



LUND UNIVERSITY

Plasma biomarkers in pulmonary arterial hypertension diagnosis, treatment response and risk stratification - utilizing Lund Cardio Pulmonary Register

Bouzina, Habib

2020

Document Version:

Publisher's PDF, also known as Version of record

[Link to publication](#)

Citation for published version (APA):

Bouzina, H. (2020). *Plasma biomarkers in pulmonary arterial hypertension diagnosis, treatment response and risk stratification - utilizing Lund Cardio Pulmonary Register*. [Doctoral Thesis (compilation), Department of Clinical Sciences, Lund]. Lund University, Faculty of Medicine.

Total number of authors:

1

General rights

Unless other specific re-use rights are stated the following general rights apply:

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

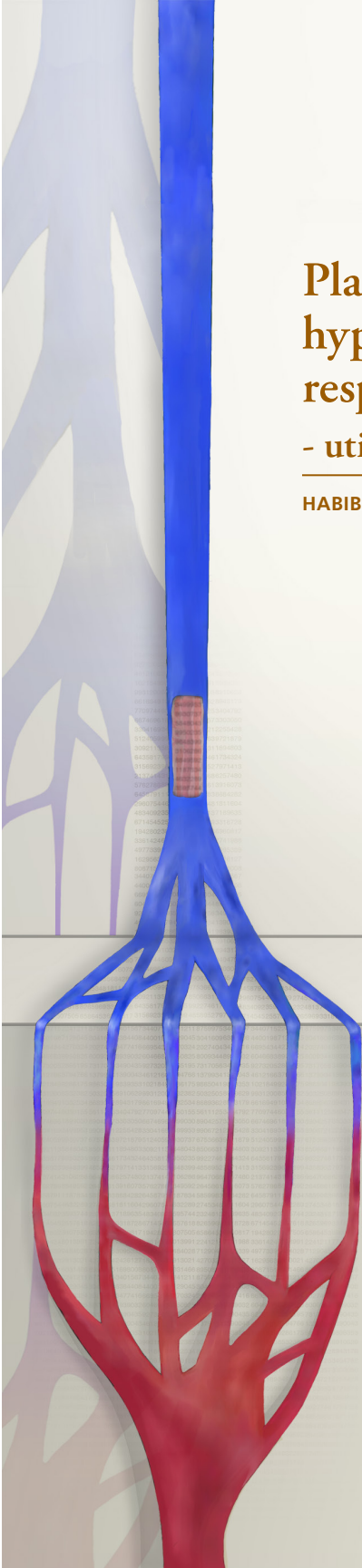
Read more about Creative commons licenses: <https://creativecommons.org/licenses/>

Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

LUND UNIVERSITY

PO Box 117
221 00 Lund
+46 46-222 00 00

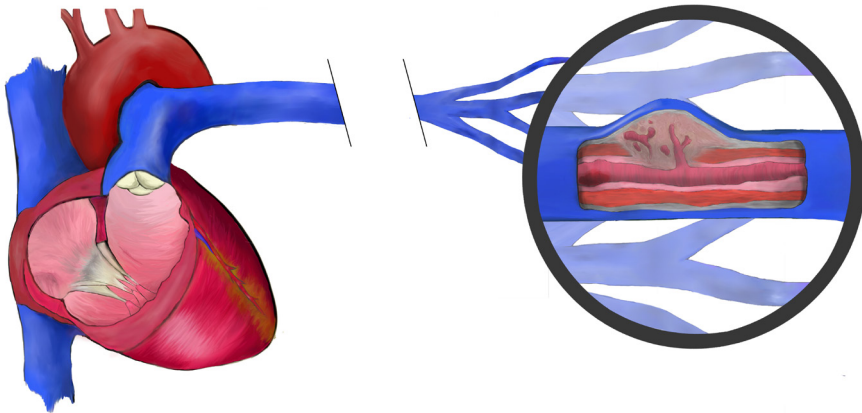


Plasma biomarkers in pulmonary arterial hypertension diagnosis, treatment response and risk stratification

- utilizing Lund Cardio Pulmonary Register

HABIB BOUZINA | CARDIOLOGY | FACULTY OF MEDICINE | LUND UNIVERSITY





Plasma biomarkers in pulmonary arterial hypertension diagnosis, treatment response and risk stratification

- utilizing Lund Cardio Pulmonary Register

Habib Bouzina



LUND
UNIVERSITY

DOCTORAL DISSERTATION

by due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended at Segerfalksalen, BMC, Lund University, Lund.

October 30, 2020, 13:15

Faculty opponent

Marc Humbert, MD, PhD, Professor
Faculté de Médecine, Université Paris-Sud and
Université Paris-Saclay, Le Kremlin-Bicêtre, France

Organization LUND UNIVERSITY Dept. of Clinical Sciences Lund, Cardiology, Faculty of Medicine Author: Habib Bouzina		Document name Dissertation	
		Date of issue October 30, 2020	
		Sponsoring organizations: ALF foundations, Actelion Pharmaceuticals Sweden AB, GlaxoSmithKline and the Swedish Society of Pulmonary Hypertension (SveFPH).	
Title and subtitle: Plasma biomarkers in pulmonary arterial hypertension diagnosis, treatment response and risk stratification - utilizing Lund Cardio Pulmonary Register.			
Abstract Pulmonary arterial hypertension (PAH) is a rare disease, where vasoconstriction and remodeling of small pulmonary arteries result in elevated pulmonary vascular resistance, and ultimately right ventricular dysfunction. Although the molecular mechanisms sustaining PAH are not completely understood, many cornerstones in PAH pathology have been identified, including processes in vasoconstriction, enhanced cell growth and apoptosis resistance, metabolic reprogramming and altered inflammation, which all constitute major vascular insults. The introduction of PAH treatment has markedly improved patient survival, but PAH management still requires further development. New and viable biomarkers in PAH have the potential to provide earlier diagnosis, evaluate therapy response and estimate patients' risk for early mortality, which can be assessed using the European Society of Cardiology and European Respiratory Society (ESC/ERS) risk model or the REVEAL risk score. This dissertation aimed to investigate plasma biomarkers that are potentially linked to different domains of PAH pathology, in treatment-naïve PAH patients from the Lund Cardio Pulmonary Register (LCPR) cohort. The first two papers focused on biomarkers related to receptor tyrosine kinase signaling, which, among others, is connected to cellular growth. The first paper showed that plasma soluble fms-like tyrosine kinase-1 (sFlt-1) levels decrease between PAH diagnosis and early treatment follow-ups. Changes in sFlt-1, between diagnosis and follow-ups, correlated with changes in some markers of clinical severity in PAH. This provides an additional treatment response perspective to the previous sFlt-1 studies in PAH, which focused on diagnosis and differentiation. The second paper showed that PAH patients at diagnosis exhibited low plasma levels of stem cell factor (SCF), which were inversely correlated to markers of clinical severity. SCF levels differentiated high-risk PAH patients from other PAH patients, where risk was assessed using the ESC/ERS risk model. A combination of plasma SCF and transforming growth factor (TGF)- α provided high sensitivity and specificity for detecting high-risk PAH patients. SCF in combination with TGF- α is, therefore, a potential multi-marker strategy in PAH risk stratification. Previous reports have underlined several similarities between tumor pathology and PAH, which include altered cellular energetics, sustained growth signaling and apoptosis resistance. For instance, cellular metabolic shifts in PAH include increased glucose uptake and consumption. The two following papers in this dissertation focused on metabolic and tumor-related biomarkers. The main findings from the third and fourth papers shed light on plasma fibroblast growth factor (FGF)-23 and insulin-like growth factor binding protein (IGFBP)-1, respectively. FGF-23 and IGFBP-1 levels were elevated in PAH compared to controls, and correlated to hemodynamics, six-minute walking distance (6MWD) and N-terminal prohormone of brain natriuretic peptide (NT-proBNP) as well as the ESC/ERS risk model and a modified REVEAL risk score. The fifth and final paper investigated plasma biomarkers linked to vaso-regulation. In particular, the results centered around adrenomedullin precursor and peptides, including mid-regional pro-adrenomedullin (MR-proADM), which collectively were referred to as ADM. ADM levels at PAH diagnosis were higher compared to healthy controls and unaltered compared to chronic thromboembolic pulmonary hypertension, but lower compared to pulmonary hypertension due to left heart disease. At PAH diagnosis, plasma ADM correlated with hemodynamics, 6MWD and NT-proBNP as well as risk according to the ESC/ERS risk model and a modified REVEAL risk score. PAH patients with higher plasma ADM levels showed worse survival compared to those with lower levels. Adrenomedullin and MR-proADM have previously been linked to PH severity. Paper V confirmed this observation and provided an additional aspect of ADM and risk assessment in PAH. Taken together, the papers in this dissertation provide new plasma biomarkers for further research in PAH, and add new insights to the previously investigated circulating biomarkers in PAH.			
Key words: pulmonary arterial hypertension, plasma biomarkers, risk stratification, treatment response, sFlt-1, stem cell factor, FGF-23, IGF binding protein, adrenomedullin.			
Classification system and/or index terms (if any)			
Supplementary bibliographical information		Language: English	
ISSN and key title 1652-8220		ISBN 978-91-7619-971-8	
Recipient's notes	Number of pages 67	Price	
	Security classification		

I, the undersigned, being the copyright owner of the abstract of the above-mentioned dissertation, hereby grant to all reference sources permission to publish and disseminate the abstract of the above-mentioned dissertation.

Signature 

Date 2020-09-17

Plasma biomarkers in pulmonary arterial hypertension diagnosis, treatment response and risk stratification

- utilizing Lund Cardio Pulmonary Register -

Habib Bouzina



LUND
UNIVERSITY

Main supervisor:

Göran Rådegran, MD, MSc Eng Phys, DMSc, Associate Professor

Assistant supervisor:

Roger Hesselstrand, MD, PhD, Associate Professor

Cover photo by Habib Bouzina

Copyright Habib Bouzina

Paper 1 © Bouzina H, et al. 2017

Paper 2 © ERS 2018

Paper 3 © Bouzina H, et al. 2019

Paper 4 © Bouzina H, et al. 2020

Paper 5 © Bouzina H, et al. 2020

Lund University, Faculty of Medicine

ISBN 978-91-7619-971-8

ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2020



Media-Tryck is a Nordic Swan Ecolabel
certified provider of printed material.
Read more about our environmental
work at www.mediatryck.lu.se


MADE IN SWEDEN 

Table of Contents

List of papers	7
Abstract	9
Summary in Swedish (Sammanfattning på svenska)	9
Abbreviations	15
Introduction	17
Pulmonary hypertension.....	17
PAH treatment.....	18
PAH prognosis	18
Risk stratification	19
Circulating biomarkers in PAH.....	19
PAH pathology.....	20
Molecular PAH pathology and related circulating biomarkers	21
Aims	29
Materials and methods	31
General method description	31
Lund Cardio Pulmonary Register and blood sampling	31
Ethics	31
Clinical evaluation and aspects.....	32
Right heart catheterization.....	32
General comments on statistical analyses and software	32
Paper I	33
Study cohort and biomarkers.....	33
Analysis approach and statistics	34
Paper II-V	35
Study cohort	35
Data collection and risk calculations	35
Biomarker selection.....	36
Biomarker analysis	36
Comments on analysis approach	37
Statistics.....	37

Results.....	39
Paper I	39
Paper II	40
Paper III and IV	42
Paper V	46
Discussion	47
Paper I	47
Paper II	48
Paper III and IV	48
Paper V	50
Strengths and limitations	51
Future perspectives.....	51
Conclusions	53
Acknowledgments.....	55
Bibliography.....	57
Paper I-V	66

List of papers

The present thesis is based on the following papers, referred to in the text by their respective Roman numerals.

- I. **Bouzina H**, Nielsen S, Scheele C, Rådegran G. Plasma sFlt-1 fluctuations in response to pulmonary arterial hypertension treatment. *Cardiovascular Pharmacology: Open Access* 2016: 6(2).
- II. **Bouzina H**, Rådegran G. Low plasma stem cell factor combined with high transforming growth factor- α identifies high-risk patients in pulmonary arterial hypertension. *ERJ Open Research*, 2018: 4(4).
- III. **Bouzina H**, Hesselstrand R, Rådegran G. Higher plasma fibroblast growth factor 23 levels are associated with a higher risk profile in pulmonary arterial hypertension. *Pulmonary Circulation*, 2019: 9(4).
- IV. **Bouzina H**, Hesselstrand R, Rådegran G. Plasma insulin-like growth factor binding protein 1 in pulmonary arterial hypertension. *Scandinavian Cardiovascular Journal* 2020:1-8.
- V. **Bouzina H**, Rådegran G. Plasma adrenomedullin peptides and precursor levels in pulmonary arterial hypertension disease severity and risk stratification. *Pulmonary Circulation* 2020: 10(3).

Scientific contributions related to, but not included in this PhD dissertation, are:

- VI. Säleby J, **Bouzina H.**, Lundgren J, Nielsen S., Scheele C, Rådegran G. Angiogenic and inflammatory biomarkers in the differentiation of pulmonary hypertension. *Scandinavian Cardiovascular Journal* 2017: 51(5).
- VII. Säleby J, **Bouzina H.**, Ahmed S, Lundgren J, Rådegran G. Plasma receptor tyrosine kinase RET in pulmonary arterial hypertension diagnosis and differentiation. *ERJ Open Research* 2019: 5(4).
- VIII. Arvidsson M, Ahmed A, **Bouzina H.**, Rådegran G. Matrix metalloproteinase 7 in diagnosis and differentiation of pulmonary arterial hypertension. *Pulmonary Circulation* 2019: 9(4).
- IX. Ahmed A, Ahmed S, Arvidsson M, **Bouzina H.**, Lundgren J, Rådegran G. Prolargin and matrix metalloproteinase-2 in heart failure after heart transplantation and their association with hemodynamics. *ESC Heart Failure* 2020: 7(1).
- X. Ahmed S, Ahmed A, Säleby J, **Bouzina H.**, Lundgren J, Rådegran G. Elevated plasma tyrosine kinases VEGF-D and HER4 in heart failure patients decrease after heart transplantation in association with improved hemodynamics. *Heart and Vessels* 2020: 35(6).
- XI. Ahmed A, Ahmed S, Arvidsson M, **Bouzina H.**, Lundgren J, Rådegran G. Elevated plasma sRAGE and IGFBP7 in heart failure decrease after heart transplantation in association with hemodynamics. *ESC Heart Failure* 2020.
- XII. Ahmed S, Ahmed A, **Bouzina H.**, Lundgren J, Rådegran G. Elevated plasma endocan and BOC in heart failure patients decrease after heart transplantation in association with improved hemodynamics. *Heart and Vessels* 2020.

Abstract

Pulmonary arterial hypertension (PAH) is a rare disease, where vasoconstriction and remodeling of small pulmonary arteries result in elevated pulmonary vascular resistance, and ultimately right ventricular dysfunction. Although the molecular mechanisms sustaining PAH are not completely understood, many cornerstones in PAH pathology have been identified, including processes in vasoconstriction, enhanced cell growth and apoptosis resistance, metabolic reprogramming and altered inflammation, which all constitute major vascular insults. The introduction of PAH treatment has markedly improved patient survival, but PAH management still requires further development. New and viable biomarkers in PAH have the potential to provide earlier diagnosis, evaluate therapy response and estimate patients' risk for early mortality, which can be assessed using the European Society of Cardiology and European Respiratory Society (ESC/ERS) risk model or the REVEAL risk score. This dissertation aimed to investigate plasma biomarkers that are potentially linked to different domains of PAH pathology, in treatment-naïve PAH patients from the Lund Cardio Pulmonary Register (LCPR) cohort.

The first two papers focused on biomarkers related to receptor tyrosine kinase signaling, which, among others, is connected to cellular growth. The first paper showed that plasma soluble fms-like tyrosine kinase-1 (sFlt-1) levels decrease between PAH diagnosis and early treatment follow-ups. Changes in sFlt-1, between diagnosis and follow-ups, correlated with changes in some markers of clinical severity in PAH. This provides an additional treatment response perspective to the previous sFlt-1 studies in PAH, which focused on diagnosis and differentiation. The second paper showed that PAH patients at diagnosis exhibited low plasma levels of stem cell factor (SCF), which were inversely correlated to markers of clinical severity. SCF levels differentiated high-risk PAH patients from other PAH patients, where risk was assessed using the ESC/ERS risk model. A combination of plasma SCF and transforming growth factor (TGF)- α provided high sensitivity and specificity for detecting high-risk PAH patients. SCF in combination with TGF- α is, therefore, a potential multi-marker strategy in PAH risk stratification.

