positive for von Willebrand factor (vWF) and Claudin-5, indicating ECs, expressed *ACAN* in neointimal and plexiform lesions. A subset of cells negative for both αSMA and vWF also expressed *ACAN*.

Increased expression of *ACAN* mRNA in laser-dissected small pulmonary arteries from IPAH patients, compared to FLD controls, was previously published for the material utilized (176). Trends of increased expression of *ADAMTS1*, *ADAMTS5* and *ADAMTS9*, as well as significant upregulation of *ADAMTS15*, was shown with qRT-PCR for the same dataset.

We successfully performed SRμCT on all the included IPAH patients, allowing for interpretation of stainings in the three-dimensional space. We observed, and demonstrated through segmentations in three separate patient cases, that aggrecan preferably accumulated in the pre-stenotic or stenotic region of remodeled pulmonary arterioles, rather than in the post-stenotic region or dilated collaterals exiting from the same vessels. This finding suggested preferable accumulation of aggrecan at sites of elevated pulmonary arterial pressure.

Study II

Similar to what had been observed for *ACAN* in Study I, the expression of both *VCAN* exon 7 and exon 8 was found in both αSMA- and vWF-positive cells, as well as double-negative cells, in PAH vasculopathy. No apparent pattern of distribution was observed on the mRNA level, however βGAG appeared more widely distributed in all lesion types. Little to no mRNA or protein signal for αGAG or βGAG was observed in healthy pulmonary arteries.

Versican globular domains G1 and G3 did not consistently colocalized in IPAH vascular lesions on immunofluorescent stainings, suggesting proteolytic cleavage which was supported by the differential patterns of DPEAAE and ARRGQF neoepitope accumulation. Within the neointima, G3 appeared closer to the endothelial lining whereas G1 was localized towards the media. Conversely, DPEAAE stained positive at the endothelial aspect of the neointima together with HA, and ARRGQF at the border between intima and media. Tenascin-C co-localized with versican G3, although not consistently across all lesion types.

Reconstructing a shunt-type (type 1) and obstructive (type 4) plexiform lesion, as per the definition previously published by our group (124), allowed for comparisons with immunofluorescent stainings. Versican and DPEAAE accumulated within plexiform lesions, whereas isolated tenascin-C stain was found in both pre- and post-stenotic collaterals with connections to the bronchial circulation.

Based on our finding of versican G3 along the endothelial lining, we hypothesized that the C-terminal end of versican was proteolytically cleaved and mobilized to the circulation. ELISAs for versican G3 demonstrated significant increased concentrations of versican G3 in IPAH/HPAH plasma from patients both with and without PAH-specific treatment, compared to healthy controls. Mass spectrometry analysis proved that assay kit standard was from the versican G3 protein domain.

Study III

Human DA samples underwent laboratory μCT and SRμCT. The median birth weight of children undergoing corrective cardiac surgery was 3130 g, 27.3% were females and the 59.0% were born at full-term. 81.8% received PGE_1 from birth to maintain DA patency.

High-resolution SRμCT imaging enabled micrometer-scale resolution imaging of the DA, PDA and Ao, allowing for distinction between the DA and PDA, as well as detailed study of DA morphology in three dimensions. Structure-tensor analysis revealed vectorial information, indicating a predominantly radial orientation of fibers in intimal cushions.

ACAN and *VCAN*, as well as *ADAMTS1*, *ADAMTS4* and *ADAMTS5* were expressed in the closing DA. Despite observed trends of increased aggrecan and versican core protein accumulation in the closing DA, there was a significant heterogeneity in lectican accumulation between specimens. We selected representative specimens from different stages of DA maturation, to create a timeline of DA development. A peak in versican and aggrecan accumulation was observed during the second stage of DA development. Versican and aggrecan were notably decreased or absent in later stages of DA development and in the LA. This peak in aggrecan and versican coincided with an increased signal of β-catenin in VSMCs, coupled with a decrease in contractile markers.

Accumulation of DPEAAE occurred at the border between media and adventitia during intrauterine DA development with centripetal progression towards the intima as the DA was closing/had closed. Aggrecan and versican were notably decreased in the PDA.

To further investigate these findings, we performed Ab/PAS and DPEAAE staining on sections of rabbit and lamb DA tissue of different gestational ages. In the rabbit, intrauterine intimal cushion formation with GAGs and DPEAAE was lacking, whereas the lamb displayed a similar pattern of centripetal progression of DPEAAE staining.

