



LUND UNIVERSITY

Modulators of hypoxic pulmonary vasoconstriction and pulmonary hypertension. Implications for new treatment strategies.

KYLHAMMAR, DAVID

2016

[Link to publication](#)

Citation for published version (APA):

KYLHAMMAR, DAVID. (2016). *Modulators of hypoxic pulmonary vasoconstriction and pulmonary hypertension. Implications for new treatment strategies*. [Doctoral Thesis (compilation), Department of Clinical Sciences, Lund]. Lund University: Faculty of Medicine.

Total number of authors:

1

General rights

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

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

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

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

Take down policy

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

LUND UNIVERSITY

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

Modulators of hypoxic pulmonary vasoconstriction and pulmonary hypertension

Implications for new treatment strategies

DAVID KYLHAMMAR | CARDIOLOGY | FACULTY OF MEDICINE | LUND UNIVERSITY



Modulators of hypoxic pulmonary vasoconstriction and pulmonary hypertension

Implications for new treatment strategies

David Kylhammar, M.D.



LUND
UNIVERSITY

DOCTORAL DISSERTATION

by due permission of the Faculty of Medicine, Lund University, Sweden.

To be defended at Segerfalksalen, BMC, Lund University, Lund.

May 26, 2016 13:30.

Faculty opponent

Clin. Assoc. Prof. Jørn Carlsen, M.D., D.M.Sc., Dept. of Clinical Medicine,
University of Copenhagen, Copenhagen, Denmark.

Organization LUND UNIVERSITY Dept. of Clinical Sciences, Cardiology, Faculty of Medicine Author(s) David Kylhammar	Document name DISSERTATION	
	Date of issue May 26, 2016	
	Sponsoring organization Medicinska Fakulteten, Skånes universitetssjukhus, ALF, Maggie Stephens stiftelse, Craafordska stiftelsen, Stiftelsen Landshövding Per Westlings Minnesfond, Ebba och Herman Bings legat, Regementsläkare Dr. Hartelli Stipendiastiftelse, Svensk Förening för Pulmonell Hypertension, Actelion Pharmaceuticals Sweden AB, Pfizer AB, Copenhagen Muscle Research Centre.	
Title and subtitle Modulators of hypoxic pulmonary vasoconstriction and pulmonary hypertension. Implications for new treatment strategies.		
Abstract <p>Pulmonary arterial hypertension (PAH) is a disease of small pulmonary arteries, characterized by excessive vasoconstriction, remodelling of the arterial wall, in-situ thrombotisation and characteristic plexiform lesions. PAH leads to an elevation in right ventricular afterload, which causes right heart failure and ultimately death. As the diagnosis is often set late and the prognosis poor there is a need for new diagnostic approaches and treatment strategies.</p> <p>In a first study, we retrospectively investigated a cohort of patients diagnosed with PAH in Lund from 2000-2010 and demonstrated that the survival of patients still is poor, but comparable to other European countries. We found that initial combination therapy could be more efficient than initial monotherapy in improving the haemodynamic status. Additionally, we identified the 6-minute walking distance, pulmonary vascular resistance index, stroke volume index and mean right atrial pressure/stroke volume index as prognostic markers at treatment follow-up. These variables may therefore potentially be used as future treatment goals.</p> <p>Hypoxia induces pulmonary vasoconstriction. This hypoxic pulmonary vasoconstriction (HPV) matches perfusion with ventilation in focal hypoxia, but in global hypoxia generalized HPV induces pulmonary hypertension (PH). Generalized HPV may additionally worsen PH when primarily of another aetiology. Studies into the mechanisms of HPV can lead to new physiological insights, but also contribute to find new complementary treatments for hypoxia-induced PH. As modulatory pathways may furthermore be common for hypoxia-induced PH and other forms of PH, including PAH, its study can also lead to better comprehension of pathogenetic mechanisms and potential treatments for other forms of PH.</p> <p>We established an <i>in vivo</i> porcine model of HPV and found that endothelin-1 is important for the sustained HPV response and that a cyclooxygenase-2-derived vasoconstrictor, probably thromboxane A₂, contributes to potentiate the HPV response. Adenosine diphosphate was not an obligatory potentiating modulator of HPV, but could be a non-mandatory contributor. Additionally, we conclude that the intravenous dual endothelin A and endothelin B receptor antagonist tezosentan, and the inotropic and pulmonary vasodilatory drug levosimendan, could be new means to modulate hypoxia-induced PH.</p> <p>Finally, in a prospective cohort of patients with systemic sclerosis-associated PAH (SSc-PAH) we identified a panel of circulating angiogenic and inflammatory biomarkers that could be used for PAH screening among SSc patients. We additionally found that circulating angiogenic and inflammatory biomarkers were deranged at time of PAH diagnosis and altered after initiation of PH-targeted treatments in a group of patients with idiopathic PAH or SSc-PAH. Plasma levels of some angiogenic and inflammatory biomarkers furthermore correlated with important clinical variables of prognostic impact, such as the 6-minute walking distance, mean right atrial pressure and NT-proBNP levels. These findings could contribute to earlier identification and better prognostication of PAH patients and thereby potentially improve their prognosis.</p>		
Key words Pulmonary arterial hypertension, systemic sclerosis, biomarker, hypoxia, endothelin-1, cyclooxygenase-2, thromboxane A ₂ , adenosine diphosphate, levosimendan, tezosentan		
Classification system and/or index terms (if any)		
Supplementary bibliographical information		Language: English
ISSN and key title:1652-8220		ISBN: 978-91-7619-291-7
Recipient's notes	Number of pages 86	Price
	Security classification	

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

Signature



Date

160420

Modulators of hypoxic pulmonary vasoconstriction and pulmonary hypertension

Implications for new treatment strategies

David Kylhammar, M.D.



LUND
UNIVERSITY

Supervisor:

Associate Professor Göran Rådegran, M.Sc.Eng.Phys., M.D., D.M.Sc.

Assistant Supervisor:

Associate Professor Roger Hesselstrand, M.D., Ph.D.

Copyright David Kylhammar

Cover: *Pulmonary artery pressure curve from a patient with pulmonary arterial hypertension.* Designed by Christian Reitan with little help from David Kylhammar.

Cardiology, Dept. of Clinical Sciences, Faculty of Medicine, Lund University

ISBN 978-91-7619-291-7

ISSN 1652-8220

Lund University, Faculty of Medicine Doctoral Dissertation Series 2016:65

Printed in Sweden by Media-Tryck, Lund University

Lund 2016



In Memory of Professor Bengt Saltin

Content

List of Publications.....	9
Summary	11
Populärvetenskaplig sammanfattning.....	13
Abbreviations	15
Introduction	17
Pulmonary Hypertension.....	17
Pulmonary Arterial Hypertension.....	18
Hypoxic Pulmonary Vasoconstriction	22
Biomarkers in Pulmonary Arterial Hypertension.....	25
Current and Future Biomarkers in Pulmonary Arterial Hypertension.....	25
Aims	27
Materials and Methods	29
Paper I	29
Study Population	29
Gathering of Data	29
Ethics	29
Statistics.....	29
Papers II-V	30
<i>In Vivo</i> Pig Model of Acute Hypoxic Pulmonary Vasoconstriction.....	30
Drugs	32
Ethics.....	33
Statistics.....	33
Paper VI	34
Study Population	34
Plasma Samples and Enzyme-linked Immunosorbent Assay.....	34
Gathering of Data	34
Ethics.....	35
Statistics.....	35
Results and Comments	37
Paper I	37

Papers II-V	42
Effects of acute hypoxia	43
Paper II	43
Paper III	48
Paper IV	51
Paper V	55
General Comments	59
Paper VI	60
Future perspectives	67
Conclusions	69
Bibliography	71
Acknowledgements	85
Papers I-VI	87

List of Publications

This thesis is based on the following papers and they will in the text be referred to by their Roman numerals.

- I. **Kylhammar D**, Persson L, Hesselstrand R, Rådegran G. Prognosis and response to first-line single and combination therapy in pulmonary arterial hypertension. *Scand Cardiovasc J*. 2014 Aug; 48(4): 223-33.
- II. Hedelin P, **Kylhammar D**, Rådegran G. Dual endothelin receptor blockade with tezosentan markedly attenuates hypoxia-induced pulmonary vasoconstriction in a porcine model. *Acta Physiol*. 2012 Mar; 204(3): 419-34.
- III. Wiklund A, **Kylhammar D**, Rådegran G. Levosimendan attenuates hypoxia-induced pulmonary hypertension in a porcine model. *J Cardiovasc Pharmacol*. 2012 May; 59(5): 441-9.
- IV. **Kylhammar D**, Rådegran G. Cyclooxygenase-2 inhibition and thromboxane A(2) receptor antagonism attenuate hypoxic pulmonary vasoconstriction in a porcine model. *Acta Physiol*. 2012 Aug; 205(4). 507-19.
- V. **Kylhammar D**, Bune LT, Rådegran R. P2Y₁ and P2Y₁₂ receptors in hypoxia- and adenosine diphosphate-induced pulmonary vasoconstriction in vivo in the pig. *Eur J Appl Physiol*. 2014 Sep; 114(9): 1995-2006.
- VI. **Kylhammar D**, Hesselstrand R, Nielsen S, Scheele C, Rådegran G. Angiogenic and inflammatory biomarkers for screening and follow-up in patients with pulmonary arterial hypertension. *In Manuscript*.

Published articles were printed with permission from the publishers.

Scientific contributions that are not included in this dissertation are as follows:

- Lundgren J, **Kylhammar D**, Hedelin P, Rådegran G. sGC stimulation totally reverses hypoxia-induced pulmonary vasoconstriction alone and combined with dual endothelin-receptor blockade in a porcine model. *Acta Physiol.* 2012 Nov; 206(3): 178-94.
- **Kylhammar D**, Rådegran G. The principal pathways involved in the in vivo modulation of hypoxic pulmonary vasoconstriction, pulmonary arterial remodelling and pulmonary hypertension (Review). *Accepted for publication with minor revisions.*
- Sandqvist A, Schneede J, Henrohn D, **Kylhammar D**, Lundgren J, Hedeland M, Bondesson U, Rådegran G, Wikström G. Differences in plasma L-arginine and dimethylarginines in diagnosis and treatment of pulmonary arterial hypertension: A prospective observational study. *In Manuscript.*

Summary

Pulmonary hypertension (PH) has many causes, whereof pulmonary arterial hypertension (PAH) is one. PAH is a disease primarily of pulmonary arteries, characterized by excessive pulmonary vasoconstriction, vascular remodelling, in-situ thrombosis and the development of plexiform lesions. PAH can be idiopathic, hereditary, drugs or toxins induced or associated to other diseases, most commonly connective tissue diseases (CTD) or congenital heart diseases. Mortality rates in PAH are high despite modern therapies and there is a definite need for new diagnostic approaches and treatment strategies.

Additionally, hypoxia causes pulmonary vasoconstriction. This is beneficial when hypoxia is focal or when ventilation is unevenly distributed, as blood flow is then directed primarily to well-ventilated lung regions. Conversely, when hypoxia is global, hypoxic pulmonary vasoconstriction (HPV) induces generalized pulmonary vasoconstriction, which may lead to PH. This can occur at high altitude where the partial pressure for oxygen is low and in chronic lung diseases. HPV may, however, also aggravate the degree of PH in PAH. The mechanisms behind HPV are not fully understood and there is a need for complementary treatments of hypoxia-induced PH besides oxygen and treatment of the underlying cause. Additionally, an acute hypoxia model can be a tool to study regulatory mechanisms of pulmonary arterial tone in general. In such a way, findings regarding the regulation of HPV may also help to better understand the pulmonary arterial processes in other forms of PH, including PAH.

The general aims of this doctoral dissertation were therefore to evaluate mechanisms of pulmonary vasoconstriction, with special emphasis on HPV, and to perform investigations into new treatment strategies and diagnostic approaches for patients with PH due to PAH or hypoxia. The long-term goal is to improve outcome for patients with PH.