Previous reports have underlined several similarities between tumor pathology and PAH, which include altered cellular energetics, sustained growth signaling and apoptosis resistance. For instance, cellular metabolic shifts in PAH include increased glucose uptake and consumption. The two following papers in this dissertation focused on metabolic and tumor-related biomarkers. The main findings from the third and fourth papers shed light on plasma fibroblast growth factor (FGF)-23 and insulin-like growth factor binding protein (IGFBP)-1, respectively. FGF-23 and IGFBP-1 levels were elevated in PAH compared to controls, and correlated to hemodynamics, six-minute walking distance (6MWD) and N-terminal prohormone of brain natriuretic peptide (NT-proBNP) as well as the ESC/ERS risk model and a modified REVEAL risk score.

The fifth and final paper investigated plasma biomarkers linked to vaso-regulation. In particular, the results centered around adrenomedullin precursor and peptides, including mid-regional pro-adrenomedullin (MR-proADM), which collectively were referred to as ADM. ADM levels at PAH diagnosis were higher compared to healthy controls and unaltered compared to chronic thromboembolic pulmonary hypertension, but lower compared to pulmonary hypertension due to left heart disease. At PAH diagnosis, plasma ADM correlated with hemodynamics, 6MWD and NT-proBNP as well as risk according to the ESC/ERS risk model and a modified REVEAL risk score. PAH patients with higher plasma ADM levels showed worse survival compared to those with lower levels. Adrenomedullin and MR-proADM have previously been linked to PH severity. Paper V confirmed this observation and provided an additional aspect of ADM and risk assessment in PAH.

Taken together, the papers in this dissertation provide new plasma biomarkers for further research in PAH, and add new insights to the previously investigated circulating biomarkers in PAH.

Summary in Swedish (Sammanfattning på svenska)

Patienter med sjukdomen pulmonell arteriell hypertension (PAH) drabbas av att blodkärlen i lungorna drar ihop sig och blir förträngda, vilket medför en belastning på höger hjärthalva. Detta leder till bland annat andfäddhet och trötthet. Intensiv forskning inom PAH fältet har lett till en fördjupad kunskap kring olika sjukliga mekanismer som driver PAH. Det handlar bland annat om hur sammandragningen i de sjuka kärlen sker, hur celltillväxten i lungornas blodkärl uppstår samt hur inflammation påverkar kärlen.

Det första preparatet mot PAH introducerades 1995. Efter detta har alternativen för PAH behandling succesivt ökat, vilket har förbättrat sjukdomens prognos markant. Trots de stora framstegen i utvecklandet av effektiv behandling, råder det fortfarande en hög dödlighet bland patienter med PAH. För att identifiera patienter med en hög risk för tidig död används idag särskilda riskmodeller. Många variabler som ingår i sådana modeller kräver dock information från krävande utredningar. Därmed behövs även enklare metoder för att förutse vilka PAH patienter som löper ökad risk för tidig död.

Proteiner som kan mätas i blodet och ge information om patienternas riskprofil är en metod som skulle kunna förbättra vården av PAH patienter. De nämnda riskmodellerna inkluderar endast ett sådant protein; N-terminal prohormone of brain natriuretic peptide (NT-proBNP). NT-proBNP avspeglar endast en aspekt av PAH sjukdomen, nämligen belastningen på hjärtat, vilket dessutom inte är unikt för PAH. Att undersöka flera olika blodburna proteiner som kan spegla olika aspekter av PAH sjukdomen skulle möjligen kunna ge bättre information om patienternas riskprofil.

Denna avhandling syftade till att undersöka proteiner, som är relativt nya inom PAH området och som är potentiellt kopplade till olika aspekter av PAH patologi, i blodmaterial från patienter med PAH. Markörerna studerades i relation till

sjukdomens svårighetsgrad samt patienternas överlevnad, risk för tidig död och svar på PAH-specifik behandling.

Delarbetena I och II fokuserade på blodburna proteiner som är kopplade till cellernas tillväxtförmåga. I studien fann vi att blodnivåerna av markören soluble fms-like tyrosine kinase-1 (sFlt-1) sjunker hos PAH patienter under de tidiga uppföljningarna efter diagnos. Förändringarna i sFlt-1 nivåer mellan diagnostillfället och uppföljningarna visade samband med förändringar i redan etablerade kliniska variabler. Tidigare studier har visat att sFlt-1 nivåer i blod är förhöjda hos patienter med PAH. Delarbete I belyser en annan aspekt av markören sFlt-1 inom PAH, nämligen förändringarna mellan diagnos och uppföljningarna efter behandlingsstart. Proteinet sFlt-1 kan även ha en roll inom PAH patologi, men detta är ännu inte studerat. Det är dock välkänt att sFlt-1 kan bidra till sjukliga förändringar i kärl i andra sjukdomar.

Delarbete II fortsätter med att undersöka proteiner besläktade med sFlt-1. Fynden i detta arbete fokuserade på blodmarkören stem cell factor, vars nivåer vid diagnostillfället var lägre hos PAH patienter jämfört med kontroller. Detta är i linje med tidigare studier som har visat att patienter med hjärt- och kärlsjukdomar uppvisar låga nivåer av stem cell factor i blodet. Markören stem cell factor i delarbete II visade också en koppling till viktiga kliniska variabler, bland annat resistensen i lungkärlen och hjärtats pumpförmåga. Vi fann dessutom att stem cell factor kunde identifiera högriskpatienter, vars risk för tidig död bedömdes enligt riskmallen från kliniska PAH riktlinjer. En kombination av stem cell factor med blodmarkören transforming growth factor α , som är ett annat protein i samma molekylära gren, verkade erbjuda en ännu bättre potential till att identifiera högriskpatienter. Denna kombination visade även en tendens till att vara kopplad med bättre överlevnad hos patienterna.

Nya studier tyder på att en störd ämnesomsättning i lungkärlens celler kan ge väsentliga konsekvenser för PAH. Lungkärl från PAH har, till exempel, visat sig förbruka mer energi än vad friska celler förväntas göra. Denna observation påminner om hur tumörceller ändrar sin ämnesomsättning för att överleva. Detta är nämligen en av många paralleller som har observerats mellan tumörer och PAH. Andra likheter är att cellerna i lungkärlen uppvisar en tendens till att överleva och föröka sig i en sjuklig takt. Delarbete III fokuserade på markörer som är kopplade till ämnesomsättning, och delarbete IV på markörer som är kopplade till tumörbiologin.

De huvudsakliga resultaten från delarbete III skildrade förhållandet mellan svårighetsgraden av PAH och markören fibroblast growth factor 23 (FGF-23), som har en välkänd koppling till njursvikt. Denna koppling är viktig då kronisk njursvikt påverkar överlevnaden vid PAH negativt. Resultaten från delarbete IV lyfte fram markören insulin-like growth factor binding protein 1 (IGFBP-1). Både FGF-23 och IGFBP-1 visade sig ha samband med kliniska parametrar och två olika riskmodeller som bedömer risken för tidig död.

Medan delarbete I-IV fokuserade primärt på markörer kopplade till sjuklig cellöverlevnad och tillväxt, har delarbete V fokuserat på proteiner kopplade till reglering av kärlens sammandragning. Regleringen av hur mycket lungans små pulsådor dras samman är viktigt inom PAH, vilket speglas i att alla nutida PAH preparat riktar sig primärt mot kärksammandragning. Resultaten från delarbete V lyfte fram adrenomedullin proteiner, som var förhöjda i blod och som visade starka samband till kliniska parametrar samt risk för tidig död. Adrenomedullin proteiner var dessutom kopplade till PAH patienternas överlevnad. Detta bekräftar vad tidigare studier har kommit fram till angående adrenomedullin proteiner och PAH, men bidrar med ytterligare en aspekt som beskriver risken för tidig död.

Sammanfattningsvis bidrar denna avhandling med nya biomarkörkandidater, som potentiellt är till nytta för riskbedömning och evaluering av behandlingsrespons hos PAH patienter. Denna avhandling sätter fokus på molekyler som är nya inom PAH fältet, och generar hypoteser kring dessa som kan utforskas vidare inom experimentell PAH. Vissa aspekter av avhandlingen bekräftar och utvecklar även resultat från tidigare biomarkörstudier inom PAH fältet.

Abbreviations

6MWD	Six-minute walking distance
ADM	Adrenomedullin peptides and precursor
ADMA	Asymmetric dimethylarginine
BMP	Bone morphogenetic protein
CI	Cardiac index
CTD-PAH	Connective tissue disease associated pulmonary arterial hypertension
CTEPH	Chronic thromboembolic pulmonary hypertension
ESC/ERS	European Society of Cardiology / European Respiratory Society
FGF	Fibroblast growth factor
GDF-15	Growth differentiation factor 15
HPAH	Hereditary pulmonary arterial hypertension
IGFBP	Insulin-like growth factor binding protein
IPAH	Idiopathic pulmonary arterial hypertension
LCPR	Lund Cardio Pulmonary Register
LVSWI	Left ventricular stroke work index
MPAP	Mean pulmonary artery pressure
MR-proADM	Mid-regional pro-adrenomedullin

n	Number of patients
NT-proBNP	N-terminal prohormone of brain natriuretic peptide
PAH	Pulmonary arterial hypertension
PAWP	Pulmonary artery wedge pressure
PDGF	Platelet-derived growth factor
PEA	Proximity extension assay
PH	Pulmonary hypertension
Pim-1	Provirus integration site for Moloney murine leukemia virus 1
PVR	Pulmonary vascular resistance
RVSWI	Right ventricular stroke work index
SCF	Stem cell factor
sFlt-1	Soluble fms-like tyrosine kinase-1
SOD	Superoxide dismutase
SPAHR	Swedish pulmonary arterial hypertension registry
STAT3	Signal transducer and activator of transcription 3
SvO ₂	Mixed venous oxygen saturation
TGF	Transforming growth factor
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

Introduction

Pulmonary hypertension

Pulmonary hypertension (PH) encompasses disease groups that exhibit high pulmonary artery pressure. These include pulmonary arterial hypertension (PAH) (group 1), PH due to left heart disease (group 2), PH due to lung disease and/or hypoxia (group 3), chronic thromboembolic PH (CTEPH) (group 4) and PH with unclear and/or multifactorial origin (group 5)¹.

PAH is a rare disease characterized by constrictive, hypertrophic, and fibrotic remodeling of distal pulmonary arteries, ultimately leading to right ventricular dysfunction². This leads to an array of unspecific symptoms, including dyspnea and fatigue. A PAH diagnosis requires a mean pulmonary artery pressure (MPAP) ≥ 25 mmHg at rest together with a pulmonary artery wedge pressure (PAWP) ≤ 15 mmHg and a pulmonary vascular resistance (PVR) > 3 Wood units, as assessed by right heart catheterization, along with the exclusion of parenchymal, hypoxic or thrombotic lung diseases, left-sided heart disease and unclear and/or multifactorial causes¹. A reduction in the MPAP diagnosis criterion, from ≥ 25 to > 20 mmHg, has been proposed in the 2018 World Symposium on PH but has still not been implemented in new clinical guidelines³.

PAH includes the subgroups idiopathic (IPAH) and hereditary PAH (HPAH), drug and toxin-induced forms as well as conditions associated with other diseases such as connective tissue diseases (CTD-PAH), human immunodeficiency virus, portal hypertension, congenital heart disease as well as schistosomiasis¹. In the Swedish PAH registry (SPAHR), IPAH and HPAH subgroups constitute around half of the PAH patients⁴.

PAH treatment

Historically, PAH patients without targeted treatment exhibited a median survival of 2.8 years, with one- and three-year survival rates of 68% and 48%, respectively⁵. Since the first PAH-targeting drug was introduced in 1995, the medical arsenal against PAH has continually been expanding. The current targeted therapies in PAH include endothelin receptor antagonists, phosphodiesterase 5 inhibitors, a soluble guanylate cyclase stimulator, as well as prostacyclin analogs and a prostacyclin receptor agonist. These drugs address the altered endothelin, nitric oxide, and prostacyclin vaso-regulatory pathways in PAH. European Society of Cardiology and European Respiratory Society (ESC/ERS) 2015 PH guidelines recommend a treatment strategy with mono- or upfront combination therapy at PAH diagnosis, depending on the patient's clinical severity and risk for early mortality¹. In combination therapy, two or more drug classes are used to address multiple vaso-regulatory pathways, aiming to achieve better treatment response and improved prognosis⁶. At follow-up, treatment escalation is recommended for patients showing inadequate treatment response¹. There is currently no cure for PAH, and the last treatment resort is still lung transplantation.

PAH prognosis

Effective medical treatment has improved disease control and survival in PAH. For example, recent reports show that survival rates for one- and three-years in PAH are 85% and 71%, respectively, in Sweden⁴. Nonetheless, these survival rates are still unsatisfactory, and PAH management requires better and more viable resources for earlier diagnosis, improved risk assessment and better evaluation of therapy response, as well as new therapies targeting the remodeling of pulmonary arteries. PAH is generally detected in later stages of the disease, where most incident patients are in World Health Organization (WHO) functional class III⁴. Indeed, a significant delay between symptom onset and diagnosis has been reported⁷.

Risk stratification

Patients' risk for early mortality can be assessed using the REVEAL risk score or the risk assessment strategy from the ESC/ERS guidelines. These are multiparametric approaches that, among others, use hemodynamics, echocardiography data, functional status, and clinical vital signs to estimate risk. The ESC/ERS model, which stratifies patients into low-, intermediate- or high-risk groups, has been extensively elaborated on in 2017, and validated for predicting one-year mortality in three independent studies⁸⁻¹⁰ (Table 1).

Table 1. Determinants of prognosis from the ESC/ERS pulmonary hypertension guidelines

Determinants of prognosis	Low risk	Intermediate risk	High risk
WHO functional class	I, II	III	IV
6MWD	> 440 m	165-440 m	< 165 m
NT-proBNP	< 300 ng/L	300-1400 ng/L	> 1400 ng/L
Right atrial area	< 18 cm ²	18-26 cm ²	> 26 cm ²
Pericardial effusion	No	No or minimal	Yes
Right atrial pressure	< 8 mmHg	8-14 mmHg	> 14 mmHg
Cardiac index	≥ 2.5 L/min/m ²	2.0-2.4 L/min/m ²	< 2.0 L/min/m ²
SvO ₂	> 65 %	60-65 %	< 60 %

Adapted from the European Society of Cardiology and European Respiratory Society (ESC/ERS) pulmonary hypertension guidelines¹. Parameters that are used in the Swedish pulmonary arterial hypertension registry (SPAHR) risk assessment model are presented herein. 6MWD: 6-minute walking distance; NT-proBNP: N-terminal prohormone of brain natriuretic peptide; SvO₂: mixed venous oxygen saturation; WHO: World Health Organization.

Circulating biomarkers in PAH

The National Institutes of Health Biomarkers Definitions Working Group has defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention"¹¹.

An ideal circulating biomarker in PAH should provide earlier diagnosis, evaluate prognosis, and detect therapy response failure in PAH. Biomarkers could serve as tools that mirror the hemodynamic state in clinics that may not offer frequent hemodynamic evaluations. Finally, biomarkers could serve as screening tools that identify early pulmonary arterial engagement in systemic sclerosis patients, relatives to HPAH patients or other populations with increased risk for PAH¹².

Remarkable biomarkers in PAH are presented in the next section, in relation to their context in pathology.

PAH pathology

In PAH, distal pulmonary arteries are subjected to vasoconstriction and remodeling, which includes intimal thickening and fibrosis, medial hypertrophy, adventitial fibrosis as well as characteristic plexiform lesion formation² (Figure 1). Increased perivascular inflammation and in situ thrombosis are also observed. Narrowed pulmonary arterial lumens in PAH generate elevated pulmonary vascular pressure and resistance that result in an elevated right ventricular load. In response, the right ventricle first becomes hypertrophic but subsequently dilates, leading to right ventricular failure, and ultimately death. Notably, the most common cause of death in PAH is right ventricular failure / sudden death¹³.