Study IV

We received 49 complete responses from 39 centers in 16 countries, where most participants self-identified as pediatric cardiologists (90%) with expertise in end-stage heart failure (84%), practicing at pHTx centers (86%) in North America (57%).

Most respondents (88%) reported that AVT was performed if baseline PVRi was elevated. The preferred method of determining CO was a combination of calculated Fick and thermodilution (47%). Inhaled NO and oxygen were often used for AVT (86%). Half (51%) of the respondents reported that they had a PVRi cut-off for pHTx eligibility, with PVRi of 6 $WU \cdot m^2$ being the most commonly reported value for cut-off. The highest accepted pre-pHTx PVRi differed, depending on if the value was pre- or post-AVT: the median for reported pre-AVT values was 9 $WU \cdot m^2$ (mean 9.7, range 3–20), whereas post-AVT median value was 6 $WU \cdot m^2$ (mean 6.4, range 3–14.4).

Hemodynamic compromise (65%) and pre-existing pulmonary edema (53%) were reported as the most common contraindications against initiating pulmonary vasodilator therapy. In children with PH-LHD on LVAD, therapy was reportedly initiated to minimize RV afterload in the intensive care unit (84%) and avoid RVAD (71%). PDE5i were most often used to treat high PVRi (65%). Notably, 31% reported to never use pulmonary vasodilators in children with PH-LHD without LVAD. The presence of an LVAD appeared to increase the likelihood of pulmonary vasodilator treatment, with PDE5i remaining the preferred drug (80%).

Three pediatric case vignettes were outlined for the respondents. Multiple choice treatment options included listing the for pHTx or combined heart-lung transplantation, as well as combinations of pulmonary vasodilator therapy and LVAD implantation, with options to repeat RHC prior to listing for pHTx, or not. Reponses on case vignettes and free text comments on these questions highlighted the significant variability in practice.

When asked if respondents believed whether an elevated PVRi above their chosen cut-off contributed to post-pHTx mortality in patients with PH-LHD, Lickert scale values ranged from 0 (disagree) to 100 (agree), with both median and mean of 50, indicating the diverging opinions in the field.

Study V

In Sweden, 254 children were listed for pHTx with or without simultaneous listing for other organs between Jan 1st 1989 and December 31st 2023. Eighteen children listed for combined heart-lung transplantation had at this point been excluded from the cohort. No patient was lost to follow-up.

During ERA I, 118 children were listed for pHTx, and 136 children were listed during ERA II (15% increase), with 44% increase in the incidence of listing for pHTx amongst children <18 years of age in Sweden. Days on the waiting list increased over time (median 45 days in ERA I, median 79 days in ERA II), paired with a decrease in absolute wait list mortality (30.5% in ERA I, 8.8% in ERA II). Kaplan-Meier estimated wait list mortality was lower during ERA II but also diverged based on age groups but not based on etiology (CHD vs CM) or sub-etiology (UVH vs non-UVH CHD, HLHS vs non-HLHS UVH and different subgroups of CM) of heart failure. After stepwise adjustment for covariates using Cox proportional hazard modeling, ERA II was a significant predictor of improved wait list survival, compared to ERA I (HR 0.21, [95% CI 0.10-0.42], p<0.001).

A total of 185 children (72.8% of all listed) reached pHTx between 1989 and 2023 in Sweden: 72 during ERA I and 113 during ERA II (57% increase), with a 96% increase in pHTx incidence amongst children <18 years of age. Post-operative mortality at 30 days was 2.2%. The 1-, 10- and 30-year post-pHTx survival over the study period was 94.5% (95% CI 90.0–97.0), 79.4% (95% CI 71.6–85.2) and 57.1% (95% CI 44.3–68.1), respectively. There was no statistically significant difference in post-pHTx Kaplan-Meier estimated mortality based on ERA of transplantation, age group, etiology/sub-etiology of heart failure.

