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Plasma extracellular matrix and inflammatory proteins as biomarkers for diagnosis, differentiation, and risk assessment in pulmonary arterial hypertension

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Plasma extracellular matrix and inflammatory proteins as biomarkers for diagnosis, differentiation, and risk assessment in pulmonary arterial hypertension

- utilizing Lund Cardio Pulmonary Registry

Mattias Arvidsson, MD

LUND UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on 15th of November 2024 at 09.00 in F5 Hall, Skåne University Hospital, Entrégatan 7, Lund, Sweden

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Title and subtitle: Plasma extracellular matrix and inflammatory proteins as biomarkers for diagnosis, differentiation, and risk assessment in pulmonary arterial hypertension - utilizing Lund Cardio Pulmonary Registry

Abstract: Pulmonary arterial hypertension (PAH) is a rare disease that affects the pulmonary vasculature. Characterized by increased pulmonary arterial pressures and vascular resistance, it causes right heart failure (HF), and premature death. The main symptom, exertional dyspnoea, is non-specific where patient's and doctor's delay often lead to diagnosis in advanced stages. Despite development of PAH specific drugs and new treatment strategies, prognosis remains poor. This dissertation aimed to screen for bloodborne biomarkers with potential to facilitate differentiation between various causes of dyspnoea for earlier diagnosis, and more accurate risk assessment in PAH.

Patients with PAH, chronic thromboembolic pulmonary hypertension, pulmonary hypertension (PH) associated with HF with preserved or reduced ejection fraction, and patients with HF without PH who underwent right heart catheterisation as part of investigation for dyspnoea, and healthy controls enrolled in the Lund Cardio Pulmonary Registry were included. Venous blood samples were analysed with proximity extension assays and quantitative PCR. Statistical analyses were used to compare proteins' levels in the different groups and evaluate proteins as discriminators of PAH.

In paper I-II, plasma matrix metalloproteinase (MMP)-7 and prolargin were identified as candidates that could discriminate PAH from controls and among the other patient groups with dyspnea.

In paper III, increased plasma levels of MMP-2, perlecan, and tissue inhibitor of metalloproteinases 4 (TIMP-4) were negative predictors of survival. MMP-2 levels at diagnosis correlated with the European 3-strata risk assessment score.

In paper IV, plasma tumor necrosis factor related apoptosis-inducing ligand (TRAIL) could identify PAH among the other dyspnea patients and controls. Additionally, annexin A1 was a prognostic marker in PAH.

The present thesis provides evidence for several new biomarkers, related to extracellular matrix or inflammation, with a diagnostic and prognostic potential in PAH. Combining multiple proteins in a biomarker panel may further improve the discriminative and/or prognostic ability.

Key words: pulmonary arterial hypertension, plasma biomarkers, diagnosis, differentiation, risk assessment, MMP-7, prolargin, MMP-2, TRAIL, ANXA1

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- utilizing Lund Cardio Pulmonary Registry

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

I. **Arvidsson, M.,** Ahmed, A., Bouzina, H., and Rådegran, G. (2019), Matrix metalloproteinase 7 in diagnosis and differentiation of pulmonary arterial hypertension. Pulmonary Circulation, 9: 1-8 2045894019895414. https://doi.org/10.1177/2045894019895414

II. **Arvidsson, M.,** Ahmed, A., Bouzina, H., and Rådegran, G. (2021) Plasma proteoglycan prolargin in diagnosis and differentiation of pulmonary arterial hypertension. ESC Heart Failure, 8: 1230–1243. https://doi.org/10.1002/ehf2.13184

III. **Arvidsson, M.,** Ahmed, A., Säleby, J., Hesselstrand, R., and Rådegran, G. (2022), Plasma matrix metalloproteinase 2 is associated with severity and mortality in pulmonary arterial hypertension. Pulmonary Circulation.; 12:e12041. https://doi.org/10.1002/pul2.12041

IV. **Arvidsson, M.,** Ahmed, A., Säleby, J., Ahmed, S., Hesselstrand, R., and Rådegran, G. (2023), Plasma TRAIL and ANXA1 in diagnosis and prognostication of pulmonary arterial hypertension. Pulm Circ.; 13:e12269. https://doi.org/10.1002/pul2.12269

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VI. Ahmed, A., Ahmed, S., **Arvidsson, M.**, Bouzina, H., Lundgren, J., and Rådegran, G. (2020) Elevated plasma sRAGE and IGFBP7 in heart failure decrease after heart transplantation in association with haemodynamics. ESC Heart Failure, 7: 2340–2353. https://doi.org/10.1002/ehf2.12772

Abstract

Pulmonary arterial hypertension (PAH) is a rare disease that affects the pulmonary vasculature. Characterized by increased pulmonary arterial pressures and vascular resistance, it causes right heart failure (HF), and premature death. The main symptom, exertional dyspnoea, is non-specific where patient's and doctor's delay often lead to diagnosis in advanced stages. Despite development of PAH specific drugs and new treatment strategies, prognosis remains poor. This dissertation aimed to screen for bloodborne biomarkers with potential to facilitate differentiation between various causes of dyspnoea for earlier diagnosis, and more accurate risk assessment in PAH.

Patients with PAH, chronic thromboembolic pulmonary hypertension, pulmonary hypertension (PH) associated with HF with preserved or reduced ejection fraction, and patients with HF without PH who underwent right heart catheterisation as part of investigation for dyspnoea, and healthy controls enrolled in the Lund Cardio Pulmonary Registry were included. Venous blood samples were analysed with proximity extension assays and quantitative PCR. Statistical analyses were used to compare proteins' levels in the different groups and evaluate proteins as discriminators of PAH.

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The present thesis provides evidence for several new biomarkers, related to extracellular matrix or inflammation, with a diagnostic and prognostic potential in PAH. Combining multiple proteins in a biomarker panel may further improve the discriminative and/or prognostic ability.

Summary in Swedish (Sammanfattning på svenska)

Pulmonell hypertension (PH) är ett sjukdomstillstånd karakteriserat av ett förhöjt tryck i lungkretsloppet. Det är ett vanligt sjukdomstillstånd som förekommer hos ca 1 procent av världens befolkning och uppemot 10% av alla människor äldre än 65 år. Det finns flera olika bakomliggande orsaker till PH och tillståndet delas in i fem grupper utifrån detta. Grupp 1, pulmonell arteriell hypertension (PAH), grupp 2, PH relaterad till vänstersidig hjärtsjukdom, grupp 3, PH relaterad till lungsjukdom eller låga syrenivåer, grupp 4 relaterat till flödeshinder i lungartären såsom kroniska blodproppar i lungkärlen (kronisk tromboembolisk PH, CTEPH), och grupp 5, PH med oklar eller multifaktoriella orsaker. Orsakerna till PH är olika vanliga. Grupp 2 PH är den vanligaste orsaken, följt av grupp 3 PH. PAH är däremot en väldigt ovanlig sjukdom med en nyinsjuknandefrekvens på ca 6-8 fall per million invånare och år, och en förekomst på ca 48-55 fall per million invånare i västvärlden. Vilket innebär att ca 500 människor i Sverige lever med PAH.

PAH är en progressiv sjukdom som leder till ökat tryck och motstånd i lungkretsloppet, beroende av att de små blodkärlen i lungorna drar ihop sig och kärlväggen blir tjockare vilket resulterar i trängre blodkärl. Detta leder till ett ökat lungkärlsmotstånd där hjärtat behöver arbeta hårdare för att pumpa blodet genom lungorna. Detta leder på sikt till högersidig hjärtsvikt och tidig död.

Under de senaste decennierna har ett antal olika PAH specifika läkemedel och behandlingsstrategier utvecklats, men då huvudsymtomen utgörs av ökad andfåddhet vid ansträngning och trötthet som inte kan vilas bort, vilket är ospecifikt för PAH, dröjer det ofta länge från det att individen får symtom till dess att den får rätt diagnos och insatt behandling. Denna avhandling syftar till att identifiera blodburna biomarkörskandidater för att kunna underlätta identifiering av PAH sjukdomen i ett tidigare skede och bidra till mer träffsäkra riskbedömningar som underlag för patientens behandling och överlevnad.

I delarbete I var fokus på matrix metalloproteinaser (MMP), en grupp proteiner som är involverade i omstrukturering av extracellulärmatrix, som är den ”byggnadsställning” som omger kärlen. Vi fann att man med hjälp av blodburet

MMP-7 kunde identifiera individer med PAH bland andra individer med PH och eller/ hjärtsvikt.

I delarbete II undersökte vi proteoglykaner och associerade protein-byggstenar i kärlens ”byggnadsställningar”, samt en mängd proteiner relaterade till inflammation. Vi fann att nivåerna av blodburet prolargin skiljde sig mellan friska kontrollpersoner jämfört med sjuka med PAH, CTEPH, PH relaterad till vänstersidig hjärtsjukdom, samt hjärtsvikt utan PH. Nivåerna av blodburet prolargin kunde dessutom användas för att identifiera PAH från de andra sjukdomsgrupperna. Det fanns även ett samband mellan nivån av prolargin och sämre hjärtfunktion.

I delarbete III utvärderades extracellulärmatrix-relaterade proteiner som prognostiska markörer vid PAH. Vi fann att ökade nivåer av MMP-2, perlecan och vävnadsinhibitor för metalloproteinas 4 (TIMP-4) i blodet var relaterade till sämre överlevnad. Nivåerna av MMP-2 var relaterade till patienternas sjukdomsbörda.

I delarbete IV studerades proteiner relaterade till tumörnekrosfaktor (TNF) som diagnostiska markörer för PAH. Därefter studerades en större grupp inflammationsrelaterade proteiner som prognostiska biomarkörer vid PAH. TNF relaterad apoptos inducerande ligand (TRAIL) kunde identifiera PAH från andra sjukdomsgrupper och ökade nivåer av annexin A1 var kopplat till sämre överlevnad.

Sammanfattningsvis presenterar denna avhandling flera proteiner med potential för att bli framtida diagnostiska eller prognostiska blodburna biomarkörer för PAH. Dessa skulle kunna, enskilt eller tillsammans med andra biomarkörer i ett större provbatteri, bidra till tidigare diagnos av PAH och mer träffsäkra riskbedömningar och därmed åstadkomma en mer precis behandlingsvägledning.

Abbreviations

6MWD	Six-minute walking distance
ANXA1	Annexin A1
AUC	Area under the curve
BSA	Body surface area
BMPR2	Bone morphogenic protein receptor type 2
CI	Confidence interval
CO	Cardiac output
COMPERA	Comparative, Prospective Registry of Newly Initiated Therapies for Pulmonary Hypertension
CTD-PAH	Connective tissue disease associated PAH
CTEPH	Chronic thromboembolic pulmonary hypertension
ECM	Extracellular matrix
ERA	Endothelin receptor antagonist
ESC/ERS	European Society of Cardiology/ European Respiratory Society
FDR	False discovery rate
HF	Heart failure
HFpEF-PH	PH associated with HF with preserved ejection fraction
HFrEF-PH	PH associated with HF with reduced ejection fraction
HR	Heart rate
IPAH	Idiopathic PAH
LCPR	Lund Cardio Pulmonary Registry
LVSWI	Left ventricular stroke work index
MAP	Mean arterial pressure
MMP	Matrix metalloproteinase

MPAP	Mean pulmonary arterial pressure
MRAP	Mean right atrial pressure
PCA	Prostacyclin analogue
PAH	Pulmonary arterial hypertension
PAWP	Pulmonary arterial wedge pressure
PDE5i	Phosphodiesterase type 5 inhibitor
PH	Pulmonary hypertension
PVR	Pulmonary vascular resistance
REVEAL	Registry to Evaluate Early and Long-Term PAH Disease Management
RVSWI	Right ventricular stroke work index
ROC	Receiver operating characteristic
RHC	Right heart catheterization
SPAHR	Swedish PAH registry
SMC	Smooth muscle cells
PASMC	Pulmonary artery smooth muscle cells
SV	Stroke volume
SVI	Stroke volume index
TNF	Tumor necrosis factor
TPG	Transpulmonary pressure gradient
TRAIL	TNF-related apoptosis-inducing ligand
WHO	World Health Organization
WHO-FC	World Health Organization functional class

Introduction

Pulmonary hypertension

Pulmonary hypertension (PH) is a common condition affecting 1% of the population worldwide,[1] and up to 10% of those >65 years of age.[2] It is a condition characterized by increased pressures in the pulmonary circulation, eventually leading to right heart failure (HF) and ultimately death.[1, 3] PH has a diverse aetiology and is divided into five different groups according to the World Health Organisation (WHO) classification, i.e. group 1, pulmonary arterial hypertension (PAH); group 2, PH associated with left heart disease; group 3, PH associated with lung diseases and/or hypoxia; group 4, PH associated with pulmonary artery obstructions, including chronic thromboembolic PH (CTEPH); and group 5, PH with unclear and/or multifactorial mechanisms.[1] The majority of patients with PH have an underlying left heart disease (LHD), including advanced HF, which is very common. There is at present, however, no specific treatment for the PH component in PH associated with LHD. PAH, on the other hand, is a rare disease, for which PAH specific treatments are available.[1]

Pulmonary arterial hypertension

PAH is a rare disease with an incidence of ~6-8 cases per million inhabitants and year, and a prevalence of ~48-55 cases per million in economically developed countries.[4, 5] PAH was according to the 2015 European Society of Cardiology/ European Respiratory Society (ESC/ERS) guidelines defined by a mean pulmonary arterial pressure (MPAP) ≥25 mmHg at rest, a pulmonary vascular resistance (PVR) >3 Wood units (WU), and a pulmonary arterial wedge pressure (PAWP) ≤15 mmHg.[6] The haemodynamic thresholds at rest for PAH were, however, redefined in the 2022 ESC/ERS PH guidelines as MPAP >20 mmHg at rest and PVR >2 WU, whereas PAWP remained as ≤15 mmHg (Table 1).[1]

The classical PH definition was based on expert opinion, and the new cut-offs for MPAP and PVR were revised based on a more scientific approach and available evidence.[1, 7]

An important systematic review (based on multinational data from 13 countries and almost 1200 healthy individuals) assessing haemodynamic values at rest found that a normal MPAP ranges from 14.0 ±3.3 (mean ± standard deviation, SD) mmHg; and increased mortality in patients with MPAP 19-24 mmHg.[7, 8] With two SDs, accounting for 95% of the population, a normal upper limit of MPAP is 20.6 mmHg. For PVR, the normal upper limit and the lowest prognostically relevant threshold have been found to be ~ 2 WU.[9, 10]

Table 1. Haemodynamic definitions of pulmonary hypertension

Definition	Haemodynamic characteristics
PH	MPAP >20 mmHg
Pre-capillary PH	MPAP >20 mmHg, PAWP ≤15 mmHg, PVR >2 WU
IpcPH	MPAP >20 mmHg, PAWP >15 mmHg, PVR ≤2 WU
CpcPH	MPAP >20 mmHg, PAWP >15 mmHg, PVR >2 WU
Exercise PH	MPAP/CO slope between rest and exercise >3 mmHg/L/min
Unclassified PH	MPAP >20 mmHg, PAWP ≤15 mmHg, PVR ≤2 WU

Reproduced from the 2022 European Society of Cardiology (ESC)/European Respiratory Society (ERS) Guidelines for the diagnosis and treatment of pulmonary hypertension with permission of the ESC.[1] Values at rest unless otherwise stated. CpcPH, combined post- and pre-capillary PH; IpcPH, isolated post-capillary PH; MPAP, mean pulmonary arterial pressure; PAWP, pulmonary arterial wedge pressure; PH, pulmonary hypertension; PVR, pulmonary vascular resistance; WU, wood units.