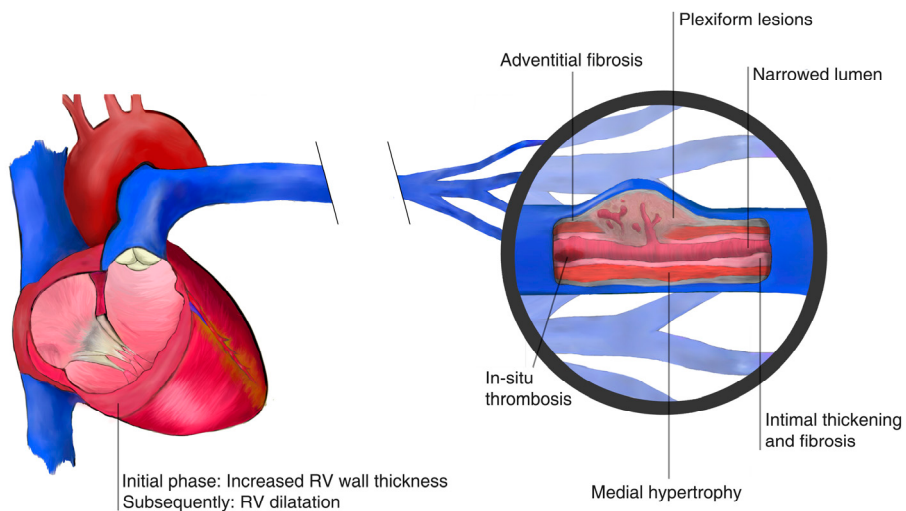


Figure 1. Morphological changes in pulmonary arterial hypertension.

RV: right ventricle.

Molecular PAH pathology and related circulating biomarkers

Although the underlying molecular pathobiology leading to and sustaining the characteristic PAH features are still not completely understood, many mechanisms have been extensively mapped out since the millennial shift. Research has moved beyond experiments that pivoted mainly on vasoconstriction, to embrace other intriguing areas of pathology as well, including cellular growth and apoptosis resistance, metabolic reprogramming, altered inflammatory and immune response as well as extracellular matrix and coagulation imbalances (Figure 2). As PAH pathology is a broad subject, this section focuses on pathology areas that are touched upon in paper I-V.

Altered vaso-regulation

Increased vasoconstriction is an important part of PAH pathology. As previously mentioned, vaso-regulatory signaling cascades are the primary targets of all available PAH-specific drugs¹, including endothelin receptor antagonists. Endothelin-1, a vasoconstrictive peptide, has a significant role in PAH pathology. Many studies report increased plasma endothelin-1 concentrations in PAH¹⁴. Endothelin-1 is involved in several abnormal signaling cascades, and its inhibition has been shown to suppress monocrotaline-induced PH¹⁵. For instance, endothelin-1 promotes apoptosis resistance in fibroblasts¹⁶, which have a role in the collagen deposition seen in PAH. Endothelin-1 may also potentiate vascular inflammation. In human vascular smooth muscle cells, endothelin-1 can induce interleukin-6 release through the pro-inflammatory transcription factor nuclear factor- κ B¹⁷.

The prostacyclin and nitric oxide pathways are also important altered vaso-regulatory mechanisms in PAH. Notably, asymmetric dimethylarginine (ADMA), which impedes nitric oxide production (Figure 2), is elevated in blood samples from IPAH patients and is associated with worse hemodynamics and survival¹⁸. Enhanced response of the renin-angiotensin-aldosterone system is also observed in PAH patients¹⁹. Pulmonary arteries from patients with PAH show increased expression of angiotensin II receptor type 1. Through this receptor, angiotensin II promotes proliferation in pulmonary artery smooth muscle cells in PAH patients. Moreover, human PAH endothelial cells exhibit increased production of angiotensin II. In line with this, renin activity and angiotensin II blood levels are elevated and associated with an increased risk of death or lung transplantation in PAH patients¹⁹.

Adrenomedullin is a potent vasodilator that has been linked to PH severity in previous biomarker research^{20, 21}. In addition to its vasodilating properties, adrenomedullin has been suggested to alleviate other pathological processes in PAH, including cell proliferation and collagen accumulation²²⁻²⁴. Interestingly, the administration of adrenomedullin attenuates PH in animal models and improves hemodynamics in human PH^{22, 25-28}.

Cellular proliferation and apoptosis resistance

Heightened cellular proliferation and apoptosis resistance are central contributors to pulmonary arterial remodeling. Receptor tyrosine kinases and their ligands are among the altered molecular domains that have been connected to these pathological changes. Several growth factors in this molecular group are connected to pulmonary arterial remodeling in PH, including fibroblast growth factor (FGF)-2, platelet-derived growth factor (PDGF) and transforming growth factor (TGF)- α . For instance, pulmonary endothelial cells from IPAH patients overproduce FGF-2, which may contribute to enhanced endothelial cell proliferation and survival, as well as smooth muscle cell hyperplasia^{29, 30}. PDGF is also known to enhance pulmonary arterial smooth muscle cell proliferation³¹. Both FGF-2 and PDGF have previously been shown to be elevated in blood samples from PAH patients^{32, 33}. Moreover, the epidermal growth factor receptor ligand TGF- α has been shown to impede pulmonary vascular development and promote pulmonary arterial remodeling in experimental PH³⁴.

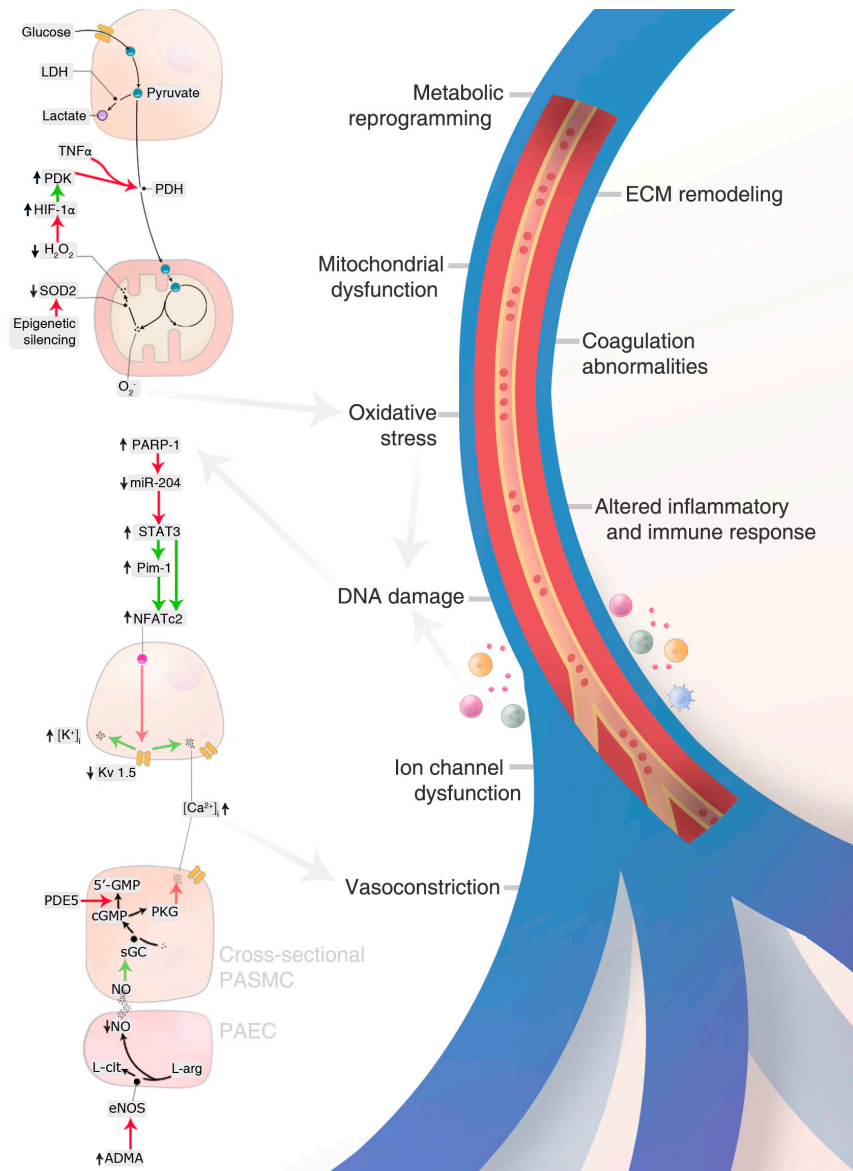


Figure 2. Mechanisms of PAH pathology

Important aspects of PAH pathology, along with some selected molecular mechanisms. Detailed descriptions are provided in the running text. Green arrow: stimulatory effect. Red arrow: inhibitory effect. Black arrows on the side of labels: increased or decreased expression in PAH. $[Ca^{2+}]_i$: Intracellular calcium concentration; $[K^+]_i$: Intracellular potassium concentration; 5'-GMP: Guanosine monophosphate; ADMA: Asymmetric dimethylarginine; cGMP: Cyclic guanosine monophosphate; DNA: deoxyribonucleic acid; ECM: Extracellular matrix; eNOS: Endothelial nitric oxide synthase; H_2O_2 : Hydrogen peroxide; HIF-1 α : Hypoxia-inducible factor-1 α ; Kv: Potassium voltage-gated channel; L-arg: L-arginine; L-cit: L-citrulline; LDH: Lactate dehydrogenase; NFATc2: Nuclear factor of activated T-cells c2; NO: Nitric oxide; O_2^- : Superoxide; PAEC: Pulmonary artery endothelial cell; PARP-1: Poly [ADP-ribose] polymerase 1; PASMCS: Pulmonary artery smooth muscle cell; PDE5: Phosphodiesterase 5; PDH: Pyruvate dehydrogenase; PDK: Pyruvate dehydrogenase kinase; Pim-1: Provirus integration site for Moloney murine leukemia virus 1; PKG: Protein kinase G; sGC: Soluble guanylyl cyclase; SOD2: Mitochondrial superoxide dismutase; STAT3: Signal transducer and activator of transcription 3; TNF- α : Tumor necrosis factor α .

In contrast to these observations, other growth factors involved in receptor tyrosine kinase signaling have been shown to exert protective effects in experimental PAH, including hepatocyte growth factor and vascular endothelial growth factor (VEGF)^{35, 36, 37}. VEGF overexpression protects against monocrotaline-induced PH³⁶. Also, VEGF administration results in vasodilation in the neonate piglet pulmonary circulation³⁸. In accordance, blocking VEGF receptors using the tyrosine kinase inhibitor Sugen (SU5416), in combination with chronic hypoxia, results in severe PH in rats. In blood circulation, levels of VEGF are elevated in PAH, presumably as a compensatory mechanism against the elevated pulmonary arterial pressure³². Whether free and active VEGF levels in PAH are diminished or not is an interesting consideration to take into account. Notably, soluble fms-like tyrosine kinase-1 (sFlt-1) (also known as soluble VEGF receptor 1), which is known to blunt VEGF signaling, is also elevated in blood samples from PAH patients³⁹⁻⁴².

The TGF- β superfamily is another central molecular domain in PAH, as reflected by the fact that many gene mutations in HPAH have a direct connection to this superfamily. These include bone morphogenetic protein receptor type II, mothers against decapentaplegic homolog 9, activin receptor-like kinase 1 and endoglin¹. Bone morphogenetic proteins (BMP), TGF- β as well as associated proteins, such as endoglin, have been reported to have pathological roles in PAH^{43, 44}. For example, the contraction of pulmonary artery smooth muscle cells elicited through endothelin-1 is counteracted by BMP-7⁴³. Plasma BMP-7 levels are increased in both HPAH and IPAH patients⁴⁵, which may mirror a compensatory release of BMP-7.

Endoglin is an accessory TGF- β receptor component, which contributes to endothelial cell proliferation in PAH, through TGF- β signaling⁴⁴. Pulmonary endothelial cells from IPAH patients express increased endoglin protein levels⁴⁴. Moreover, endoglin protein levels are elevated in human PAH serum^{40, 44}. One study reported, furthermore, that soluble endoglin concentrations are more specific and sensitive for differentiating PAH from controls than N-terminal prohormone of brain natriuretic peptide (NT-proBNP)⁴⁰.

Another interesting member of the TGF- β superfamily is growth differentiation factor 15 (GDF-15, also known as macrophage inhibitory cytokine-1) that, among others, has been shown to have important roles in tumor biology⁴⁶. Rhodes et al. showed that blood-borne GDF-15 is elevated in IPAH patients⁴⁷. Another study

showed that circulating GDF-15 levels in more than half of the studied IPAH population surpassed a defined upper reference limit for GDF-15 and that this biomarker may hold a potential prognostic role⁴⁸. Zelniker et al. showed that PAH patients who did not survive a four year follow-up period manifested with higher circulating GDF-15 levels than those who survived⁴⁹. Moreover, plasma GDF-15 levels are significantly higher in systemic sclerosis-associated PAH compared to systemic sclerosis patients without PAH⁵⁰.

Metabolic reprogramming

In the last decade, a metabolic theory in PAH has additionally emerged, describing the negative impact of abnormal glucose and lipid processing, mitochondrial dysfunction, increased oxidative stress as well as iron and vitamin D deficiencies⁵¹.

An elevated glycolytic rate has been observed in pulmonary arterial endothelial cells from PAH patients⁵². Moreover, increased glucose uptake, as assessed through positron emission tomography imaging, has been noted in human PAH lungs⁵³. Pyruvate dehydrogenase, an enzyme that permits further pyruvate processing in the mitochondria, also seems to be an interesting contributor to PAH pathology. Pyruvate dehydrogenase inhibition and other abnormalities disrupting pyruvate from being processed in the mitochondrion favor glycolysis^{54, 55}. Pyruvate dehydrogenase kinase and tumor necrosis factor α are among the negative regulators of pyruvate dehydrogenase, and could, therefore, have a significant role in the altered cellular metabolism observed in PAH (Figure 2). The pyruvate dehydrogenase kinase inhibitor dichloroacetate has been shown to both prevent and reverse PAH in monocrotaline rat models⁵⁴. Dichloroacetate also increased both right ventricular function and glucose oxidation in monocrotaline rats⁵⁶. Interestingly, dichloroacetate is known to reduce proliferation in tumors⁵⁷.

Imbalances in lipid metabolism are also connected to PAH progression. For instance, oxidized lipids may, among other processes, add to the smooth muscle cell proliferation and inflammation seen in PAH⁵⁸. Moreover, mitochondrial dysfunction, including an imbalance in the tricarboxylic acid cycle, seems to have a key role in PAH⁵⁹. Many metabolic intermediates, including those included in the tricarboxylic acid cycle and glycolysis, are altered in human PAH lungs⁵⁹. For instance, human PAH lungs exhibit increased gene expression of isocitrate dehydrogenase 1, which catalyzes isocitrate to α -ketoglutarate⁵⁹. Plasma levels of several metabolites are also altered in PAH patients, including tricarboxylic acid

cycle intermediaries, purine metabolites, tryptophan breakdown products, fatty acids, sphingomyelins and steroids^{60, 61}.

Oxidative stress and DNA damage

Blood analyses in PAH also show imbalances in several markers related to oxidative stress. These include increased activity of xanthine oxidase and 15-F_{2t}-isoprostane as well as decreased superoxide dismutase (SOD)⁶²⁻⁶⁴.

Superoxide is produced during physiological electron transport chain activity in the mitochondria but is normally rapidly detoxified through the mitochondrial SOD (SOD2). Nonetheless, reduced SOD2 expression is observed in pulmonary artery smooth muscle cells⁶⁵ and endothelial cells⁶⁶ derived from IPAH patients. In accordance with these observations, epigenetic silencing of SOD2 has been proposed as a mechanism in PAH⁶⁷. SOD reduction can be critical as it could favor the accumulation of reactive oxygen species, which in turn can contribute to an intracellular disturbance in pulmonary arterial cells. For instance, decreased hydrogen peroxide levels, as a result of diminished SOD2 activity, upregulates hypoxia-inducible factor-1 α ⁶⁵. This in turn can inhibit pyruvate dehydrogenase kinase, contributing to the altered cellular metabolism in PAH (Figure 2)⁶⁵.

Long-lasting oxidative stress may also result in deoxyribonucleic acid (DNA) damage. Indeed, DNA damage markers are reported to be increased in both endothelial cells⁶⁸ and smooth muscle cells⁶⁹ of pulmonary arteries from PAH patients. DNA damage can have significant effects on PAH pathology. For instance, DNA damage in PAH promotes poly [ADP-ribose] polymerase 1 (PARP-1) expression⁶⁹, which has been linked with decreased mi-R204. Decreased miR-204 in PAH may subsequently upregulate the signal transducer and activator of transcription 3 (STAT3)-nuclear factor of activated T-cells c2 (NFATc2) pathway, leading to ion channel abnormalities, vasoconstriction as well as cellular proliferation and apoptosis resistance⁶⁹⁻⁷¹ (Figure 2).

Lessons from tumor biology

Previous reports in PAH have underlined numerous similarities between PAH and tumor biology (Figure 3)⁷²⁻⁷⁴. Some of these have already been presented in previous sections, including altered cellular metabolism, impaired oxidative balance, sustained growth signaling and apoptosis resistance.