pHTx from VAD were more common in ERA II (17.4% in ERA I, 32.7 in ERA II) and did not show worse post-pHTx survival compared to other listed children, over the entire study period. Frequency of both pre- and post-operative ECMO, as well as post-operative VAD, remained stable over time but were associated with poorer post-transplant outcomes. Recipients with elevated baseline PVRi (≥6 $WU \cdot m^2$) at pre-AVT RHC did not display significantly worse survival compared to those under the chosen cut-off, although a trend towards worse survival was observed. There was no correlation between post-transplant survival time and levels of pre-AVT or post-AVT PVRi. Only two patients with PVRi ≥6 $WU \cdot m^2$, post-AVT, underwent pHTx. The frequency of ABOi pHTx increased over time (2.9% in ERA I and 13.3% in ERA II) and was non-inferior compared to ABO-compatible pHTx. During the contemporary era, recipients deemed at high immunological risk based on the given classification had both higher width of immunization and cumulative MFI, and all developed or displayed DSA. DSA were present, or developed, in 22.1% of all recipients during the contemporary era and HLA-DQ DSA constituted 45.9% of all clinically significant *de novo* DSA detected in recipients.

Discussion

Spatiotemporal dynamics of lectican accumulation and turnover

The dedicated work of Wight and colleagues has been fundamental in deciphering the role of PGs in systemic vascular remodeling and disease, such as atherosclerosis and restenosis (22, 185-189). Their work has highlighted the importance of the early, provisional, ECM that is generated as a result of vascular injury to facilitate cell migration, signaling and dedifferentiation (186). Versican and ADAMTS proteases have been demonstrated to be key players in the provisional ECM in disease and throughout development (24, 33, 190), exerting biological functions through the G1 and G3 domain and through generating biologically active fragments such as versikine (46). Aggrecan and its turnover, on the other hand, has been less extensively studied in the vascular milieu (20). Studies in aortic aneurysms and dissection have suggested that aggrecan accumulation plays a major role in disrupting lamellar integrity through its hydrophilic capabilities (39, 50, 52).

Pulmonary arterial vasculopathy

Study I and II showed that lecticans were locally expressed and accumulated in vascular lesions of IPAH to a much greater extent than in healthy pulmonary arteries or pulmonary veins, preferably in early provisional matrices. Our observations were recently supported by the work from Mutgan *et al* (191). Multiple growth factors and morphogens known to influence PH development interact with GAGs (192, 193) and lectican-HA complexes have been shown to support a pro-proliferative ECM (87, 192, 194), suggesting that abundant lectican accumulation is a target for therapeutic intervention in PAH.

We demonstrated that *VCAN* exon 7, *VCAN* exon 8 and *ACAN* mRNA was expressed *in situ* by both VSMCs and ECs in PAH. Interestingly, we observed that lecticans were preferentially expressed by VSMCs in medial hypertrophy, whereas ECs expressed lecticans in intimal hyperplasia and plexiform lesions. Consequently, multiple stimuli and pathways may be involved in regulating lectican accumulation in pulmonary vascular remodeling. Thenappan *et al* proposed that induction of EC dysfunction and ECM proteolysis by an unknown circulating serum factor is a key

mechanism in the initiation of vasculopathy in PAH (195). Proteolysis of resident lecticans resulting in the release of morphogens retained in the ECM (192). and in generation of bioactive fragments (33, 35), could potentially trigger further cell and ECM expansion. Prior studies in human vein grafts have shown upregulation of versican in the intima and media in response to grafting to the arterial circulation (35), harmonizing well with our findings of increased expression and accumulation of lecticans in the hypertensive setting. VSMCs are highly mechanosensitive and increased PG production is a known consequence of increased cyclic mechanical stretch in VSMCs (196, 197). This may provide a complementary pathway regulating lectican expression within the tunica media. Cells expressing lectican mRNA, negative for both VSMC and EC markers, were observed in IPAH. We currently hypothesize that these cells might be dedifferentiated or transdifferentiated chondrocyte-like VSMCs (198), although this warrants further investigation.

When studying vascular lesions in the three-dimensional setting with SRμCT in Study I and II we showed that versican and aggrecan preferentially accumulated within, or proximal to, stenotic lesions but rarely distally to obliterative vascular remodeling or in collateral vessels exiting such lesions. Tenascin-C, an interacting partner of the G3 domain, was however observed in collateral vessels. The selective accumulation of lecticans further supports the involvement of VSMC mechanosensing, as well as shear stress, in the regulation of lectican expression in PAH.