Diagnostic delay

The symptoms in patients with PAH are often non-specific and characterized by unclear dyspnoea, fatigue, and/or reduced exercise tolerance (Figure 1).[11] Diagnosis is therefore often hampered by both a patient's and a doctor's delay.[12] The mean time between onset of symptoms and PAH diagnosis have not improved significantly from the reported mean time to diagnosis of 2 years and a median of 1.3 years in the 1980s.[11, 13]

Studies from the US, German, French, Spanish, and Austrailian registries between 2002 and 2017 indicate a mean time between onset of symptoms and PAH diagnosis ranging from 2.3-3.9 years.[13-18]

For example, Khou et al reported a mean and median delay from onset of symptoms to PAH diagnosis of 2.5 years and 1.2 years respectively, which is nearly the same as the mean 2.0 years and median 1.3 years reported in the 1980s.[11, 13] Likewise, a study of American insurance claims between 2007 and 2020 reported that the median time to PAH diagnosis was 2.3 years from the onset of chronic unexplained dyspnea, and 2.5 years from the onset of any PH symptoms (Figure 1).[19]

Longer diagnostic delay is associated with a significantly lower 5-year survival,[13] and the lack of reduction in time to PAH diagnosis underscores the importance to

facilitate a faster diagnosis.[12] Moreover, most PAH patients are still diagnosed in advanced stages of the disease, where ~80% were in World Health Organization functional class (WHO-FC) III or IV at diagnosis.[5, 20, 21] According to the 2022 annual report of the Swedish PAH registry (SPAHR), 70% of the patients diagnosed in Sweden between 2018 and 2022 were in WHO FC III and 9% in WHO-FC IV.[20] Corresponding numbers for 2000-2008 were 70% in WHO-FC III and 10% in WHO-FC IV.[5, 20] Comparatively, for incident PAH cases of the German part of "Comparative, Prospective Registry of Newly Initiated Therapies for Pulmonary Hypertension"(COMPERA) in 2014, 71% were reported to be in WHO-FC-III and 12% in WHO-FC IV.[21] For incident cases in the French PAH registry 2002-2003 75% were in WHO-FC III (63%) and IV (12%).[14] The "Registry to Evaluate Early and Long-Term PAH Disease Management" (REVEAL) reported 74% in WHO-FC III or IV at PAH diagnosis, whereof 61.3% in FC III and 12.3% in WHO-FC IV.[18]

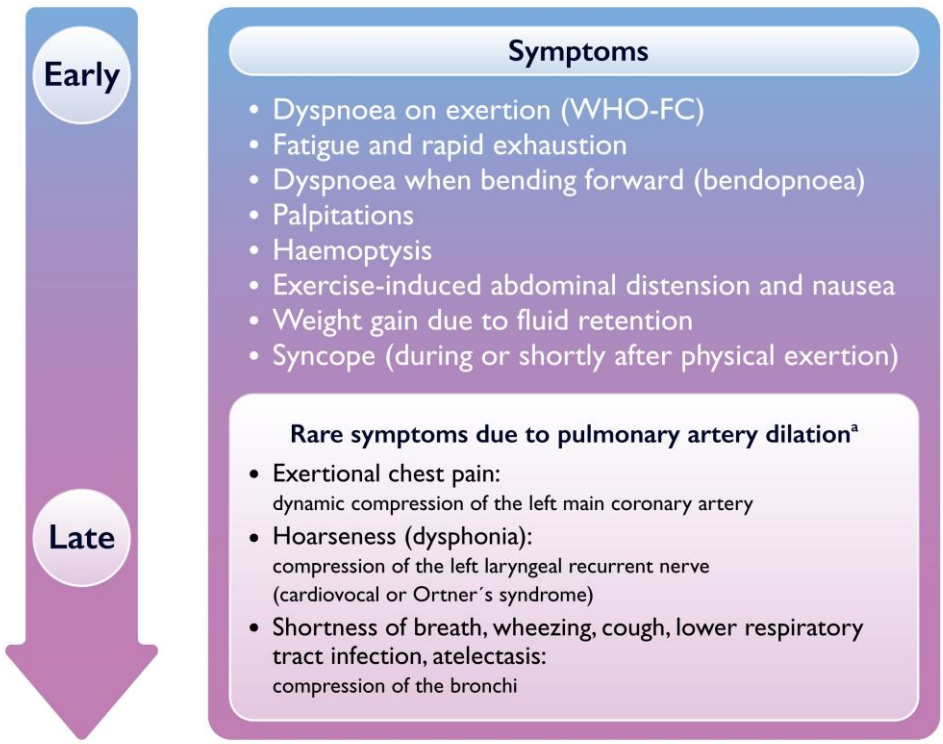

Figure 1. Symptoms of pulmonary hypertension
Reproduced from the 2022 European Society of Cardiology (ESC)/European Respiratory Society (ERS) Guidelines for the diagnosis and treatment of pulmonary hypertension with permission of the ESC.[1] Progression of symptoms in PH beginning with the non-specific dyspnoea on exertion and fatigue. WHO-FC, World Health Organisation functional class.

Altogheter, it is imperative to shorten the diagnostic delay, and a means to accomplish this could be the development of an easily accessible diagnostic biomarker or a biomarker panel for PAH.

Prognosis

Patients with PAH have a poor prognosis, with a median survival of 2.8 years from diagnosis without treatment, and only 11.8 months if associated with rheumatologic disease.[6, 22] In the pre-treatment era, the 1-, 3-, and 5-year survival in the NIH registry were 68%, 48%, and 34%, respectively.[22]

Patients with incident PAH in SPAHR with a baseline diagnosis from 2008-2014 showed a 1-, 3- and 5-year survival of 85%, 71%, and 59%, respectively.[5] For patients diagnosed from 2015-2022 in SPAHR, the corresponding survival were 90%, 71% and 60%.[20] Data from the US PH association registry 2015-2020 showed a 1-year survival of 92% and a 3-year survival of 79%.[23] Five-year survival in COMPERA from 2009-2016 was 69.7%. Thus, the five-year survival has roughly doubled compared to before the introduction of targeted PAH treatment and new treatment regimens, where the 5-year survival has increased from 34% to 60-79%.[5, 22-24]

Development of PAH specific medications and initial up-front combination therapy regimens have thus improved survival, but PAH prognosis remains unsatisfactory with a median transplant free survival of 6.2 years.[25-29] In fact, one study comparing survival in PAH from 2005-2019 did not find any difference in survival despite evolving treatment options.[29] A diagnostic delay where diagnosis is firstly made in advanced stages of PAH may thus contribute to the halted improvements in survival.

Pathology of PAH

PAH is a rare vasculopathy affecting pulmonary vessels, mainly distal muscular-type arteries ranging from 500 µm down to 70 µm, with vascular remodelling, including medial hypertrophy/hyperplasia, fibrosis of the intimal and adventitial layers, in situ thrombotic-, and plexiform lesions; but the disease also affects small pre-capillary pulmonary arteries ranging from 70 µm down to 20 µm (arterioles), with perivascular inflammation, abnormal muscularisation and obliteration.[30]

PAH is characterized by pulmonary endothelial dysfunction, resulting in an imbalance of vasodilating and vasoconstricting factors favouring vasoconstriction.[30]

PAH have many mechanisms including endothelial dysfunction and smooth muscle cell (SMC) proliferation.[31] Several gene mutations can lead to development of PAH, including germline mutations in bone morphogenic protein receptor type 2 (BMPR2), TBX4, ACVRL1, ENG, SMAD9, and KCNK3 among others.[32] The most common is a mutation of the BMPR2 gene, encoding BMPR2, a member of the transforming growth factor-β (TGF-β) superfamily.[33] It is the cause of around 70-80% of familial cases of PAH and 10-20% of idiopathic PAH (IPAH).[32] The BMPR2 mutation leads to reduced antiproliferative BMP-Smad 1/5/8 signal transduction and increased proliferative response to TGF-β signaling via Smad2/3, contributing to the development of PAH.[34]

In PAH, a pro-inflammatory phenotype of pulmonary endothelial cells characterized by an increase in surface expression of adhesion molecules, E-selectin, intracellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1) and excessive release of various cytokines and chemokines have been reported.[35] In addition, the cytokine TNF-α suppress BMPR2 signaling via "a disintegrin and metalloproteases" (ADAM) 10 and ADAM 17 in pulmonary artery SMCs (PASMC) favoring BMP mediated proliferation via activin receptors.[36]

Remodeling of the extracellular matrix (ECM) have been suggested to play a central role in PAH development.[37] ECM remodeling and pulmonary vascular stiffness have been found to promote further remodeling via YAP-TAZ pathway.[38] The composition of ECM is regulated by balance of proteolytic enzymes including matrix metalloproteinases (MMPs) and ADAMs and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs).[37] Multiple MMPs, ADAMs, TIMPs and collagen levels have been found to be altered in the intima and media of pulmonary arteries in PAH.[39, 40] MMPs contribute to processes such as ECM turnover, phenotype switching, hyperplasia, cell migration, apoptosis and have been proposed to play an important role in pulmonary vascular remodeling and development of PAH.[41]

PAH treatment

In the past three decades, several PAH specific treatments have been developed along with new treatment strategies **(**Figure 2**)**.[34]

Patients with IPAH, heritable PAH/familial PAH (FPAH) or drug induced PAH and a positive response by vasoreactivity testing, can be treated by high dose calcium channel blockers i.e. amlodipine, felodipine, diltiazem, and nifedipine. Roughly 10% of PAH patients are acute responders at vasoreactivity testing and display a reduction in MPAP by ≥10 mmHg, to absolute values of MPAP ≤40mmHg, with increased or unchanged cardiac output (CO). Only few are long term responders to calcium channel blockers.[1, 42]

Epoprostenol- a prostacyclin analogue (PCA) - the first PAH specific treatment available to all PAH patients was approved in 1995.[43, 44] In 2002, the first endothelin receptor antagonist (ERA) bosentan was introduced,[45] followed by sildenafil, a phosphodiesterase type 5 inhibitor (PDE5i) in 2005.[46] Thus, three different mechanisms of action for PAH treatment emerged. The endothelin, the nitric oxide and the prostacyclin pathways.[1] There are currently four routes of administration, i.e subcutaneus, intravenous, oral, and inhalational.[1] The prostacyclin pathway includes PCA and prostacyclin receptor agonists. PCA include apart from epoprostenol for i.v. use, iloprost[47] for inhalation and treprostenil[48] for s.c., i.v., or p.o. use. It has also been approved in the US for inhalation in patients with PAH and interstitial lung disease.[49] In 2015, the oral intake prostacyclin receptor agonist selexipag was introduced.[25]

The endothelin pathway is targeted by ERAs, and bosentan was the first dual endothelin receptor antagonist introduced in 2002.[45] It was followed by ambrisentan in 2008, an ERA which is more selective to the endothelin A receptor,[50] and 2013 by the dual ERA, macitentan.[51]

The nitric oxide pathway is targeted by the PDE5i, sildenafil, available for the indication PAH since 2005,[46] tadalafil from 2009,[52] and the soluble guanulate cyclase stimulator riociguat since 2013.[53]

In addition to a growing arsenal of treatments, new treatment strategies have been developed along with risk stratification strategies.[1] Initial dual combination therapy of ERA and PDE5i was introduced in the AMBITION trial in 2015.[26] Initial p.o. triple combination therapy with ERA, PDE5i, and the prostacyclin analogue selexipag have since been evaluated in the TRITON trial 2021, but was not found superior to initial dual combination therapy.[27] Thus, initial combination therapy is recommended for low and intermediate risk PAH patients. (Recommendation level 1 and evidence level B by the 2022 ECS/ERS guidelines). However, for high-risk patients, the ESC/ERS 2022 guidelines, recommend initial triple combination therapy with s.c./i.v. PCA, ERA and a PDE5i.[1] Unless a low-risk status is achieved at follow-up, treatment can be further escalated with addition of prostacyclin receptor agonist for intermediate-low risk and i.v. or s.c. PCA or evaluation for lung transplant if in intermediate-high or high risk.[1]

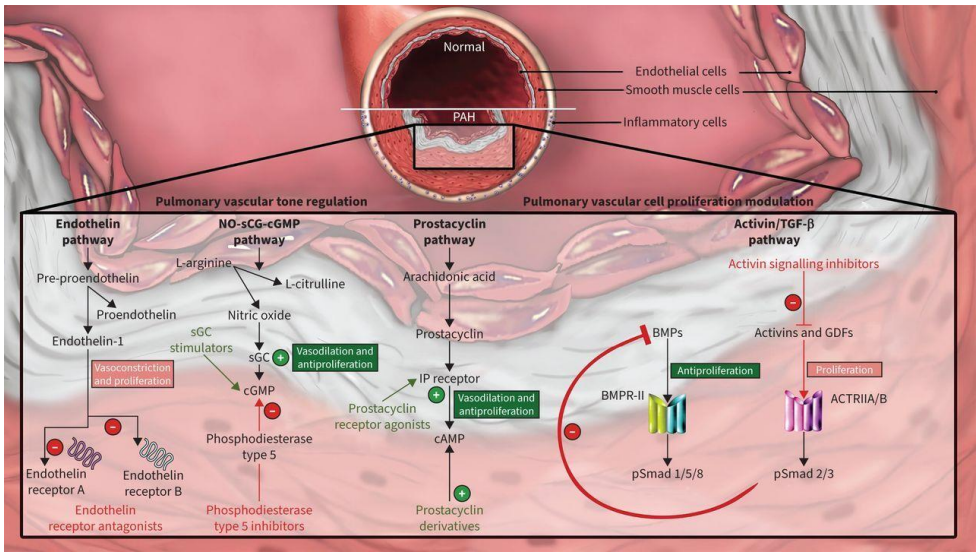

Figure 2. Drug targets in PAH. Classical pathways of endothelin, nitric oxide, prostacyclin, and the emerging activin/TGF-β pathway

The top picture show a small pulmonary artery (<500 µm in diameter) with signs of pulmonary arterial hypertension (PAH) including intimal proliferation and medial hypertrophy. Dysfunctional pulmonary arterial endothelial cells have decreased production of prostacyclin and nitric oxide (NO), with increased production of endothelin-1 and increased activin signaling promoting endothelial cell dysfunction and proliferation of pulmonary vascular endothelial cells. Sotatercept degrade ligands for the activin receptor II complex leading to reduced Smad2/3 signaling and resulting in less inhibition of the antiproliferative signaling of BMPRII/smad1/5/8 pathway. [54] Green boxes indicate a desirable effect; red boxes indicate an undesirable effect; Plus signs indicate a stimulatory effect; Minus signs indicate an inhibitory effect. Abbreviations: BMP, bone morphogenetic protein; BMPR-II, BMP receptor type II; cGMP, cyclic guanosine monophosphate; GDFs, growth differentiation factors; IP, prostacyclin receptor; sGC, soluble guanylate cyclase; pSmad1/5/8, phosphorylated Smad1/5/8; pSmad2/3, phosphorylated Smad2/3; TGF-β, transforming growth factor-β. Reproduced with permission of the ERS 2024: European Respiratory Journal[34]