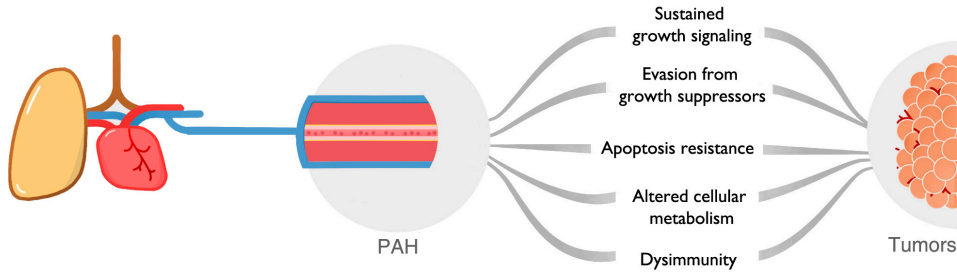


Figure 3. Pathological similarities between PAH and tumors.

PAH: pulmonary arterial hypertension.

Altered metabolism is a hallmark of cancer⁷⁵, which is suggested to drive tumor growth. Important metabolic shifts in cancerous cells include increased glucose uptake, glycolysis and lactate production⁷⁶. As mentioned in the previous section, a similar metabolic shift is present in PAH. Notably, the growth effect of PDGF in PAH may partly be mediated through an intracellular metabolic shift that includes increased glucose consumption and elevated lactate production³¹. In human pulmonary artery smooth muscle cells, another study also showed that PDGF increases sphingosine kinase 1⁷⁷; an enzyme involved in tumor proliferation and survival. Sphingosine kinase 1 has, moreover, been reported to contribute to pulmonary artery smooth muscle cell proliferation in PAH⁷⁸. Another tumor-related protein that has gained interest in PAH is the proto-oncogene Pim-1 (provirus integration site for Moloney murine leukemia virus 1)⁷¹. Pim-1 lung tissue expression is increased in PAH patients and is correlated positively with PVR⁷¹. Moreover, Pim-1, which is triggered by STAT3, is suggested to promote proliferation and resistance to apoptosis in PAH pulmonary artery smooth muscle cells⁷¹. As a blood-borne biomarker, Pim-1 holds a promising potential as well⁷⁹. Its plasma levels are elevated in PAH and show some correlation to clinical variables of PAH severity, including 6MWD and NT-proBNP⁷⁹. Pim-1 overexpression in plasma is also associated with worse survival⁷⁹.

Aims

Paper I

The aim of the first paper (paper I) was to assess the changes in various angiogenic and inflammatory plasma biomarkers between PAH diagnosis and two early treatment follow-ups. Furthermore, the changes in plasma biomarkers between baseline and the two early follow-ups were observed in relation to the changes in hemodynamics, six-minute walking distance (6MWD) and NT-proBNP.

Paper II-V

The biomarkers studied in paper II-V are linked to different areas of pathology that are relevant to PAH. The aims of paper II-V were:

- To assess whether plasma biomarkers at PAH diagnosis are correlated with hemodynamics, 6MWD, NT-proBNP and early mortality risk scores.
- To assess whether plasma biomarkers change after PAH treatment initiation, at an early follow-up after diagnosis.
- To assess plasma protein levels in treatment-naïve PAH patients in relation to healthy subjects.
 - In paper V, biomarker levels in PAH were, moreover, compared to levels in patients with CTEPH, PH due to left heart failure with reduced or preserved ejection fraction as well as heart failure without PH.

Materials and methods

General method description

In this section follows general descriptions and comments of materials and methods, that are relevant for all the papers included in the present dissertation.

Lund Cardio Pulmonary Register and blood sampling

Lund Cardio Pulmonary Register (LCPR) is a patient blood sample cohort, initiated by Dr. Göran Rådegran in September 2011. LCPR includes blood samples from patients evaluated for PH or severe heart failure at the Hemodynamic Lab at Skåne University Hospital in Lund, Sweden. Patients included in the register are assessed with right heart catheterization, where venous blood samples are obtained through an introducer, predominantly from the internal jugular vein. Plasma is thereafter collected after centrifugation and stored at -80°C in the LCPR cohort of Region Skåne's biobank.

The register aims to advance biomarker research within the PH and heart failure domains, particularly in the areas of diagnosis, treatment response evaluation and risk assessment. Paper I-V have been among the first efforts to initiate research with recourses from LCPR.

Ethics

All subjects included in LCPR have received oral and written information regarding the purpose of the blood sampling, and have given their written consent to participate. The local ethical board in Lund has approved the studies included in the present dissertation (Dnr 2015/270, Dnr 2011/777, Dnr 2011/368, Dnr 2010/114, Dnr 2010/442).

Clinical evaluation and aspects

PAH baseline was defined as the time of diagnosis, as established by right heart catheterization, where patients were naïve to all PAH-specific treatment.

PH patients were diagnosed by experienced cardiologists at the national PH and transplantation reference center at Skåne University Hospital in Lund, where PH subgroups were differentiated using techniques that, besides right heart catheterization, included computer tomography, spirometry, pulmonary scintigraphy, echocardiography and/or magnetic resonance tomography.

Right heart catheterization

Right heart catheterizations were performed using Swan-Ganz catheters. Thermodilution was used to measure cardiac output. Body surface area, mean right atrial pressure (MRAP), mean arterial pressure, MPAP, PAWP, stroke volume index and cardiac output were used to calculate cardiac index (CI), PVR as well as right (RVSWI) and left (LVSWI) ventricular stroke work indexes.

The formulae used were: $CI = \text{cardiac output} / \text{body surface area}$, $PVR = (\text{MPAP} - \text{PAWP}) / \text{cardiac output}$, $RVSWI = (\text{MPAP} - \text{MRAP}) \times \text{SVI}$ and $LVSWI = (\text{mean arterial pressure} - \text{PAWP}) \times \text{SVI}$.

General comments on statistical analyses and software

Throughout all papers, R statistical software (R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism (GraphPad Software, La Jolla, California, USA) were used for statistical computing and graphics. In general, non-parametric tests were used, as appropriate. Normal distribution was primarily assessed graphically, using methods such as QQ-plots and histograms. Statistical significance was defined as raw $p < 0.05$ unless multiple comparison adjustments were applied. Values are generally reported as median (upper-lower quartile) for continuous parameters unless otherwise stated.

Paper I

Study cohort and biomarkers

Incident treatment-naïve PAH patients in LCPR that had two consecutive early right heart catheterization follow-ups, constituted the cohort (n = 21) for paper I (Table 2). At baseline, the median age was 70 years and 71% were female. Ninety percent of the patients were in WHO functional class III at baseline. Median times from baseline to the first and second follow-ups were 112 and 264 days, respectively. Thirteen patients had IPAH/HPAH and eight had CTD-PAH. PAH patients at the first follow-up were on initial mono (n = 16) or initial combination (n = 5) therapy, which constituted analysis subgroups in this study. Among the initial monotherapy group, nine patients were escalated to combination therapy at the first follow-up.

Six angiogenic proteins linked to receptor tyrosine kinase signaling, as well as three inflammatory biomarkers, were assessed in plasma using multiplex sandwich immunoassays (Meso Scale Discovery, Rockville, MD, USA). These were VEGF-A and -D, sFlt-1, placental growth factor, FGF-2, angiopoietin-1 receptor, interleukin-6 and -8, as well as tumor necrosis factor α .

Table 2. Baseline patient characteristics

	PAH cohort in paper I	PAH cohort in paper II-V
Sample size n (% females)	21 (71)	48 (83)
Age years	70 (57-75)	72 (64-76)
BSA m ²	1.8 (1.7-2.1)	1.8 (1.6-2)
IPAH/HPAH n	13	23
CTD-PAH n	8	25
WHO-FC* n		
I	0	1
II	1	9
III	19	28
IV	1	2
6MWD m	214 (143-259)	242 (176-348)
SvO ₂ %	NA	59 (51-66)
Hemodynamics		
MPAP mmHg	45 (39-55)	43 (37-54)
PAWP mmHg	6 (5-7)	8 (6-11)
MRAP mmHg	7 (6-9)	7 (4-11)
CI l·min ⁻¹ ·m ⁻²	2.0 (1.8-2.4)	2.2 (1.8-2.8)
PVR Wood units	10 (8-12)	10 (7-12)

Baseline

characteristics for pulmonary arterial hypertension (PAH) cohorts in paper I-V. Continuous values are presented as median (lower-upper quartiles). BSA, body surface area; IPAH/HPAH: idiopathic or hereditary PAH; CTD-PAH: Connective tissue disease-associated PAH; WHO-FC: World Health Organization functional class; 6MWD: 6-minute walking distance; SvO₂: mixed venous oxygen saturation; MPAP: mean pulmonary arterial pressure; PAWP: pulmonary artery wedge pressure; MRAP: mean right atrial pressure; PVR: pulmonary vascular resistance; NA: not assessed. *The values may not add up to the total cohort size due to missing functional class data.

Analysis approach and statistics

Using the Friedman test together with Dunn's test for multiple comparisons, all investigated biomarkers were compared between baseline and respective follow-up, in all patients, as well as in mono- and combination therapy subgroups separately. The correlations between changes in studied biomarkers were tested against the changes in selected hemodynamic parameters, 6MWD and NT-proBNP in all PAH patients pooled together using the Spearman's rank correlation method.

Paper II-V

Study cohort

Throughout paper II-V, a cohort of 48 incident and treatment-naïve patients with PAH was studied. The median age was 72 years and the proportion being female was 83% at PAH diagnosis (Table 2). PAH patients had IPAH/HPAH (n = 23) or CTD-PAH (n = 25). Seventy percent of the patients were in WHO functional class III. The most common comorbidities at diagnosis were systemic hypertension (35%), diabetes mellitus (25%) and thyroid disease (23%). Among the incident PAH cohort, 33 patients had blood samples at an early treatment follow-up (median time from baseline was 116 days). In the subgroup with early clinical follow-ups, the proportions on initial monotherapy and initial combination therapy were 27% and 67%, respectively, whereas the remaining 6% were on calcium channel blockers with or without PAH-specific treatment.

To investigate PH differentiation in paper V, blood samples were additionally assessed in patients with CTEPH (n = 20) and PH due to left heart failure with reduced (n = 36) or preserved (n = 33) ejection fraction, as well as in heart failure patients without PH (n = 15). Reduced and preserved ejection fractions were defined as < 50% and ≥ 50%, respectively.

Moreover, peripheral venous blood samples were obtained from 16 controls considered to be healthy (median age = 47 years, 69% were female), which were used throughout paper II-V.

Data collection and risk calculations

Data, such as hemodynamics, 6MWD, NT-proBNP, WHO functional class and medical treatment, were collected from patient records.

ESC/ERS guideline risk scores, based on the Kylhammar et al. SPAHR model, were calculated using MRAP, CI, WHO functional class, 6MWD, NT-proBNP and mixed venous oxygen saturation (SvO₂)⁹. Due to a high share of missing values, echocardiographic parameters were not included in the risk score calculations. Each parameter was graded with a score from 1 to 3, according to the cut-offs provided in the risk assessment instrument from the 2015 ESC/ERS PH guidelines¹ (Table

1), where 1 corresponded to “low”, 2 to “intermediate” and 3 to “high” risk⁹. In paper II, the most commonly occurring score among the six parameters defined the patient’s risk class. In paper III-V, a mean of the parameter scores defined a patient’s risk score, as this was more in line with the study design of these papers.

REVEAL risk scores (version 1.0) were moreover calculated for all PAH patients⁸⁰. These scores were slightly modified to account for missing values, according to the descriptions in paper II-V.

Biomarker selection

Proseek multiplex cardiovascular II, cardiovascular III and oncology II 96-plex proximity extension assay (PEA)-based immunoassays (Olink Proteomics, Uppsala, Sweden) were used for all biomarker analyses in paper II-V. Each panel encompassed 92 biomarkers. Before measuring the biomarkers, the proteins were divided into different categories, based on their main roles in pathology. Some of these categories constituted the foci of paper II-V, whereas other categories were investigated in other studies⁸¹⁻⁸³.

Following the same path as the first paper (paper I), the second article (paper II) focused on biomarkers related to receptor tyrosine kinase signaling and cellular growth. Paper III and IV, continued focusing on biomarkers linked to abnormal cellular growth, investigating metabolic proteins and tumor markers, respectively.

While the first four papers (paper I-IV) focused on biomarkers that reflect cellular growth and energetics, the fifth and final paper (paper V) shed light on biomarkers connected to vaso-regulation. These included renin, angiotensin-converting enzyme 2 as well as adrenomedullin peptides and precursor. The adrenomedullin peptides and precursor were measured collectively, and were together referred to as ADM. The ADM assay utilized polyclonal antibodies that could detect the following peptides and pro-peptides of ADM: pro-adrenomedullin N-20 terminal peptide, mid-regional pro-adrenomedullin (MR-proADM), adrenomedullin, as well as the unprocessed ADM protein.

Biomarker analysis

In summary, the PEA technique uses oligonucleotide-labeled antibody pairs to detect the targeted biomarkers, in order to avoid unspecific antibody binding and potential cross-reactivity events⁸⁴. As two related probes are brought into close

proximity, the oligonucleotides hybridize in a pair-wise manner. The addition of DNA polymerase results in a proximity-dependent DNA polymerization event, which creates a unique polymerase chain reaction target sequence. The DNA sequence is then detected and quantified using a microfluidic real-time polymerase chain reaction instrument (Biomark HD; Fluidigm, San Francisco, CA, USA). Data quality control and normalization are performed utilizing an internal extension control and an inter-plate control, to adjust for intra- and inter-run variation. Assay validation data and panel information are available at www.olinlink.com.

Comments on analysis approach

Paper II

All biomarkers were tested for correlations against selected hemodynamic parameters, NT-proBNP and 6MWD. Plasma stem cell factor (SCF) showed the strongest correlations to the studied parameters, making it the focus of paper II. SCF was selected to be compared between risk classes, as assessed according to the ESC/ERS risk assessment strategy.

A SCF to TGF- α ratio was, at the analysis stage, detected and studied further in relation to the modified REVEAL risk score, prognosis and identification of high-risk PAH patients, as assessed with the ESC/ERS risk stratification model.

Paper III–V

Biomarkers that showed to be altered in PAH patients at diagnosis compared to the healthy controls were qualified for correlation analysis against selected hemodynamic parameters, NT-proBNP and 6MWD in PAH patients at diagnosis. Plasma biomarkers that showed the strongest correlations were further studied in relation to risk scores at PAH diagnosis.

As paper V focused on PAH, correlation analyses were only performed on the PAH group and not on the other studied patients in paper V, who only served as disease controls in the study.

Statistics

The Mann Whitney U-test was used to compare biomarker levels between PAH and healthy controls (in paper II-IV). The Wilcoxon signed-rank test was used to assess

biomarker level changes between diagnosis and treatment follow-up in PAH patients.

To assess the false discovery rate, the Benjamini-Hochberg procedure was used for the statistical tests comparing PAH patients to healthy controls (and disease groups in paper V). The Q value of the false discovery rate was set to 5%.

Correlations between biomarkers and clinical parameters were assessed using the Spearman's rank correlation method. Survival was illustrated using the Kaplan-Meier method and differences in survival between groups were assessed using the log-rank test.

Further details regarding statistics are presented below for paper II and V separately:

Paper II

Using the Kruskal-Wallis test along with Dunn's test for multiple comparisons, baseline plasma SCF levels were compared among low-, intermediate- and high-risk PAH groups, defined according to the ESC/ERS risk model.

Decision tree analysis was used to detect a marker combination that could identify PAH patients that are high-risk, according to the ESC/ERS risk model. The detected pattern was then assessed using Fisher's exact test.

Receiver operating characteristics analysis was used to assess the performance of the SCF/TGF- α ratio as well as SCF alone as predictors of high-risk PAH. Using the optimal cut-offs (according to Youden's index), survival analysis for SCF/TGF- α , as well as SCF alone, were performed according to the methods described above. An event was defined as patient death.

Paper V

Biomarker levels were compared between PAH and the other study groups using the Kruskal-Wallis test along with the Benjamini-Hochberg test as subsequent multiple comparison testing.

Survival was compared between PAH patients with supra- versus infra-median baseline ADM. An event was defined as patient death or lung transplantation. ROC analysis was performed to evaluate whether plasma ADM levels can differentiate PAH patients that meet versus those that do not meet the defined event within one year after diagnosis.

Results

The most relevant statistical measures are provided in tables and figures. For simplicity, the running text contains no specific statistical measures. These are provided in paper I-V. Baseline characteristics for the PAH cohorts in paper I-V are provided in Table 2.