In **Paper I**, it was demonstrated that survival is poor for patients with idiopathic PAH and even worse for those with PAH associated to a CTD. One reason for this could be that the treatment strategies used for PAH patients were not sufficient, as we found that the recommended approach of initial monotherapy was in most cases not satisfactory. Instead, our results indicated that initial combination therapy could be a means to more rapidly and potently improve the pulmonary haemodynamic status and cardiac performance. Another reason for the poor survival rates might be that the diagnosis of PAH is often set late in the disease. In **Paper**

VI, we identified a panel of circulating angiogenic and inflammatory biomarkers, including placenta growth factor (PlGF), vascular endothelial growth factor (VEGF)-D, soluble VEGF receptor-1 (sVEGFR-1) and tumour necrosis factor (TNF)- α , which could in different ways contribute to the screening process for an earlier diagnosis of PAH among patients with systemic sclerosis (SSc). Plasma levels of these circulating biomarkers were either higher in SSc patients who would eventually develop PAH, as compared to SSc patients who did not develop PAH during a follow-up of over a decade, or increased in SSc patients as PAH developed. SSc is the most common CTD associated with PAH. We also found that several circulating angiogenic and inflammatory biomarkers, namely PlGF, VEGF-A, VEGF-D, sVEGFR-1, interleukin-6 and TNF- α , were elevated at PAH diagnosis in patients with either idiopathic or SSc-associated PAH. Such biomarkers could potentially aid in identifying what patients with PH, or symptoms of PH, that have PAH.

At present, it is recommended that the treatment of patients with PAH should be aimed at reaching pre-defined treatment goals. The current treatment goals are however primarily based on “expert opinion” and there is only sparse data regarding prognostic markers at follow-up after initiation of PAH-targeted therapies. In **Paper I**, we identified a higher pulmonary vascular resistance index and a lower 6-minute walking distance at treatment follow-up to be associated with worse outcome in PAH. In complementary analyses, we additionally show that lower stroke volume index and higher mean right atrial pressure/stroke volume index at treatment follow-up are associated with worse outcome. Additionally, in **Paper VI** we found that plasma levels of PlGF, VEGF-D, interleukin-6 and TNF- α at treatment follow-up correlated with clinical variables of prognostic impact and that higher plasma levels of PlGF and TNF- α were indeed associated with an increased mortality risk in a univariate COX proportional hazards model.

With respect to the modulation of HPV, in **Papers II and IV** it was demonstrated that endothelin-1 and a cyclooxygenase-2 derived vasoconstrictor contribute to potentiate HPV *in vivo* in pigs and seem to be of specific importance for the sustained HPV response. It was suggested that the cyclooxygenase-2 derived vasoconstrictor was thromboxane A₂. In **Paper V**, we found that adenosine diphosphate, through activation of P2Y₁ and P2Y₁₂ receptors, may be a non-obligatory potentiating modulator of HPV, but a definite role for adenosine diphosphate in modulating the HPV response was not proven in our *in vivo* porcine model. The results in **Paper II** furthermore implicated that the dual endothelin receptor antagonist tezosentan could be used both to reverse and to prevent acute hypoxic PH. In **Paper III** levosimendan was additionally shown to be a potential treatment for acute hypoxic PH, as the drug both reduced right ventricular afterload and prevented a cardio-depressive effect of acute hypoxia *in vivo* in pigs.

Populärvetenskaplig sammanfattning

Förhöjt blodtryck i lungkretsloppet kan ha många orsaker. Oavsett vad som ligger bakom så medför det förhöjda blodtrycket att höger hjärthalva, som pumpar blodet genom lungkretsloppet, får arbeta mot ett högre motstånd. Det resulterar på sikt i att höger hjärthalva börjar svikta i sin funktion, vilket kan leda till döden. En viktig orsak till förhöjt blodtryck i lungkretsloppet är den ovanliga lungkärslsjukdomen pulmonell arteriell hypertension (PAH). Dödligheten vid PAH är hög och diagnosen ställs ofta sent i sjukdomsförloppet. Det finns därför ett behov av nya behandlingsstrategier och nya diagnostiska metoder vid PAH.

När syrgashalten i omgivningen är låg drar lungkretsloppets blodådror ihop sig. Det är ändamålsenligt om den låga syrgashalten är begränsad till en del av lungan. Då omfördelas blodflödet till områden av lungan där syrgashalten är god. När syrgashalten är låg i hela eller stora delar av lungan blir kärlsammandragningen emellertid så utbredd att blodtrycket i lungkretsloppet kan stiga till förhöjda nivåer. Detta kan ske t ex på hög höjd, vid lungsjukdom eller PAH. De mekanismer som medför att lungkretsloppets blodådror drar sig samman vid låga syrgashalter är ofullständigt utredda. Det behövs också, som komplement till syrgas och behandling av den underliggande orsaken, nya behandlingar för förhöjt blodtryck i lungkretsloppet då detta orsakas av låg syrgashalt. Genom att bättre förstå de mekanismer som ligger till grund för att lungkretsloppets blodådror drar sig samman vid låg syrgashalt kan vi på sikt möjligen också lära mer om vad som kan orsaka förhöjt blodtryck i lungkretsloppet vid andra tillstånd, såsom PAH, eftersom mekanismerna för kärlsammandragning kan vara gemensamma för olika former av PH.

Syftet med det aktuella avhandlingsarbetet har varit att utvärdera mekanismer, som kan bidra till att lungkretsloppets blodådror drar ihop sig vid låg syrgashalt samt att hitta nya behandlingsstrategier och nya diagnostiska metoder vid förhöjt blodtryck i lungkretsloppet av olika orsaker. På lång sikt syftar avhandlingsarbetet till att förbättra prognosen för patienter med förhöjt blodtryck i lungkretsloppet.

I **delstudie I** bekräftades att dödligheten vid PAH är hög. Det visade sig att majoriteten av patienterna inte klarade sig med endast ett läkemedel mot det förhöjda blodtrycket i lungkretsloppet och att behandling med flera läkemedel redan från början skulle kunna vara ett sätt att snabbare och mer effektivt sänka blodtrycket och förbättra hjärtats pumpförmåga. De patienter som hade lägre motstånd i lungcirkulationen och som hade en bättre fysisk prestationsförmåga efter

att behandling påbörjats hade en bättre prognos. Att patienter med PAH diagnostiseras sent i sjukdomsförloppet kan vara en bidragande orsak till den dåliga prognosen. I **delstudie VI** sökte vi därför efter nya markörer i blodet, som skulle kunna bidra till att tidigare ställa diagnosen PAH. I studien fann vi flera sådana markörer, som var förhöjda hos patienter med bindvävssjukdomen systemisk skleros och som senare skulle komma att utveckla PAH. Dessa markörer skulle kunna användas vid screening för PAH hos patienter med systemisk skleros. PAH är vanligare vid bindvävssjukdomar och särskilt vid systemisk skleros. Patienter med systemisk skleros och PAH har dessutom den allra högsta dödligheten. De markörer vi identifierade speglar en störd kärltillväxt och en aktiv inflammation. Det är processer som kan bidra till utvecklingen av PAH. Vi visade också att flera sådana markörer var förhöjda vid PAH-diagnosen hos patienter med PAH av okänd anledning och på basen av systemisk skleros. Dessa markörer skulle kunna bidra till tidigare diagnos av PAH även när orsaken inte är systemisk skleros. Avslutningsvis så fann vi att flera av de markörer vi identifierat som förhöjda vid PAH korrelerade till graden av hjärtsvikt och fysisk prestationsförmåga hos patienterna. Högre halter av ett par markörer var dessutom förknippade med sämre överlevnad. Dessa markörer skulle kunna användas för att identifiera patienter i behov av mer aggressiv behandling.

I **delstudierna II, IV och V** undersökte vi om endotelin, cyclooxygenase-2, tromboxane A₂ och adenosindifosfat bidrog till att blodådrorna i lungkretsloppet drog ihop sig vid låg syrgashalt. Endotelin, tromboxane A₂ och adenosindifosfat är ämnen som drar ihop blodådrorna i lungkretsloppet medan cyclooxygenase-2 är ett enzym som kan producera tromboxane A₂. Vi undersökte detta i en grismodell. Vi visade att såväl endotelin, som cyclooxygenase-2 och tromboxane A₂ bidrog till att förstärka den kärlsammandragande effekten av låg syrgashalt samt att de verkar spela en roll särskilt då den låga syrehalten kvarstår under en längre tid. Adenosindifosfat kan vara en icke obligatorisk faktor, som bidrar till kärlsammandragningen i lungkretsloppet vid låg syrgashalt, men vi kunde inte med säkerhet visa det i vår grismodell. Resultaten i **delstudie II** talade vidare för att läkemedlet tezosentan, som blockerar effekten av endotelin, skulle kunna vara verksamt för att effektivt behandla förhöjt blodtryck i lilla kretsloppet när detta orsakats av låg syrgashalt. I **delstudie III** visade vi att det kärlvidgande och hjärtstärkande läkemedlet levosimendan också skulle kunna användas för att behandla förhöjt blodtryck i lungcirkulationen orsakat av låg syrgashalt då levosimendan sänkte trycket och motståndet i lungkretsloppet samtidigt som hjärtats pumpförmåga stärktes.

Abbreviations

6MWD: 6-minute walk distance
ADP: Adenosine diphosphate
ANOVA: Analysis of variance
AO: Aorta
ATP: Adenosine triphosphate
BNP: Brain natriuretic peptide
CI: Cardiac index
CO: Cardiac output
CO₂: Blood O₂ content
COX: Cyclooxygenase
CTD: Connective tissue disease
CTD-PAH: Connective tissue disease-associated Pulmonary Arterial Hypertension
DPG: Diastolic pressure gradient
ET: Endothelin
FGF-2: Fibroblast growth factor-2
F_iO₂: Inspiratory oxygen fraction
Hb: Haemoglobin
hPAH: hereditary pulmonary arterial hypertension
HPV: Hypoxic pulmonary vasoconstriction
HR: Heart rate
IL: Interleukin
iPAH: idiopathic pulmonary arterial hypertension
LCPR: Lund Cardio Pulmonary Register
PAP: Pulmonary artery pressure
n: Number of patients
NO: Nitric oxide
NT-proBNP: N-terminal pro-hormone of brain natriuretic peptide
pO₂: Blood partial O₂ pressure
PA: Pulmonary artery
PAH: Pulmonary arterial hypertension
PAP: Pulmonary artery pressure
PAWP: Pulmonary arterial wedge pressure
PIGF: Placenta growth factor
PH: Pulmonary hypertension

PVR: Pulmonary vascular resistance
RA: Right atrium
RAP: Right atrial pressure
RHC: Right heart catheterization
 r_s : Spearman's correlation coefficient
SD: Standard deviation
SEM: Standard error of the mean
SO₂: Blood O₂ saturation
SPAHR: Swedish Pulmonary Arterial Hypertension Register
SSc: Systemic sclerosis
SSc-PAH: Systemic sclerosis-associated Pulmonary Arterial Hypertension
SV: Stroke volume
SvO₂: Mixed venous oxygen saturation
sVEGFR-1: soluble vascular endothelial growth factor receptor-1
SVR: Systemic vascular resistance
TNF- α : Tumour necrosis factor- α
TXA₂: Thromboxane A₂
VEGF: Vascular endothelial growth factor
VE/VCO₂: Ventilator equivalents for carbon dioxide
VO₂: Oxygen uptake
WHO: World Health Organization
WU: Wood units

Introduction

Pulmonary Hypertension

Pulmonary hypertension (PH) is defined by a mean pulmonary artery pressure (PAP) ≥ 25 mmHg at rest as measured by right heart catheterization (RHC). PH can be pre-capillary, post-capillary, or combined pre- and post-capillary, depending on the magnitude of the pulmonary arterial wedge pressure (PAWP), diastolic pressure gradient (DPG) and pulmonary vascular resistance (PVR). Patients with pre-capillary PH exhibits PAWP ≤ 15 mmHg, whereas patients with post-capillary PH exhibits PAWP > 15 mmHg. Isolated post-capillary PH is defined by a DPG < 7 mmHg and/or a PVR ≤ 3 WU, whereas combined pre- and post-capillary PH is defined by a DPG ≥ 7 mmHg and/or a PVR > 3 Wood units (WU) (Table 1) (1).

Table 1 Haemodynamic Definitions of Pulmonary Hypertension

Adapted from Galie et al., 2015 (1).

DEFINITION	CHARACTERISTICS
Pulmonary hypertension	mean PAP ≥ 25 mmHg
Pre-capillary pulmonary hypertension	mean PAP ≥ 25 mmHg PAWP ≤ 15 mmHg
Post-capillary pulmonary hypertension	mean PAP ≥ 25 mmHg PAWP > 15 mmHg
Isolated post-capillary pulmonary hypertension	DPG < 7 mmHg and/or PVR ≤ 3 WU
Combined post-capillary and pre-capillary pulmonary hypertension	DPG ≥ 7 mmHg and/or PVR > 3 WU

According to aetiology, PH is classified into five different groups; (i) pulmonary arterial hypertension (PAH), (ii) PH due to left heart disease, (iii) PH due to lung diseases and/or hypoxia, (iv) chronic thromboembolic PH and other pulmonary artery obstructions, and (v) PH with unclear and/or multifactorial mechanisms (Table 2) (2).