In addition to the three classical pathways, a new fourth pathway has been proposed. In the PULSAR and STELLAR trials, the activin signalling inhibitor Sotatercept reduced PVR and increased six-minute walking distance (6MWD) when given in addition to background therapy.[54, 55]

Sotatercept is a fusion protein with a FC domain of human immunoglobin G1 linked to the extracellular domain of human activin receptor type IIa (ActRIIa) which acts as a ligand trap for activins and growth differentiation factors.[34] The proposed mechanism of action involves rebalancing of growth promoting and growth-inhibiting signalling pathways. Mutation in BMPR2 leads to downregulation of the BMPR2-Smad1/5/8 pathway which promotes production of activin ligands i.e activin A, growth differentiation factor 8 and 11, which leads to up-regulation of ActRIIA-Smad2/3 pathway and production of endogenous BMP antagonists gremlin-1 and noggin which further reduce BMP-Smad1/5/8 signalling.[54]

Altogether this leads to a shift toward proproliferative signalling of activin-Smad2/3. Sotatercept acts to degrade excess ActII ligands to reduce ActRIIA-Smad2/3 signalling, and thus, rebalance the growth promoting and growth inhibiting pathways.[54] Sotatercept received Food and Drug Administration approval in Q1, 2024 and has recently received European Medicines Agency approval in Q3, 2024. Since the 2024 7th World Symposium on PH, activin-signalling inhibitors are recommended as an alternative treatment escalation in PAH with intermediate-low risk or greater risk at follow-up.[56]

Biomarkers

The National Institutes of Health's Biomarkers Definition Working Group established the BEST (Biomarkers, EndpointS, and other Tools) resource to harmonize the definition and classification of biomarkers across different disciplines in medicine. As such, they define 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."[57, 58]

According to the BEST resource, biomarkers, based on clinical utility, are divided into several different classes, e.g. diagnostic, monitoring, prognostic, susceptibility/risk biomarkers, and clinical endpoint biomarkers. The biomarker definitions according to BEST are listed in table 2.[58] A recent review have summarized biomarker studies in the field of PAH according to BEST definitions.[59]

Biomarkers could be useful in several different ways in PAH. A diagnostic biomarker could confirm PAH at an early stage when the patient start exhibiting early symptoms of the disease. At present, there is no clinically available biomarker that is diagnostic of PAH.[1] Thus, development of a diagnostic biomarker for PAH could lead to earlier diagnosis.

There are, however susceptibility biomarkers that can be used to screen for PAH before development of symptoms., e.g. the DETECT algorithm which use a combination of FVC% predicted/DLCO% predicted, telangiectasias, anti-centromere antibodies, N-terminal pro BNP (NT-proBNP), urate and electrocardiogram to predict development of PAH in patients with systemic sclerosis.[60]

Table 2. Biomarker definitions accordning to BEST[58]

Type of biomarker	Definition
Susceptibility/Risk Biomarker	A biomarker that indicates the potential for developing a disease or medical condition in an individual who does not currently have clinically apparent disease or the medical condition.
Diagnostic Biomarker	A biomarker used to detect or confirm presence of a disease or condition of interest or to identify individuals with a subtype of the disease.
Monitoring Biomarker	A biomarker measured repeatedly for assessing status of a disease or medical condition or for evidence of exposure to (or effect of) a medical product or an environmental agent.
Response Biomarker	A biomarker used to show that a biological response, potentially beneficial or harmful, has occurred in an individual who has been exposed to a medical product or an environmental agent.
Predictive Biomarker	A biomarker used to identify individuals who are more likely than similar individuals without the biomarker to experience a favorable or unfavorable effect from exposure to a medical product or an environmental agent.
Prognostic Biomarker	A biomarker used to identify likelihood of a clinical event, disease recurrence or progression in patients who have the disease or medical condition of interest.
Safety Biomarker	A biomarker measured before or after an exposure to a medical product or an environmental agent to indicate the likelihood, presence, or extent of toxicity as an adverse effect.

Various biomarkers differ in invasiveness and in time and resource consuming measures. A hemodynamic measure by right heart catheterizations (RHC), schematic of the procedure shown in figure 3,[61] would be both time and resource consuming and would be difficult to use for screening, unless in a very high-risk population. A bloodborne biomarker would have advantages of being minimally invasive and readily available. While initially it would be resource intensive as it would need a clinical and later an analytical validation,[58] a simple blood sample would take much less resources when established, and could be of use in a wider population, such as patients suffering from dyspnoea and acting as a diagnostic biomarker. Screening is defined as a systematic approach to detect asymptomatic individuals, generally or in populations at risk of disease. The screening approach is in practice extended to patients with mild symptoms or early disease.[62]

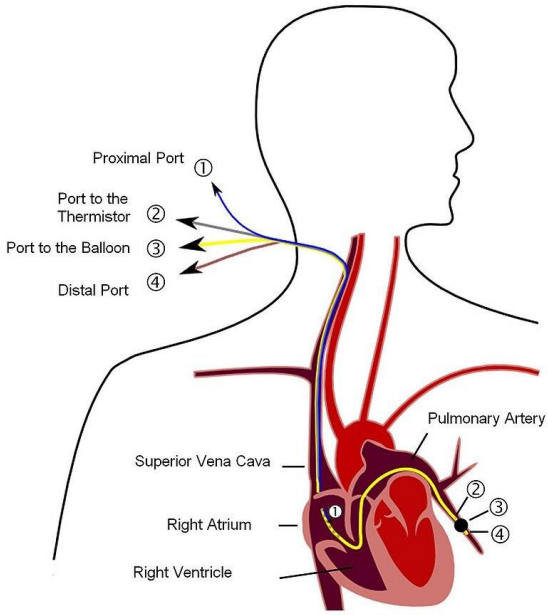

Figure 3. Right heart catheterization
Introduced via the right internal jugular vein and the balloon tip wedged into a distal pulmonary vessel.[61] Required for hemodynamic measurements and PAH diagnosis.

It is important to note in what population a test would be used. PAH is a rare disease, and it may not be meaningful to screen the entire asymptomatic population for PAH, since the low prevalence will lead to low positive predictive values and a lot of unnecessary investigations. However, in a selected population with greater risk, e.g. patients with exertional dyspnoea, the positive predictive value of a test would increase with the increased prevalence. There are high-risk populations for PAH, including those with connective tissue disease, congenital heart disease and genetic alterations. Currently screening for PAH is routinely performed in systemic sclerosis patients using the DETECT algorithm.[60] With readily available, and hopefully cost-efficient biomarker(s), it could be possible to screen patients with dyspnoea to facilitate an earlier PAH diagnosis.

Several potential plasma biomarkers for PAH have been tested throughout the years, and there is no available clinical diagnostic biomarker for PAH. The natriuretic peptides, i.e brain natriuretic peptide (BNP) and NT-proBNP are however, routinely used in clinical practice, as prognostic biomarkers in the current risk stratification strategies, with the addition of renal dysfunction which could be assessed with creatinine as in the REVEAL risk stratification strategy.[1, 63-65] Urate and anti-centromere antibody are part of the DETECT algorithm which is used in systemic

sclerosis patients to screen for PAH.[60] BNP and NT-proBNP have also been used as clinical endpoints in pharmacological studies in PAH but have not been validated as such.[66] Natriuretic peptides are non-specific of PAH and can be influenced by other factors i.e. comorbidities.[1] Development of new prognostic markers could improve risk assessment by allowing greater precision. Current biomarkers in risk stratification strategies are associated with cardiac function rather than vascular alterations, and biomarkers associated with the basis of the disease rather than the result may potentially lead to better risk assessment as well as earlier detection.

Risk assessment

Providing individualised risk assessment for each PAH patient to assess mortality risk and guide treatment decisions has become an integral part of PAH management.[1, 6, 67, 68]

During the past decades, several different risk assessment models for PAH have been introduced. In the US, a risk score calculator was developed based on the REVEAL, using 12 weighted non-modifiable and modifiable parameters.[69] Later refined into the REVEAL 2.0 risk calculator which use 13 parameters,[63] and complemented by the abridged REVEAL lite 2 risk calculator, using only 6 weighted non-invasive and modifiable variables, based on REVEAL 2.0.[64]

In Europe, the risk stratification strategy of the 2009 ESC/ERS PH guidelines, according to number of parameters in the range associated with "better prognosis" or "worse prognosis", was extended and refined into a risk assessment table grading risk parameters into "low", "intermediate" and "high" risk in the 2015 ESC/ERS guidelines.[6, 68] The ESC/ERS risk stratification strategy was subsequently validated independently by SPAHR,[70] followed by COMPERA, which used the SPAHR validation strategy,[24] and in the French PH registry.[71]

Kylhammar et al developed the original SPAHR model to calculate the overall risk score at baseline diagnosis and early follow-up based on the variables and thresholds defined by the 2015 ESC/ERS guidelines' risk assessment table,[70] and was the first to validate the 2015 ESC/ERS risk assessment table.[70] This has subsequently been modified in the updated SPAHR model, as well as validated at follow-up, up to after five years, where the "intermediate risk" group was divided into "intermediate low" and "intermediate high" risk, allowing calculation of a four strata model from the three strata model utilizing defined thresholds for "intermediate-low" and "intermediate-high" values.[72, 73]

The COMPERA strategy, utilizing the original SPAHR model of calculation, was developed in 2022 into COMPERA 2.0, a simplified four strata risk assessment model, including only three non-invasive parameters; WHO-FC, 6MWD, and BNP

or NT-proBNP,[74] resolving the intermediate risk group into intermediate-low and intermediate-high risk. The four strata COMPERA model have externally been validated by the French PH registry.[75]

In the 2022 ESC/ERS guidelines, the risk assessment table from 2015 was further refined with the addition of new parameters. A comprehensive three strata model (Table 3) is recommended at baseline and a simplified four strata (Table 4) risk assessment model based on COMPERA 2.0 is recommended at follow-up, with additional variables included as needed.[1] In the 2022 ESC/ERS risk assessment table, the 1-year mortality was updated to 5-20% for intermediate risk and >20% for high risk, whereas intermediate- and high risk in the previous risk assessment table was 5-10% and >10% respectively, while low risk remained at <5%, as per previous guidelines,[1, 6] Additionally several new parameters were introduced in the comprehensive three strata table including tricuspid annular plane systolic excursion (TAPSE)/sPAP ratio calculated by echocardiography, cardiac magnetic resonance imaging (MRI) measurements of RVEF%, SVI and right ventricular end-systolic volume index (RVESVI), and RHC assessed SVI.[1]

Table 3. ESC/ERS 2022 Comprehensive three strata risk assesment in PAH

Determinants of prognosis (estimated 1-year mortality)	Low risk (<5%)	Intermediate risk (5–20%)	High risk (>20%)
Clinical observations and modifiable variables			
Signs of right HF	Absent	Absent	Present
Progression of symptoms and clinical manifestations	No	Slow	Rapid
Syncope	No	Occasional syncope	Repeated syncope
WHO-FC	I, II	III	IV
6MWD	>440 m	165–440 m	<165 m
CPET	Peak VO2 >15 mL/min/kg (>65% pred.) VE/VCO2 slope <36	Peak VO2 11–15 mL/min/kg (35–65% pred.) VE/VCO2 slope 36–44	Peak VO2 <11 mL/min/kg (<35% pred.) VE/VCO2 slope >44
Biomarkers: BNP or NT-proBNP	BNP <50 ng/L NT-proBNP <300 ng/L	BNP 50–800 ng/L NT-proBNP 300–1100 ng/L	BNP >800 ng/L NT-proBNP >1100 ng/L
Echocardiography	RA area <18 cm^2 TAPSE/sPAP >0.32 mm/mmHg No pericardial effusion	RA area 18–26 cm^2 TAPSE/sPAP 0.19–0.32 mm/mmHg Minimal pericardial effusion	RA area >26 cm^2 TAPSE/sPAP <0.19 mm/mmHg Moderate or large pericardial effusion
cMRI	RVEF >54% SVI >40 mL/m^2 RVESVI <42 mL/m^2	RVEF 37–54% SVI 26–40 mL/m^2 RVESVI 42–54 mL/m^2	RVEF <37% SVI <26 mL/m^2 RVESVI >54 mL/m^2
Haemodynamics	RAP <8 mmHg CI ≥2.5 $L/min/m^2$ SVI >38 mL/m^2 SvO_2 >65%	RAP 8–14 mmHg CI 2.0–2.4 $L/min/m^2$ SVI 31–38 mL/m^2 SvO_2 60–65%	RAP >14 mmHg CI <2.0 $L/min/m^2$ SVI <31 mL/m^2 SvO_2 <60%

Reproduced from the 2022 European Society of Cardiology(ESC)/European Respiratory Society(ERS) Guidelines for the diagnosis and treatment of pulmonary hypertension with permission of the ESC.[1] Abbreviations: 6MWD, six-minute walking distance; BNP, brain natriuretic peptide; CI, cardiac index; cMRI, cardiac magnetic resonance imaging; CPET, cardiopulmonary exercise testing; HF, heart failure; NT-proBNP, N-terminal pro-brain natriuretic peptide; PAH, pulmonary arterial hypertension; pred., predicted; RA, right atrium; RAP, right atrial pressure; sPAP, systolic pulmonary arterial pressure; SvO2, mixed venous oxygen saturation; RVESVI, right ventricular end-systolic volume index; RVEF, right ventricular ejection fraction; SVI, stroke volume index; TAPSE, tricuspid annular plane systolic excursion; VE/VCO2, ventilatory equivalents for carbon dioxide; VO2, oxygen uptake; WHO-FC, World Health Organization functional class.

Table 4. Simplified four strata risk assesment

Determinants of prognosis	Low risk	Intermediate–low risk	Intermediate–high risk	High risk
Points assigned	1	2	3	4
WHO-FC	I or II[a]	-	III	IV
6MWD, m	>440	320–440	165–319	<165
BNP, ng/L or	<50	50–199	200–800	>800
NT-proBNP, ng/L	<300	300–649	650–1100	>1100

Reproduced from the 2022 European Society of Cardiology(ESC)/European Respiratory Society(ERS) Guidelines for the diagnosis and treatment of pulmonary hypertension with permission of the ESC.[1] Risk is calculated by dividing the sum of all grades by the number of variables and rounding to the next integer. Abbreviations: 6MWD, six-minute walking distance; BNP, brain natriuretic peptide; NT-proBNP, N-terminal pro-brain natriuretic peptide; WHO-FC, World Health Organization functional class; superscript [a], WHO-FCII and II are assigned 1 point as both are associated with long-term survival.