Paper I

Plasma sFlt-1 decreased between diagnosis and follow-ups (Table 3, Figure 4). Between diagnosis and the first follow-up, changes in plasma sFlt-1 correlated positively with changes in PVR and NT-proBNP, and inversely with changes in LFSWI. Between diagnosis and second follow-up, sFlt-1 changes correlated positively with changes in PVR, and inversely with changes in 6MWD and LFSWI.

When observing initial mono- and initial combination therapy groups separately, plasma sFlt-1 decreased only in the initial combination therapy group between baseline and first follow-up.

Plasma sFlt-1 decreased in the initial monotherapy group, between baseline and the second follow-up. The initial combination therapy group showed a tendency towards decreased sFlt-1 levels between baseline and the second follow-up, which, however, did not meet statistical significance.

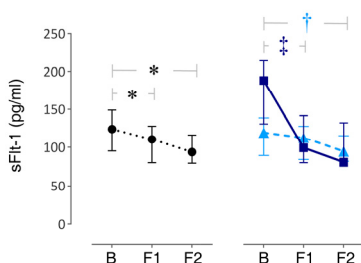


Figure 4. Plasma sFlt-1 changes in pulmonary arterial hypertension.

Plasma sFlt-1 concentrations in pulmonary arterial hypertension patients at baseline (B), first (F1) and second (F2) follow-up. Patients are pooled (●), or divided into initial combination (■) and initial monotherapy (▲) groups. * $p < 0.05$ for the changes between the marked periods. ‡ $p < 0.05$ for the change in the initial combination therapy group, during the marked period. † $p < 0.05$ for the change in the initial monotherapy group, during the marked period.

Table 3. Highlighted plasma biomarkers from paper I-V

	Pulmonary arterial hypertension				
	Controls	Baseline	Baseline subset	First / early follow-up [§]	Second follow-up
Paper I					
sFlt-1 (pg/ml)	NA	124 (95-149)	NA	110 (80-128) [#]	94 (79-116) [#]
NT-proBNP (ng/l)	NA	2359 (1667-3089)	NA	1455 (642-2469) [#]	958 (421-3265) [#]
Paper II					
SCF (AU)	8.5 (8.3-8.8)	8.1 (7.4-8.4)*	8.2 (7.5-8.4)	8.3 (8.0-8.4) [#]	NA
TGF- α (AU)	1.3 (0.9-1.4)	1.8 (1.6-2.1)*	1.8 (1.6-2.1)	1.7 (1.5-2.0)	NA
Paper III					
FGF-23 (AU)	3.8 (3.6-4.0)	5.5 (4.9-6.2)*	5.6 (4.6-6.4)	4.7 (4.2-5.9) [#]	NA
Paper IV					
IGFBP-1 (AU)	3.1 (2.3-4.3)	4.7 (4.2-5.8)*	4.7 (4.2-5.6)	5.0 (4.4-5.5)	NA
Paper V					
ADM (AU)	6.0 (5.8-6.2)	7.2 (7.0-7.6)*	7.2 (7.0-7.5)	7.2 (7.0-7.6)	NA
Paper II-V					
NT-proBNP (AU)	0.2 (0.1-0.2)	3.1 (2.1-3.8)*	2.9 (2.1-3.4)	2.1 (1.3-2.9) [#]	NA

§ First follow-up for paper I and early (i.e. not necessarily first) follow-up for paper II-V.

* p<0.05 compared to controls. # p<0.05 compared to baseline in paper I and to baseline subset in paper II-V.

ADM: adrenomedullin peptides and precursor; AU: arbitrary units; FGF-23: Fibroblast growth factor 23; IGFBP-1: Insulin-like growth factor binding protein 1; NT-proBNP: N-terminal prohormone of brain natriuretic peptide; sFlt-1: soluble fms-like tyrosine kinase-1; SCF: stem cell factor; TGF- α : Transforming growth factor α .

Paper II

The main results showed that plasma SCF levels were lower in PAH patients at diagnosis, compared to healthy controls (Table 3, Figure 5A). Plasma SCF at baseline correlated, among others, inversely to PVR, MRAP and NT-proBNP, and positively to CI and SvO₂ (Figure 5B and 5C). PAH patients at high risk exhibited lower SCF levels compared to those with intermediate or low risk of early mortality, as classified according to the risk stratification tool from ESC/ERS PH guidelines. Nonetheless, SCF could not differentiate low- from intermediate-risk patients (Figure 5A).

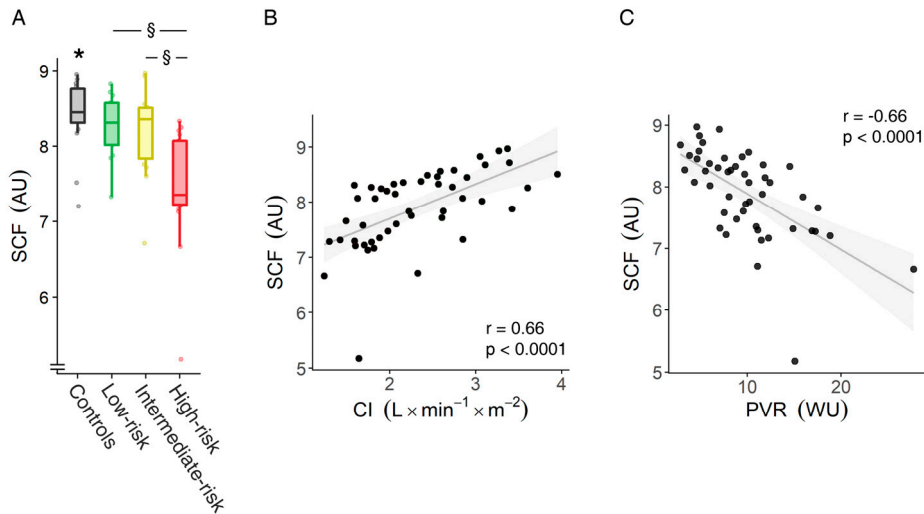


Figure 5. Plasma stem cell factor at PAH diagnosis.

Plasma stem cell factor (SCF) is lower in treatment-naïve PAH patients at diagnosis compared to healthy controls (A). SCF levels are lower in high-risk patients compared to low- and intermediate-risk PAH patients (A). Moreover, plasma SCF levels are correlated with hemodynamics at diagnosis, including cardiac index (CI) (B) and pulmonary vascular resistance (PVR) (C). * $p < 0.05$ comparing versus all PAH patients. § $p < 0.05$ comparing highlighted groups. Patients' risk was classified according to the 2015 European Society of Cardiology/European Respiratory Society PH guidelines. AU: arbitrary units; WU: Wood units.

TGF- α was another focus of the study, which was higher in plasma from PAH patients compared to healthy controls. TGF- α showed moderate correlations to NT-proBNP, 6MWD, MRAP, SvO₂ and CI. TGF- α levels were elevated in high- compared to low-risk PAH patients and tended to be higher in high- compared to intermediate-risk patients (although the latter was statistically non-significant).

Aiming to investigate whether a multi-biomarker approach could provide an edge in identifying high-risk PAH patients, decision tree analysis was performed on all studied biomarkers. This analysis identified SCF and TGF- α as a potential combination for identifying high-risk from low- or intermediate-risk patients. Thus, a SCF/TGF- α ratio was further investigated.

The SCF/TGF- α ratio resulted in a sensitivity of 86% and a specificity of 82% for detecting PAH patients at high risk, using an optimal cut-off (a ratio of 4.4). PAH patients with SCF/TGF- α at or below this cut-off tended to have a worse overall survival in comparison to those above the cut-off (statistically non-significant) (Figure 6A). Finally, SCF/TGF- α levels (but not SCF alone) at PAH diagnosis correlated to a modified REVEAL risk score.

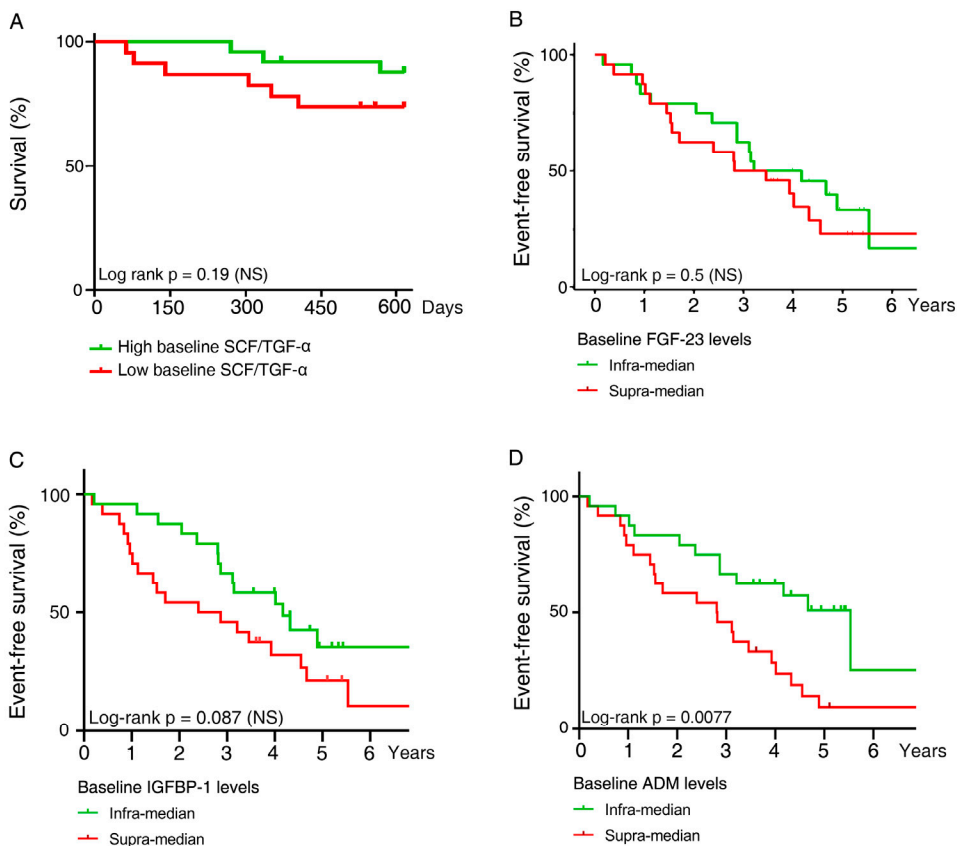


Figure 6. Survival curves of highlighted plasma biomarkers investigated in paper II-V.

Pulmonary arterial hypertension (PAH) patients with a low stem cell factor to transforming growth factor α (SCF/TGF- α) ratio (≤ 4.4) showed a statistically non-significant (NS) trend for worse survival compared to those with a high ratio (>4.4) (A). Fibroblast growth factor (FGF)-23 levels showed no association with survival (B). PAH patients with supra-median IGFBP-1 levels at diagnosis tended to have worse overall event-free survival than patients with infra-median levels (C). PAH patients with supra-median adrenomedullin peptides and precursor (ADM) levels at diagnosis exhibit worse overall event-free survival than those with infra-median levels. An event in B-D was defined as death or lung transplantation.

SCF increased in PAH patients between diagnosis and follow-up (Table 3), where changes in SCF were mainly correlated to changes in PVR and 6MWD.

Paper III and IV

Paper III studied metabolic markers, whereof plasma FGF-23 (Table 3, Figure 7A), FGF-21, fatty acid binding protein 4 and lectin-like oxidized low-density lipoprotein

receptor 1 were increased and paraoxonase-3 was decreased in PAH patients at diagnosis compared to healthy controls. Paper IV studied tumor-related biomarkers where, among others, baseline IGFBP-1 (Table 3, Figure 8A), -2 and -7 as well as vimentin, carbonic anhydrase 9 and human epididymis protein 4 were elevated in PAH patients compared to controls.

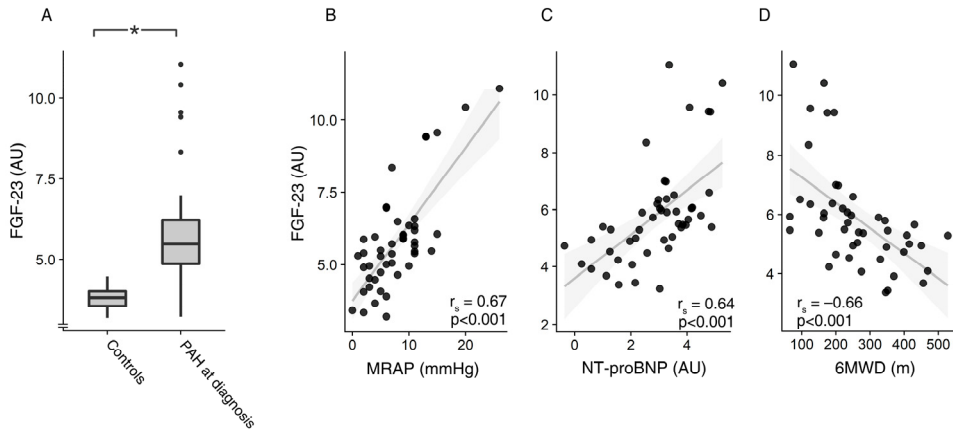


Figure 7. Plasma fibroblast growth factor-23 at PAH diagnosis.

Plasma fibroblast growth factor-23 (FGF-23) levels at diagnosis are elevated in pulmonary arterial hypertension (PAH) patients (A). Baseline FGF-23 correlated to mean right atrial pressure (MRAP) (B), N-terminal prohormone of brain natriuretic peptide (NT-proBNP) (C) and 6-minute walking distance (6MWD) (D). AU: arbitrary units. * $p < 0.05$.

Among the biomarkers in paper III and IV, FGF-23 and IGFBP-1 showed the most notable correlations to the studied clinical parameters and were therefore selected for further analyses. In specific, both baseline FGF-23 and IGFBP-1 correlated positively to MRAP and NT-proBNP, and inversely to SvO₂, 6MWD and CI (Figure 7B-D, 8B and 8C). FGF-23 correlated moreover positively to PVR.

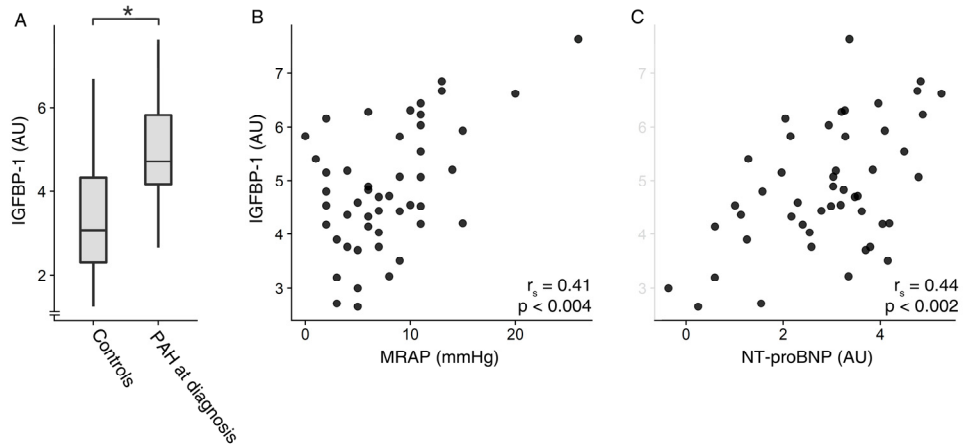


Figure 8. Plasma insulin-like growth factor binding protein 1 at PAH diagnosis.

Elevated plasma insulin-like growth factor binding protein 1 (IGFBP-1) at diagnosis (A) correlated to mean right atrial pressure (MRAP) (B) and N-terminal prohormone of brain natriuretic peptide (NT-proBNP) (C). AU: arbitrary units.

The ESC/ERS risk model correlated to both FGF-23 and IGFBP-1 at baseline. Likewise, the modified REVEAL risk score was associated with plasma FGF-23 and IGFBP-1 baseline levels (Figure 9A-D). FGF-23 levels showed no association with survival (this was not analyzed when paper III was conducted), whereas PAH patients with supra-median IGFBP-1 levels at diagnosis tended to have worse overall event-free survival than patients with infra-median levels (statistically non-significant), where an event was defined as death or lung transplantation (Figure 6B and 6C).

From diagnosis to an early treatment follow-up, plasma FGF-23 levels decreased, with changes in FGF-23 mainly correlating to changes in 6WMD, SvO₂, and PVR. Moreover, changes in FGF-23 correlated positively to changes in ESC/ERS risk score. No significant changes in plasma IGFBP-1 levels were observed between baseline and follow-up (Table 3).