Isoforms, interacting partners and proteolysis of versican was the focus of Study II. We showed distinct patterns of DPEAAE and ARRGQF accumulation in PAH, suggesting turnover of versican isoforms V0 and V1 and motility of fragments. We were not able to distinguish between V0- and V1-derived βGAG, however the presence of αGAG indicated that full-length V0 was indeed present as V2 is limited to the CNS (27). Versican core proteins G1 and G3 did not consistently colocalize, indicating the presence of fragmented and intact versican isoforms. Our findings, however, raised suspicion of additional proteolysis *in situ*. If solely cleaved at the Glu^{441}-Ala^{442} bond of βGAG, the core protein domains and their corresponding fragment neoepitopes (G1-DPEAAE and ARRGQF-G3) would colocalize. Isolated staining for DPEAAE and ARRGQF, without the corresponding core protein domain, indicated that additional proteolysis occurred. It is likely that multiple sites of proteolytic cleavage within lecticans remain to be identified (46).

ADAMTS-mediated cleavage of lecticans was supported by the results from Study I, where upregulation of multiple versicanases/aggrecanases was shown in small pulmonary arteries, albeit only significant for *ADAMTS15*. Pooling of pulmonary arteries with lesions at different stages of vascular remodeling may have been the cause of insignificant data in this analysis. Although shown to proteolytically cleave both versican (199, 200) and recombinant aggrecan (201), information on ADAMTS15 is scarce. Targeted myocyte and VSMC deletion of *ADAMTS8*, a protease whose gene locus is tightly linked with that of *ADAMTS15*, was associated

with reduced RV hypertrophy and systolic pressure in mice subjected to hypoxia-induced PAH (202). Studies to investigate the temporal differences in ADAMTS protease expression throughout PAH disease development could shed light on mechanisms underlying early vascular remodeling.

Closure of the ductus arteriosus

The largest muscular artery in the human body, the DA, has a unique function and biological faith as it is a transient structure. Previous studies have investigated its anatomical remodeling in two dimensions (78, 82) and have formulated hypotheses on three-dimensional morphology, but no definitive confirmative work has been presented to date.

Dividing the cohort into DA segments with more homology to great (elastic) artery structure versus segments with well-developed ductal features indicated that both aggrecan and versican were present in the mature DA to a greater extent. The case-based timeline of human specimens suggested an early peak in lectican accumulation, with centripetal advancement of versican degradation at later stages. Intriguingly, versican degradation was absent in the tunica intima and tunica media in early stages, perhaps allowing the full hydrophilic capabilities of aggrecan and versican to expand the provisional ECM. The peak in lectican accumulation coincided with downregulation of several contractile markers, as well as increased β-catenin signal in VSMCs of the tunica intima. Dedifferentiated, less contractile, VSMCs were previously described within parts of the tunica intima and associated with increased cytolytic necrosis and apoptosis in the human DA (203). β-catenin is a part of the Wnt signaling pathway, which is active in VSCMs, and was shown to be vital in mediating neointima formation after arterial injury in mice (204). Despite the lack of evidence to prove a causal relationship, the findings of Study III and previous work showing that β-catenin is the main promoter of *VCAN* expression in VSMCs (205, 206), together suggests that a this pathway is involved in early stages of intimal cushion formation. The importance of EC-derived versican for VSMC migration was studied *in vitro* (87), however our data indicate that DA VSMCs are also involved in the build-up of versican, perhaps in a complementary fashion.

Centripetal degradation of versican V0 and V1 by ADAMTS proteases was present throughout development of the lamb DA. In the rabbit DA, which did not display intimal cushions but rather postnatal intimal proliferation, both GAGs, HA and DPEAAE accumulation were notably absent. Our findings, albeit in a small cohort of animals, suggest that versican regulation may be a key differentiator in DA functional closure of small and large mammals.

Albeit not the first to image the human Ao and DA (207, 208), the results presented in Study III expand the methodological applications of SRμCT and integrate semi-automated segmentation and analyses.

The two-dimensional morphological characteristics of the Ao, DA, PDA and LA were apparent and highly homologous to corresponding histological sections, and through investigations in the 3rd dimension we were able to visualize the DA in its anatomical position in the E18.5 murine torso, highlighting SRμCT as a key tool for future studies of cardiovascular development in animal models. Currently, no facility in Europe offers in vivo SRμCT imaging for animals, however, infrastructures centered around the MAXIV facility in Lund, Sweden, hold promise for its development.