In addition, web calculators have been introduced to facilitate the clinical implementation of the risk stratification models using three and four strata i.e. https://www.svefph.se/risk-stratification.[73]

The addition of novel biomarkers to current risk assessment models could be a means to improve their prognostic accuracy. For example, a study by Rhodes et al utilized a panel of nine circulating proteins with prognostic value independent of NT-proBNP in conjunction with the original REVEAL risk score for prediction of survival in a mixture of prevalent and incident cases, which increased the area under the curve (AUC) from 0.83 to 0.91.[76] Additionally, a panel consisting of six proteins with prognostic value have been evaluated compared to NT-proBNP, and when used in addition to NT-proBNP improved the AUC for prediction of 5-year outcomes from 0.76 to 0.82.[77]

Aims

The overall aim of the present thesis was to investigate various ECM and inflammatory proteins to establish novel plasma biomarker candidates for diagnosis, differentiation, and risk assessment of PAH.

The specific aims of each paper:

Paper I aimed to investigate the plasma levels of MMPs as biomarkers for diagnosis and differentiation of PAH.

Paper II aimed to investigate the potential of plasma levels of proteoglycans and a selection of inflammatory proteins as biomarkers for diagnosis and differentiation of PAH.

Paper III aimed to investigate plasma ECM related proteins as prognostic markers in PAH in relation to survival and the 2015 ESC/ERS risk stratification score.

Paper IV aimed to investigate plasma proteins associated with inflammation, including TNF as biomarkers for: i) diagnosis of PAH in dyspnoea populations, and as ii) prognostic markers for PAH in relation to the 2015 ESC/ERS risk assessment table.

Materials and methods

Lund Cardio Pulmonary Registry and plasma sampling

The Lund Cardio Pulmonary Registry (LCPR) is a blood sample cohort in the biobank of Region Skåne, initiated by Göran Rådegran in September 2011.

LCPR includes adult individuals ≥18 years of age who have been referred to the Haemodynamic Lab at Skåne University Hospital (SUS) in Lund for investigation of unclear dyspnoea. LCPR include those with suspected PH, advanced HF, and patients before- and after heart transplantation, requiring a RHC. LCPR comprises also cardiopulmonary healthy individuals who serve as control subjects, but in whom a RHC is not conducted.

Blood was drawn from the venous introducer predominantly inserted in the right internal jugular vein. The samples were centrifuged at 2000 g at room temperature, and the plasma were thereafter stored in -80° Celsius, in Region Skåne Biobank, according to regional biobank practice. Healthy controls made a routine visit with physical examination and non-fasting venous blood samples were collected from peripheral veins and processed as aforementioned.

The study populations of the included papers have been derived from LCPR. In papers I, II, and IV**,** the study population consisted of adult patients (≥18 years) investigated at the Haemodynamic Lab at SUS in Lund for unclear dyspnoea between September 2011 and March 2017 with RHC at baseline diagnosis. The study population consisted of PAH (n = 48), CTEPH (n = 20), PH associated with HF with preserved ejection fraction (EF), (HFpEF-PH, n = 33), or reduced EF (HFrEF-PH, n = 36), and HF without PH (HF-non-PH, n = 15, consisting of HFpEF, n = 7, HFrEF, n = 8). In addition, 20 cardiopulmonary heathy individuals without RHC were included. Patients without RHC data at baseline, or having an unclear diagnosis were excluded. Patients with chronic obstructive pulmonary diseases or group III PH were excluded in all the papers. Likewise, PH with multifactorial and/ or unclear causes were excluded. This was due to the very small number of such patient included in the LCPR. The possibility of including these groups or patients with functional dyspnoea would have increased the generalisability of the results.

The study population in paper III consisted of 48 adult PAH patients (≥18 years) with RHC at baseline diagnosis, enrolled between September 2011 and September

2016. Out of 48 included patients, 33 had plasma samples from an early follow-up (median: 116; min-max range: 18-289 days) with RHC.

Population characteristics

In papers I-IV the median age of the patients with PAH was 71.5 (interquartile range 64-76) years. Of the PAH patients, 83.3% were female, and 66.6% were in WHO-FC III or IV at diagnosis. Baseline characteristics of papers I, II, IV are displayed in table 5. There were five cases of immune suppressants, mycophenolate mofetil, or high dosage corticosteroids in PAH patients, one with PAH associated with connective tissue disease other than systemic sclerosis, and the rest with systemic sclerosis associated PAH. Characteristics of the PAH patients in paper III are displayed in table 6.

Protein analyses

Plasma samples from LCPR were analysed with Olink proteomics Proseek Multiplex technique using the commercially available Cardiovascular II, Cardiovascular III, and Oncology II, 96 plex proximity extension assay (PEA) panels. In brief, PEA utilize antibodies with tails of complementary oligonucleotide strands which bind a target protein. When tails of corresponding antibodies come into proximity they bind, hybridize, and create a DNA tag which is elongated with a DNA polymerase and relative protein concentrations can be read out by quantitative PCR (Figure 4).[78] Data is normalised by four internal controls added to each sample, to reduce intra-assay variability. Three external negative control samples and three inter-plate control samples per assay are also included to reduce inter-assay variability.[79] Protein levels were reported in normalized protein expression (NPX) values which is an arbitrary unit (AU) on a log2 scale. The use of PEA allowed for a large number of proteins to be analysed simultaneously.

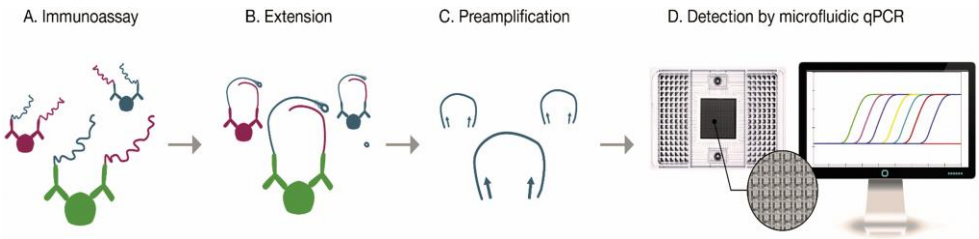

Figure 4. Proximity extension assay
Image courtesy of Olink Protemics AB. A) Antibodies with oligonucliotide tails bind a target protein. B) Correspondning oligonucleotide tails form antibody pairs come in proximity, hybridise and the DNA tag is extended by DNA polymerase co create a PCR template. C) All DNA tags are preamplificated by universal primers. D)The individual DNA tags is then detected and quantified by microfluidic qPCR.[78]

Protein selection

For the papers included in this thesis, subsets of proteins from the aforementioned Olink panels, potentially related to the PAH mechanisms of vascular remodelling and inflammation, were selected, for each respective hypothesis. The proteins included in the analysed panels were grouped according to mutual relevance and separate hypotheses. Paper I included 10 MMPs and associated proteins related to the ECM. Paper II included 12 proteoglycans and a range of 65 inflammatory and adhesion proteins. For paper III, a combined group of 14 extracellular proteins including MMPs and proteoglycans were selected. For paper IV, a group of 25 tumour necrosis factor (TNF) related, and 28 other inflammatory proteins with known altered levels (paper III) were selected. Resulting in a total of 113 proteins investigated, including NT-proBNP.

Table 5. Baseline characteristics papers I-II, IV

	Control	PAH	CTEPH	HFpEF-PH	HFrEF-PH	HF-non-PH
Sample size, n (% females)	20 (50)	48 (83.3)	20 (65)	33 (63.6)	36 (19.4)	15 (53.3)
Age, years	41 (26.8-50.5)	71.5 (64-76)	75 (70.8-77.8)	75 (68.5-83)	54 (47.3-59.5)	60 (46-76)
BSA, m^2	1.9 (1.8-2)	1.7 (1.6-2)	1.8 (1.8-2)	1.9 (1.7-2.1)	2 (1.9-2.1)	2 (1.7-2.1)
Creatinine, µmol/L	-	90 (71-114)	88 (73-123)	99 (79-117)	121 (90-145)	93 (81-123)
NT-proBNP, AU	-	3.1 (2.1-3.8)	2.6 (1.0-4.2)	2.9 (2.4-3.3)	4.9 (4.1-5.4)	3.2 (1.3-4.4)
6MWD, m	-	242 (173-349)	300 (238-325) (n-3)	-	-	-
WHO-FC, n, I/II/III/IV/NA	-	1/9/28/4/8	0/6/13/0/1	-	-	-
PAH risk scores, n, low/intermediate/high	-	8/26/14	-	-	-	-
Haemodynamics						
CI, L/min/m^2	-	2.2 (1.8-2.8)	2.3 (1.9-2.5)	2.4 (2.1-2.8)	1.6 (1.4-1.9)	1.9 (1.6-2.2)
CO, L/min	-	3.8 (3.0-5.1)	4 (3.5-4.7)	4.5 (3.7-5.7)	3.2 (2.8-4.0)	3.3 (3.0-4.4)
HR, beats/min	-	77.5 (70-94.3)	75 (69.5-88)	70 (61.5-82.5)	71 (68.3-86)	72 (60-84)
LWSWI, mmHg × mL/m^2	-	2488 (2045-3213)	2508 (2330-3187)	2664 (2189-3308)	1152 (957-1636) (n-1)a	2168 (1650-2716)
MAP, mmHg	-	96 (89.4-104)	98.5 (94-110.3)	98 (91.5-104.5)	79.5 (75.3-88.8)	89 (80-96)
MPAP, mmHg	-	43 (37-54.8)	42 (35-54.3)	34 (28.5-46)	34.5 (29-40.8)	20 (17-22)
MRAP, mmHg	-	7 (4-11)	5.5 (3.3-8)	10 (6.5-14)	14.5 (9-17)	6 (2-16)
PAWP, mmHg	-	8 (6-11)	9.5 (7-13)	18 (16-22.5)	25 (19-28) (n-1)a	15 (9-18)
PVR, WU	-	9.5 (6.2-11.8)	9.3 (5.9-10.8)	3.6 (2.4-4.9)	3 (2.3-3.7) (n-1)a	1.5 (1.0-2.0)
RVSWI, mmHg × mL/m^2	-	991 (807-1246)	1111 (845-1298)	832 (671-1140)	440 (306-650)	382 (196-495)
SV, mL/beat	-	51.2 (40.8-56.3)	56.3 (45.8-65.1)	61.7 (48.8-83.7)	45.1 (36.0-54.5)	54.8 (44.8-58.8)
SVI, mL/beat/m^2	-	28.7 (35-22.6)	30.5 (32.5-26.3)	33.8 (42.3-28.1)	22.5 (27.2-18.2)	29 (31.9-25.2)
SvO_2, %	-	59.3 (51.1-66.2)	62.5 (54.9-67.9)	64.1 (57.8-66.8)	50.3 (46.5-55.2)	61.2 (58.5-69.2)

Continues

Table 5. Continued	Control	PAH	CTEPH	HFpEF-PH	HFrEF-PH	HF-non-PH
PAH subgroup, n (%)						
IPAH	-	21 (43.8)	-	-	-	-
FPAH	-	2 (4.2)	-	-	-	-
SSc-PAH	-	21 (43.8)	-	-	-	-
CTD-PAH	-	4 (8.3)	-	-	-	-
Comorbidities, n (%)						
Atrial fibrillation	-	4 (8.3)	3 (15.0)	25 (78.1) (n-1)	14 (38.9)	8 (61.5) (n-2)
Diabetes mellitus	-	12 (25.0)	0 (0)	11 (36.7) (n-3)	4 (11.1)	3 (21.4) (n-1)
Ischemic heart disease	-	7 (14.6)	2 (5.0)	6 (23.1) (n-7)	6 (16.7)	6 (42.9) (n-1)
Hypertension	-	17 (35.4)	11 (55.0)	22 (75.9) (n-4)	7 (19.4)	7 (53.8) (n-2)
Stroke	-	2 (4.2)	3 (5.0)	6 (22.2) (n-6)	4 (11.1)	2 (15.4) (n-2)
Thyroid disease	-	11 (22.9)	1 (5.0)	2 (7.7) (n-7)	3 (8.3)	3 (21.4) (n-1)
Medications, n (%)						
ACEi	-	10 (21.3) (n-1)	2 (11.1) (n-2)	12 (36.4)	19 (52.8)	3 (20)
ARB	-	4 (8.5) (n-1)	7 (38.9) (n-2)	10 (30.3)	14 (38.9)	5 (33.3)
β-blockers	-	16 (34) (n-1)	9 (50) (n-2)	25 (75.8)	35 (97.2)	11 (73.3)
Immunosuppressants	-	5 (10.6) (n-1)	1 (5.6) (n-2)	0 (0)	1 (2.8)	3 (20)
MRA	-	11 (23.4) (n-1)	3 (16.7) (n-2)	9 (27.3)	21 (58.3)	7 (46.7)

Adapted from paper IV. Categorical variables are presented as numbers and percentage, n (%). Continuous variables are presented as median (25 – 75 percentile). Superscript (n-x) indicates number of missing values. The immunesuppresants consist of mycofenolat mofetil and high dose corticosteroids and were used in reumathological patients. Abbreviations: -, data not available; 6MWD, six-minute walking distance; ACEi, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; AU, arbitrary units; BSA, body surface area; CI, cardiac index; CO, cardiac output; CTD-PAH, PAH associated with connective tissue disease other than systemic sclerosis; CTEPH, chronic thromboembolic pulmonary hypertension; FPAH, familial PAH; HR, heart rate; HF-non-PH, heart failure (HF) without PH; HFpEF-PH, PH associated with HF with preserved ejection fraction (EF); HFrEF-PH, PH associated with HF with reduced EF; IPAH, idiopathic PAH; LVSWI, left ventricular stroke work index; MAP, mean arterial pressure; MPAP, mean pulmonary arterial pressure; MRA, mineral corticoid receptor antagonist; MRAP, mean right atrial pressure; NT-proBNP, N-terminal pro b- type natriuretic peptide; PAH, pulmonary Arterial Hypertension; PAWP, pulmonary arterial wedge pressure; PVR, pulmonary vascular resistance; RVSWI, right ventricular stroke work index; SSc-PAH, systemic sclerosis associated PAH; SV, stroke volume; SVI, stroke volume index; SvO2, mixed venous oxygen saturation; WHO-FC, the World Health Organization functional class; a, one patient unable to go through complete testing.