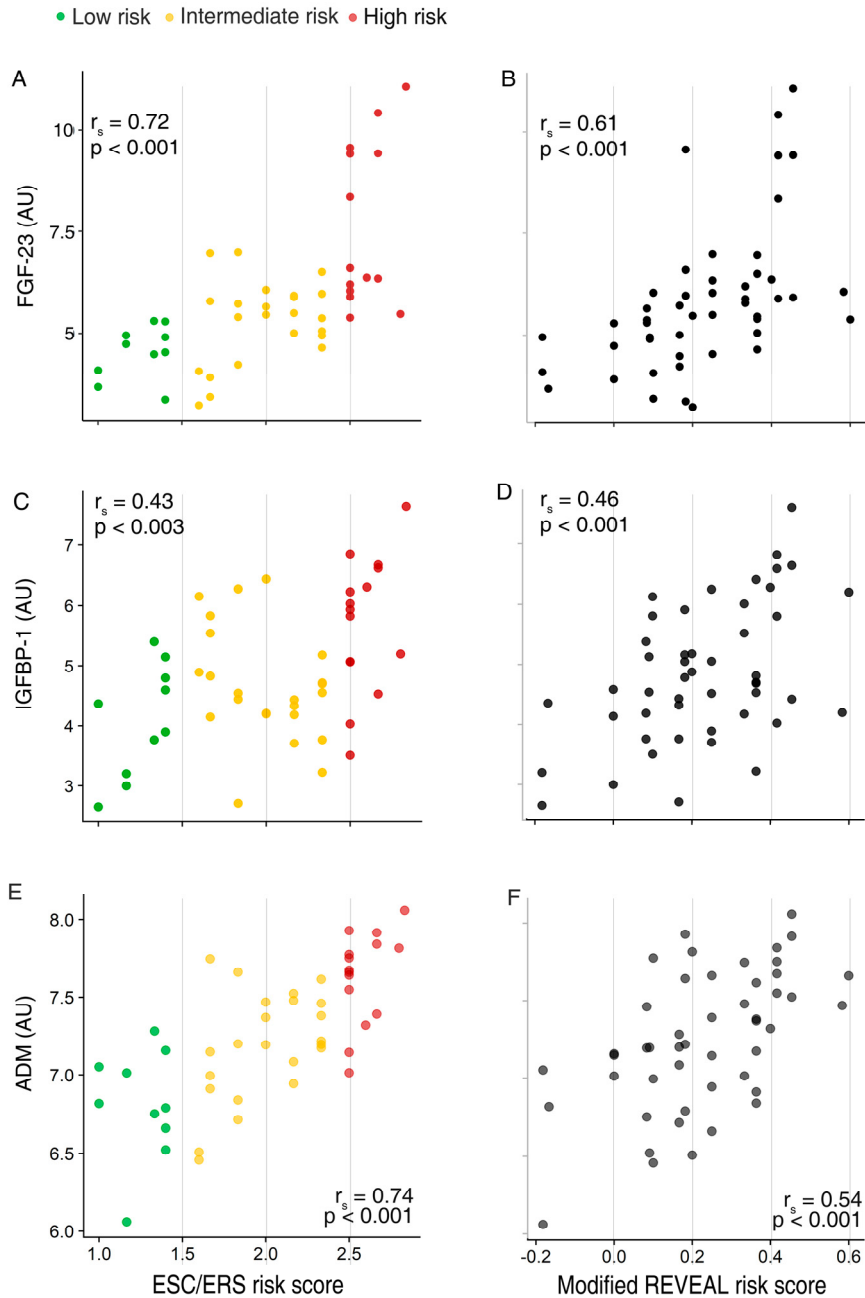


Figure 9. Correlations between highlighted plasma biomarkers and risk scores of early mortality.

Plasma fibroblast growth factor-23 (FGF-23), insulin-like growth factor binding protein 1 (IGFBP-1) and adrenomedullin peptides and precursor (ADM) correlated with risk scores calculated based on the European Society of Cardiology/European Respiratory Society (ESC/ERS) PAH guidelines (A, C and E, respectively) and the REVEAL risk score (B, D and F, respectively). AU: arbitrary units.

Paper V

Paper V focused on ADM since it showed the strongest correlations to clinical parameters among the biomarkers studied in paper V.

Plasma ADM levels in PAH were higher than control levels, similar to those in CTEPH, but were lower in comparison to PH due to heart failure (Table 3, Figure 10A). In PAH patients, ADM levels at diagnosis showed relatively strong correlations to baseline MRAP, NT-proBNP (Figure 10B), 6MWD and SvO₂. Also, plasma ADM levels correlated with the ECS/ERS- and modified REVEAL risk scores at PAH diagnosis (Figure 9E and 9F).

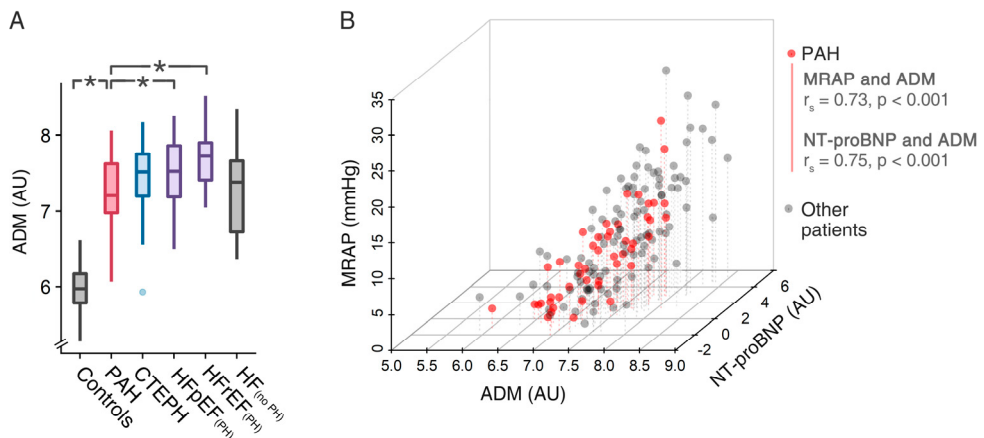


Figure 10. Plasma ADM at PAH diagnosis.

Plasma adrenomedullin peptides and precursor (ADM) levels across investigated study groups (A). ADM correlated to mean right atrial pressure (MRAP) and N-terminal prohormone of brain natriuretic peptide (NT-proBNP) (B). * $p < 0.05$. CTEPH: chronic thromboembolic pulmonary hypertension; HF_{rEF (PH)}: pulmonary hypertension due to left heart failure with reduced ejection fraction; HF_{pEF (PH)}: pulmonary hypertension due to left heart failure with preserved ejection fraction; HF_(no PH): heart failure without pulmonary hypertension; PAH: pulmonary arterial hypertension (at diagnosis).

PAH patients with supra-median ADM levels at diagnosis showed worse overall survival than those with infra-median levels (difference in median survival was 32 months) (Figure 6D). Plasma ADM levels did not differentiate PAH patients who died within a year from diagnosis (7 of 48) and those who survived past one year.

Plasma ADM levels in PAH patients were unaltered between diagnosis and the early treatment follow-up (Table 3).

Discussion

Paper I

The main results in paper I show that plasma sFlt-1 in PAH patients decreased after the administration of PAH-specific treatment. The results also suggest that changes in sFlt-1 are correlated to changes in some important clinical parameters. Nonetheless, the results are inconsistent for NT-proBNP and 6MWD, which is likely related to the small study cohort. A larger cohort is required to draw a firm conclusion regarding this. Moreover, the results generate the hypothesis that initial combination therapy can have a more pronounced effect on circulating sFlt-1 than initial monotherapy. Taken together, plasma sFlt-1 may be an interesting candidate for a treatment response biomarker in PAH. Notably, sFlt-1 has previously been studied as a blood-borne biomarker in PAH, but mainly in the context of PAH diagnosis and differentiation³⁹⁻⁴¹. These studies showed elevated circulating sFlt-1 levels in PAH compared to healthy and disease controls. Kylhammar et al. have, furthermore, shown that plasma sFlt-1 is higher in systemic sclerosis patients that develop PAH compared to those who do not⁴². Paper I adds a treatment response perspective to sFlt-1 in PAH. Together, the studies that have investigated sFlt-1 in PAH indicate that blood-borne sFlt-1 may be a potential multifunctional biomarker in PAH.

However, it should be noted that the studies on sFlt-1 in PAH are somewhat conflicting in some respects. Malhotra and colleagues showed that sFlt-1 was associated with New York Heart Association functional class and survival, but not with right atrial pressure⁴⁰. In contrast, Säleby et al. reported correlations between sFlt-1 and hemodynamics, including MRAP, in PAH patients⁴¹. Finally, Tiede and colleagues found no associations between sFlt-1 and hemodynamics, 6MWD or survival³⁹.

Roles for sFlt-1 in PAH pathology have at present not been investigated. It is, however, of interest to mention that enhanced sFlt-1 is suggested to contribute to the endothelial dysfunction seen in preeclampsia, through the depletion of VEGF-

A and placental growth factor⁸⁵. For instance, sFlt-1 infusion has been demonstrated to result in a rise in blood pressure, through an endothelin-1-mediated vasoconstriction⁸⁶.

Paper II

Paper II shows that low plasma SCF levels in PAH are inversely correlated with increased hemodynamic severity and that SCF can discriminate high-risk PAH patients from other PAH risk classes, assessed with the ESC/ERS risk assessment model. Pursuing the search for a multi-marker approach for risk stratification in PAH, the low SCF levels were combined with the high TGF- α levels in PAH patients. This combination showed good sensitivity and specificity for detecting high-risk PAH patients, and appears to provide an edge over the performance that SCF provides alone when identifying high-risk patients. Although statistically non-significant, a modest association between this ratio and patients' survival was noted. Relatively small study groups limiting the statistical power for analysis may be what is blunting this observation, but the statistical non-significance could also signify a weak or no prognostic value for this marker combination.

Low plasma SCF levels have previously been linked to worse outcome in patients with cardiovascular diseases^{87,88}. Paper II provides initial evidence linking low SCF levels to clinical severity in PAH.

SCF is sparsely investigated in PH. In chronic hypoxia mice models, inhibiting signaling of the SCF receptor, c-Kit, reduces pulmonary vascular remodelling⁸⁹. Plasma-soluble c-Kit is increased in PAH patients, and both SCF and c-Kit gene expressions are elevated in small pulmonary arteries and surrounding lung tissues from animal PAH models^{90,91}. Moreover, c-Kit positive progenitor cells have been shown to accumulate in remodeled human PAH vessels⁹⁰. In contrast to these findings, another paper has shown that exogenous SCF may promote angiogenesis and attenuate PH in rats with neonatal hyperoxia-induced lung injury⁹².

Paper III and IV

Like paper I and II, paper III and IV explored plasma biomarkers that are related to cellular growth and apoptosis, mainly in relation to risk assessment. These papers

focused on metabolism and tumor-related processes – both, fields that have previously been of interest in PAH^{51, 72-74}.

Numerous studies have shown a clear relationship between impaired renal function and increased mortality rates in PAH^{93, 94}. Therefore, it is not surprising that the analyses from paper III showed that increased plasma FGF-23 was associated with clinical severity in PAH patients. Results from paper IV mainly shed light on IGFbps, particularly IGFBP-1. Plasma IGFBP-1 showed moderate correlations to hemodynamics, 6MWD, NT-proBNP and risk scores.

In paper III, the relationship between FGF-23 levels and clinical parameters did not account for patients' impaired renal function, as the statistical assumptions for a regression model were not met. However, the study did not aim to adjust for renal function. Instead, the biomarkers in paper III, as well as those in paper I-II and IV-V, should be viewed as initial evidence of new molecules in the PAH field, and FGF-23 should be seen as another molecular aspect in the important relationship between impaired renal function and PAH. Adjusting for renal function, and other clinical variables, to study independent effects of FGF-23 can be of interest in future studies, since this protein has a plethora of effects that might be relevant to PAH pathology. Such pathological processes include cardiomyocyte hypertrophy, vasoconstriction and decreased nitric oxide availability⁹⁵⁻⁹⁷.

In contrast to our results, Kaiser et al. showed no association between FGF-23 and hemodynamics in a pooled study sample encompassing the PH groups 1, 3 and 4⁹⁸. This can be ascribed to the difference in the methods utilized and the FGF-23 sequences targeted, as described in paper III. However, it can also be related to the different composition of study samples; where our sample only included group 1 PH, whereas that of Kaisers et al. also included group 3 and 4 PH. Finally, we only included incident patients, whereas Kaiser et al. included both incident and prevalent patients. Notably, we found that FGF-23 levels decreased slightly at an early treatment follow-up, with changes in FGF-23 correlating to changes in key clinical parameters.

IGFBP-1, which is a carrier protein that can bind to and regulate insulin-like growth factor 1 signaling⁹⁹, may be an interesting player in PAH pathology. Insulin-like growth factor 1 signaling has previously been suggested to have a role in PH pathology¹⁰⁰⁻¹⁰². Depletion of insulin-like growth factor 1 may attenuate right ventricular hypertrophy and vascular remodeling in small pulmonary arteries of neonatal hypoxic PH models¹⁰¹. In a proteomic study by Rhodes and colleagues,

plasma IGFBP-1 was demonstrated to predict survival in PAH patients¹⁰³. Higher plasma IGFBP-1 levels in our PAH cohort showed a trend for worse survival.

Paper V

Adrenomedullin and MR-proADM have previously been linked to PH severity^{20,21}. Paper V has confirmed this and has provided an additional risk assessment aspect of adrenomedullin peptides in PAH. In paper V, elevated plasma ADM levels at PAH diagnosis were highly correlated to both the modified REVEAL risk score as well as the ESC/ERS risk model. Also, we showed that plasma ADM levels in PAH were lower compared to PH due to left heart disease, but were not different from ADM levels in CTEPH.

Moreover, plasma ADM levels at baseline were, in paper V, associated with overall survival in PAH, where patients with supra-median ADM levels showed worse survival compared to those with infra-median levels. A previous study from Kolditz and colleagues showed that plasma MR-proADM is related to one-year mortality²⁰. In contrast, in paper V, ADM did not discriminate between PAH patients that survived past one year from those that did not. This might be related to the relatively small groups used for analysis. However, there are some differences between the two studies that should be addressed. While only incident PAH patients were included in paper V, the study from Kolditz et al. used mostly prevalent along with few incident patients. Also, our measuring technique assessed plasma adrenomedullin variants other than MR-proADM.

Adrenomedullin is a potent vasodilator. In addition, adrenomedullin has been suggested to impede pulmonary artery smooth muscle cell proliferation and to lessen pulmonary arterial collagen accumulation in PAH²²⁻²⁴. Moreover, adrenomedullin administration has been observed to result in attenuated PH in animal models and improved hemodynamics in human PH^{22, 25-28}.

Strengths and limitations

The inclusion criteria were restricted to only include PAH patients that were treatment-naïve at baseline. Although this results in a relatively small study sample, it can be viewed as a strength in the present studies, as many of the included biomarkers have previously not been thoroughly investigated in PAH.

Present PAH patients were older than what is considered as the typical age for PAH. In paper II-V, the studied cohort was, furthermore, older than the control group. However, these papers mainly focused on PAH patients' risk scores. According to SPAHR, the median age for PAH patients in Sweden is almost 70 years⁴, similar to the study cohort utilized in paper II-V. This median age is somewhat high, especially when compared to some other registries such as the REVEAL registry¹⁰⁴. That said, the COMPERA registry also shows that many IPAH patients exhibit advanced age¹⁰⁵.

Future perspectives

The biomarker field in PAH is still in somewhat early development. The aim of this PhD dissertation was therefore directed towards screening for new potential biomarkers in PAH. The present papers can be viewed as initial research that provides interesting results on new potential biomarkers, to be further studied in larger future projects and cohorts. Moreover, the results have generated new hypotheses that can advance research in PAH pathology.

Paper I and II underline the role of growth factors and receptor tyrosine kinase signaling in PAH and introduce sFlt-1, SCF and TGF- α as proteins that deserve further attention in PAH pathology. Future studies are also needed to elaborate on the specific pathological role of these markers in PH. For instance, sFlt-1 has been studied in several biomarker studies in PH but has not been specifically studied in relation to PAH pathology. Plasma FGF-23 in paper III provides a potential and additional aspect of the connection between PAH and renal disease, but whether FGF-23 has pathological effects independent of renal function in PH is yet to be investigated.

Conclusions

The present projects provide information on new potential biomarkers in PAH, with a potential utility in risk assessment or treatment response evaluation, that deserve further investigation in studies with larger cohorts.