Specific PH-targeted medications are approved for treating patients with PAH or chronic thromboembolic PH. However, patients with chronic thromboembolic PH should preferentially be treated by thrombendarterectomy and pharmacological treatment is only proven beneficial for patients with distal and inoperable lesions or

recurrent alternatively persistent PH after surgery. Lung transplantation is a final resort for patients with end-stage PAH or chronic thromboembolic PH (1).

Table 2 Clinical Classification of Pulmonary Hypertension

Adapted from Simonneau et al., 2013 (2).

1. PULMONARY ARTERIAL HYPERTENSION
<ul style="list-style-type: none"> 1.1. Idiopathic 1.2. Heritable (bone morphogenetic protein receptor 2 mutation or other mutations) 1.3. Drugs and toxins induced 1.4. Associated with connective tissue diseases, human immunodeficiency virus infection, portal hypertension, congenital heart disease or schistosomiasis
1'. PULMONARY VENO-OCCLUSIVE DISEASE AND/OR PULMONARY CAPILLARY HEMANGIOMATOSIS
1''. PERSISTENT PULMONARY HYPERTENSION OF THE NEWBORN
2. PULMONARY HYPERTENSION DUE TO LEFT HEART DISEASE
<ul style="list-style-type: none"> 2.1. Left ventricular systolic dysfunction 2.2. Left ventricular diastolic dysfunction 2.3. Valvular disease 2.4. Congenital/acquired left heart inflow/outflow tract obstruction and congenital cardiomyopathies 2.5. Congenital/acquired pulmonary veins stenosis
3. PULMONARY HYPERTENSION DUE TO LUNG DISEASES AND/OR HYPOXIA
<ul style="list-style-type: none"> 3.1. Chronic obstructive pulmonary disease 3.2. Interstitial lung disease 3.3. Other pulmonary diseases with mixed restrictive and obstructive pattern 3.4. Sleep-disordered breathing 3.5. Alveolar hypoventilation disorders 3.6. Chronic exposure to high altitude 3.7. Developmental lung diseases
4. CHRONIC THROMBOEMBOLIC PULMONARY HYPERTENSION AND OTHER PULMONARY ARTERY OBSTRUCTIONS
5. PULMONARY HYPERTENSION WITH UNCLEAR AND/OR MULTIFACTORIAL MECHANISMS

Pulmonary Arterial Hypertension

PAH is a rare disease, which is haemodynamically defined by the presence of pre-capillary PH with a PAWP \leq 15 mmHg and PVR $>$ 3 WU in the absence of other causes of pre-capillary PH (1). In European populations, the prevalence varies between 15 and 26 cases per million inhabitants and the annual incidence varies between 2.4 and 7.6 cases per million inhabitants (3-5). PAH can be idiopathic (iPAH), hereditary (hPAH), drugs and toxins induced or associated with other diseases, such as congenital heart diseases or connective tissue diseases (CTD) (Table 2) (2). The most common CTD associated with the development of PAH is systemic sclerosis (SSc) (3, 5). Patients with PAH are predominately diagnosed in late stages of the disease, most often in functional class III-IV (3, 5, 6). This may be due to the rarity, but also the progressive worsening of the disease.

PAH is due to narrowing and obstruction of the pulmonary arterial lumen by pulmonary vasoconstriction and pulmonary vascular remodelling, which involves intimal and medial proliferation, inflammatory infiltrates and fibrosis, as well as in-situ thrombosis and the development of characteristic plexiform lesions (7). Recent data furthermore suggests that PAH may additionally include pulmonary venous remodelling (8).

PH imposes an increased afterload on the right ventricle leading to increased right ventricular wall tension, which in turn causes a compensatory hypertrophy of the right ventricular walls. As the disease progresses, the right ventricle subsequently dilates and begins to fail. The most common cause of death in PAH is right heart failure (9). Untreated subjects with PAH display a median survival of only 2.8 years, or if associated with SSC no longer than approximately one year (10). Survival for patients with PAH is still poor and three-year survival rates of approximately 50-60 % have been reported in incident cohorts despite modern PH-targeted drugs (11, 12). In Sweden, an incident cohort of PAH patients included in the Swedish PAH Register (SPAHR) from 2008 and onwards had three-year survival rates of 71 % (Rådegran et al. Characteristics and survival of adult Swedish PAH and CTEPH patients 2000-2014, *Submitted manuscript*).

The pathogenesis of PAH is multifactorial, complex and incompletely understood. Endothelial dysfunction with increased production of vasoconstrictor/pro-proliferative substances, such as endothelin (ET) and thromboxane A₂ (TXA₂), and decreased production of vasodilator/anti-proliferative substances, such as nitric oxide and prostacyclin, contributes. Inflammation, metabolic reprogramming, circulating bone marrow-derived cells and altered levels of vascular growth factors and angiostatic factors, as well as micro ribonucleic acids, may also add to the pathogenic process. The genetic background, including mutations in the genes for bone morphogenic protein type II receptor and activin receptor-like kinase-1, can contribute to the susceptibility and severity of disease. Environmental factors, such as hypoxia, may also influence the severity of PH (8, 13).

Before 2001, intravenous administration of the pulmonary vasodilator epoprostenol, a synthetic prostacyclin, was the only treatment available for patients with PAH. Over the past 15 years, several pulmonary vasodilatory drugs, available for per oral use, have, however, been developed and approved for the treatment of PAH. In Europe, these PH-targeted drugs include single ET_A- (ambrisentan) and dual ET_A and ET_B (bosentan, macitentan) receptor antagonists, phosphodiesterase-5 inhibitors (sildenafil, tadalafil) and a stimulator of soluble guanylate cyclase (riociguat). Additional synthetic prostacyclins have furthermore been approved for inhalation (iloprost), subcutaneous and intravenous (treprostenil) use (1). The administration of parental prostacyclin analogues has become less cumbersome due to modern technologies. A randomized controlled trial investigating the effect of a

per oral prostacyclin receptor agonist (selexipag) in PAH was furthermore recently published with positive results (14). A small proportion of patients, with better survival, defined as vasoreactive upon RHC, may moreover be treated with calcium channel blockers (15, 16). Nevertheless, PAH is still incurable and lung transplantation is a last resort (1).

Table 3 Risk Assessment in Pulmonary Hypertension

Adapted from Galiè et al., 2015 (1). Abbreviations not defined in text include: BNP, brain natriuretic peptide; RA, right atrium; SvO₂, mixed venous oxygen saturation; VE/VCO₂, high ventilator equivalents for carbon dioxide; VO₂, oxygen uptake.

Determinants of prognosis (Estimated 1-year Mortality)	Low Risk < 5 %	Intermediate Risk 5-10 %	High Risk > 10 %
Clinical signs of right heart failure	Absent	Absent	Present
Progression of symptoms	No	Slow	Rapid
Syncope	No	Occasional syncope	Repeated syncope
WHO functional class	I, II	III	IV
6MWD	> 440 m	165-440 m	< 165 m
Cardiopulmonary exercise testing	Peak VO ₂ > 15 ml/min/kg (> 65 % of predicted) VE/VCO ₂ slope < 36	Peak VO ₂ 11-15 ml/min/kg (35-65 % of predicted) VE/VCO ₂ slope 36-44.9	Peak VO ₂ < 11 ml/min/kg (< 35 % of predicted) VE/VCO ₂ slope ≥ 45
NT-proBNP levels	< 300 ng/l (BNP < 50 ng/l)	300-1400 ng/l (BNP 50-300 ng/l)	> 1400 ng/l (BNP > 300 ng/l)
Imaging (echocardiography, cardiac magnetic resonance imaging)	RA area < 18 cm ² No pericardial effusion	RA area 18-26 cm ² No or minimal pericardial effusion	RA area > 26 cm ² Pericardial effusion
Haemodynamics	RAP < 8 mmHg CI ≥ 2.5 l/min/m ² SvO ₂ > 65 %	RAP 8-14 mmHg CI 2.0-2.4 l/min/m ² SvO ₂ 60-65 %	RAP > 14 mmHg CI < 2.0 l/min/m ² SvO ₂ < 60 %

With respect to the treatment of PAH patients with PH-targeted medications, guidelines from the European Society of Cardiology and European Respiratory Society previously recommended initial monotherapy with sequential addition of further drugs or lung transplantation in case of an “inadequate clinical response” (17). In August 2015, the AMBITION study, could for the first time demonstrate superiority for initial combination therapy over initial monotherapy in terms of time to clinical failure in a randomized controlled trial (18). This year, new guidelines were published, which advocate either initial monotherapy or initial combination therapy with sequential addition of further drugs or lung transplantation in case of an “inadequate clinical response” (1). Initial monotherapy is solely recommended

as an alternative for patients in WHO functional class II or III with a low to intermediate risk (1).

According to these most recent guidelines, an adequate clinical response is for most patients considered the achievement of a stable, “low risk” profile, according to a risk assessment tool (Table 3) (1). A similar strategy was advocated for in the former guidelines (17). The risk assessment is based on a number of factors, including clinical presentation, exercise capacity, laboratory workup, imaging and haemodynamic parameters. The variables and cut-off levels included are, however, largely based on expert opinion (1). The utility of the parameters as prognostic markers at time of diagnosis are well validated, whereas only a few, smaller studies have confirmed their value for prognostication at follow-up after treatment initiation (19). Only one study has retrospectively confirmed the efficacy of some variables, namely functional class, cardiac index (CI), mixed venous oxygen saturation and plasma levels of the N-terminal pro-hormone of brain natriuretic peptide (NT-proBNP), as treatment goals (20).

As PAH is still associated with a poor prognosis there is a need for the development of new treatments and treatment strategies. Specifically, investigations into variables of prognostic value at follow-up are warranted to improve the risk stratification on which therapeutic decisions are based. Further studies are also necessary to better characterize the response to combinations of PH-targeted medications, especially as first-line treatment.

Hypoxic Pulmonary Vasoconstriction



Figure 1. The Club Andino Boliviano Ski Lodge at 5270 m Above Sea Level at Chacaltaya in the Bolivian Andes.

The partial pressure for oxygen in air at this altitude corresponds to a F_iO_2 of approximately 0.10. Photographed by and printed with the permission of Göran Rådegran.

The Swedish physiologists Ulf von Euler and Göran Liljestrand described hypoxic pulmonary vasoconstriction (HPV) in 1946. In an *in vivo* cat model they demonstrated that lowering of the inspiratory oxygen fraction (F_iO_2) from 0.21 (normoxia) to 0.10 (hypoxia) resulted in a prompt increase in PAP, which was fully and immediately reversible when normoxia was resumed (Figure 2). They elegantly concluded that it should lead to; “an adequate distribution of blood flow through various parts of the lung according to the efficiency of aeration” (21). One year later, the phenomenon of HPV was demonstrated in humans (22). The major determinant of HPV is the alveolar oxygen tension, but mixed venous oxygen tension additionally contributes (23). It is possible that arterial oxygenation of the bronchial circulation may also add to the response (24). The degree of HPV reflects the severity of the hypoxic stimulus down to very low oxygen tensions at which the HPV response may be blunted (25, 26). The mechanisms that underlie HPV are despite 70 years of research still not fully elucidated, but important insights have been made.

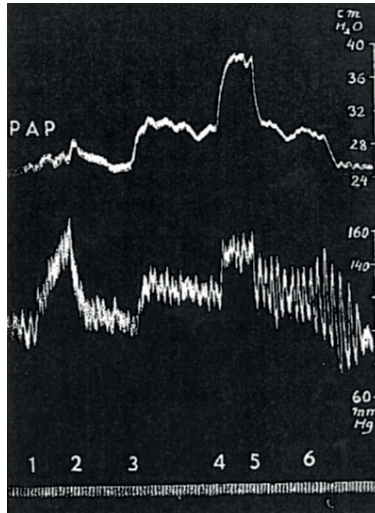


Figure 2. Increase in PAP Evoked by Acute Hypoxia *In Vivo* in the Cat.