Table 6. Characteristics of the patients with PAH in paper III

	All PAH patients [a]	IPAH/FPAH [a]	CTD-PAH [a]	PAH before treatment	PAH after treatment
Sample size, n (% females)	48 (88.3)	23 (73.9)	25 (92)	33 (87.9)	33 (87.9)
Age, years	71.5 (64-76)	73 (57-77)	71 (64.5-76)	71 (60.5-76.5)	NA
BSA, m^2	1.75 (1.59-1.97)	1.77 (1.59-1.98)	1.70 (1.60-1.80)	1.73 (1.58-1.79)	1.73 (1.58-1.79)
Haemodynamics					
MPAP, mmHg	43 (37-54.8)	51 (42-56)	39 (30-43.5)	43 (37-55)	36 (32-48)
PAWP, mmHg	8 (6-11)	9 (6-12)	8 (5-10)	6 (5-9.5)	8 (5-11)
PVR, WU	9.5 (6.23-11.83)	11.47 (8.86-14.52)	6.88 (4.73-9.92)	9.56 (6.95-12.06)	5.79 (4.3-8.69)
CI, l/min/m^2	2.19 (1.75-2.82)	1.9 (1.69-2.24)	2.62 (1.92-3.06)	2.25 (1.8-2.85)	2.7 (2.14-3.45)
MRAP, mmHg	7 (4-11)	9 (6-11)	6 (2.5-9)	6 (3-9.5)	6 (3-9.5)
SvO_2, %	59.3 (51.1-66.2)	55.2 (49-61)	64.9 (54.5-71.3)	62.3 (54.5-66.2)	63.4 (58.4-72.2)
Clinical parameters					
6MWD, m	242 (173-349) [(n-2)]	225 (150-280) [(n-2)]	267 (180-352)	242 (184-346) [(n-1)]	270 (222-338) [(n-3)]
NT-proBNP	2149 (865-3631) [(n-2)]	2213 (1678-4747) [(n-1)]	1169 (411-3370) [(n-1)]	2104 (767-3139) [(n-1)]	695 (243-1797) [d]
WHO-FC, I/II/III/IV/NA, n	1/9/28/2/8	1/3/16/0/3	0/6/12/2/5	1/6/22/2/2	2/10/15/0/6
Comorbidities, n (%)					
Thyroid disease	11 (22.9)	5 (21.7)	6 (24)	10 (30.3)	NA
Ischemic heart disease	7 (14.6)	4 (17.4)	3 (12)	5 (15.2)	NA
Stroke	2 (4.2)	2 (8.7)	0 (0)	2 (6.1)	NA
Atrial fibrillation	4 (8.3)	2 (8.7)	2 (8)	3 (6.1)	NA
Diabetes mellitus	12 (25)	10 (43.5)	2 (8)	8 (24.2)	NA
Systemic hypertension	17 (35.4)	12 (52.2)	5 (20)	9 (24.2)	NA

Adapted from paper III. Categorical variables are presented as numbers and percentage, n (%). Continuous variables are presented as median (interquartile range: 25 percentile-75 percentile). Superscript (n-x) indicates number of missing values. Abbreviations: BSA, body surface area; CI, cardiac index; CTD-PAH, connective tissue disease associated PAH; NA, not available; MPAP, mean pulmonary arterial pressure; MRAP, mean right atrial pressure; PAH, pulmonary arterial hypertension; PAWP, pulmonary arterial wedge pressure; PVR, pulmonary vascular resistance; SvO_2, mixed venous oxygen saturation; WHO-FC, World Health Organization functional class; WU, wood units; 6MWD, six-minute walking distance; superscript [a], at baseline before PAH specific treatment.

Clinical workup and diagnosis

Patients were evaluated and diagnosed by experienced cardiologists at the regional PH centre at SUS, Lund. The clinical workup included, in addition to RHC, echocardiography, MRI, to identify left ventricular dysfunction, classify HF and exclude intracardiac shunts; pulmonary scintigraphy, high resolution computer tomography (HRCT), and spirometry with diffusion capacity was used to exclude PH associated with hypoxia and/or lung disease. The clinical workup to differentiate the various PH groups followed the guidelines at the time of diagnosis.[6] Initial RHC was considered baseline for PAH and all PAH patients were treatment-naïve for PAH specific treatment at enrolment.

In papers I-IV, PAH was defined according to the prevailing guidelines at the time of analysis, i.e. by MPAP ≥25 mmHg at rest, PAWP ≤15 mmHg, and PVR >3WU. Pre-capillary PH was defined by MPAP ≥25 at rest and a PAWP ≤15 mmHg. Post-capillary PH was defined by MPAP ≥25 at rest and a PAWP >15 mmHg, in accordance with guidelines at the time of diagnosis.[6, 68] HF was divided into preserved EF ≥50% or reduced EF <50% in accordance with guidelines at the time of sample retrieval.[80]

Haemodynamics

Haemodynamic parameters were registered by RHC using Swan-Ganz catheters. Registered parameters include MPAP, mean right atrial pressure (MRAP) and PAWP. During RHC, CO was assessed with thermodilution, heart rate (HR) was measured by electrocardiogram, mean arterial pressure (MAP) calculated from non-invasive blood-pressure measurements, and mixed venous saturation (SvO_2) was measured using ABL 90 Flex by Radiometer. 6MWD and WHO-FC were collected from medical records.

Body surface area (BSA), cardiac index, stroke volume (SV), left ventricular stroke work index (LVSWI), right ventricular stroke work index (RVSWI), PVR, and transpulmonary pressure gradient were calculated by the following formulae: BSA = $weight^{0.425}$ x $height^{0.725}$ x 0.007184,[81] Cardiac index = CO/BSA, SV = CO/HR, SVI = SV/BSA, LVSWI = (MAP-PAWP) x SVI, RVSWI = (MPAP-MRAP) x SVI, PVR = transpulmonary pressure gradient /CO, transpulmonary pressure gradient = MPAP-PAWP.

Risk scores calculation

Risk scores were calculated according to the 2015 ESC/ERS risk table, using the SPAHR model developed by Kylhammar et al.[6, 70] Risk scores of patients with PAH were calculated at diagnosis. Each available risk parameter among a maximum of 8 validated variables i.e. cardiac index, MRAP, NT-proBNP, 6MWD, SvO_2, WHO-FC, pericardial effusion, and right atrial area, was graded into "low risk", "intermediate risk", and "high risk" corresponding to "1", "2", and "3" points, respectively. Data on right atrial area and pericardial effusion was not available. Resulting values were added into a sum and divided by the number of available parameters. The resulting number were rounded off to the nearest integer creating an overall risk score.[70] Exact risk scores, before rounded off, were utilized in correlations and Cox regression analyses.[70, 82, 83]

General statistical considerations

Normality was assessed visually with histograms. Non-parametric testing has generally been used as appropriate, and P values <0.05 have been considered statistically significant unless false discovery rate (FDR) analysis was applied. FDR analysis was used to limit the number of false positives due to large number of statistical tests conducted. Benjamini and Hochberg FDR with a Q value of 5% was chosen to reduce the number of false positive results (type I errors), while simultaneously not being too strict considering the screening character of the studies. Q value is the desired probability of a positive result being a false positive. When FDR was used, the raw P values were displayed throughout the papers.

Statistical analysis and graphing were performed using GraphPad Prism for windows, GraphPad Software; https://www.graphpad.com/, and R, a language and environment for statistical computing, R foundation for statistical computing, https://www.R-project.org/.

Paper I-II

In paper I and II, Kruskal-Wallis test was used to analyse the differences between the patient groups and controls. A Benjamini and Hochberg FDR analysis (Q = 5%) was used on the overall Kruskal-Wallis P-values to reduce the number of false positives due to multiple testing.[84] Kruskal-Wallis test with P values below the threshold set by FDR were further analysed with Dunn's multiple comparison tests, between all included disease group and controls, and again followed by an FDR analysis.

In paper I, the threshold for the ANOVAs and multiple comparisons was set to P <0.021. In paper II, the threshold was set to P <0.033 for the ANOVAs and P <0.016 for the following multiple comparisons. For subgroup analyses, the Mann-Whitney U test was used.

Receiver operating characteristic (ROC) was used to evaluate the selected proteins' diagnostic potential in identifying PAH from a pooled dyspnoea group of patients with CTEPH, HFpEF-PH, HFrEF-PH and HF-non-PH. Youden's index (formula: sensitivity + specificity – 1) was used to determine the optimal biomarker-level-specific cut-off values to determine sensitivity and specificity.

Correlations between proteins' levels and haemodynamic parameters were performed with Spearman's corelation coefficient.

Paper III

In Paper III, the Wilcoxon matched-pairs signed ranks test was used to compare the proteins' levels at baseline and at an early follow-up (median 116 (min-max range: 18-289)) days. Mann-Whitney's test was used for sub-analysis of different PAH aetiologies. ROC analysis determined the proteins' discriminatory ability for the composite outcome of death or lung transplantation, and Youden's index was used to define the optimal cut-off points. Proteins with an AUC $\neq 0.5$ were selected for further analysis. Kaplan-Meier plots with log-rank tests were used to assess the time to death or lung transplantation with proteins' levels dichotomised according to the identified cut-off points. Survival data was censored on April 9th, 2021, with a median follow-up time of 3.33 (interquartile range 1.54-4.64) years. Logarithmic NPX values were converted to a linear scale.

Univariable Cox proportional hazards models were used to assess the prognostic value of the six proteins plotted in the Kaplan Meier curves, age as a continuous variable, and sex as a dichotomous variable. Significant proteins were further analysed in separate multivariable Cox proportional hazards models adjusted for age and sex.

Spearman correlations were used to investigate associations between the proteins' levels and the SPAHR model risk scores calculated from the 2015 ESC/ERS risk assessment table. Spearman correlations were in addition, used between proteins' levels and the available parameters included in the risk assessment model.

Paper IV

In paper IV, the analyses were divided into a diagnostic arm and a prognostic arm (Figure 5).

Diagnostic arm

The diagnostic arm, found in figure 5, included 25 TNF-related proteins. Similar to paper I-II, initial Kruskal-Wallis tests were conducted, followed by an initial FDR (Q = 5%) where significant proteins were further analysed with uncorrected Dunn's multiple comparison test, followed by a second FDR analysis (Q = 5%). Proteins with a difference in levels between PAH and controls, CTEPH, HFpEF-PH, HFrEF-PH, as well as HF-non-PH were selected for further ROC analysis.

Prognostic arm

For the prognostic arm, found in figure 5, TNF-related proteins without difference between PAH and controls in the diagnostic arm were excluded. Thus, 16 TNF-related proteins progressed to the prognostic arm. In addition, 28 inflammatory proteins with altered levels from controls in paper II were included. Logarithmic NPX values were converted to a linear scale. ROC analysis and Youden's index were used to create cut-offs for proteins' levels to stratify patients by survival $\leq$3 years or >3 years. Mann-Whitney U- test was used to determine the difference in proteins' levels between the strata. Seven proteins displayed a difference between the survival groups and underwent further analyses. Kaplan-Meier with Log-rank tests, univariable Cox regression, including age, sex, risk score, and significant protein candidates in the univariable model were analysed with multivariable Cox regression models adjusted for age, female sex (male as reference), and SPAHR risk assessment scores.

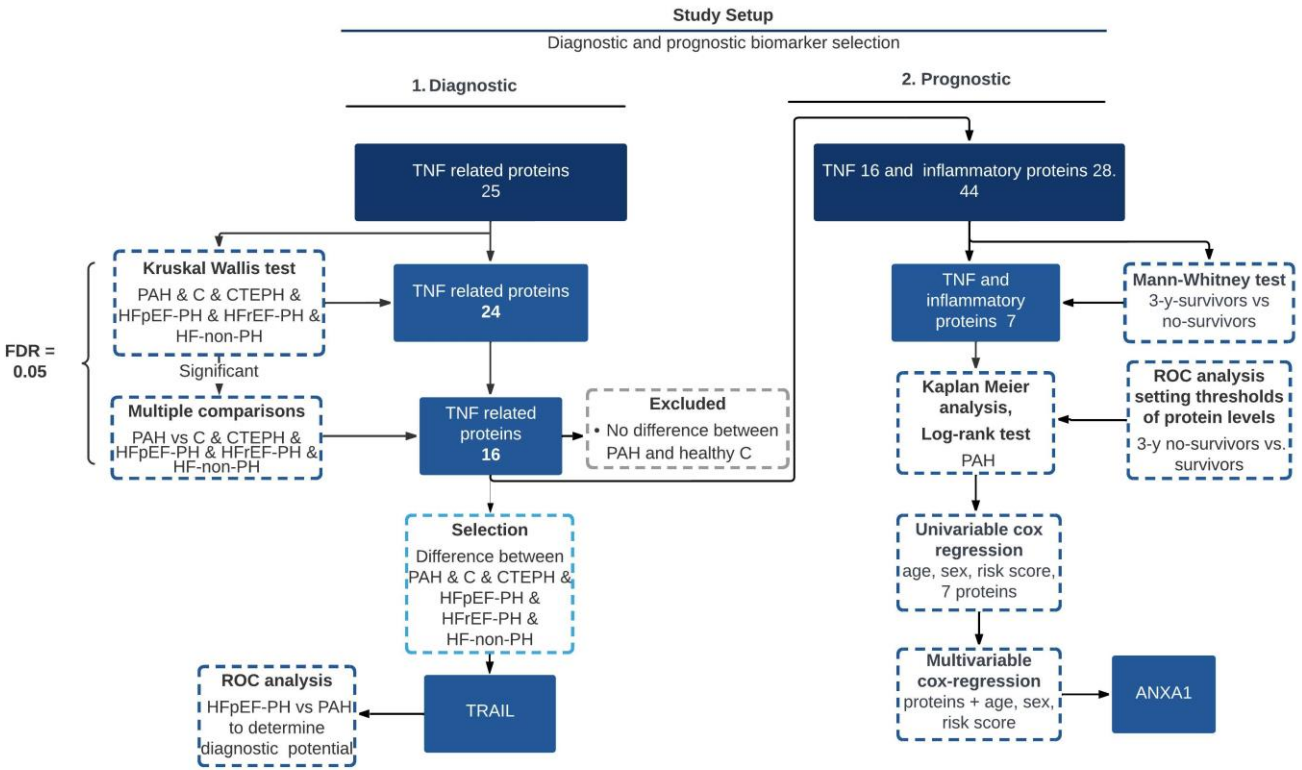

Figure 5. Paper IV study setup

1) Diagnostic arm, investigating 25 TNF- related proteins as plasma biomarkers for PAH resulting in TRAIL selected for further analysis for diagnostic potential. 2) TNF-related proteins (n = 16) and inflammatory proteins (n = 28) from an earlier study, with a significant difference in levels in PAH compared to controls were analysed in the prognostic arm of the study. Resulting in prognostic marker candidate ANXA1

Ethics

Paper I-IV were approved by the regional ethics committee in Lund (diary numbers 2010/114, 2010/248, 2010/442, 2011/368, 2015/270) and were conducted in accordance with the declarations of Helsinki and Istanbul. All participants provided informed written consent before inclusion. Blood samples and haemodynamic data were collected from patients while undergoing RHC as part of clinical investigation for dyspnoea, and follow-up visits. No participant was subjected to RHC for research purposes alone, and thus not exposed for greater medical risks than non-participating patients. Peripheral blood samples were collected from volunteers used as control subjects, and sampling of peripheral blood is considered a minimal risk. A nurse employed at the heart clinic administered study enrolment to minimize potential therapeutic misconceptions regarding participation in the research. Information given to potential study participants is standardised to ensure equal distribution of information. Other data is collected from medical records. Inclusion of blood samples with corresponding medical data in a registry for research could constitute a risk for breach of personal integrity. To minimise this risk, personal identifiers as social security numbers were stored securely separate from the data files used for analyses, and participants were anonymized and only referred to by assigned LCPR numbers.

Results

Paper I

Matrix metalloproteinase 7

Plasma levels of MMP-7 in PAH were higher than controls (P <0.0001), and lower than the other included disease groups i.e. CTEPH, HFpEF-PH, HFrEF-PH, and HF-non-PH (P <0.0081). A ROC analysis of MMP-7 in PAH versus the other disease groups resulted in an AUC of 0.75 (95% CI = 0.67-0.83) (Figure 6). No significant differences in the plasma levels of MMP-7 were observed in a post-hoc subgroup analysis of PAH subgroups or between genders. Table 7 present the main biomarkers and their plasma levels from the included papers.