We performed structure-tensor analysis using an implementation developed for studies of fiber directionality in material sciences (183) and were able to demonstrate differential orientation of the medial and intimal layers of the closing DA. Further studies utilizing this and similar constructions will be vital to advance our knowledge of vascular biomechanics.

Diagnostic and pharmacological implications

The separation of versican fragments observed in Study II led us to believe that specific epitopes might be identified at elevated concentrations in plasma of PAH patients, as was shown previously (191). Indeed, we successfully validated these findings in two separate cohorts of IPAH/HPAH, both with and without PAH-targeted therapy, and confirmed that the identified epitope came from the versican G3 domain. With regards to conclusions on the relationship between circulating versican G3 and PAH disease severity, we are limited by a small sample number, a single time point, and the lack of information on confounding factors that might have influenced the concentration of circulating versican.

Disease-modifying therapies to halt or reverse pulmonary vascular remodeling are lacking in current practice. To our knowledge, there are no available therapies directly targeting versican or aggrecan, however several modifiers of MMP- or ADAMTS-mediated proteolytic activity have been studied in the setting of osteoarthritis (209) and cancer (210) and might be interesting to consider for PAH.

In the absence of each other, versican and HA were shown to be less stable (211). Attempts to inhibit HA synthesis in chronic diseases have been theorized, and to some extent tested, with hymecromone (4- methylumbelliferone), a drug used to treat biliary dyskinesia (212). Similar trials in the PAH setting have not yet been undertaken. The targeted degradation or blockage of the G1 HA-binding domain, would theoretically destabilize lectican-HA supramolecular complexes and impair function.

Influencing the post-transcriptional modification of CS chains and the GAG-binding domain were suggested targets for immunomodulating cancer therapies (22, 213), where an ECM rich in certain CS sulfation patterns and βGAG has been associated with malignant potential. However, we are currently limited in our understanding of

the molecular relationships between lecticans, ADAMTS proteases and PAH remodeling and DA closure, and thus unable to pinpoint a target that could modify vascular remodeling to our advantage.

It is our firm belief that through advancing the knowledge of the versatile lecticans in obliterative vascular remodeling, pivotal mechanisms underlying disease development can be identified, which could be deciphered to generate targeted therapies.

Pre-transplant PH in children – ascending to new heights?

Clinical practice in pediatrics, perhaps within the subspecialty of pediatric cardiology in particular, is heterogenous by nature: as lifestyle and environmentally acquired cardiopulmonary diseases are relatively rare, the majority of practice is centered around congenital malformations, where detailed genotyping and phenotyping are used to tailor treatments to the growing child. However, Study IV indicated that discrepancies in clinical practice might be the result of more profound differences in the view of PH-LHD and hemodynamic measurements in this patient group.

Half of the respondents in Study IV reported to have a PVRi cut-off for pHTx eligibility, with the majority using the lowest value obtained following AVT. Values ranged between 4-8 $WU \cdot m^2$, with >50% reporting their cut-off value at 6 $WU \cdot m^2$, possibly reflecting the sustained influence of the 24th Bethesda Conference (214) held in 1993. Several retrospective cohort studies have investigated pre-pHTx cut-offs for PVRi since then. Rigorous work published in the contemporary era stems from Richmond *et al* (215) and Chiu *et al* (152, 153) who concluded that mortality at 30 days, and up to 5 years post-pHTx, was un-affected by an elevated PVRi <9 $WU \cdot m^2$. These findings are contrasted by early work (151, 216-218) where strong correlations between elevated PVRi and post-pHTx mortality were reported.

Although early reports managed to find strong negative correlations despite smaller cohort sizes, more recent larger studies seem to generally have reported more positive post-pHTx outcomes of elevated pre-transplant PVRi (112). This could reflect advancements in pre- and post-pHTx management of PH-LHD, CHD and RV failure in the contemporary era. In Sweden, children listed between 1989 and 2008 were implicated to present with a worse overall clinical status at listing, than those listed in the contemporary era (2009-2023). This was indicated by the combination of increased incidence, decreased mortality and longer duration until pHTx during this period.