First description of HPV by Ulf von Euler and Göran Liljestrand in *Acta Physiologica Scandinavica* in 1946. In the figure, "4" indicates a switch from F_{iO_2} 0.205 (normoxia) to F_{iO_2} 0.105 (hypoxia) and "5" the return to F_{iO_2} 0.205 (normoxia). Hypoxia promptly increased PAP, which immediately returned to normal when normoxia was resumed. Printed with permission from John Wiley and Sons.

The pulmonary resistance arteries are the major site of the HPV response (27), although pulmonary veins also constrict in response to hypoxia (28). There is moreover a general consensus that the vascular smooth muscle cells of the pulmonary resistance arteries possess the ability to independently contract in response to hypoxia. Hence, mechanisms for oxygen sensing, as well as for mediation and effectuation of HPV, are located within the pulmonary arterial smooth muscle cell. The exact mechanisms are intensely debated and the current hypotheses were recently extensively reviewed (29). Briefly, an oxygen sensor may be located within the electron transport chain of the mitochondria, where hypoxia leads to an altered redox and/or energy state of the cell, which through activation of intracellular signalling pathways results in increased intracellular calcium levels and activation of the contractile apparatus. Nonetheless, in the intact animal, the HPV response is influenced by factors extrinsic to the pulmonary arterial smooth muscle cell, such as endothelial-derived vasoactive substances (30), erythrocytes (31, 32), the sympathetic nervous system (33), extracellular pH and partial pressure for carbon dioxide (34). Additionally, there is great variability in the HPV response between species (35-41), with age (42, 43) and even between adult individuals of the same species (25, 44-46). Recently, Sylvester et al. (29) proposed that the HPV response was characterized by different phases in the intact animal. First, an acute phase of HPV lasting 0-30 min, and secondly, a more gradually developed phase of sustained HPV at 30-120 min. There may possibly also be a third phase of

augmented HPV beyond 120 min of sustained hypoxia. Of note, the first acute phase of HPV may not persist if hypoxia is severe. Importantly, the underlying mechanisms may vary between these different phases and in general the acute phase of the HPV response has received more attention, as compared to the sustained phases. I and Göran Rådegran recently authored a review article that characterize the principal pathways of HPV modulation *in vivo*, with special reference to these temporal phases, species and age, which is currently accepted for publication with minor revisions. In this review, we additionally discuss the role of the same modulators in hypoxic pulmonary arterial remodelling and PH, as well as in PH due to lung diseases.

HPV is beneficial in focal hypoxia or when ventilation is unevenly distributed, such as in atelectasis or various chronic lung diseases. HPV then improves ventilation-perfusion matching to improve oxygen uptake and increase arterial oxygenation (47-52). Additionally, HPV is of great importance in the foetus where it redistributes CO to other organs than the lungs, as gas exchange is confined to the placenta (53). In global hypoxia *ex utero*, however, e.g. upon high altitude ascent or in acute deterioration of chronic obstructive pulmonary disease, HPV is generalized and may induce PH, which can lead to acute right heart failure (54, 55). HPV may due to uneven distribution that leads to regional overperfusion additionally contribute to the development of high altitude pulmonary oedema (56). The increase in right ventricular afterload induced by generalized HPV may also add to the reduction in maximum CO and limitation of exercise capacity in hypoxia (57, 58). Finally, generalized HPV may aggravate PH in for instance PAH (8).

Further studies concerning the regulation of pulmonary vascular tone in hypoxia are warranted to additionally clarify the mechanisms behind HPV. In specific, *in vivo* studies are a priority to test hypotheses for relevance (29) and to clarify the modulation of the various phases of the HPV response. The sustained phases of HPV are probably of greater importance in the context of pulmonary pathophysiological processes involving hypoxia, whereas the acute phase is important for instant regulation of ventilation-perfusion matching.

A model of acute hypoxia-induced PH can also serve to assess the effect of new vasodilatory treatments for PH related to acute hypoxia and potentially also for PH of other aetiologies, especially in the setting of acute PH. Additionally, an acute hypoxia model can be a tool to study regulatory mechanisms of pulmonary arterial tone. In that way, findings regarding the regulation of HPV may by extension also help to better understand the pulmonary arterial processes in other forms of PH, including PAH.

Biomarkers in Pulmonary Arterial Hypertension

According to the definition of the National Institute of Health Biomarker Definition Working Group, a biomarker is “a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention”. Biomarkers can be used as diagnostic tools, for staging or classification of the extent of disease, as indicators of prognosis or as predictors of or tools to assess treatment response (59).

Current and Future Biomarkers in Pulmonary Arterial Hypertension

Biomarkers are widely used in the study and clinical care of patients with PAH. Until recently, surrogate end-points, i.e. 6-minute walk distance (6MWD) or PVR, were used to assess the effect of treatment in all clinical trials with PH-targeted drugs. Upon evaluation of PAH patients in the clinic, moreover, biomarkers such as haemodynamic and echocardiographic parameters, 6MWD and natriuretic peptides are assessed to value and steer therapeutic decisions (1). The only circulating biomarkers in clinical use are natriuretic peptides, i.e. brain natriuretic peptide and NT-proBNP, as they are proven to be reliable prognostic factors and retrospectively validated as therapeutic targets (20, 60-62).

In later years, a growing interest to find new circulating biomarkers associated to PAH has evolved. The need for such biomarkers is emphasised by the necessity for earlier diagnosis and enhanced tools to determine treatment response and for prognostication in order to improve survival. Also, the need for new biomarkers is stressed by the dependence on specific expertise for proper echocardiographic evaluation and the invasive nature and sometimes poor availability of RHC for follow-up assessments. Furthermore, as NT-proBNP levels reflect myocardial stress they do not increase until late stages of the disease. In addition to the moderate sensitivity, NT-proBNP has low specificity as it may be affected by a number of different conditions. It was recently suggested that the optimal circulating biomarker for use in PAH should; (i) be observer independent, (ii) non-invasive, (iii) widely available, (iv) disease-specific, (v) a sign of disease activity, and (vi) an optimal treatment target (63).

Circulating biomarkers for PAH screening are of specific interest with respect to patients with SSc, who have a high incidence of PAH and a particularly poor prognosis (64). Screening for PAH in SSc is at present recommended using transthoracic echocardiography, pulmonary function testing with determination of the diffusion capacity for carbon monoxide and NT-proBNP measurements (65). Additional circulating biomarkers could aid in the screening process by making it simpler and by increasing its sensitivity and specificity.

Several circulating biomarkers, including ET-1 and C-terminal pro-ET-1, various interleukins (IL), C-reactive protein, angiotensin, troponins, von Willebrand factor and some micro ribonucleic acids, have over the past years been studied in patients with PAH (63). Even though some results are promising, there is yet no new circulating biomarker, which has been adopted into clinical use. There are moreover very sparse data with respect to potential screening biomarkers in SSc (66), alterations in circulating biomarkers over time and in relation to treatment in patients with PAH (67, 68). Importantly, it has been stressed that a multiple biomarker approach is probably necessary (63, 69).

Aims

The general aims of this doctoral dissertation were to evaluate mechanisms of pulmonary vasoconstriction, with special emphasis on HPV, and to find new treatment strategies and diagnostic approaches for patients with PH of various causes.

The specific aims of each paper were:

- I.** To in a cohort of patients with iPAH, hPAH or CTD-associated PAH (CTD-PAH) estimate survival, identify prognostic variables at time of diagnosis and at follow-up after initiation of PH-targeted treatments, as well as to retrospectively determine the haemodynamic responses to initial mono or combination therapy, respectively.
- II.** To determine the pulmonary haemodynamic effects of the intravenous dual ET_A and ET_B receptor antagonist tezosentan in an *in vivo* porcine model of acute hypoxic PH, with special reference to the temporal aspects and the potential to prevent acute hypoxic PH, to investigate the contribution of ET-1 to the modulation of HPV and tezosentan as a potential treatment for acute hypoxic PH.
- III.** To determine the pulmonary haemodynamic effects of the intravenous inotropic and pulmonary vasodilatory drug levosimendan in an *in vivo* porcine model of acute hypoxic PH to investigate its potential as a treatment for acute hypoxic PH.
- IV.** To determine the contribution of COX-2 and TXA₂ to the modulation of HPV in an *in vivo* porcine model.
- V.** To determine if adenosine diphosphate (ADP), via activation of P2Y₁ and P2Y₁₂ receptors, contributes to the modulation of HPV in an *in vivo* porcine model.
- VI.** To investigate the potential use of a panel of angiogenic and inflammatory biomarkers for earlier PAH diagnosis, evaluation of treatment response and prognostication in patients with SSc-associated PAH (SSc-PAH) or iPAH.

Materials and Methods

Paper I

Study Population

All 77 patients that were ≥ 18 years of age and diagnosed with isolated iPAH, hPAH or CTD-PAH in Lund from 2000 to 2010, and subsequently included in the SPAHR were included in the study. 39 patients had iPAH or hPAH. 38 patients had CTD-PAH, whereof 84 % had SSc-PAH.

Gathering of Data

Medical journal data on age, sex, haemodynamic variables, 6MWD, World Health Organization (WHO) functional class, PH-targeted treatments, clinical events and survival status were gathered from 1 January 2000 to 31 December 2011.

Ethics

The study was ethically approved by the local ethics board in Lund, Sweden (Dnr. 2011/777, Dnr. 2011/368, Dnr. 2010/114).

Statistics

Parametric or non-parametric statistics were used depending on the distribution of data. Differences in haemodynamic variables before and after treatment start were compared using a paired t-test or Wilcoxon Signed Rank test. When comparing two groups, a t-test or Rank Sum test was performed. Survival rates were estimated using the Kaplan-Meier method and differences between groups were investigated using the Log-Rank test. COX regression was used to calculate age-adjusted hazard ratios for standardized values of potential prognostic markers. Values are presented as mean \pm standard deviation (SD) for continuous variables and absolute for

categorical variables. $P < 0.05$ was considered statistically significant. Statistical analyses were performed in SigmaPlot 11 (Systat Software Inc., San José, CA, USA) and SPSS (IBM SPSS Statistics 20, IBM Corp., Armonk, NY, USA).

Papers II-V

***In Vivo* Pig Model of Acute Hypoxic Pulmonary Vasoconstriction**

We established an *in vivo* pig model for HPV at the Department of Experimental Medicine and Surgery at the Panum Institute, University of Copenhagen, Copenhagen, Denmark (Figure 3). Animal care and surgical procedures were performed by trained animal technicians.



Figure 3. *In Vivo* Pig Model for Acute HPV.

The pig is intubated and connected to a respirator where the F_{iO_2} is lowered to 0.10. A Swan-Ganz catheter is inserted for measurements of pulmonary haemodynamics and CO by thermodilution. A catheter for systemic blood pressure measurement is inserted into the aorta. Blood gases are drawn from the pulmonary artery and the aorta. Drugs are infused into the right atrium by an infusion pump. Photographed by and printed with the permission of Göran Rådegran.

Anaesthesia and Ventilation

Prior to the experiments, all pigs were fasted overnight with free access to water. All pigs were pre-medicated intramuscularly with 1 ml/10 kg of a mixture of 6.25 ml Narcoxyl vet (Xylazin 20 mg/ml; Intervet, Roskilde, Denmark), 1.25 ml Ketaminol vet (Ketamin 100 mg/ml; Intervet, Roskilde, Denmark), 2 ml Metadon (10 mg/ml; Nycomed, Roskilde, Denmark) and 2 ml Turbogesic (Butorphanoltartrat

10 mg/ml; Scanvet, Fredensborg, Denmark) in Zoletil 50 vet Tørstof (Virbac, Kolding, Denmark). Anaesthesia was maintained with 1.5 ml/kg/h of Propofol (10 mg/ml; B Braun Medical A/S, Frederiksberg, Denmark) and 1 ml/10 kg/h of Fentanyl (50 µg/ml; Janssen-Cilag, Birkerød, Denmark) administered intravenously throughout the experiments with a B Braun Infusomate® Space (B Braun Medical A7S, Frederiksberg, Denmark) and a Braun Perfusor® Compact infusion pump (B Braun Medical A/S, Frederiksberg, Denmark), respectively. After intubation the lungs were mechanically ventilated with a respirator (Dameca, Rødovre, Denmark), and the F_iO₂ monitored using an oxygen sensor and a medical oxygen apparatus OM871 (Dameca, Rødovre, Denmark). After the experiments, the animals were euthanized by a 10-15 ml mixture of Pentobarbital and Lidocainhydrochloride (200 and 20 mg/ml, respectively; Veterinærapoteket, University of Copenhagen, Copenhagen, Denmark).