Table 7. Highlighted diagnostic biomarkers in the papers

Proteins (AU)	Control	PAH	CTEPH	HFpEF-PH	HFrEF-PH	HF-non-PH
Paper I						
MMP-7	8.09 (7.79-8.54)*	9.23 (8.90-9.50)	9.66 (9.37-10.0)*	9.69 (9.33-10.1)*	9.64 (9.18-9.93)*	9.52 (9.31-10.1)*
Paper II						
Prolargin	6.00 (5.88-6.16)*	6.38 (6.16-6.57)	6.71 (6.59-6.83)*	6.76 (6.57-6.94)*	6.84 (6.66-7.10)*	6.76 (6.47-7.00)*
Paper IV						
TRAIL	146 (119-164)*	113 (97-135)	148 (118-177)*	139 (119-159)*	135 (117-171)*	151 (119-177)*

*Significantly different from PAH; AU, arbitrary units; CTEPH, chronic thromboembolic PH; HF, heart failure; HFpEF-PH, PH associated with HF with preserved ejection fraction (EF); HFrEF-PH, PH associated with HF with reduced EF; MMP, matrix metalloproteinase; PAH, pulmonary arterial hypertension; PH, pulmonary hypertension; TRAIL, TNF-related apoptosis-inducing ligand.

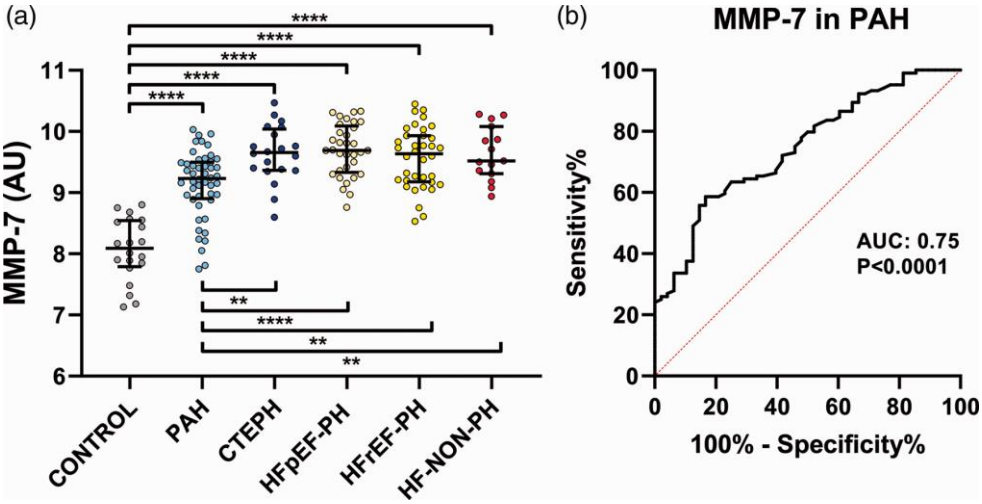

Figure 6. Plasma MMP-7 in PAH diagnosis
a) MMP-7 levels in PAH is higher than controls (P <0.0001) and lower than the other included disease groups (P <0.0081) b) ROC curve of MMP-7 as a discriminator of PAH from the other disease groups. Abbreviations: **, P <0.01; ****, P <0.0001; AU, arbitrary units; AUC, Area under the ROC curve; CTEPH, chronic thromboembolic PH; HF, heart failure; HFpEF-PH, PH associated with HF with preserved ejection fraction (EF); HFrEF-PH, PH associated with HF with reduced EF; MMP, matrix metalloproteinase; PAH, pulmonary arterial hypertension; PH, pulmonary hypertension.

Paper II

Prolargin

The main results were that plasma prolargin could differentiate PAH from the other disease groups and controls (Figure 7). Prolargin levels were lower (P = 0.003) in controls compared to patients with PAH, and prolargin levels were higher (P <0.0001) in the disease groups compared to PAH. A ROC analysis of prolargin as a differentiator of PAH among the included dyspnoea groups yielded an AUC of 0.84 (95% CI, 0.77-0.91, P <0.001) with a sensitivity of 74% and a specificity 83.3%. Plasma prolargin levels correlated with several haemodynamic parameters associated with worse survival in PAH (Figure 8). Subgroup analysis did not identify any association (P = 0.13) between prolargin levels, and idiopathic, familial or connective tissue disease associated PAH.

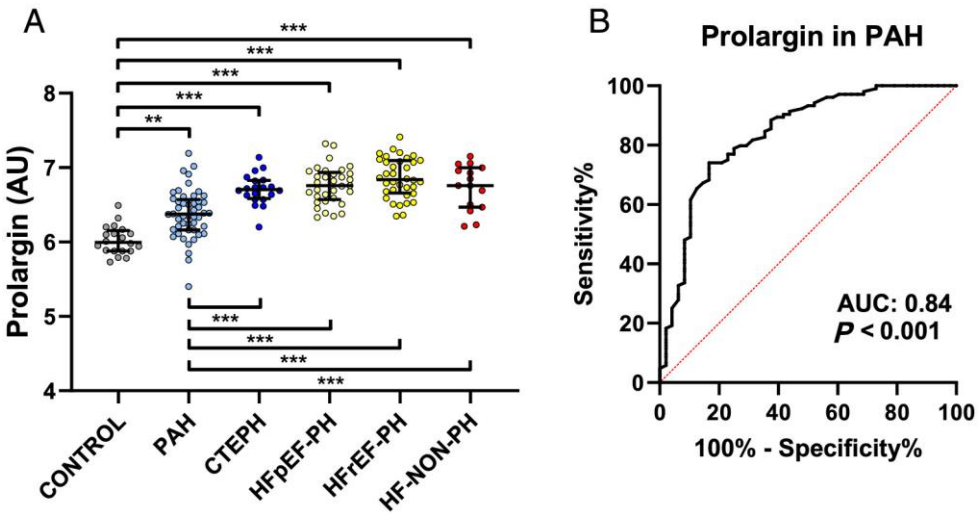

Figure 7. Plasma Prolargin in PAH diagnosis

a) Prolargin levels in PAH are higher than controls (P <0.003), and lower than all the other included disease groups (P <0.001). b) ROC curve of prolargin as a disciminator of PAH from the other disease groups. Abbreviations: **, P <0.01; ***, P <0.001; AU, arbitrary units; AUC, Area under the ROC curve; CTEPH, chronic thromboembolic PH; HF, heart failure; HFpEF-PH, PH associated with HF with preserved ejection fraction (EF); HFrEF-PH, PH associated with HF with reduced EF; PAH, pulmonary arterial hypertension; PH, pulmonary hypertension.

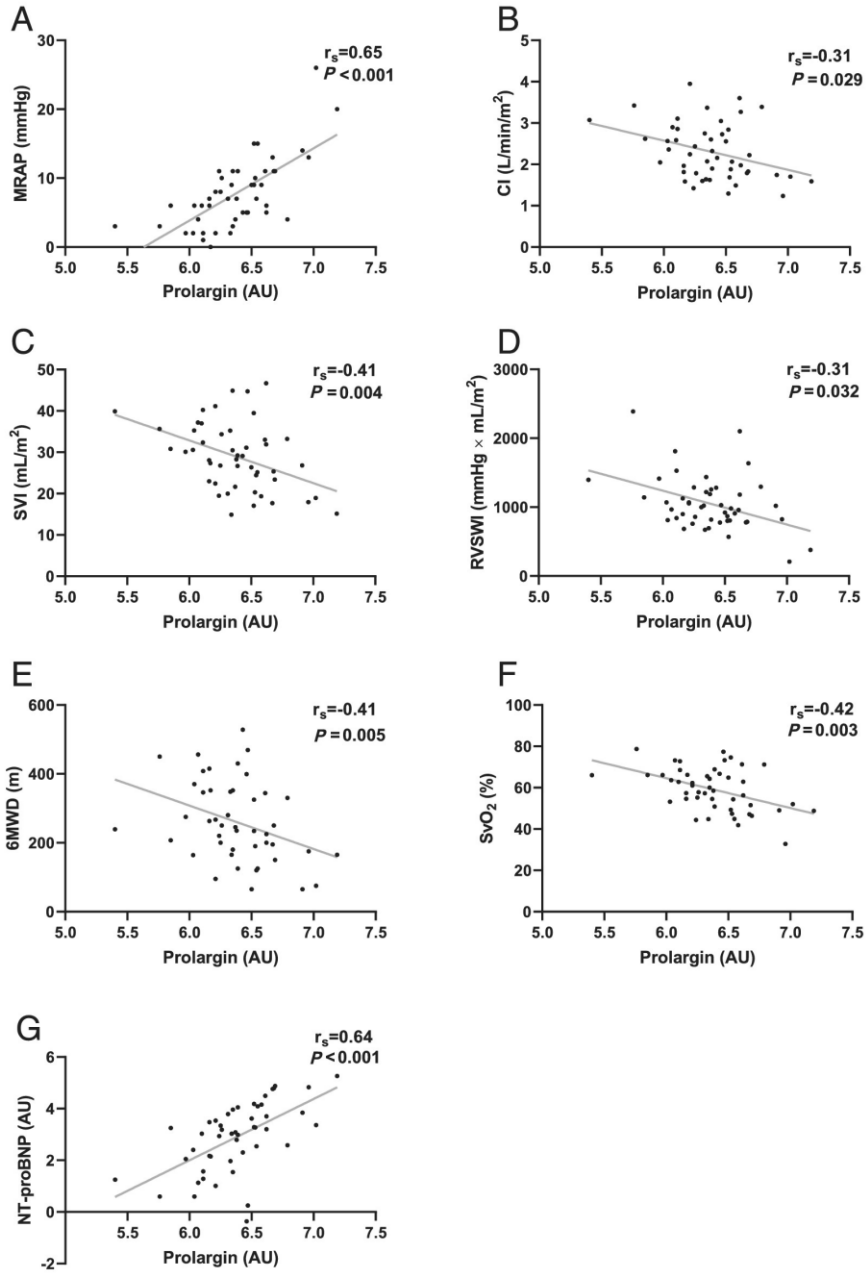

Figure 8. Significant correlations of prolargin and haemodynamic parameters
A, G) Prolargin correlated positively with MRAP and NT-proBNP. B-F) Prolargin correlated negatively with cardiac index (in this figure, CI), SVI, RVSWI, 6MWD and SvO2. Abbreviations: 6MWD, six-minute walking distance; AU, arbitrary units; MRAP, mean right atrial pressure; NT-proBNP, N-terminal pro brain natriuretic peptide; RVSWI; right ventricular stroke work index; SVI; stroke volume index; SVO_2, mixed venous saturation.

Paper III

Changes from baseline to early follow-up in PAH

Glypican-1 was the only protein among the 15 investigated proteins that displayed a difference, with higher levels at follow-up (P =0.048) and higher levels in connective tissue disease associated PAH (CTD-PAH) versus idiopathic/familial PAH at baseline (P = 0.029).

Survival and prognostic models in PAH

In ROC analysis, MMP-2, -7, -9, -12, and perlecan displayed AUCs in which the CI ≠0.5 and was further assessed with Kaplan-Meier analysis. During the observation period, 30 (62.5%) patients with PAH died, and 3 (6.3%) patients underwent lung transplantation.

Protein levels above the attained thresholds for non-survivors (including transplanted) identified with ROC analyses for each protein were all associated with worse transplant-free survival in the Kaplan-Meier estimates (Figure 9).

In univariable Cox regression model, MMP-2, MMP-7, perlecan, TIMP-4, age, and sex were significant predictors of transplant free-survival, of which MMP-2 had the largest increase in hazard ratio per increase in linear AU, with hazard ratio 1.14 (95% CI 1.033-1.23). Moreover, female sex was a strong predictor of survival displaying a hazard ratio of 0.36 (95% CI 0.15-0.84). In the multivariable model, increasing levels of MMP-2, perlecan, and TIMP-4 remained negative predictors of survival when adjusted for age and sex (Table 8).

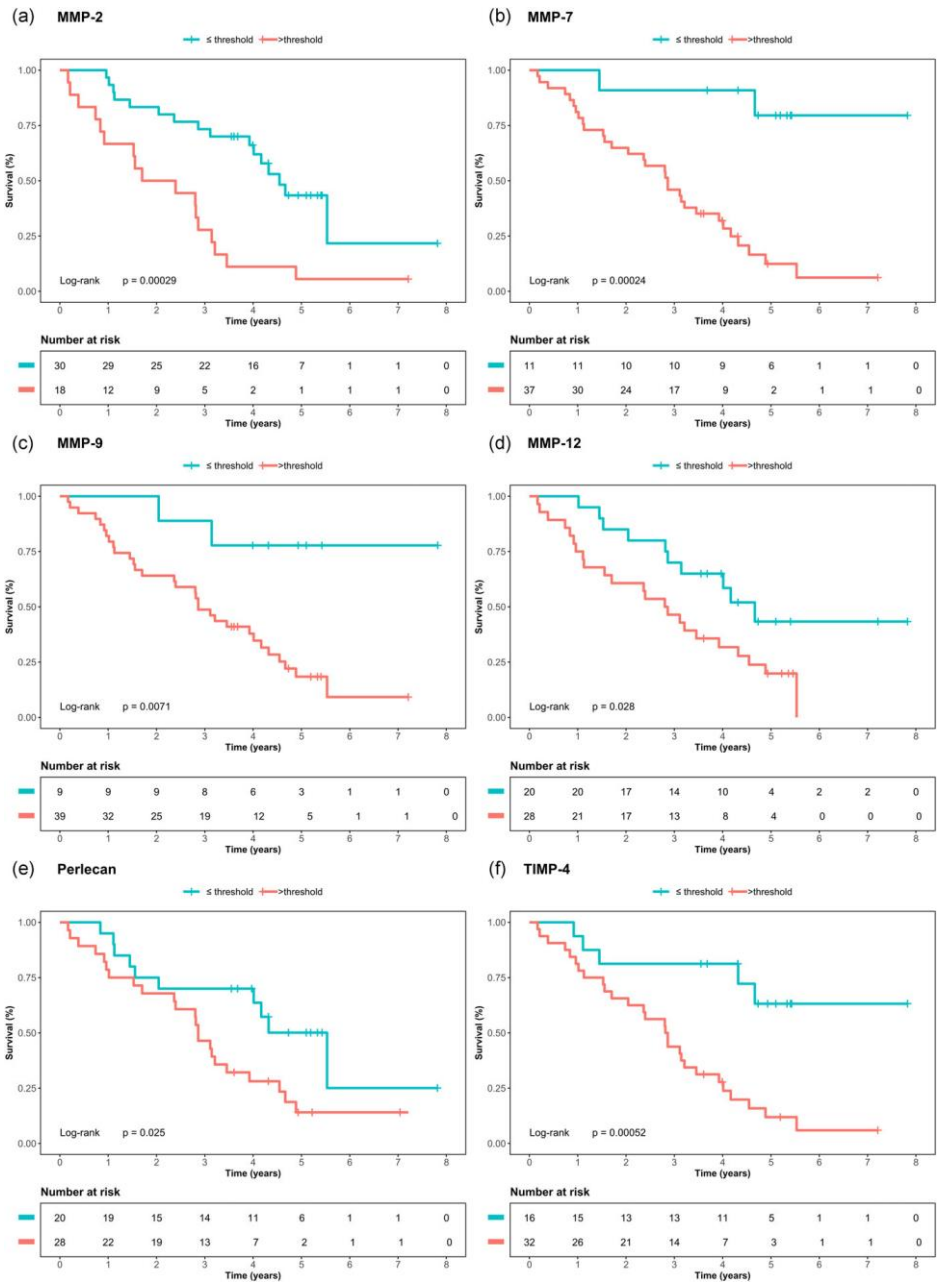

Figure 9. Kaplan-Meier curves of transplant-free survival of patients with PAH in relation to ECM related proteins levels

Transplant-free survival of PAH patients with plasma levels above (teal) the ROC determined thesholds for survival were significantly higher (P <0.03) than for PAH patients with levels below (red) the treshold. a) MMP-2, b) MMP-7, c) MMP-9, d) MMP-12, e) perlecan, f) TIMP-4. Abbreviations:MMP, matrix metalloproteinase; TIMP-4, tissue inhibitor of metalloproteinases 4.