Study V additionally highlighted the increase in the use of preoperative VAD in Sweden, without impaired long-term survival, although the use amongst children with PH was not specifically investigated. A majority of practitioners in Study IV were willing to treat patients with PH-LHD on VAD with pulmonary vasodilators but seemed more restrictive with vasodilators in the absence of durable MCS. Few respondents considered listing for combined heart-lung transplantation an option for any of the three cases presented, perhaps reflecting a general awareness of the improved outcomes of pre-pHTx VAD treatment (219-222). More importantly, improved wait list outcomes in children with VAD support have been demonstrated to be even more pronounced in children with elevated PVRi ≥6 (223), underscoring the importance of durable MCS for unloading of the LV.

Despite a successive build-up of evidence to support higher PVRi cut-offs, merely 49% of the participants in Study IV reported to have transplanted patients with pre-AVT PVRi ≥6 WU·m^2 in the last five years. This indicates that current practice lags behind the evidence presented during the last decade. In Sweden, only two children with reported hemodynamic data and post-AVT PVRi ≥6 WU·m^2 had been transplanted between 1989-2023, as reported in Study V. Simultaneously, patients with a pre-AVT PVRi well above 6 had been accepted liberally when reaching below this value following AVT. In Study V we did not manage to demonstrate any significant correlation between elevated PVRi (pre- and post-AVT) and survival time, and estimated survival for the dichotomized PVRi variable did not show a statistically significant difference in post-pHTx survival.

Pioneering work by Addonizio *et al* (150) in a small cohort of children (n=30) suggested that elevated PVR was only associated with increased post-pHTx mortality if coinciding with inotrope dependance. The impact of additive high-risk factors on post-pHTx outcomes has been studied by others more recently (154, 224), with similar outcomes. This may suggest that elevated PVRi may have been a surrogate for aggravated heart failure in early studies, and that improved management of non-PH-related factors in the modern era allows for a wider span of acceptable pre-pHTx PVRi. It may also be the case that most pHTx centers today are very well aware of the risk for adverse outcomes in patients with high PVRi, so that the condition is managed successfully through active monitoring and treatment during the post-pHTx period. This would make it difficult in contemporary cohorts to prove that PVRi remains a risk factor for mortality.

The recently updated ISHLT Guidelines for the Evaluation and Care of Cardiac Transplant Candidates (October 2024) (148), harmonized pediatric and adult practice guidelines for the first time and suggested that a PVRi ≤9 WU·m^2 obtained after "*unloading with diuresis and vasodilation (with vasoactive agents or MCS as needed)*" would be acceptable for isolated pHTx (Class of Recommendation 2a and Level of Evidence B-NR) (148). This recommendation better reflects our current understanding of PVRi in relationship to end-stage heart disease in children (225) and has the impact to significantly alter practice. However, the derivation and

reliability of PVRi remains controversial, as clearly visible in Lickert scale responses provided in Study IV.

As previously outlined, multiple factors influence the calculation of PVRi and limits applicability in the pediatric population. AVT offers an inter-individual comparison, which partially addresses these limitations, however, remains dependent on the operator and the drugs used. A great portion of respondents in Study IV indicated that AVT was performed if baseline PVRi was elevated, with a combination of calculated Fick and thermodilution being the preferred method to determine CO. At our center, the utility of magnetic resonance imaging combined with computational fluid dynamics for pulmonary hemodynamic estimations in Fontan patients was demonstrated (226). Utilizing advanced imaging modalities in combination with computational modeling may potentially provide improved estimations of pulmonary blood flow and PVRi in the future. The use of implantable devices for continuous measurement of pulmonary hemodynamics (227-229) holds promise but warrants further investigation in the pediatric population.

The diastolic transpulmonary pressure gradient (dTPG) was suggested as a physiologically sound alternative to estimate the severity of pulmonary vascular disease in PH-LHD (230). It is calculated by subtracting PAWP from the diastolic PA pressure and is thus less dependent of flow metrics, however more sensitive to heart rate variability (231, 232). dTPG ≥7 mmHg has been suggested as a cutoff to differentiate combined pre- and post-capillary PH from isolated post-capillary PH. One study in children showed a relationship between elevated dTPG, but not PVRi, and increased risk of early graft loss (233). Currently, data is lacking to support dTPG as a superior alterative to PVRi for the estimation of pre-pHTx vascular resistance in the pulmonary circulation (112).