Invasive Procedures, Measurements and Haemodynamic Surveillance

Two small skin incisions were made, one along the medial line of the throat and one in the groin, to locate the internal jugular vein and the common carotid artery, as well as the femoral artery, respectively. An infusion catheter (Baby feeding tube No. 8, Unomedical, Birkerød, Denmark) was inserted through the internal jugular vein and advanced into the right atrium. Catheters for arterial pressure measurements (Baby feeding tube No. 8, Unomedical, Birkerød, Denmark) were positioned in the aortic arch, through the common carotid artery, as well as in the femoral artery. The femoral arterial catheter was used as a back-up if problems with the aortic catheter would occur.

A Swan Ganz catheter (Edwards Lifesciences, Irvine, CA, USA), introduced through the internal jugular vein, was used to measure mean right atrial pressure (RAP), mean PAP, PAWP, as well as CO in triplicates by thermodilution, infusing 10 ml boluses of iced saline (70). CO was calculated using a Baxter-Vigilance® monitor (Edwards Critical-Care, Saint-Prex, Switzerland). PAWP and CO was monitored intermittently.

Electrocardiogram, heart rate (HR), mean aortic blood pressure, mean RAP and mean PAP were monitored on a surveillance screen (Hewlett Packard, Berkshire, UK). The pressures were measured with pressure transducer sets (Edwards Lifesciences LLC, Irvine, CA, USA) connected to water filled connecting tubes. The data were recorded using a PowerLab 8/30 system (AD Instruments Ltd., Oxfordshire, UK). Saturation was monitored by a pulse oximetry probe (Philips M1193A, Philips Medico A/S, Copenhagen, Denmark) positioned on the tails of the pigs, as a continuous feedback during the experiments. PVR, systemic vascular resistance (SVR) and stroke volume (SV) were calculated according to the following formulas: $PVR = (\text{mean PAP} - \text{PAWP})/\text{CO}$, $SVR = (\text{mean aortic blood pressure} - \text{mean RAP})/\text{CO}$, and $SV = \text{CO}/\text{HR}$.

Blood samples from the pulmonary artery and aortic arch were drawn for immediate blood gas analysis with an ABL System 605 and an OSM 3 Hemoximeter apparatus or an ABL 700 Series Hemoximeter apparatus (Radiometer, Copenhagen, Denmark). Determining blood aorta (AO) and pulmonary artery (PA) O₂ saturation (S_{AO}O₂, S_{PA}O₂), haemoglobin concentration (Hb_{AO}, Hb_{PA}), O₂ pressure (p_{AO}O₂, p_{PA}O₂) and pH (pH_{AO}, pH_{PA}). This allowed for calculation of aorta and pulmonary artery O₂ content (C_{AO}O₂, C_{PA}O₂), where C_{AO}O₂ = (Hb_{AO} · 1.34 · S_{AO}O₂) + (0.0031 · p_{AO}O₂), and C_{PA}O₂ = (Hb_{PA} · 1.34 · S_{PA}O₂) + (0.0031 · p_{PA}O₂), O₂ extraction = C_{AO}O₂ – C_{PA}O₂, and O₂ consumption = CO · (C_{AO}O₂ – C_{PA}O₂). In the formulas; SO₂ is expressed as a fraction of 1.0, Hb as g/dl, pO₂ as mmHg, and the correction factors 1.34 as ml/g and 0.0031 as ml/dl/mmHg.

Induction of Hypoxia and Initiation of Treatments

During the establishment of our *in vivo* hypoxia pig model we noticed that the pigs could develop a circulatory collapse with falling blood pressure, a high pulse, and occasionally ventricular arrhythmias leading to death, if hypoxia was induced too rapidly. We therefore induced hypoxia gradually by lowering the F_iO₂ first to approximately 0.15 and then to approximately 0.10, which was our target F_iO₂. We also administered fluids, i.e. isotonic sodium chloride or Ringer solution, during the induction of hypoxia. Initially, fluids were administered in relation to the needs of each pig to remain stable circulatory conditions, but we subsequently standardized the administration of fluids to 1000 ml per pig. The respiratory frequency and the tidal volumes were kept constant. Baseline hypoxia measurements were made after 15 min at a F_iO₂ of approximately 0.10. Thereafter hypoxia was maintained at a F_iO₂ of approximately 0.10 during control experiments (Paper IV-V) or administration of the pharmacological compounds (Paper II-V) or placebo (Paper III). In Paper II, intravenous tezosentan was also administered during normoxia prior to the induction of hypoxia.

Drugs

In Paper II, the dual ET_A and ET_B receptor antagonist tezosentan (Actelion, Allschwill, Switzerland) was administered as a manual bolus injection of 5 mg/kg in the right atrium.

In Paper III, levosimendan (Simdax; Orion Pharma, Espoo, Finland) at a concentration of 2.5 mg/ml was administered in the right atrium by an infusion pump (Model 11 Plus; Harvard Apparatus, Holliston, MA, USA). Levosimendan was administered at a dose of 6 mg/kg for 10 minutes, and thereafter 0.1 mg/kg/min for 10 minutes, followed by 0.2 mg/kg/min throughout the experiment. The placebo solution was administered at the same infusion rate.

In Paper IV, the COX-2 inhibitor nimesulide (Tocris Bioscience, Bristol, UK) was administered at a dose of 3-10 mg/kg body weight in pilot experiments with no trend for a different response. A dose of 10 mg/kg was chosen for the rest of the experiments. The TXA₂ receptor antagonist daltroban was given at total doses of 5-10 mg/kg estimated lung weight (approximated to 1 kg) in pilot experiments and found to give a similar response. A dose of 5 mg/kg estimated lung weight was chosen for the following experiments. Infusions were made in the right atrium by an infusion pump (Model 11 Plus; Harvard Apparatus, Holliston, MA, USA).

In Paper V, the P2Y₁ receptor antagonist MRS2500 (Tocris Bioscience, Bristol, UK) was administered as a loading dose of 1 mg during 2 min, followed by a continuous infusion over 8 min. The total dose was 2.5 mg for five pigs and 5 mg for two pigs, with no trend for a different response. Physiological sodium chloride was administered at the same infusion rate as MRS2500 to evaluate if there was a shear stress induced effect on mean PAP by the infusion. The P2Y₁₂ receptor antagonist cangrelor (The Medicines Company, Parsippany, NJ, USA) was administered first as a loading dose of 30 µg/kg/min for 10 min, and then as a maintenance infusion at a dose of 4 µg/kg/min throughout the experiment. ADP (A2754; Sigma-Aldrich Danmark A/S, Brøndby, Denmark) was administered at a dose of 70 µg/kg/min. Infusions were made in the right atrium by an infusion pump (Model 11 Plus; Harvard Apparatus, Holliston, MA, USA or B Braun Infusomate® Space; B Braun Medical A/S, Frederiksberg, Denmark).

Ethics

The studies were ethically approved by Dyreforsøgstilsynet (2009/561-1621) in Copenhagen, Denmark, and performed according to the guidelines of the Department of Experimental Medicine and Surgery at the Panum Institute, Copenhagen, Denmark.

Statistics

Parametric or non-parametric statistics were used depending on the distribution of data. For repeated measurements; paired t-test, Wilcoxon Signed Rank Test, One Way Repeated Measures Analysis of Variance (ANOVA) or Friedman Repeated Measures ANOVA on Ranks were used. For group comparisons; t test, Mann-Whitney Rank Sum Test, one-way ANOVA or ANOVA on Ranks were used. Post hoc analyses were performed using Tukey's HSD test or Student-Newman-Keuls' Method. The significance level was set at $p < 0.05$. Data are presented as mean \pm standard error of the mean (SEM). Statistical analyses were performed in SigmaPlot 11 (Systat Software Inc., San José, CA, USA).

Paper VI

Study Population

20 patients with PAH (9 iPAH, 11 SSc-PAH) were included in the study together with ten SSc patients who did not develop PAH during a median follow-up period of 12 (interquartile range 10-13) years. Blood samples from eight controls without PAH or SSc had previously been gathered (71).

Plasma Samples and Enzyme-linked Immunosorbent Assay

For PAH patients, plasma samples were retrieved at PAH diagnosis and at follow-up after a median of 4 (interquartile range 3-9.8) months after initiation of PH-targeted treatments. For ten SSc-PAH patients, additional plasma samples were retrieved a median of 2 (interquartile range 0.8-3) years *before* the diagnosis of PAH was set. For SSc patients who did not develop PAH, plasma samples were collected at two time-points separated by a median of 12 (interquartile range 10-13) years.

Plasma was collected after centrifugation of venous blood sampled from the pulmonary artery or a peripheral vein. Plasma samples were stored in the Lund Cardio Pulmonary Register (LCPR) at the Section for Heart Failure and Valvular Disease or in the Systemic Sclerosis Biobank at the Section for Rheumatology, Skåne University Hospital, Lund, Sweden, at -80°C until the analyses were performed.

Placenta growth factor (PlGF), angiopoietin-1 receptor, vascular endothelial growth factor (VEGF)-A, VEGF-D, soluble VEGF receptor 1 (sVEGFR-1), IL-6, IL-8 and tumour necrosis factor- α (TNF- α) were analysed in venous plasma using multiplex sandwich immunoassays manufactured by Meso Scale Discovery (Rockville, MD, USA). Each substance was measured in duplicates and the mean was calculated. Samples with a coefficient variation > 20 were excluded.

Gathering of Data

Complementary data, including age, sex, haemodynamic variables, 6MWD, NT-proBNP, PH-targeted treatments and survival status, was gathered from medical journals.

Ethics

The study was ethically approved by the local ethics board in Lund (Dnr. 2011/777, Dnr. 2011/368, Dnr. 2010/114, Dnr. 2008/590). Informed consent was retrieved from the study participants.

Statistics

Non-parametric statistics were used. For group comparisons, a Mann-Whitney Rank Sum Test was performed. For paired comparisons, a Wilcoxon signed-rank test was performed. Spearman's test was used for correlation analysis. To investigate associations with risk of death, a COX proportional hazards model was used. Continuous variables are expressed as median (interquartile range) and categorical variables are expressed as absolute values (per cent). A p value < 0.05 was considered statistically significant. Statistical analyses were performed in SigmaPlot 11 (Systat Software Inc., San José, CA, USA).

Results and Comments

Paper I

Results

1, 2 and 3-year survival rates for patients with iPAH or hPAH were 89, 78 and 71 %, respectively, and significantly better ($p<0.004$) than for patients with CTD-associated PAH among whom 1, 2 and 3-year survival rates were 84, 55 and 40 %, respectively (Figures 4A and 4B).

For the entire study cohort, it was shown that the proportion of patients that started on first-line monotherapy and that still were alive on monotherapy were 58, 41 and 24 %, respectively, after 1, 2 and 3 years (Figures 4C and 4D). 37.5 % of patients who received first-line monotherapy were escalated to combination therapy already at the first follow-up assessment. Moreover, in a retrospective analysis, first-line combination therapy more greatly decreased ($p<0.018$) PVR than first-line monotherapy and only patients that had received first-line combination therapy had significantly improved ($p<0.043$) CI at the first follow-up assessment (Figure 5). There was no difference in survival between patients who had received first-line monotherapy or combination therapy.

At the baseline assessment, higher mean RAP ($p<0.019$), mean RAP/CI ($p<0.022$) and WHO functional class ($p<0.001$), as well as lower 6MWD ($p<0.002$) were associated with worse outcome. At the first follow-up assessment, higher PVR index ($p<0.009$) and lower 6MWD ($p<0.005$) were associated with worse outcome. Additionally, worse survival rates were found for patients in WHO functional class III/IV ($p<0.003$ vs. WHO functional class I/II) or with a 6MWD < 250 m ($p<0.003$ vs. 6MWD ≥ 250 m) at the baseline assessment and for patients with a 6MWD < 325 m ($p<0.027$ vs. 6MWD ≥ 325 m) at the first follow-up assessment.

Comments

At the time when the present study was performed, initial monotherapy was recommended for the treatment of PAH. Combination therapy was endorsed when the response to monotherapy was not satisfactory (17). We showed that over a 3-year follow-up period only 24 % of patients survived on monotherapy and that 37.5 % of patients were escalated to combination therapy already at the first haemodynamic follow-up assessment. The patients that were escalated to

combination therapy had a more compromised haemodynamic status at the first follow-up. This demonstrates that monotherapy is not sufficient for the majority of patients with PAH.