Table 8. Univariable and multivariable Cox regression analysis in paper III

Explanatory variable	HR (95% CI)	P value
Univariable Cox regression		
Age, years	1.038 (1.003-1.074)	**0.032**
Female	0.36 (0.15-0.84)	**0.019**
MMP-2 (AU)	1.14 (1.033-1.26)	**0.009**
MMP-7 (AU)	1.002 (1.0003-1.004)	**0.023**
MMP-9 (AU)	1.018 (0.992-1.044)	0.17
MMP-12 (AU)	1.002 (0.999-1.005)	0.26
Perlecan (AU)	1.010 (1.002-1.019)	**0.02**
TIMP-4 (AU)	1.038 (1.007-1.069)	**0.015**
Multivariable Cox regression		
MMP-2 (AU)	1.13 (1.011-1.26)	**0.031**
Age, years	1.038 (1.00-1.078)	0.057
Female	0.213 (0.084-0.541)	**0.001**
MMP-7 (AU)	1.002 (1.000-1.004)	0.098
Age, years	1.04 (1.001-1.08)	**0.046**
Female	0.197 (0.075-0.52)	**0.001**
Perlecan (AU)	1.01 (1.0004-1.02)	**0.041**
Age, years	1.045 (1.006-1.086)	**0.023**
Female	0.226 (0.09-0.566)	**0.001**
TIMP-4 (AU)	1.037 (1.003-1.071)	**0.031**
Age, years	1.044 (1.004-1.086)	**0.032**
Female	0.201 (0.079-0.513)	**<0.001**

Age, female sex, MMP-2, MMP-7, perlecan and TIMP-4 were predictors of transplant-free survival. Bold indicates statistical significance (P <0.05). Abbreviations, AU, arbitrary unit; CI, confidence interval; HR, Hazard ratio. MMP, matrix metalloproteinase; TIMP-4, tissue inhibitor of metalloproteinases 4.

Correlation with the ESC/ERS risk scores and haemodynamics

Increasing levels of MMP-2 correlated with worsening ESC/ERS risk scores, MRAP, NT-proBNP and 6MWD (Figure 10).

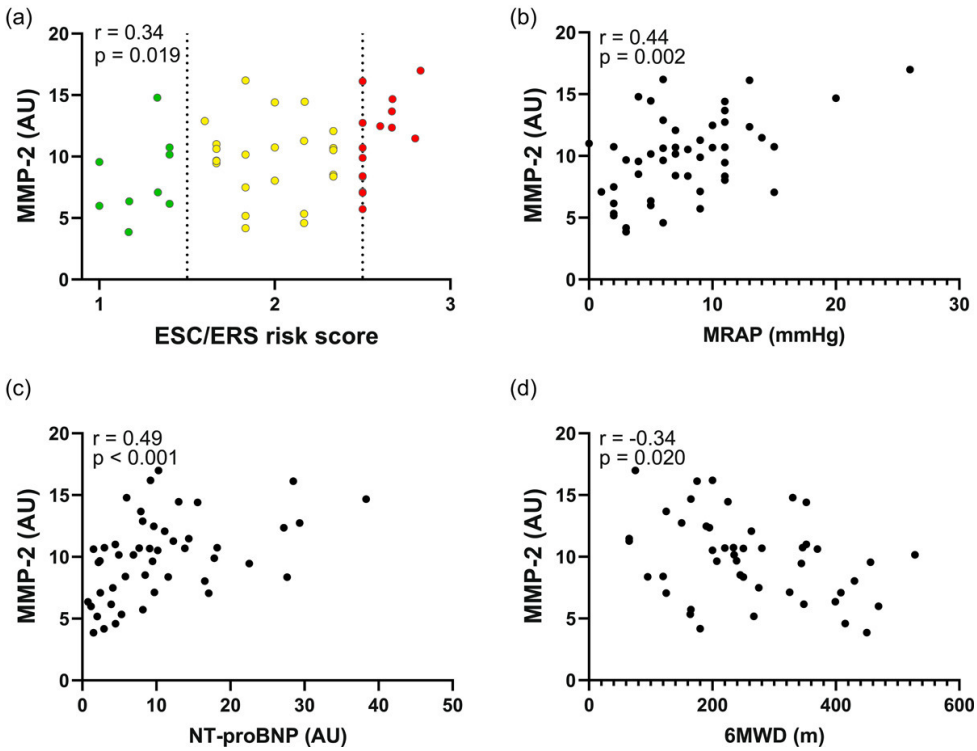

Figure 10. Correlations of MMP-2 and ESC/ERS risk score and haemodynamic parameters
Increasing plasma levels of MMP-2 correlated with a) worsening of ESC/ERS risk scores. b) increasing MRAP. c) increasing NT-proBNP, and d) shorter 6MWD. 6MWD, six-minute walking distance; AU, arbitrary units. MMP, matrix metalloproteinase; MRAP, mean right atrial pressure, NT-proBNP, N-terminal pro brain natriuretic peptide.

Paper IV

Diagnostic Testing

TRAIL levels were significantly lower in PAH compared to controls (P = 0.0061) and the other included disease groups (P <0.0082). TRAIL could discriminate PAH from the other studied dyspnoea populations AUC 0.70 (CI 0.61-0.79, P <0.0001) with a sensitivity of 81% and a specificity of 53% (Figures 11, 12).

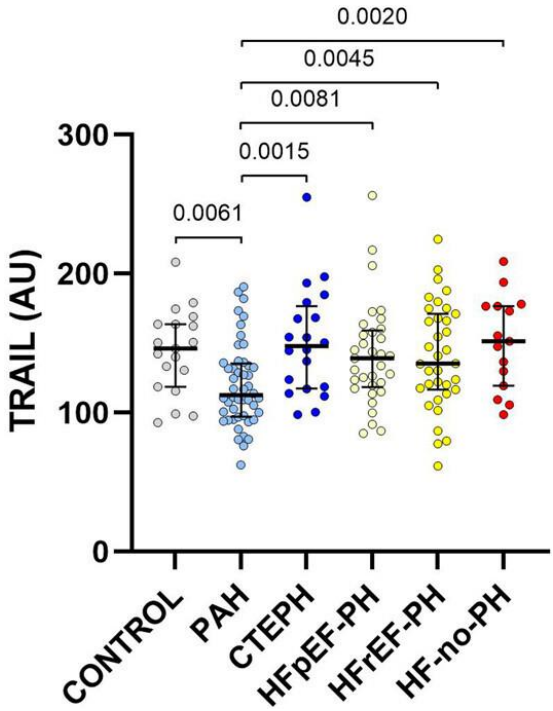

Figure 11. Difference in TRAIL levels compared to PAH

Abbreviations: AU, arbitrary units; AUC, Area under the curve; CTEPH, chronic thromboembolic PH; HF, heart failure; HFpEF-PH, PH associated with HF with preserved ejection fraction (EF); HFrEF-PH, PH associated with to HF with reduced EF; PAH, pulmonary arterial hypertension; PH, pulmonary hypertension; TRAIL, TNF-related apoptosis-inducing ligand

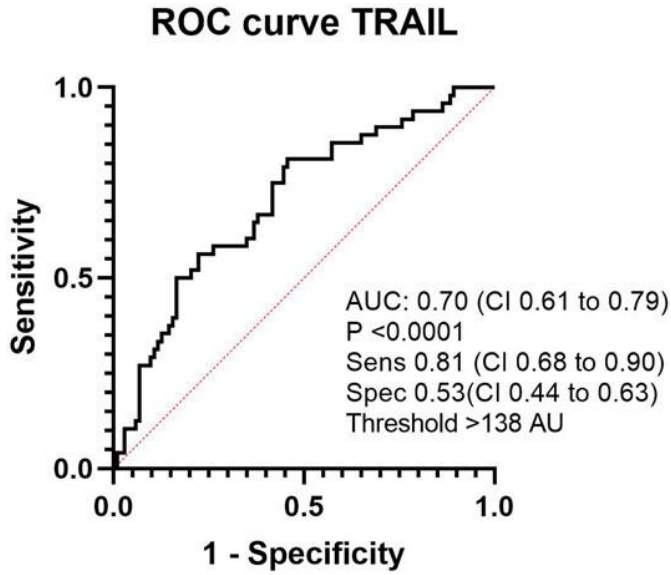

Figure 12. TRAIL as a dicriminator of PAH from CTEPH, HFpEF-PH, HFrEF-PH and HF-non-PH
Abbreviations: AU, arbitrary units; AUC, Area under the ROC curve; CI, confidence interval; CTEPH, chronic thromboembolic PH; HF, heart failure; HFpEF-PH, PH associated with HF with preserved ejection fraction (EF); HFrEF-PH, PH associated with HF with reduced EF; PAH, pulmonary arterial hypertension; PH, pulmonary hypertension; Sens, sensitivity; Spec, specificity; TRAIL, TNF-related apoptosis-inducing ligand.

Prognostic testing

A total of seven proteins, annexin A1(ANXA1), CEACAM8, CXCL-17, GDF-15, IL-6, PSP-D, and TRAIL-R2 showed significant differences in plasma levels between PAH patients with ≤3 and >3 years of transplant-free survival post diagnosis during the follow-up time. In the following Kaplan-Meier and log rank tests, all proteins displayed a significant difference in survival between above and below the proteins´ threshold levels (Figure 13).

ANXA1, CEACAM8, CXCL17, IL-6, PSP-D, TRAIL-R2, age, female sex, and risk scores had a significant prognostic value in the univariable Cox regression analysis. In the multivariable Cox regression models including age, female sex, and ESC/ERS risk scores, only ANXA1 (P = 0.044, hazard ratio 1.34 (95% CI 1.008-1.77)) and CEACAM8 (P = 0.048, hazard ratio 1.060 (95% CI 1.0004-1.12) remained prognostic (Table 9).

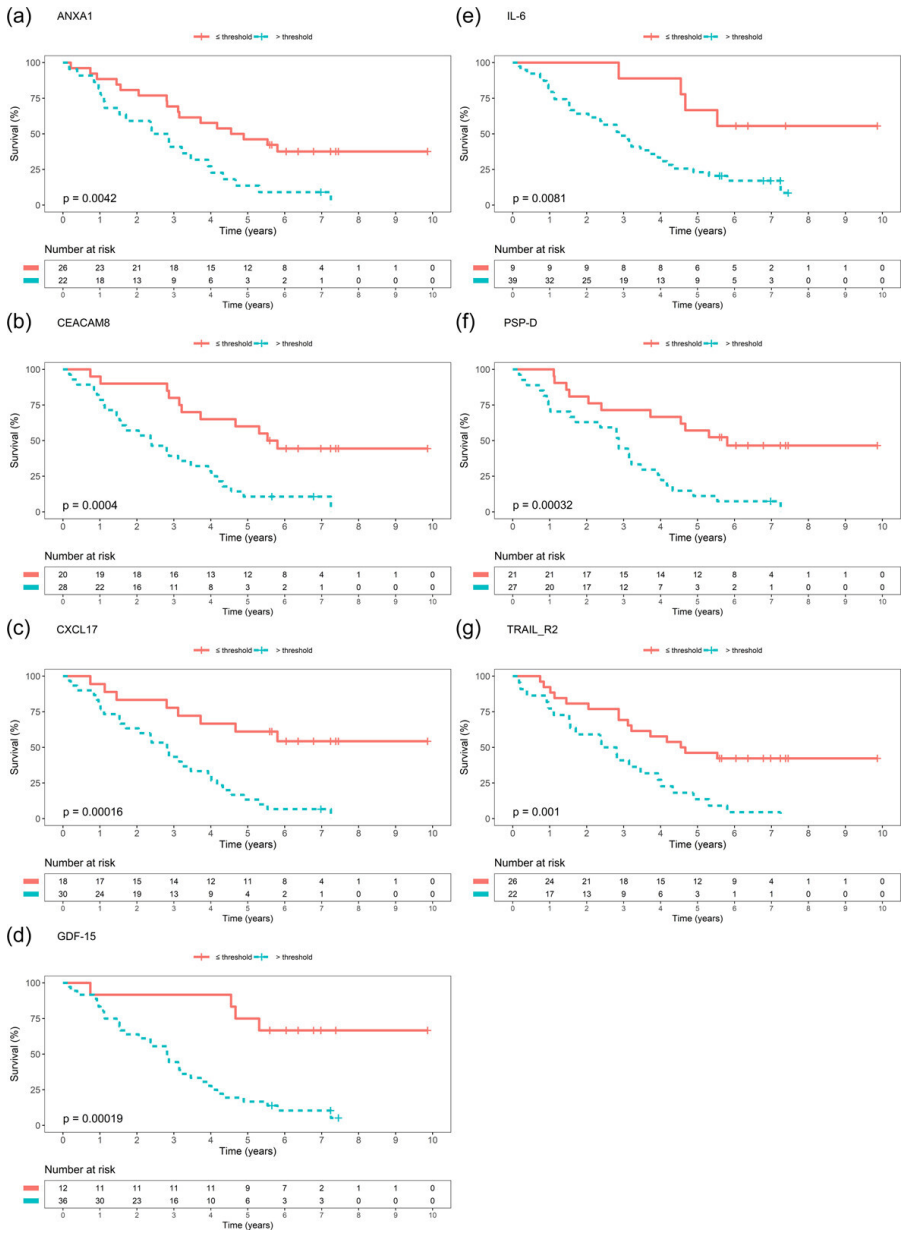

Figure 13. Kaplan-Meier curves of transplant free survival in PAH patients in relation to protein levels

Transplant-free survival of PAH patients with plasma levels above (teal) the ROC determined thesholds for survival were significantly higher (P <0.01) than for PAH patients with levels below (red) the treshold. ANXA1, annexin A1; CEACAM8, carcinoembryonic antigen-related cell adhesion molecule 8; CXCL17, C–X–C motif chemokine 17; GDF-15, growth/differentiation factor 15; IL-6, interleukin 6; PSP-D, pulmonary surfactant-associated protein D; TRAIL-R2, tumor necrosis factor-related apoptosis-inducing ligand receptor 2.