The major conclusions of each study are:

- I** Plasma sFlt-1 levels decrease between diagnosis and early treatment follow-ups. Between diagnosis and follow-ups, the changes in plasma sFlt-1 correlate to changes in some important clinical parameters. This provides an additional dimension to the previous biomarker research on circulating sFlt-1 in PAH, where sFlt-1 has a potential use in treatment response evaluation in PAH.
- II** PAH patients exhibit low plasma SCF levels that are correlated with increased hemodynamic severity. Plasma SCF levels discriminate high-risk PAH patients from other PAH patients, as assessed with the ESC/ERS risk assessment model. SCF combined with TGF- α detected high-risk patients with good sensitivity and specificity.
- III and IV** Several metabolic proteins and tumor-related biomarkers are altered in PAH. The main results show that elevated FGF-23 and IGFBP-1 levels are associated with disease severity. In specific, plasma FGF-23 levels at diagnosis correlated with worse hemodynamics, 6MWD and NT-proBNP, as well as to risk scores for early mortality, assessed with the ESC/ERS and modified REVEAL risk score models. Likewise, plasma IGFBP-1 showed moderate correlations with hemodynamics, 6MWD, NT-proBNP, and risk scores for early mortality.
- V** Plasma ADM levels in incident PAH patients are higher compared to healthy controls, lower compared to PH due to left

heart disease, but are not different from ADM levels in CTEPH patients. Elevated plasma ADM at PAH diagnosis are highly correlated to key clinical parameters as well as to both the ESC/ERS and modified REVEAL risk scores. ADM is associated with overall survival in PAH. Paper V confirms results from previous studies and adds a risk assessment perspective to ADM in PAH.

Acknowledgments

My thanks go to all who supported me during my PhD studies. Special thanks to:

Dr. Göran Rådegran, my supervisor. Without your efforts to initiate LCPR and your genuine enthusiasm, this PhD dissertation would not have been possible. Thank you for introducing me to this amazing field of research, for believing in me and for always being available.

Dr. Roger Hesselstrand, my assistant supervisor. Thank you for the great support and valuable feedback throughout the years.

My fellow PhD students **Abdulla Ahmed, Salaheldin Ahmed, Mattias Arvidsson, Joanna Säleby, Eveline Löfdahl** and **Anna Werther-Evaldsson**. Your presence during these years will give a brighter color to my memories as a PhD student. Thank you for the encouragement, the laughs and all the interesting and wise discussions.

Former PhD students **David Kylhammar, Jakob Lundgren, Carl Söderlund** and **Moman Mohammad**. Thank you for your guidance and advice, which have been very helpful during my early years as a PhD student.

The nursing staff at the Hemodynamic Lab, for helping me out with the data collection and welcoming me into your workplace. A special thanks to **Anneli Ahlqvist**, for your immense support and your fantastic work with LCPR.

The members of the Swedish Society of PH (SveFPH). A special thanks to **Barbro Kjellström** and **Bodil Ivarsson**, for the great support throughout my PhD studies.

Monica Magnusson and **Lena Lindén**, for your help and advice regarding administrative tasks, and for all the lovely coffee breaks.

Finally, thanks to my parents and siblings **Sorya Merani, Salah Bouzina, Iman Bouzina, Selma Bouzina** and **Mohammad Bouzina**. You have a special place in my heart.

Bibliography

1. Galie N, Humbert M, Vachiery JL, et al. 2015 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension: The Joint Task Force for the Diagnosis and Treatment of Pulmonary Hypertension of the European Society of Cardiology (ESC) and the European Respiratory Society (ERS): Endorsed by: Association for European Paediatric and Congenital Cardiology (AEPC), International Society for Heart and Lung Transplantation (ISHLT). *European heart journal* 2016; 37: 67-119. 2015/09/01. DOI: 10.1093/eurheartj/ehv317.
2. Tuder RM, Marecki JC, Richter A, et al. Pathology of pulmonary hypertension. *Clinics in chest medicine* 2007; 28: 23-vii. DOI: 10.1016/j.ccm.2006.11.010.
3. Simonneau G, Montani D, Celermajer DS, et al. Haemodynamic definitions and updated clinical classification of pulmonary hypertension. *European Respiratory Journal* 2019; 53: 1801913. DOI: 10.1183/13993003.01913-2018.
4. Radegran G, Kjellstrom B, Ekmeahag B, et al. Characteristics and survival of adult Swedish PAH and CTEPH patients 2000-2014. *Scandinavian cardiovascular journal : SCJ* 2016; 50: 243-250. 2016/05/06. DOI: 10.1080/14017431.2016.1185532.
5. D'Alonzo GE, Barst RJ, Ayres SM, et al. Survival in patients with primary pulmonary hypertension. Results from a national prospective registry. *Annals of internal medicine* 1991; 115: 343-349. 1991/09/01. DOI: 10.7326/0003-4819-115-5-343.
6. Galie N, Barberà JA, Frost AE, et al. Initial Use of Ambrisentan plus Tadalafil in Pulmonary Arterial Hypertension. *New England Journal of Medicine* 2015; 373: 834-844. DOI: 10.1056/NEJMoa1413687.
7. Badesch DB, Raskob GE, Elliott CG, et al. Pulmonary Arterial Hypertension: Baseline Characteristics From the REVEAL Registry. *CHEST* 2010; 137: 376-387. DOI: 10.1378/chest.09-1140.
8. Boucly A, Weatherald J, Savale L, et al. Risk assessment, prognosis and guideline implementation in pulmonary arterial hypertension. *European Respiratory Journal* 2017; 50. DOI: 10.1183/13993003.00889-2017.
9. Kylhammar D, Kjellstrom B, Hjalmarsson C, et al. A comprehensive risk stratification at early follow-up determines prognosis in pulmonary arterial hypertension. *European heart journal* 2017 2017/06/03. DOI: 10.1093/eurheartj/ehx257.
10. Hoepfer MM, Kramer T, Pan Z, et al. Mortality in pulmonary arterial hypertension: prediction by the 2015 European pulmonary hypertension guidelines risk stratification model. *The European respiratory journal* 2017; 50 2017/08/05. DOI: 10.1183/13993003.00740-2017.

11. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clinical pharmacology and therapeutics* 2001; 69: 89-95. 2001/03/10. DOI: 10.1067/mcp.2001.113989.
12. Frost A, Badesch D, Gibbs JSR, et al. Diagnosis of pulmonary hypertension. *European Respiratory Journal* 2019; 53: 1801904. DOI: 10.1183/13993003.01904-2018.
13. Tonelli AR, Arelli V, Minai OA, et al. Causes and circumstances of death in pulmonary arterial hypertension. *American journal of respiratory and critical care medicine* 2013; 188: 365-369. 2013/04/23. DOI: 10.1164/rccm.201209-1640OC.
14. Pezzuto B, Badagliacca R, Poscia R, et al. Circulating biomarkers in pulmonary arterial hypertension: update and future direction. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* 2015; 34: 282-305. 2015/02/16. DOI: 10.1016/j.healun.2014.12.005.
15. Miyauchi T, Yorikane R, Sakai S, et al. Contribution of endogenous endothelin-1 to the progression of cardiopulmonary alterations in rats with monocrotaline-induced pulmonary hypertension. *Circulation research* 1993; 73: 887-897. 1993/11/01.
16. Kulasekaran P, Scavone CA, Rogers DS, et al. Endothelin-1 and transforming growth factor-beta1 independently induce fibroblast resistance to apoptosis via AKT activation. *American journal of respiratory cell and molecular biology* 2009; 41: 484-493. 2009/02/04. DOI: 10.1165/rcmb.2008-0447OC.
17. Browatzki M, Schmidt J, Kubler W, et al. Endothelin-1 induces interleukin-6 release via activation of the transcription factor NF-kappaB in human vascular smooth muscle cells. *Basic research in cardiology* 2000; 95: 98-105. 2000/05/29.
18. Kielstein JT, Bode-Boger SM, Hesse G, et al. Asymmetrical dimethylarginine in idiopathic pulmonary arterial hypertension. *Arteriosclerosis, thrombosis, and vascular biology* 2005; 25: 1414-1418. 2005/04/30. DOI: 10.1161/01.ATV.0000168414.06853.f0.
19. de Man FS, Tu L, Handoko ML, et al. Dysregulated renin-angiotensin-aldosterone system contributes to pulmonary arterial hypertension. *American journal of respiratory and critical care medicine* 2012; 186: 780-789. 2012/08/04. DOI: 10.1164/rccm.201203-0411OC.
20. Kolditz M, Seyfarth HJ, Wilkens H, et al. MR-proADM Predicts Exercise Capacity and Survival Superior to Other Biomarkers in PH. *Lung* 2015; 193: 901-910. 2015/09/14. DOI: 10.1007/s00408-015-9802-y.
21. Kakishita M, Nishikimi T, Okano Y, et al. Increased plasma levels of adrenomedullin in patients with pulmonary hypertension. *Clinical science (London, England : 1979)* 1999; 96: 33-39. 1998/12/19.
22. Pang L, Qi J, Gao Y, et al. Adrenomedullin alleviates pulmonary artery collagen accumulation in rats with pulmonary hypertension induced by high blood flow. *Peptides* 2014; 54: 101-107. 2014/02/01. DOI: 10.1016/j.peptides.2014.01.013.
23. Li W, Kong QY, Zhao CF, et al. Adrenomedullin and adrenotensin regulate collagen synthesis and proliferation in pulmonary arterial smooth muscle cells. *Brazilian journal of medical and biological research = Revista brasileira de pesquisas*

- medicas e biologicas* 2013; 46: 1047-1055. 2013/12/19. DOI: 10.1590/1414-431x20132882.
24. Upton PD, Wharton J, Coppock H, et al. Adrenomedullin expression and growth inhibitory effects in distinct pulmonary artery smooth muscle cell subpopulations. *American journal of respiratory cell and molecular biology* 2001; 24: 170-178. 2001/02/13. DOI: 10.1165/ajrcmb.24.2.4210.
 25. Yoshihara F, Nishikimi T, Horio T, et al. Chronic infusion of adrenomedullin reduces pulmonary hypertension and lessens right ventricular hypertrophy in rats administered monocrotaline. *European journal of pharmacology* 1998; 355: 33-39. 1998/10/01.
 26. Harada-Shiba M, Takamisawa I, Miyata K, et al. Intratracheal gene transfer of adrenomedullin using polyplex nanomicelles attenuates monocrotaline-induced pulmonary hypertension in rats. *Molecular therapy : the journal of the American Society of Gene Therapy* 2009; 17: 1180-1186. 2009/04/02. DOI: 10.1038/mt.2009.63.
 27. Nagaya N, Kyotani S, Uematsu M, et al. Effects of adrenomedullin inhalation on hemodynamics and exercise capacity in patients with idiopathic pulmonary arterial hypertension. *Circulation* 2004; 109: 351-356. 2004/01/14. DOI: 10.1161/01.Cir.0000109493.05849.14.
 28. Nagaya N, Nishikimi T, Uematsu M, et al. Haemodynamic and hormonal effects of adrenomedullin in patients with pulmonary hypertension. *Heart (British Cardiac Society)* 2000; 84: 653-658. 2000/11/18. DOI: 10.1136/heart.84.6.653.
 29. Tu L, Dewachter L, Gore B, et al. Autocrine fibroblast growth factor-2 signaling contributes to altered endothelial phenotype in pulmonary hypertension. *American journal of respiratory cell and molecular biology* 2011; 45: 311-322. 2010/11/03. DOI: 10.1165/rcmb.2010-0317OC.
 30. Izikki M, Guignabert C, Fadel E, et al. Endothelial-derived FGF2 contributes to the progression of pulmonary hypertension in humans and rodents. *The Journal of clinical investigation* 2009; 119: 512-523. 2009/02/09. DOI: 10.1172/JCI35070.
 31. Xiao Y, Peng H, Hong C, et al. PDGF Promotes the Warburg Effect in Pulmonary Arterial Smooth Muscle Cells via Activation of the PI3K/AKT/mTOR/HIF-1 α Signaling Pathway. *Cellular Physiology and Biochemistry* 2017; 42: 1603-1613. DOI: 10.1159/000479401.
 32. Selimovic N, Bergh CH, Andersson B, et al. Growth factors and interleukin-6 across the lung circulation in pulmonary hypertension. *The European respiratory journal* 2009; 34: 662-668. 2009/03/28. DOI: 10.1183/09031936.00174908.
 33. Benisty JI, McLaughlin VV, Landzberg MJ, et al. Elevated basic fibroblast growth factor levels in patients with pulmonary arterial hypertension. *Chest* 2004; 126: 1255-1261. 2004/10/16. DOI: 10.1378/chest.126.4.1255.
 34. Le Cras TD, Hardie WD, Fagan K, et al. Disrupted pulmonary vascular development and pulmonary hypertension in transgenic mice overexpressing transforming growth factor-alpha. *American journal of physiology Lung cellular and molecular physiology* 2003; 285: L1046-1054. 2003/08/05. DOI: 10.1152/ajplung.00045.2003.

35. Guo Y, Su L, Li Y, et al. The synergistic therapeutic effect of hepatocyte growth factor and granulocyte colony-stimulating factor on pulmonary hypertension in rats. *Heart and vessels* 2014; 29: 520-531. 2013/08/13. DOI: 10.1007/s00380-013-0395-1.
36. Campbell AI, Zhao Y, Sandhu R, et al. Cell-based gene transfer of vascular endothelial growth factor attenuates monocrotaline-induced pulmonary hypertension. *Circulation* 2001; 104: 2242-2248. 2001/10/31.
37. Partovian C, Adnot S, Raffestin B, et al. Adenovirus-mediated lung vascular endothelial growth factor overexpression protects against hypoxic pulmonary hypertension in rats. *American journal of respiratory cell and molecular biology* 2000; 23: 762-771. 2000/12/06. DOI: 10.1165/ajrcmb.23.6.4106.
38. Janvier A, Nadeau S, Baribeau J, et al. Role of vascular endothelial growth factor receptor 1 and vascular endothelial growth factor receptor 2 in the vasodilator response to vascular endothelial growth factor in the neonatal piglet lung. *Critical care medicine* 2005; 33: 860-866. 2005/04/09.
39. Tiede SL, Gall H, Dörr O, et al. New potential diagnostic biomarkers for pulmonary hypertension. *European Respiratory Journal* 2015; 46: 1390-1396. DOI: 10.1183/13993003.00187-2015.
40. Malhotra R, Paskin-Flerlage S, Zamanian RT, et al. Circulating angiogenic modulatory factors predict survival and functional class in pulmonary arterial hypertension. *Pulmonary circulation* 2013; 3: 369-380. 2013/09/10. DOI: 10.4103/2045-8932.110445.
41. Säleby J, Bouzina H, Lundgren J, et al. Angiogenic and inflammatory biomarkers in the differentiation of pulmonary hypertension. *Scandinavian cardiovascular journal : SCJ* 2017; 51: 261-270. 2017/08/05. DOI: 10.1080/14017431.2017.1359419.
42. Kylhammar D, Hesselstrand R, Nielsen S, et al. Angiogenic and inflammatory biomarkers for screening and follow-up in patients with pulmonary arterial hypertension. *Scandinavian journal of rheumatology* 2018; 47: 319-324. 2018/03/13. DOI: 10.1080/03009742.2017.1378714.
43. Din S, Sarathchandra P, Yacoub MH, et al. Interaction between bone morphogenetic proteins and endothelin-1 in human pulmonary artery smooth muscle. *Vascular pharmacology* 2009; 51: 344-349. 2009/09/30. DOI: 10.1016/j.vph.2009.09.001.
44. Gore B, Izikki M, Mercier O, et al. Key role of the endothelial TGF-beta/ALK1/endoglin signaling pathway in humans and rodents pulmonary hypertension. *PLoS one* 2014; 9: e100310. 2014/06/24. DOI: 10.1371/journal.pone.0100310.
45. Liu D, Wu BX, Sun N, et al. Elevated levels of circulating BMP7 predict mortality in pulmonary arterial hypertension. *Chest* 2016 2016/03/24. DOI: 10.1016/j.chest.2016.03.007.
46. Mimeault M and Batra SK. Divergent molecular mechanisms underlying the pleiotropic functions of macrophage inhibitory cytokine-1 in cancer. *Journal of cellular physiology* 2010; 224: 626-635. 2010/06/26. DOI: 10.1002/jcp.22196.
47. Rhodes CJ, Wharton J, Howard LS, et al. Red cell distribution width outperforms other potential circulating biomarkers in predicting survival in idiopathic pulmonary