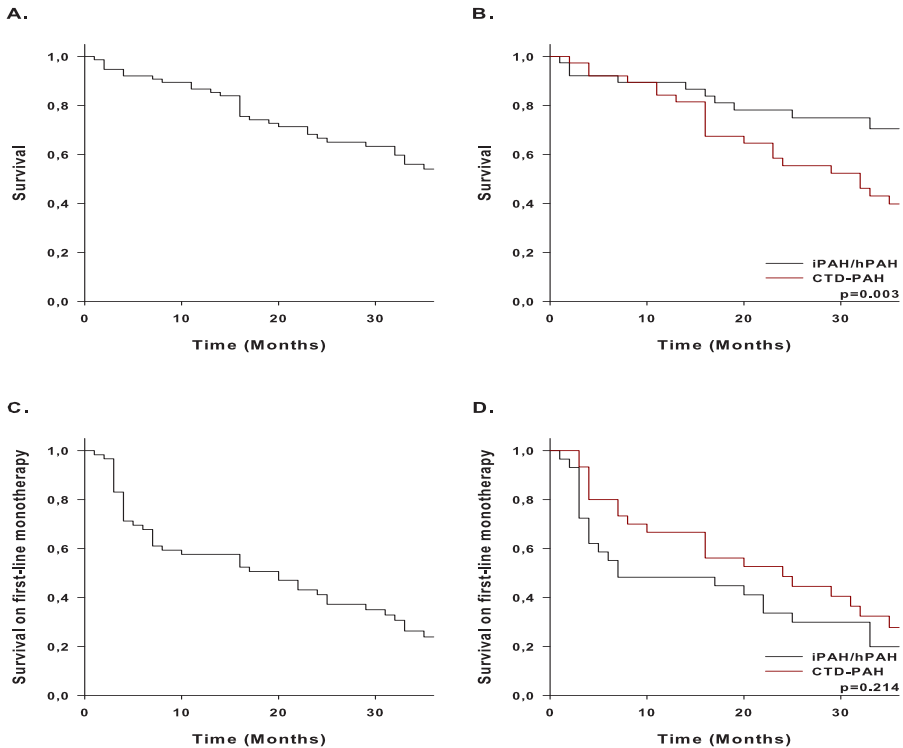


Figure 4. Survival and survival on first-line monotherapy in iPAH/hPAH and CTD-PAH patients at Skåne University Hospital 2000-2011.

1-, 2- and 3-year survival rates for (A) iPAH/hPAH and CTD-PAH combined, (B) iPAH/hPAH and CTD-PAH separately, as well as the proportion of patients that started on monotherapy and were alive on monotherapy after 1, 2 and 3 years for (C) iPAH and CTD-PAH combined and (D) iPAH/hPAH and CTD-PAH separately. Survival rates were significantly worse ($p=0.003$) for patients with CTD-PAH.

Initial combination therapy presented as a theoretically appealing alternative, but there was at the time only two small studies that had investigated effects of initial combination therapy. In the BREATHE-2 trial, which included 33 patients with PAH, there was a non-significant trend for the combination of i.v. epoprostenol and bosentan to result in greater haemodynamic improvements than i.v. epoprostenol alone (72). Additionally, in a retrospective analysis of 23 patients that received first-line i.v. epoprostenol and bosentan, a greater improvement in PVR was demonstrated, as compared to a matched historic cohort that received only i.v. epoprostenol (73). Likewise, we found that the improvement in PVR index to a first treatment follow-up was greater for patients who received first-line combination therapy. We additionally found that CI had only improved significantly at the first treatment follow-up for those who received combination therapy. In contrast to the previous studies, our patient cohort was mostly treated with per oral PH-targeted drugs and some patients were treated with a triple combination of PH-targeted drugs. The analysis of treatment response, however, holds limitations. It was a retrospective analysis with unmatched, small cohorts and the follow-up data may be biased due to loss of more severely ill patients. Nonetheless, the results indicated, in a time when first-line combination therapy was not yet recommended and sparsely used, that first-line combination therapy may more rapidly and potently than first-line monotherapy improve the haemodynamic status of patients with PAH.

Since our study was published, the AMBITION study, comparing first-line combination therapy with ambrisentan and tadalafil to first-line monotherapy with either ambrisentan or tadalafil, found that the time to a first event of clinical failure (defined as a composite of death, hospitalization for worsening of PAH, disease progression or unsatisfactory long-term clinical response) was delayed with first-line combination therapy (18). Consequently, first-line combination therapy is now an option in the present guidelines from the European Society of Cardiology and European Respiratory Society (1).

With respect to prognostication, we identified a higher absolute PVR index and a lower absolute 6MWD at treatment follow-up to be associated with worse outcome. A 6MWD below 325 m at treatment follow-up was furthermore found to discriminate patients with worse survival rates. These findings suggest that PVR index and 6MWD could potentially be used as treatment targets in PAH. Only few studies have previously assessed prognostic markers at treatment follow-up, especially with respect to haemodynamic variables, and the present treatment goals are mainly based on expert opinion (1, 19). Higher absolute PVR at follow-up had previously been shown to be associated with worse outcome in a study comprising patients on i.v. epoprostenol treatment (74) and a lesser magnitude of the change in total pulmonary resistance from baseline to follow-up was associated with worse outcome in two studies comprising patients on i.v. epoprostenol or bosentan, respectively (75, 76). In contrast, our study population was not selected with

reference to a certain treatment, but identified prognostic markers in a real-life population of PAH patients. It is noted that measures of PVR recur as a prognostic marker at treatment follow-up, but is not yet included in the risk assessment tool in the current guidelines (1).

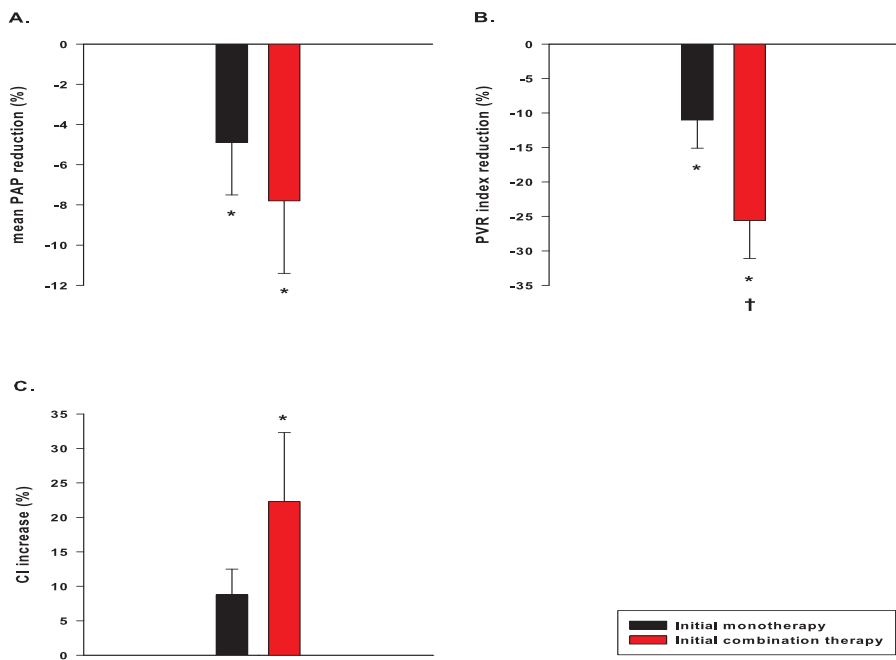


Figure 5. Mean percentage changes in (A) mean PAP, (B) PVR index and (C) CI with initial monotherapy or combination therapy in patients with iPAH or CTD-PAH. Both initial monotherapy and combination therapy significantly ($p < 0.05$) decreased (A) mean PAP and (B) PVR index. The decrease in (B) PVR index was greater with initial combination therapy. Only initial monotherapy ($p < 0.05$) significantly increased CI. A * indicates statistical significance, as compared to baseline; and a † indicates statistical significance, as compared to initial monotherapy.

The 6MWD is a strong predictor of mortality at baseline (77-79). In the present study it was demonstrated that a lower 6MWD at treatment follow-up was also associated with worse outcome and a 6MWD < 325 m at treatment follow-up discriminated patients with better or worse survival rates. Previous studies by Sitbon et al. (75) and Provencher et al. (76) also identified the 6MWD at treatment follow-up as a prognostic marker in populations of PAH patients treated with i.v. epoprostenol or bosentan, respectively. In specific, Sitbon et al. (75) showed that a 6MWD of < 380 m and Provencher et al. (76) that a 6MWD of < 378 m at treatment follow-up were associated with worse survival rates.

It is important to note that the results of the few previous studies (20, 74-76), including our own, which have assessed prognostic markers at treatment follow-up are quite diverse even though common parameters were included in the analyses. This underlines that a broad perspective, including a combination of prognostic markers, is still necessary when evaluating patients with PAH.

Surprisingly, CI was not associated with outcome in our study. To reduce interference by HR, we have now analysed the association of SV index with outcome and found that a lower SV index at diagnosis as well as at treatment follow-up were associated with worse outcome (Table 4). We furthermore found that a higher mean RAP/CI quote, which increases with right ventricular backward and/or forward failure, was associated with worse outcome at baseline, but unfortunately not a stronger prognostic marker than mean RAP alone. In analogy with the previous analysis of SV index, we have now analysed the association of mean RAP/SV index with outcome. We then found that a higher mean RAP/SV index at diagnosis as well as at treatment follow-up were associated with worse outcome (Table 4) and that mean RAP/SV index, in contrast to mean RAP/CI, appeared to be a stronger prognostic marker than mean RAP alone. In summary, these additional results indicate that SV index may be a better variable for prognostication than CI, since there is no interference by HR, and that mean RAP/SV index may be a new prognostic marker with additive information, as it takes into account the presence of both forward and backward failure.

Table 4. Age-Adjusted HR/SD for Death or Transplantation for SV Index and Mean RAP/SV Index at Baseline and Treatment Follow-Up in Patients with iPAH or SSc-PAH

Lower SV index as well as higher mean RAP/SV index were associated with worse outcome both at baseline and at treatment follow-up. Abbreviations not defined in the text include: HR, hazard ratio; and n, number of patients.

Parameter	Baseline			First RHC Follow-Up		
	n	HR/SD	95 % CI	n	HR/SD	95 % CI
SV index	69	0.69	0.48-0.98	55	0.48	0.30-0.78
mean RAP/SV index	57	1.79	1.23-2.60	45	1.52	1.03-2.25

Our results are, in conformance with previous studies on prognostic markers at treatment follow-up (20, 74-76), however, limited by the small number of patients

included. For instance, the number of events in our study restricted us from performing a complete multivariate analysis. We are currently performing a register-based larger, national study in which we explore prognostication at follow-up and potential therapeutic targets in PAH.

Papers II-V

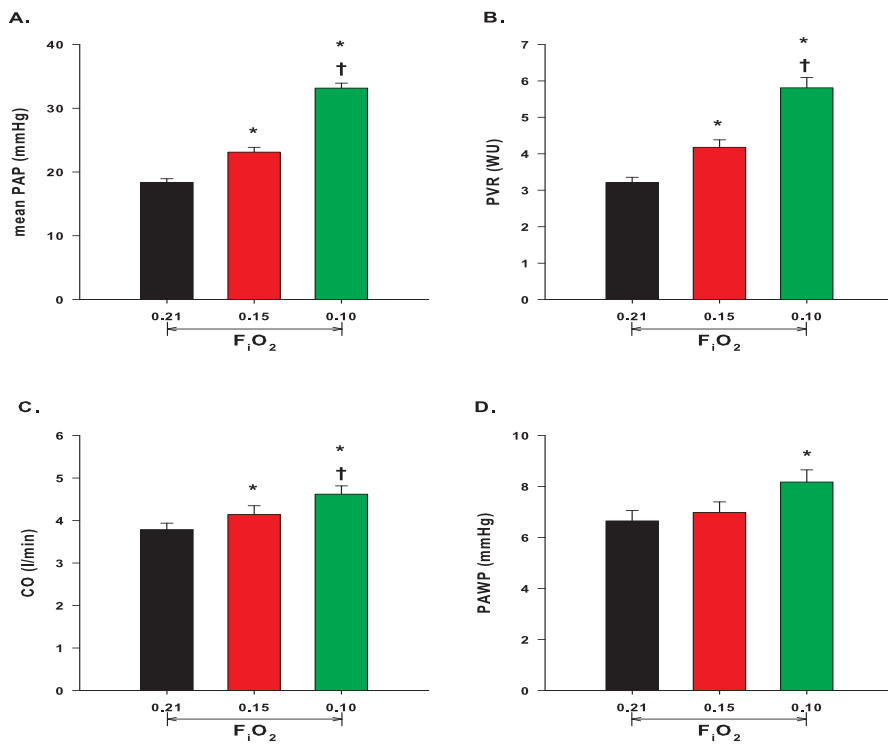


Figure 6. Effects of Acute Normobaric Hypoxia on Pulmonary Haemodynamics During Acute Hypoxia. Acute hypoxia increased ($p < 0.05$) (A) mean PAP, (B) PVR, (C) CO and (D) PAWP. A * indicates statistical significance, as compared to F_iO_2 0.21; and a † indicates statistical significance, as compared to F_iO_2 0.15.