Prospects for children with advanced heart failure in Sweden

Short- and long-term outcomes for Swedish children listed for pHTx, outlined in Study V, were comparable to the most recent reports from the United Network for Organ Sharing (234), ISHLT (235) and Pediatric Heart Transplant Society (236) databases. Characteristics and outcomes following the first two decades of pHTx in Sweden were outlined in earlier work (237, 238), however Study V gave a comprehensive update, and included the most recent 10 years of practice with multiple variables not reported previously.

Listing, but not pHTx, during ERA II was a significant determinant of post-pHTx outcomes in our stepwise Cox proportional hazard analysis, indicating that early pHTx performed in Sweden have had excellent long-term outcomes. The etiology

of heart failure, age at pHTx and presence of VAD support did not affect post-pHTx outcomes. Availability of free, accessible, health care and well-organized pediatric heart failure follow-up programs are factors that most likely have contributed to these results.

The importance of the Scandiatransplant collaboration cannot be underestimated. Maintaining a common wait list for children and optimizing organ allocation has been instrumental to enable pHTx for infants and critically ill children in the region, although high wait list mortality for infants remains a challenge in Sweden and internationally (156, 239). The efficiency of the organ exchange is highlighted by the net organ export that has occurred from the Scandiatransplant region in recent years (240).

Important differences between contemporary American and Swedish cohorts can be outlined: infant listing and pHTx are less common in Sweden and a smaller proportion of children listed for pHTx suffer from CHD (234, 237). Differences in strategies to manage complex CHD and UVH in infants might provide an explanation for both these discrepancies.

Although ABOi pHTx were scarce in our cohort, we report no post-pHTx death following ABOi pHTx during the follow-up period, supporting previous data of non-inferior outcomes in this group (174, 175). Detailed descriptions of HLA immunization and presence of DSA post-transplant in pediatric cohorts are rare, although repeated post-pHTx monitoring was suggested to be an important tool for risk stratification of pHTx recipients as well as a prognostic factor predicting CAV (241, 242). From our immunological risk stratification, based on the presence of single or a few HLA antibodies DSA with high MFI, we suggest that a substantial percentage of immunization might be attributable to a limited number of strong antibodies.

Graft failure, cardiovascular events and malignancy remain the three main causes of death in Swedish pHTx recipients, in line with reports from the ISHLT (243). Development in the field of immunosuppressive treatments, monitoring of DSA and donor-derived cell-free DNA (244) as well as the implementation of immunoadsorption protocols to combat clinically significant sensitization all have the potential to further improve the outcomes for Swedish children listed for heart transplantation.

Limitations

Aside from the limitations discussed above, the work presented in this thesis suffers from both inevitable and avertable flaws.

Human tissue sampling for Study I-III was conducted as part of surgical procedures or with a diagnostic intention and was thus not standardized between individuals or cohorts with regards to sample location, size or timing. Due to the rare nature of the conditions investigated, specimens were collected over several years and stored at biobanks. Tissue processing protocols likely varied between institutions and over time. Sampling was standardized for animal tissue, and a greater insight was provided into processing, however differences in protocols between laboratories were observed.

More specifically, the available specimens for Study I and II were from end-stage PAH, which made us unable to decipher if lectican accumulation and turnover was the cause of PAH or a secondary finding resulting from the diseased state. Such investigations would require an animal model which reflects the human disease, where the extended Sugen 5416/hypoxia rat model has emerged as a promising candidate (123, 245, 246). Specimens for Study III were taken from corrective cardiac surgeries, where differences in both genotype and phenotypes had the potential to influence lecticans, their degradation and our interpretation of these stainings. The inclusion of animal DAs in Study III partially addressed this issue.

Self-selection criteria for inclusion in Study IV had the potential to generate a heterogenous cohort of respondents with different experience, training and references. Limited depth in our investigation and lack of flexibility in the choice of predominantly multiple-choice questions, are other drawbacks of the chosen format. The intention to gather international data is desirable and could highlight differences across multiple continents, however, the reported data needs to be related to the conditions of clinical practice in each country. Heterogeneity in reported practice may, in part, have been the result of this broad inclusion criteria.

Insufficient data granularity was a limitation for Study V, where additional data on hemodynamics, perioperative and intensive care course and rejections would have allowed for improved accuracy of our descriptions, analyses and conclusions. Stratifying our analyses by additional time periods would potentially have provided more detailed information on the changes observed in pHTx care over time in Sweden but could have limited our statistical analyses. Lack of hemodynamic data for listed patients that did not undergo pHTx may have introduced selection bias.