- arterial hypertension. *Heart (British Cardiac Society)* 2011; 97: 1054-1060. 2011/05/12. DOI: 10.1136/hrt.2011.224857.
48. Nickel N, Kempf T, Tapken H, et al. Growth differentiation factor-15 in idiopathic pulmonary arterial hypertension. *American journal of respiratory and critical care medicine* 2008; 178: 534-541. 2008/06/21. DOI: 10.1164/rccm.200802-235OC.
 49. Zelniker T, Uhlmann L, Spaich S, et al. Novel biomarkers for risk stratification in pulmonary arterial hypertension. *ERJ Open Research* 2015; 1. DOI: 10.1183/23120541.00008-2015.
 50. Meadows CA, Risbano MG, Zhang L, et al. Increased expression of growth differentiation factor-15 in systemic sclerosis-associated pulmonary arterial hypertension. *Chest* 2011; 139: 994-1002. 2010/09/11. DOI: 10.1378/chest.10-0302.
 51. Sutendra G and Michelakis Evangelos D. The Metabolic Basis of Pulmonary Arterial Hypertension. *Cell Metabolism* 2014; 19: 558-573. DOI: <https://doi.org/10.1016/j.cmet.2014.01.004>.
 52. Xu W, Koeck T, Lara AR, et al. Alterations of cellular bioenergetics in pulmonary artery endothelial cells. *Proceedings of the National Academy of Sciences of the United States of America* 2007; 104: 1342-1347. 2007/01/18. DOI: 10.1073/pnas.0605080104.
 53. Zhao L, Ashek A, Wang L, et al. Heterogeneity in lung (18)FDG uptake in pulmonary arterial hypertension: potential of dynamic (18)FDG positron emission tomography with kinetic analysis as a bridging biomarker for pulmonary vascular remodeling targeted treatments. *Circulation* 2013; 128: 1214-1224. 2013/08/01. DOI: 10.1161/circulationaha.113.004136.
 54. McMurtry MS, Bonnet S, Wu X, et al. Dichloroacetate prevents and reverses pulmonary hypertension by inducing pulmonary artery smooth muscle cell apoptosis. *Circulation research* 2004; 95: 830-840. 2004/09/18. DOI: 10.1161/01.RES.0000145360.16770.9f.
 55. Sutendra G, Dromparis P, Bonnet S, et al. Pyruvate dehydrogenase inhibition by the inflammatory cytokine TNFalpha contributes to the pathogenesis of pulmonary arterial hypertension. *Journal of molecular medicine (Berlin, Germany)* 2011; 89: 771-783. 2011/08/03. DOI: 10.1007/s00109-011-0762-2.
 56. Piao L, Fang YH, Cadete VJ, et al. The inhibition of pyruvate dehydrogenase kinase improves impaired cardiac function and electrical remodeling in two models of right ventricular hypertrophy: resuscitating the hibernating right ventricle. *Journal of molecular medicine (Berlin, Germany)* 2010; 88: 47-60. 2009/12/02. DOI: 10.1007/s00109-009-0524-6.
 57. Tataranni T and Piccoli C. Dichloroacetate (DCA) and Cancer: An Overview towards Clinical Applications. *Oxidative medicine and cellular longevity* 2019; 2019: 8201079. 2019/12/13. DOI: 10.1155/2019/8201079.
 58. Sharma S, Ruffenach G, Umar S, et al. Role of oxidized lipids in pulmonary arterial hypertension. *Pulmonary circulation* 2016; 6: 261-273. DOI: 10.1086/687293.
 59. Zhao Y, Peng J, Lu C, et al. Metabolomic Heterogeneity of Pulmonary Arterial Hypertension. *PLoS one* 2014; 9. DOI: 10.1371/journal.pone.0088727.

60. Lewis GD, Ngo D, Hemnes AR, et al. Metabolic Profiling of Right Ventricular-Pulmonary Vascular Function Reveals Circulating Biomarkers of Pulmonary Hypertension. *Journal of the American College of Cardiology* 2016; 67: 174-189. 2016/01/23. DOI: 10.1016/j.jacc.2015.10.072.
61. Rhodes CJ, Ghataorhe P, Wharton J, et al. Plasma Metabolomics Implicates Modified Transfer RNAs and Altered Bioenergetics in the Outcomes of Pulmonary Arterial Hypertension. *Circulation* 2017; 135: 460-475. 2016/11/25. DOI: 10.1161/circulationaha.116.024602.
62. Gabrielli LA, Castro PF, Godoy I, et al. Systemic oxidative stress and endothelial dysfunction is associated with an attenuated acute vascular response to inhaled prostanoid in pulmonary artery hypertension patients. *Journal of cardiac failure* 2011; 17: 1012-1017. 2011/11/30. DOI: 10.1016/j.cardfail.2011.08.008.
63. Zhang R, Sun ML, Fan YF, et al. Plasma 15-F2t-isoprostane in idiopathic pulmonary arterial hypertension. *International journal of cardiology* 2014; 175: 268-273. 2014/06/01. DOI: 10.1016/j.ijcard.2014.05.014.
64. Zhang S, Yang T, Xu X, et al. Oxidative stress and nitric oxide signaling related biomarkers in patients with pulmonary hypertension: a case control study. *BMC pulmonary medicine* 2015; 15: 50. 2015/05/03. DOI: 10.1186/s12890-015-0045-8.
65. Bonnet S, Michelakis ED, Porter CJ, et al. An abnormal mitochondrial-hypoxia inducible factor-1 α -Kv channel pathway disrupts oxygen sensing and triggers pulmonary arterial hypertension in fawn hooded rats: similarities to human pulmonary arterial hypertension. *Circulation* 2006; 113: 2630-2641. 2006/06/01. DOI: 10.1161/circulationaha.105.609008.
66. Fijalkowska I, Xu W, Comhair SAA, et al. Hypoxia Inducible-Factor1 α Regulates the Metabolic Shift of Pulmonary Hypertensive Endothelial Cells. *The American journal of pathology* 2010; 176: 1130-1138. DOI: 10.2353/ajpath.2010.090832.
67. Archer SL, Marsboom G, Kim GH, et al. Epigenetic attenuation of mitochondrial superoxide dismutase 2 in pulmonary arterial hypertension: a basis for excessive cell proliferation and a new therapeutic target. *Circulation* 2010; 121: 2661-2671. 2010/06/10. DOI: 10.1161/circulationaha.109.916098.
68. Federici C, Drake KM, Rigelsky CM, et al. Increased Mutagen Sensitivity and DNA Damage in Pulmonary Arterial Hypertension. *American journal of respiratory and critical care medicine* 2015; 192: 219-228. 2015/04/29. DOI: 10.1164/rccm.201411-2128OC.
69. Meloche J, Pflieger A, Vaillancourt M, et al. Role for DNA damage signaling in pulmonary arterial hypertension. *Circulation* 2014; 129: 786-797. 2013/11/26. DOI: 10.1161/circulationaha.113.006167.
70. Courboulin A, Paulin R, Giguere NJ, et al. Role for miR-204 in human pulmonary arterial hypertension. *J Exp Med* 2011; 208: 535-548. 2011/02/16. DOI: 10.1084/jem.20101812.
71. Paulin R, Courboulin A, Meloche J, et al. Signal transducers and activators of transcription-3/pim1 axis plays a critical role in the pathogenesis of human pulmonary arterial hypertension. *Circulation* 2011; 123: 1205-1215. 2011/03/09. DOI: 10.1161/circulationaha.110.963314.

72. Rai PR, Cool CD, King JAC, et al. The cancer paradigm of severe pulmonary arterial hypertension. *American journal of respiratory and critical care medicine* 2008; 178: 558-564. 2008/06/12. DOI: 10.1164/rccm.200709-1369PP.
73. Boucherat O, Vitry G, Trinh I, et al. The cancer theory of pulmonary arterial hypertension. *Pulmonary circulation* 2017; 7: 285-299. DOI: 10.1177/2045893217701438.
74. Guignabert C, Tu L, Le Hires M, et al. Pathogenesis of pulmonary arterial hypertension: lessons from cancer. *European Respiratory Review* 2013; 22: 543. DOI: 10.1183/09059180.00007513.
75. Hanahan D and Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; 144: 646-674. 2011/03/08. DOI: 10.1016/j.cell.2011.02.013.
76. Liberti MV and Locasale JW. The Warburg Effect: How Does it Benefit Cancer Cells? *Trends in biochemical sciences* 2016; 41: 211-218. 2016/01/19. DOI: 10.1016/j.tibs.2015.12.001.
77. Sysol JR, Natarajan V and Machado RF. PDGF Induces SphK1 Expression via Egr-1 to Promote Pulmonary Artery Smooth Muscle Cell Proliferation. *American journal of physiology Cell physiology* 2016: ajpcell.00059.02016. 2016/04/22. DOI: 10.1152/ajpcell.00059.2016.
78. Chen J, Tang H, Sysol JR, et al. The sphingosine kinase 1/sphingosine-1-phosphate pathway in pulmonary arterial hypertension. *American journal of respiratory and critical care medicine* 2014; 190: 1032-1043. 2014/09/03. DOI: 10.1164/rccm.201401-0121OC.
79. Renard S, Paulin R, Breuils-Bonnet S, et al. Pim-1: A new biomarker in pulmonary arterial hypertension. *Pulmonary circulation* 2013; 3: 74-81. DOI: 10.4103/2045-8932.109917.
80. Benza RL, Gomberg-Maitland M, Miller DP, et al. The REVEAL Registry risk score calculator in patients newly diagnosed with pulmonary arterial hypertension. *Chest* 2012; 141: 354-362. 2011/06/18. DOI: 10.1378/chest.11-0676.
81. Arvidsson M, Ahmed A, Bouzina H, et al. Matrix metalloproteinase 7 in diagnosis and differentiation of pulmonary arterial hypertension. *Pulmonary circulation* 2019; 9: 2045894019895414. 2020/01/08. DOI: 10.1177/2045894019895414.
82. Ahmed A, Ahmed S, Arvidsson M, et al. Prolargin and matrix metalloproteinase-2 in heart failure after heart transplantation and their association with haemodynamics. *ESC Heart Fail* 2020; 7: 223-234. 2019/12/19. DOI: 10.1002/ehf2.12560.
83. Ahmed S, Ahmed A, Bouzina H, et al. Elevated plasma endocan and BOC in heart failure patients decrease after heart transplantation in association with improved hemodynamics. *Heart and Vessels* 2020. DOI: 10.1007/s00380-020-01656-3.
84. Assarsson E, Lundberg M, Holmquist G, et al. Homogenous 96-plex PEA immunoassay exhibiting high sensitivity, specificity, and excellent scalability. *PLoS one* 2014; 9: e95192. 2014/04/24. DOI: 10.1371/journal.pone.0095192.
85. Maynard SE, Min J-Y, Merchan J, et al. Excess Placental Soluble fms-like Tyrosine Kinase 1 (sFlt1) Could Contribute to Endothelial Dysfunction, Hypertension, and Proteinuria in Preeclampsia. *Obstetrical & Gynecological Survey* 2003; 58: 564-565. DOI: 10.1097/01.Ogx.0000083700.80693.Da.

86. Amraoui F, Spijkers L, Hassani Lahsinoui H, et al. SFlt-1 Elevates Blood Pressure by Augmenting Endothelin-1-Mediated Vasoconstriction in Mice. *PLoS one* 2014; 9: e91897. DOI: 10.1371/journal.pone.0091897.
87. Bjorkbacka H, Yao Mattisson I, Wigren M, et al. Plasma stem cell factor levels are associated with risk of cardiovascular disease and death. *Journal of internal medicine* 2017; 282: 508-521. 2017/08/27. DOI: 10.1111/joim.12675.
88. Wigren M, Rattik S, Hultman K, et al. Decreased levels of stem cell factor in subjects with incident coronary events. *Journal of internal medicine* 2016; 279: 180-191. 2015/10/16. DOI: 10.1111/joim.12443.
89. Young KC, Torres E, Hehre D, et al. Antagonism of Stem Cell Factor/c-kit Signaling Attenuates Neonatal Chronic Hypoxia-Induced Pulmonary Vascular Remodeling. *Pediatric research* 2016; 79: 637-646. DOI: 10.1038/pr.2015.275.
90. Montani D, Perros F, Gambaryan N, et al. C-kit-positive cells accumulate in remodeled vessels of idiopathic pulmonary arterial hypertension. *American journal of respiratory and critical care medicine* 2011; 184: 116-123. 2011/04/08. DOI: 10.1164/rccm.201006-0905OC.
91. Zhang T, Kawaguchi N, Hayama E, et al. High expression of CXCR4 and stem cell markers in a monocrotaline and chronic hypoxia-induced rat model of pulmonary arterial hypertension. *Exp Ther Med* 2018; 15: 4615-4622. DOI: 10.3892/etm.2018.6027.
92. Miranda LF, Rodrigues CO, Ramachandran S, et al. Stem cell factor improves lung recovery in rats following neonatal hyperoxia-induced lung injury. *Pediatr Res* 2013; 74: 682-688. 2013/10/23. DOI: 10.1038/pr.2013.165.
93. Chakinala MM, Coyne DW, Benza RL, et al. Impact of declining renal function on outcomes in pulmonary arterial hypertension: A REVEAL registry analysis. *The Journal of Heart and Lung Transplantation* 2018; 37: 696-705. DOI: 10.1016/j.healun.2017.10.028.
94. Hjalmarsson C, Rådegran G, Kylhammar D, et al. IMPACT OF AGE AND COMORBIDITY ON RISK STRATIFICATION IN IDIOPATHIC PULMONARY ARTERIAL HYPERTENSION. *European Respiratory Journal* 2018. DOI: 10.1183/13993003.02310-2017.
95. Faul C, Amaral AP, Oskouei B, et al. FGF23 induces left ventricular hypertrophy. *The Journal of clinical investigation* 2011; 121: 4393-4408. 2011/10/12. DOI: 10.1172/jci46122.
96. Silswal N, Touchberry CD, Daniel DR, et al. FGF23 directly impairs endothelium-dependent vasorelaxation by increasing superoxide levels and reducing nitric oxide bioavailability. *American journal of physiology Endocrinology and metabolism* 2014; 307: E426-436. 2014/07/24. DOI: 10.1152/ajpendo.00264.2014.
97. Six I, Okazaki H, Gross P, et al. Direct, Acute Effects of Klotho and FGF23 on Vascular Smooth Muscle and Endothelium. *PLoS one* 2014; 9: e93423. DOI: 10.1371/journal.pone.0093423.
98. Kaiser R, Seiler S, Held M, et al. Prognostic impact of renal function in precapillary pulmonary hypertension. *Journal of Internal Medicine* 2014; 275: 116-126. DOI: 10.1111/joim.12131.

99. Kasprzak A, Kwasniewski W, Adamek A, et al. Insulin-like growth factor (IGF) axis in cancerogenesis. *Mutation research Reviews in mutation research* 2017; 772: 78-104. 2017/05/23. DOI: 10.1016/j.mrrev.2016.08.007.
100. Connolly M, Garfield BE, Crosby A, et al. miR-322-5p targets IGF-1 and is suppressed in the heart of rats with pulmonary hypertension. *FEBS open bio* 2018; 8: 339-348. 2018/03/08. DOI: 10.1002/2211-5463.12369.
101. Sun M, Ramchandran R, Chen J, et al. Smooth Muscle Insulin-Like Growth Factor-1 Mediates Hypoxia-Induced Pulmonary Hypertension in Neonatal Mice. *American journal of respiratory cell and molecular biology* 2016; 55: 779-791. 2016/07/21. DOI: 10.1165/rcmb.2015-0388OC.
102. Shi L, Kojonazarov B, Elgheznawy A, et al. miR-223-IGF-IR signalling in hypoxia- and load-induced right-ventricular failure: a novel therapeutic approach. *Cardiovascular research* 2016; 111: 184-193. 2016/03/26. DOI: 10.1093/cvr/cvw065.
103. Rhodes CJ, Wharton J, Ghataorhe P, et al. Plasma proteome analysis in patients with pulmonary arterial hypertension: an observational cohort study. *The Lancet Respiratory Medicine* 2017; 5: 717-726. DOI: [https://doi.org/10.1016/S2213-2600\(17\)30161-3](https://doi.org/10.1016/S2213-2600(17)30161-3).
104. Farber HW, Miller DP, Poms AD, et al. Five-Year outcomes of patients enrolled in the REVEAL Registry. *Chest* 2015; 148: 1043-1054. 2015/06/13. DOI: 10.1378/chest.15-0300.
105. McGoon MD, Benza RL, Escribano-Subias P, et al. Pulmonary arterial hypertension: epidemiology and registries. *Journal of the American College of Cardiology* 2013; 62: D51-59. 2013/12/21. DOI: 10.1016/j.jacc.2013.10.023.