Papers II, IV and V aimed at investigating modulatory mechanisms in HPV. Papers II and III additionally aimed at investigating the effects of two drugs that could potentially be of use as complementary treatments for acute hypoxia-induced PH.

Effects of acute hypoxia

In a pooled analysis of all pigs (n=52) from Papers II-V that were exposed to acute hypoxia without a prior pharmacological intervention, acute hypoxia gradually increased ($p<0.05$) mean PAP by maximally 14.8 ± 0.5 mmHg, PVR by maximally 2.6 ± 0.2 WU and CO by maximally 0.8 ± 0.1 l/min at F_iO_2 0.10 (Figure 6). PAWP (Figure 6), mean RAP and HR increased ($p<0.05$) at F_iO_2 0.10, as compared to normoxia, by 1.5 ± 0.2 mmHg, 2.2 ± 0.2 mmHg and 17.7 ± 3.3 beats/min, respectively. Acute hypoxia gradually decreased ($p<0.001$) SVR by maximally 6.2 ± 0.7 WU at F_iO_2 0.10. Mean aortic blood pressure and SV were not significantly altered by the induction of acute hypoxia. During prolonged hypoxia at F_iO_2 0.10 for 120 min in the control experiments (n=6) there were no significant alterations in any of the measured haemodynamic variables.

Paper II

Results

The dual ET_A and ET_B receptor antagonist tezosentan, administered intravenously during acute hypoxia, was demonstrated to rapidly, within 15 min, decrease ($p<0.05$) mean PAP and PVR and the response stabilized within 30-60 min when tezosentan had decreased mean PAP by 7.5 ± 0.8 mmHg and fully normalized PVR (Figure 7).

Furthermore, intravenous tezosentan administered prior to the induction of acute hypoxia was found to reduce ($p<0.002$) the initial hypoxia-induced increase in right ventricular afterload by attenuating the elevation in mean PAP and fully preventing an increase in PVR above the normoxic level. Moreover, tezosentan decreased mean PAP and PVR by 4.6 ± 0.9 mmHg and 1.9 ± 0.4 WU, respectively, also during normoxia (Figure 8). The systemic circulatory effects of tezosentan are shown in the published article.

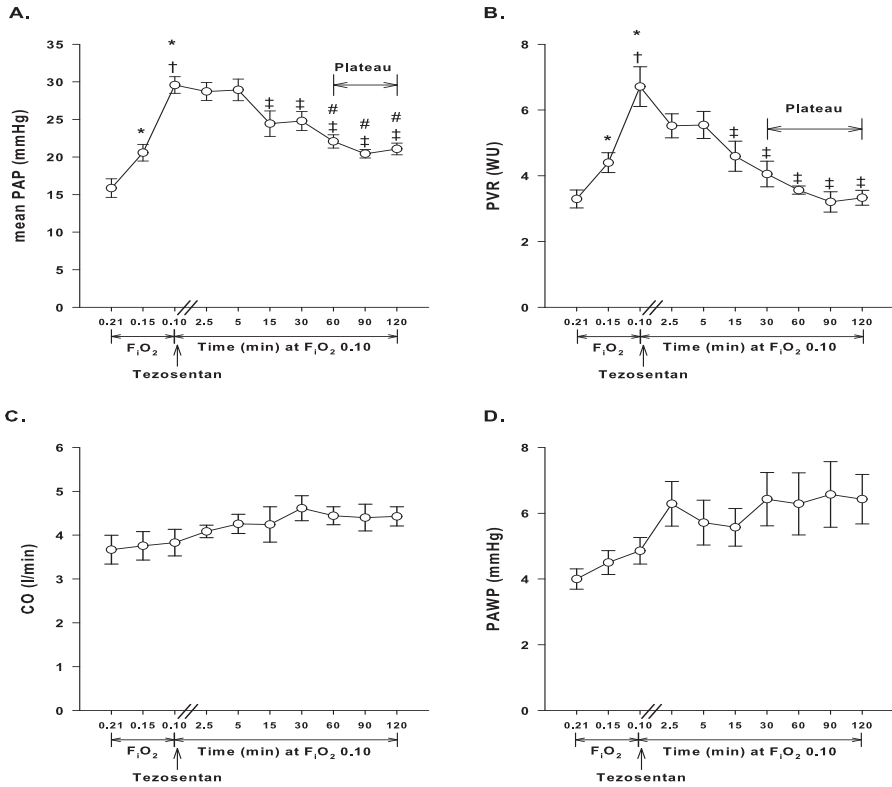


Figure 7. Effects of the dual ET_A and ET_B receptor antagonist Tezosentan on Pulmonary Haemodynamics During Acute Hypoxia.

Hypoxia increased (A) mean PAP ($p < 0.001$) and (B) PVR ($p < 0.05$). Tezosentan, administered during acute hypoxia, decreased (A) mean PAP ($p < 0.05$) to stabilize within 30-60 min and decreased (B) PVR ($p < 0.05$) to fully normalize as in normoxia. (C) CO and (D) PAWP were not altered by tezosentan administered during hypoxia. A * indicates statistical significance, as compared to F_iO₂ 0.21; a † indicates statistical significance, as compared to F_iO₂ 0.15; a ‡ indicates statistical significance, as compared to hypoxia baseline at F_iO₂ 0.10; and a # indicates statistical significance, as compared to F_iO₂ 0.21 after stabilization with tezosentan during acute hypoxia.

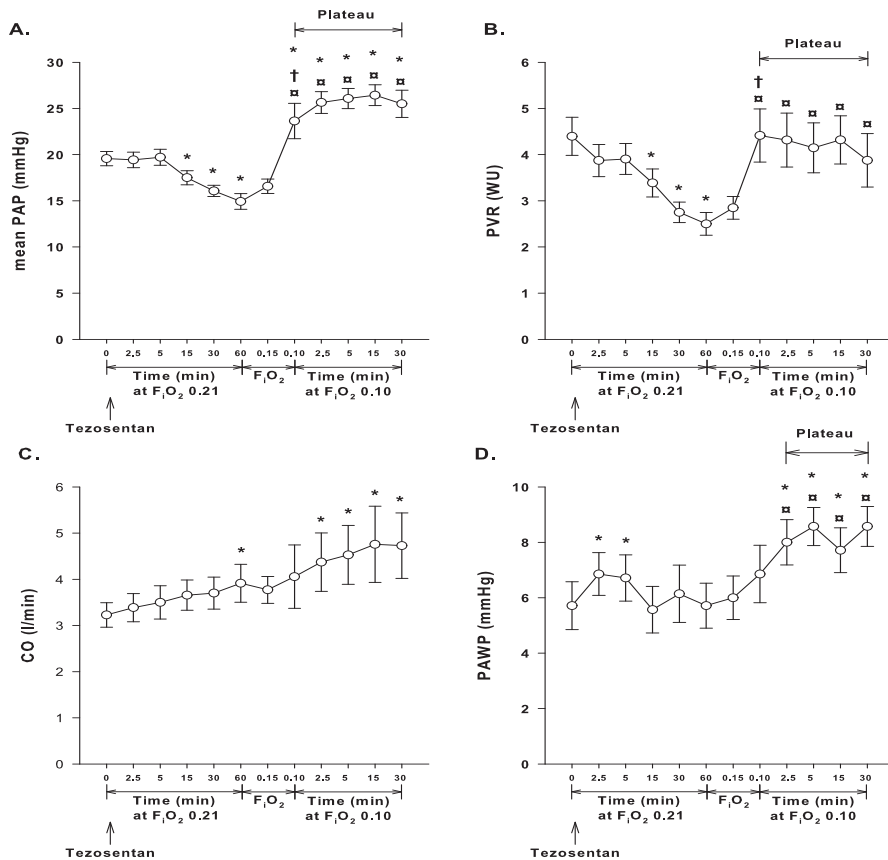


Figure 8. Effects of the dual ET_A and ET_B receptor antagonist Tezosentan on Pulmonary Haemodynamics when Administered Before Acute Hypoxia.

Tezosentan decreased (A) mean PAP ($p < 0.02$) and (B) PVR ($p < 0.001$) when administered during normoxia. Subsequent induction of acute hypoxia increased (A) mean PAP ($p < 0.001$) to a level higher ($p < 0.04$) than the baseline at F_{iO_2} 0.21 and increased (B) PVR ($p < 0.05$) to a level not different from the baseline at F_{iO_2} 0.21. After tezosentan administration during normoxia, (C) CO furthermore increased ($p < 0.02$) and (D) PAWP transiently increased ($p < 0.05$). After induction of acute hypoxia, (C) CO ($p < 0.05$) and (D) PAWP ($p < 0.03$) further increased. A * indicates statistical significance, as compared to the baseline at F_{iO_2} 0.21; a † indicates statistical significance, as compared to F_{iO_2} 0.15; and a □ indicates statistical significance, as compared to the measurement at 60 min during normoxia with tezosentan treatment.

Comments

There are three different ETs, whereof ET-1 is of greatest importance in the cardiovascular system (80, 81). ET-1 is a potent vasoconstrictor (80), which is produced by cleavage of preproendothelin-1 to proendothelin-1, which is further processed to ET-1 by endothelin converting enzymes, primarily in endothelial cells (82). ET-1 production and release is stimulated by a variety of factors, including hypoxia (83-88). ET-1 is released mainly abuminally (89), where it stimulates vasoconstriction by binding to ET_A and ET_B receptors on vascular smooth muscle cells (90). Stimulation of ET_B receptors on endothelial cells, on the other hand, induces vasodilatation by NO and prostacyclin (91, 92). ET_B receptors also mediate lung clearance of ET-1 (93).

The contribution of ET-1 in the modulation of the acute phase of the HPV response appears to vary between species, as ET receptor antagonists reduced the acute phase of HPV in rats (94-96) and pigs (97, 98), but not in dogs (99) or humans (100). Whether ET-1 contributes to potentiate the acute phase of the HPV response or not may also vary with age (101, 102). With prolonged hypoxia for 60-180 min, however, ET-1 has invariably potentiated the sustained HPV response in rats (94, 96), piglets (103, 104), pigs (105), lambs (84) and humans (57, 58, 106, 107). In humans, ET-1 was additionally found to amplify the sustained HPV response for up to several weeks (58) and to limit hypoxic exercise capacity by increasing right ventricular afterload (57, 58). Our findings underline that ET-1 is indeed a strong potentiating modulator of the sustained HPV response in pigs.

With reference to the roles of the ET_A and the ET_B receptor, respectively, ET_B receptors do not appear as important for the modulation of the immediate pulmonary vasoconstrictor response during the acute phase of HPV, as selective ET_B receptor antagonism was without effect on this response in both rats (95, 96) and pigs (97). Additionally, the effect of selective ET_A and dual ET_A and ET_B receptor antagonism on the sustained HPV response in humans were comparable (57, 58), indicating that ET_B receptors did not play a major role. In rats, however, ET_B receptor activation partly mediated a transient pulmonary vasodilatory phase, which followed the immediate pulmonary vasoconstrictor response to hypoxia (96). Our findings in pigs underline that vasoconstrictor ET receptors are far more important in the modulation of the sustained HPV response than are vasodilator ET_B receptors.

From a therapeutic perspective, both single ET_A and dual ET_A and ET_B receptor blockade have been demonstrated to alleviate right ventricular afterload when administered during established acute hypoxic PH (84, 96, 97, 102-105, 108, 109). These studies were, however, mostly performed either in a small rodent model (96), in neonates (84, 102, 103), with an inhaled ET receptor blocker (104), with subacute per oral administration of the ET receptor blocker (109) or in dogs (108), a species with a particularly weak HPV response (35, 38, 39). Franco-Cereceda & Holm (97), however, investigated the effects of intravenous administration of two single ET_A receptor antagonists and the dual ET_A and ET_B receptor antagonist bosentan on

established acute hypoxic PH in the adult pig. All three drugs were shown to alleviate right ventricular afterload during acute hypoxic PH. However, the effects of the endothelin receptor antagonists were only investigated during the first 15 min of hypoxic exposure.