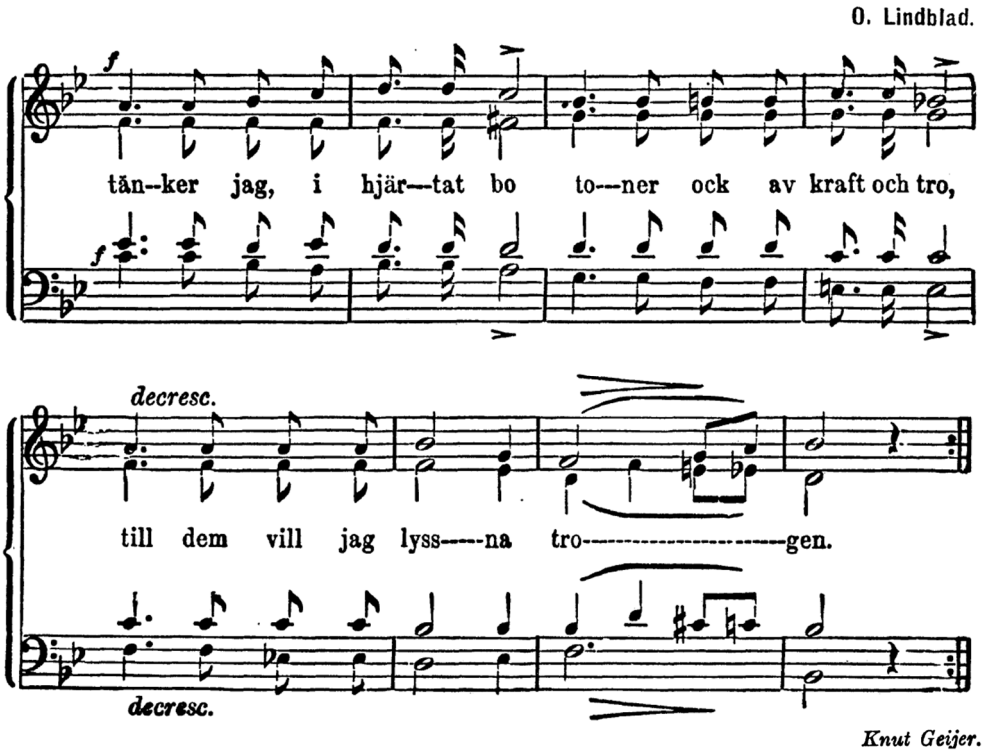
Concluding remarks

The work presented in this thesis addresses vital aspects of physiological and pathological pulmonary and fetal vascular remodeling and clinical management of neonates and children with heart disease, all relating to the elevation of vascular resistance in the pulmonary circulation. Based on Study I-V, the following conclusions are suggested:

Lectican production by VSMCs and ECs occurs in early vascular remodeling of PAH, with significant accumulation at sites of elevated flow or hypertensive injury. Aggrecan is identified as a novel component of the pulmonary vascular ECM. Proteolysis of versican by members of the ADAMTS family is likely to be a key mechanism inciting further vascular remodeling and versican G3 is strengthened as a promising candidate plasma biomarker for PAH.

Versican and aggrecan are dynamically regulated throughout DA maturation, and to a lesser extent present in the PDA at critical stages of development. The relationship between β-catenin signaling and lectican accumulation is suggested as an important target for future studies of intimal cushion formation. SRμCT enables three-dimensional investigation, segmentation and computed analyses of pulmonary and fetal vascular remodeling with high precision and minimal tissue interference.

The current clinical management of children with PH and end-stage heart disease shows little international consensus, in part due to the uncertainty and variability of methods used to quantify pulmonary hemodynamic variables for PVRi calculations. In Sweden, children with elevated PVRi undergoing pHTx did not fare worse off than their peers. Although PVRi is an imperfect estimate of pulmonary vascular disease severity, suggestions to raise cut-offs to preclude children from isolated pHTx harmonize well with the data presented in this thesis. Overall, short- and long-term results from Swedish pHTx practice are encouraging, most likely due to tight-knit teams and collaboration within each center, at the national level and in the Scandiatransplant region.



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