Table 9. Uni- and multivariable Cox regressions in paper IV

Explanatory variable	HR (95% CI)	P value
Univariable Cox regression		
Age	1.037 (1.006-1.068)	0.019
Female	0.38 (0.16-0.87)	0.023
Risk score	1.96 (1.012-3.79)	0.046
ANXA1	1.42 (1.12-1.79)	0.003
CEACAM8	1.084 (1.036-1.13)	<0.001
CXCL17	1.029 (1.012-1.047)	<0.001
GDF-15	1.002 (0.998-1.007)	0.28
IL-6	1.017 (1.001-1.033)	0.033
PSP-D	1.069 (1.017-1.12)	0.009
TRAIL-R2	1.014 (1.005-1.024)	0.004
Multivariable Cox regression		
ANXA1	1.34 (1.008-1.77)	0.044
Age	1.033 (1.001-1.067)	0.044
Female	0.26 (0.102-0.65)	0.004
Risk score	1.26 (0.59-2.69)	0.55

Abbreviations: ANXA1, annexin A1; CEACAM8, carcinoembryonic antigen-related cell adhesion molecule 8; CI, confidence intervall; CXCL17, C–X–C motif chemokine 17; GDF-15, growth/differentiation factor 15; HR, hazard ratio; IL-6, interleukin 6; PSP-D, pulmonary surfactant-associated protein D; TRAIL-R2, tumor necrosis factor-related apoptosis-inducing ligand receptor 2.

Discussion

Paper I

Paper I shows that plasma MMP-7 levels in PAH was different in PAH from controls, CTEPH, HFpEF-PH, HFrEF-PH, and HF-non-PH. Plasma MMP-7 had an acceptable AUC for differentiation of PAH from CTEPH, HFpEF-PH, HFrEF-PH, and HF-non-PH. MMP-7 may therefore be a future biomarker for PAH identification, and the discriminative ability can likely be increased by it being a part of a multi-biomarker panel.

MMP-7 among MMP-1, -3, and -13 have the ability to cleave connective tissue growth factor (CTGF) and release vascular endothelial growth factor (VEGF) inhibited by the CTGF.[85] VEGF can also be inhibited by binding of the soluble fms-like tyrosine kinase-1 (sFlt-1, also known as soluble VEGF receptor 1).[86] Circulating levels of sFlt-1 are elevated in PAH.[87, 88]MMP-7 degrade sFLT-1 thereby prevents its inhibitory effect on VEGF and restore VEGF stimulated angiogenesis in human umbilical vein endothelial cells (HUVEC).[89] Consequently MMP-7 may play a protective role in PAH since increased VEGF-A levels have protective effects in rodent models of PH.[90, 91]

Paper II

The main result of paper II was that plasma prolargin levels in PAH was higher than controls and lower than CTEPH, HFpEF-PH, HFrEF-PH and HF-non-PH. Prolargin displayed a good AUC for identification of PAH among CTEPH, HFpEF-PH, HFrEF-PH and HF-non-PH and could be a useful tool in discriminating PAH in a patient group exhibiting dyspnoea. Prolargin exhibited strong correlations with MRAP, NT-proBNP, and moderate negative correlations with other haemodynamic parameters. Therefore, it could be a negative predictor of right heart function and may indirectly reflect right heart/ventricular decompensation.

Prolargin mediates cell adhesion to the ECM through binding of cell surface glycosaminoglycans.[92] Prolargin inhibits membrane attack complex formation by reducing C9 polymerization, and in addition inhibits the alternative pathway by interaction with C3, and thus acts as an inhibitor on the complement system.[93]

Complement C3 have previously been found increased in serum of PAH patients, and suggested as a biomarker of PAH.[94] Bauer et al found that C3d complement deposition is increased in the vascular wall in lungs of IPAH patients and in a mouse chronic hypoxia PH model.[95] This is in line with the findings that the alternate pathway of the complement system is overactive in HF.[96] Genetic deletion of C3 reduced pulmonary vascular remodelling in a murine chronic hypoxia PAH model.[95] Accordingly, prolargin may be a negative regulator of complement activation in PAH and via inhibition of C3 activation reduce vascular remodelling in PAH.

A phase II study investigating treatment of PAH with tocilizumab, an IL-6 receptor antagonist, found that IL-6 where reduced. However, there was no effect on PVR or secondary treatment goals, (defined as improvement in 6MWD, WHO-FC, NT-proBNP, BORG dyspnoea score or quality of life scores.[97, 98] In paper I, IL-6 levels were increased generally across the disease groups but the increase was not specific to PAH, and thus the increase in IL-6 levels in PAH may be a result of inflammation but not the driving factor for PAH development and progression.

Paper III

The main result of paper III is that MMP-2 is a negative prognostic predictor in PAH and correlates with worsening of known risk parameters and is consequently a potential future prognostic marker for PAH.

MMP-2 is a gelatinase that degrade collagen and gelatins.[99] It has functions in angiogenesis, proliferation, and migration of vascular SMC,[100] and of pulmonary artery endothelial cells (PAEC) in a murine hypoxia model of PAH.[101]

PAH specific treatments influence MMP-2 expression including the PCA iloprost, PDE5i sildenafil and the dual ERA bosentan.[102-104] Schermuli et al reported a reduction in MMP-2 expression and vascular remodelling in response to inhaled iloprost in a monocrotaline PAH model.[103] In a study by Sun et al, MMP-2 levels in PASMC increased in response to endothelin-1 (ET-1), and treatment with the PDE5i sildenafil inhibited the MMP-2 increase.[104] In another monocrotaline model of PAH, bosentan treatment reduced gene expression but not serum levels of MMP-2.[102] In addition to increase in response to ET-1, MMP-2 cleaves the ET precursor big ET into the vasoactive ET-1.[104, 105]

MMP-2 have been evaluated as a prognostic marker for PH in a study by Tiede et al. where MMP-2 levels above the median were associated with worse survival.[106] An MMP-2/TIMP-4 ratio has been investigated as prognostic predictor in IPAH, where a high MMP-2/TIMP-4 ratio was associated with worse prognosis and higher MPAP, PVR, eGFR and TAPSE.[107] Paper III indicated worse prognosis in PAH for

both increased MMP-2 and TIMP-4, which would result in a stationary MMP-2/TIMP -4 ratio. It is possible that the different result could be due to that paper III also included CTD-PAH. There was however no apparent difference in levels of MMP2 or TIMP-4 between IPAH/FPAH and CTD-PAH at baseline.

Paper IV

Paper IV suggests TRAIL as potential biomarker for PAH with lower plasma levels in PAH compared with controls, HFpEF-PH, HFrEF-PH and HF-non-PH, and an acceptable AUC as a discriminator of PAH among others investigated for unclear dyspnoea.

TRAIL is widely expressed, most commonly in the lung, prostate and spleen.[108] It can be membrane bound or cleaved into its soluble form and bind 5 different receptors; TRAIL-receptor 1-4, and the soluble receptor osteoprotegerin which can inhibit TRAIL induced apoptosis.[109]

TRAIL is increased in PASMC,[110] and promotes migration an proliferation of vascular SMC.[111] It has increased expression in the endothelium and SMC in both concentric and plexiform IPAH lesions.[112]

In three independent rodent models, TRAIL was required for development of PAH, and TRAIL blockage reduced pulmonary vascular remodelling by reduced proliferation and increased apoptosis.[110]

PAH and cancer shares several hallmarks including metabolic changes, excessive proliferation signals and cell death resistance.[113] Dulanermin, a recombinant human TRAIL has been investigated as a potential therapy in cancer therapy trials.[114] Thus it is possible that a TRAIL pathway could be a potential target for treatment of PAH in future investigations.

Sweatt et al have found increased circulating levels of TRAIL to be part of a high-risk phenotype PAH,[115] however, paper IV did not display a difference in TRAIL levels between 3- year survivors vs non-survivors and was not selected for analysis as a prognostic marker.

Paper IV also demonstrates that ANXA1 is a strong predictor of prognosis in patients with PAH independent of age, sex and 2015 risk scores. ANXA1 is glucocorticoid regulated protein with anti-inflammatory effects and a mediator for resolution of inflammation.[116, 117] In PASMCs, upregulation of ANXA1 leads to GATA4 dependent downregulation of the apoptosis inhibitor Bcl-xL.[118] Contrary, ANXA1 is downregulated by ET-1 and chronic hypoxia.[118] Interestingly, we found that increased circulating levels of ANXA1 predicted poor prognosis in PAH, which would fit with a downregulation of apoptosis. Alternatively downregulating of

ANXA1 by ET-1 could be limited to the local pulmonary vasculature or that extensive vascular inflammation and remodelling could drive a compensatory increase in ANXA1 levels.

General limitations of the included papers

The patients with PAH in the LCPR have an older age than traditional PAH patients, but the PAH population is in line with other contemporary PAH registries i.e. the European COMPERA,[119] and the Swedish SPAHR,[5] which have been acknowledged as part of the emerging change in PAH epidemiology in the Western world.[120]

We did not have access to matched controls, and the age of the available control group is closer to the age of the classic PAH patients compared to the included patients. As a result of non-matched controls, a difference in proteins' levels compared to controls may in part be attributable to other factors than PAH such as normal ageing. However, in Paper III and IV, we were able to adjust for age and sex in the Cox regression models.

PAH is a rare disease, and the included papers are single centre studies, based on one regional referral centre for unclear dyspnoea, i.e. the Haemodynamic Lab in Lund. Thus, the sample sizes have been somewhat limited. Patients have been included during a few years' time, during which treatment regimens were evolving which could have influenced survival in the prognostic studies paper III and IV. In addition, with an older patient cohort some patients would not tolerate upfront combination therapy due to comorbidities, where a limitation in treatment options could also have influenced survival.

The study populations of the included papers have not included patients with PH associated with lung disease and/or hypoxia, group 3 PH, nor PH associated with multifactorial mechanisms, group 5 PH and can thus, not tell if the studied proteins can differentiate PAH from these PH groups.

The PEA blood analysis technique has been useful to analyse large amounts of proteins with little cross reactivity, but the output of protein levels in arbitrary units, relative within the same analysis run, and not absolute values may limit the direct transfer into the clinical setting. Some of the included clinical data that were used to calculate risk scores have been collected from journals retrospectively and is bound to have some missing data as common with retrospective studies.

The "ideal" cut off in these papers have been calculated by Youden's index resulting in the greatest combined values of sensitivity and specificity. This is, however, not always desirable.[58] PAH is a rare disease and as such positive predictive values for a test applied in the general population would be low. and not feasible. Even in a

dyspnoea population the prevalence of PAH would be low but more common than in the general population. By using a selected population of dyspnoea patients, one could increase the positive predictive value of the test. In the setting of screening a selected population of dyspnoea patients one could favour high sensitivity to catch the patients that need an expedited investigation because the positive predictive value is higher than for the general population while if a test would be applied to the general population the positive predictive value would be low and the negative predictive value increased. Thus, a high specificity and positive predictive value could be favoured to avoid a large number of false positive results that would require time and resource consuming investigations. On the other hand, in high-risk populations e.g. with family history of PAH or with connective tissue disease high sensitivity and negative predictive values could be favoured so to rule out PAH in patients with negative tests. Since PAH has a low prevalence, it may be hard to achieve high positive predictive values but easier to achieve high negative predictive values. By applying a test on a more selected population the positive predictive value of the test would increase.

Strengths include that the PAH patient are incident cases and treatment naïve and thus, PAH specific treatment cannot have influenced the composition of proteins expressed in the plasma of PAH patients. It has also allowed for a prognostic estimation at baseline diagnosis without interference of PAH treatment. The proximity extension assay analysis allowed for analysis of a large number of proteins with little cross reactivity and low sample consumption. This increased the number of proteins that was able to be included in the analyses and increase the number of times the collected plasma samples can be utilized for analysis.

Conclusions

The present thesis presents several potential plasma biomarkers for differential diagnosis and prognostication of patients with PAH. Future validation of these potential biomarkers in large multi-centre studies, with access to external validation cohorts, is encouraged.

The major conclusions of each study are as follows:

Paper I: Plasma MMP-7 levels were higher in PAH compared to controls and lower than the other included PH groups and HF without PH. The MMP-7 levels in PAH were able to identify PAH among PH and HF and could be a biomarker to identify PAH in a dyspnoea population.

Paper II: Plasma prolargin levels were lower in PAH patients than the other included PH groups and HF without PH, and higher than healthy controls. The plasma levels of prolargin discriminated PAH from the other PH groups and HF and could be a biomarker for identifying PAH in a dyspnoea population. Plasma prolargin levels correlated with worsening in MRAP, NT-proBNP, cardiac index and SVI, and may reflect impaired heart function or decompensation.

Paper III: High plasma levels of MMP-2, perlecan and TIMP-4 were associated with worse transplant-free survival in PAH. Increased plasma MMP-2 levels at diagnosis correlated with worse ESC/ERS risk scores and worsening of right heart function including the parameters MRAP, NT-proBNP, and 6MWD, and indicate that MMP-2 could be a negative prognostic marker in PAH.

Paper IV: Plasma levels of TRAIL in PAH patients were lower than in controls, the other PH groups, and in HF without PH. Plasma TRAIL levels could distinguish PAH from the other PH groups, and HF without PH. This paper identify TRAIL as a potential biomarker with good accuracy for diagnosis of PAH. Patients with high plasma ANXA1 levels had worse transplant-free survival and increased ANXA1 levels in PAH were associated with poor prognosis independent of age, sex and 2015 risk scores. Thus, ANXA1 could be a prognostic marker in PAH.

Future perspectives

The present thesis presents several potential diagnostic and prognostic biomarker candidates in PAH which would benefit from further validation in lager collaborative international studies. Large multicentre studies could allow for separate validation cohorts, and with more events, there is a possibility to adjust for more parameters in regression modelling, including combining several markers, and using a more matched control, and disease groups.

The present thesis investigated the proteins' discriminative ability for PAH in a group that has been referred to RHC as part of investigation for dyspnoea. It would be of interest to investigate their discriminative ability of PAH in a wider dyspnoea group, as it would present at the general practitioner, at a timepoint much earlier in the diagnostic chain than RHC referral. If a biomarker for PAH were to be found useful at that stage, the time to RHC referral and diagnosis could be shortened.

The use of PEA blood analysis technique resulting in arbitrary units have been reasonable in the research setting, screening for new biomarker candidates. Protein levels were expressed in AU, which is adequate for comparison of expression of one protein between different patients' groups in but is not useful to compare actual protein levels between different proteins. Utilizing a method that yield absolute values would be more practical in the clinic. Thus, in the future these results would benefit from being replicated by another analysis method with absolute values.

In the papers of the present thesis, individual potential plasma biomarkers have been identified for diagnosis as well as prognostication of PAH. Future studies could investigate if the potential biomarkers proposed in this thesis could in combination with each other, or with other promising biomarker candidates, form a multi-marker panel to increase the diagnostic, and prognostic potential to achieve higher sensitivity and specificity for PAH.

The prognostic biomarker candidates could be included in addition to the different risk score calculations and tried in a multi-marker approach in conjunction with other promising prognostic biomarkers.

It is imperative to continue the development of new diagnostic and prognostic biomarkers with relevance to the pathology of PAH to reduce time to diagnosis and to guide precise treatment decisions.

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