In contrast to bosentan, tezosentan is a water-soluble dual ET_A and ET_B receptor antagonist with short half-life, which should allow for a rapid plateau-effect, and it was developed specifically for intravenous use (110). Tezosentan is accordingly of specific interest with respect to the treatment of acute PH of various aetiologies. Geiger et al. found that tezosentan attenuated acute hypoxic PH in the adult pig (105), but the rapidity of the response and the time until the effect plateaued remained unclear. Furthermore, Geiger et al. (105) did not address the efficacy of tezosentan in preventing acute hypoxic PH. In the present study, we demonstrated that an intravenous bolus dose of tezosentan rapidly, within 15 min, reduces acute hypoxic PH. We additionally show that a stable level exhibiting a 62 % lower mean PAP value and fully normalized PVR is reached after 30-60 min. The maximum reductions in mean PAP and PVR with tezosentan during acute hypoxic PH in our study were greater than those reported at 15 min after bosentan administration during acute hypoxic PH *in vivo* in the pig (97). Additionally, we show that tezosentan administered prior to acute hypoxic exposure prevents an increase in mean PAP above a level corresponding to borderline or mild PH in humans and totally prevents an increase in PVR above what is seen in normoxia. The rapid onset and thereafter stable, positive effect of a tezosentan bolus dose on pulmonary haemodynamics during acute hypoxic PH, as well as its ability to prevent a pronounced increase in right ventricular afterload in response to acute hypoxia, makes it a good candidate as a complementary treatment and for prevention of acute hypoxic PH, for instance in an intensive care setting.

Of note, intravenous tezosentan has also been demonstrated to acutely improve pulmonary haemodynamics in animal models of PH due to infusion of the TXA₂ analogue U-46619 (111), *in utero* placement of an aorto-pulmonary graft (111), meconium aspiration (112), endotoxemic shock (113), oleic acid-induced lung injury (114) and monocrotaline treatment (115). The TACTICS trial, however, did not demonstrate efficacy for tezosentan in reducing the proportion of patients with PH who developed right ventricular failure during weaning from cardiopulmonary bypass after cardiothoracic surgery (116). Importantly, this trial encompassed only patients with PH due to left heart disease and pulmonary vasodilatory drugs may in such a population lead to an increase in left ventricular preload and subsequently elevated filling pressure. Our findings, together with those of others, indicate that tezosentan may be a good alternative for the treatment of acute pre-capillary PH, or as a substitute for per oral endothelin receptor antagonists in chronic PH, not least due to the rapid and stable effects that were confirmed in our study.

Paper III

Results

Intravenous infusion of levosimendan for 90 min during acute hypoxia was found to within 10 min decrease ($p<0.05$) mean PAP and PVR by 2.8 ± 1.0 mmHg and 0.9 ± 0.2 WU, respectively, whereas mean PAP remained at a stable elevated level and PVR increased ($p<0.05$) by maximally 1.0 ± 0.2 WU over the same 90 min time period of acute hypoxia in the placebo group (Figure 9). Moreover, in the placebo group, as compared to the hypoxia baseline measurement, CO decreased ($p<0.02$) within 10 min of acute hypoxic exposure and stabilized after 30 min at a level 0.5 ± 0.1 l · min⁻¹ lower than the hypoxia baseline (Figure 9). Likewise, in the placebo group, SV tended to successively decrease, as compared to the hypoxia baseline, but it only reached statistical significance at the 60-90 min measurements when it had maximally decreased ($p<0.05$) by 4.5 ± 1.7 ml. On the contrary, with levosimendan treatment, CO was not affected by 90 min of acute hypoxia and SV tended to successively increase, as compared to the hypoxia baseline, before it reached statistical significance at the 60-90 min measurements, when it had increased ($p<0.02$) by 6.6 ± 1.6 ml (Figure 9).

Comments

Levosimendan is a calcium sensitizer with positive inotropic actions, which may also induce systemic and pulmonary vasodilatation. The positive inotropic effect is mediated by binding of levosimendan to troponin C in cardiomyocytes, thereby increasing the affinity of troponin C for calcium. Myocardial contractility is thus enhanced without increasing intracellular calcium concentration and myocardial oxygen demand or interfering with diastolic relaxation (117). The vasodilatory effect of levosimendan is mediated by opening of adenosine triphosphate (ATP)-sensitive potassium channels in vascular smooth muscles, and possibly also by inhibition of phosphodiesterase 3 (118, 119). Levosimendan has a relatively short half-life of approximately one hour, however, its active metabolite OR-1896, has a longer half-life of approximately 80 hours and is probably responsible for the sustained haemodynamic effects, which are observed after levosimendan administration (120, 121).

No study had, to our knowledge, prior to our work assessed the effect of levosimendan in acute hypoxic PH. Our findings indicate that levosimendan may alleviate right ventricular afterload and prevent a cardio-depressive effect of hypoxia. Acute hypoxic PH indeed presents as a suitable setting for levosimendan treatment due to the drug's dual effects of pulmonary vasodilatation and inotropy.

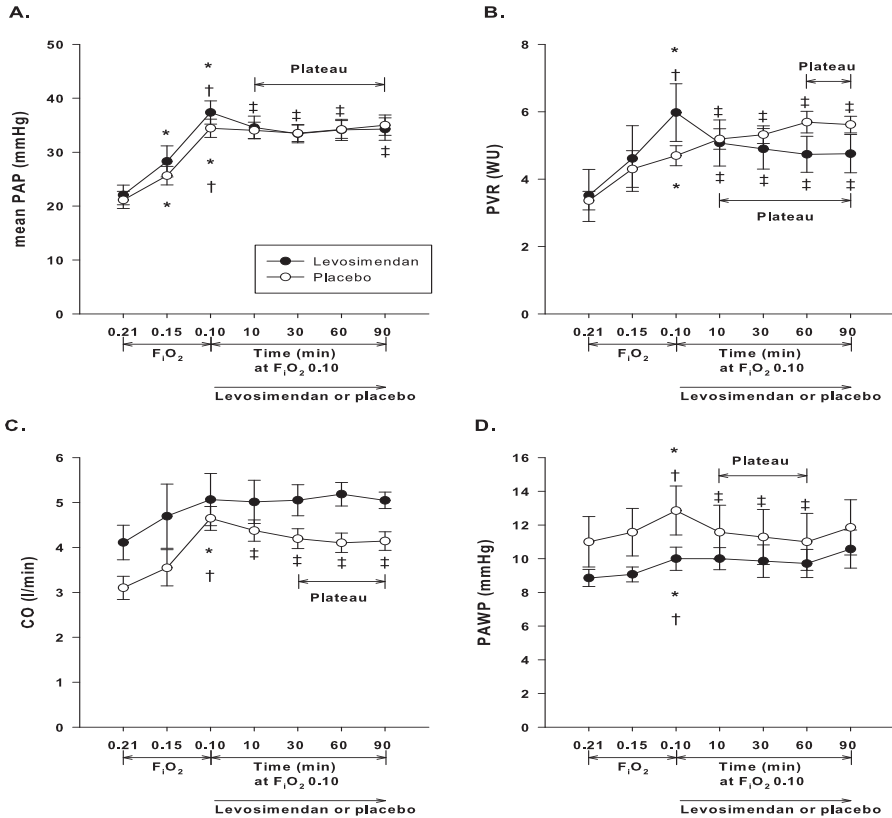


Figure 9. Effects of Levosimendan on Pulmonary Haemodynamics During Acute Hypoxia. Hypoxia increased (A) mean PAP ($p < 0.001$) and (B) PVR. Levosimendan decreased (A) mean PAP ($p < 0.05$) and (B) PVR ($p < 0.05$) to stabilize within 10 min. With placebo infusion (B) PVR further increased ($p < 0.05$) and (C) CO successively decreased ($p < 0.02$) to stabilize within 30 min. With levosimendan, (C) CO was left unaltered. With placebo infusion PAWP transiently decreased.

Indeed, acute hypoxia is known to increase CO at rest due to an increase in HR, but as hypoxia withstands, CO and SV at rest decreases (122) and this is due both to a reduced preload because of a hypoxia-induced reduction in plasma volume and the increase in right ventricular afterload because of HPV (122). In the present study, CO and SV successively decreased during 90 min hypoxic exposure in the placebo protocol. A decrease in CO and SV was accordingly observed earlier in the placebo protocol of our pig model than is normally seen in healthy humans at rest and also as compared to the control protocols from Paper IV and V. The reductions of CO and SV could be due for instance to the increase in right ventricular afterload or myocardial ischemia. Acute PH in the pig may additionally affect left ventricular function by a reduction in flow over the pulmonary circulation and by right ventricular dilatation with subsequent compression of the left ventricle, both leading to reduced left ventricular preload, as well as by impaired left ventricular contractility due to disturbed ventricular geometry and a dyssynchronous contraction pattern (123). Even though the SV decrease was observed earlier in our pig model than is normally seen in humans, the present study demonstrates that a hypoxia-induced decrease in SV can be prevented by levosimendan that even increased SV during acute hypoxia. Our findings warrants for further assessment of the effects of levosimendan treatment in acute hypoxic PH, such as occurs for instance at high altitude, in acute exacerbations of chronic obstructive pulmonary disease and when PAH is exaggerated by hypoxia.

With respect to PH of other aetiologies, levosimendan has been found to induce pulmonary vasodilatation when pulmonary vascular tone is elevated by a thromboxane A₂ analogue (119). Levosimendan has also been found to restore ventricular-arterial coupling, due to a dual effect on right ventricular contractility and right ventricular afterload, in experimental models of acute pressure overloaded right ventricular failure due to transient pulmonary arterial occlusion (124) or acute pulmonary embolism (125). In experimental models of sepsis, however, levosimendan has in different settings variably been shown to attenuate endotoxin-induced PH (126) or not (127, 128). In pilot studies incorporating patients with either chronic PH due to PAH or left heart disease (129) or acute respiratory distress syndrome associated with septic shock of extra-pulmonary origin (130), levosimendan also improved pulmonary haemodynamics.

Paper IV

Results

Intravenous administration of the selective COX-2 inhibitor nimesulide was demonstrated to decrease mean PAP ($p<0.013$) by 3.7 ± 1.3 mmHg within 45 min and PVR ($p<0.05$) by 0.8 ± 0.2 WU within 15 min to then remain steadily lowered for the remainder of the experiment (Figure 10). Furthermore, nimesulide decreased ($p<0.05$) SVR by 3.0 ± 1.5 WU within 15 min to then stabilize at the lower level. At the same time, there was a non-significant trend for a decrease in mean aortic blood pressure.

The effect of the TXA₂ receptor antagonist daltroban, administered intravenously, was also investigated. Daltroban initially and transiently increased mean PAP ($p<0.001$), and mean RAP ($p<0.001$) by maximally 7.2 ± 1.2 mmHg after 96 ± 9 s and 2.7 ± 0.4 mmHg after 85 ± 9 s, respectively. Mean RAP returned to a level not different from the hypoxia baseline within 5 min and remained unaltered thereafter. Mean PAP also returned to the hypoxia baseline within 5 min, but mean PAP then further decreased to a level 4.2 ± 1.6 mmHg lower ($p<0.005$) than the hypoxia baseline measurement (Figure 11).

In the control protocol, all haemodynamic variables remained at a stable level, as compared to the hypoxia baseline, over the corresponding time-period (Figures 9-10).

Comments

COX catalyses the first step in the conversion of free essential fatty acids to active prostaglandins, which are important regulators of vascular tone (131). There are two isoforms of COX. COX-1 is constitutively expressed and the dominant COX in healthy endothelial and vascular smooth muscle cells. COX-2 is also constitutively expressed, but may additionally be induced by a variety of stimuli, including hypoxia (131, 132). In specific, and with respect to the regulation of vascular tone, COXs catalyse the conversion of arachidonic acid to prostaglandin H₂, which is converted to TXA₂ by TXA₂ synthase or prostacyclin by prostacyclin synthase (131). By binding to their specific receptors on vascular smooth muscle cells TXA₂ and prostacyclin induce vasoconstriction and vasodilatation, respectively (133, 134).

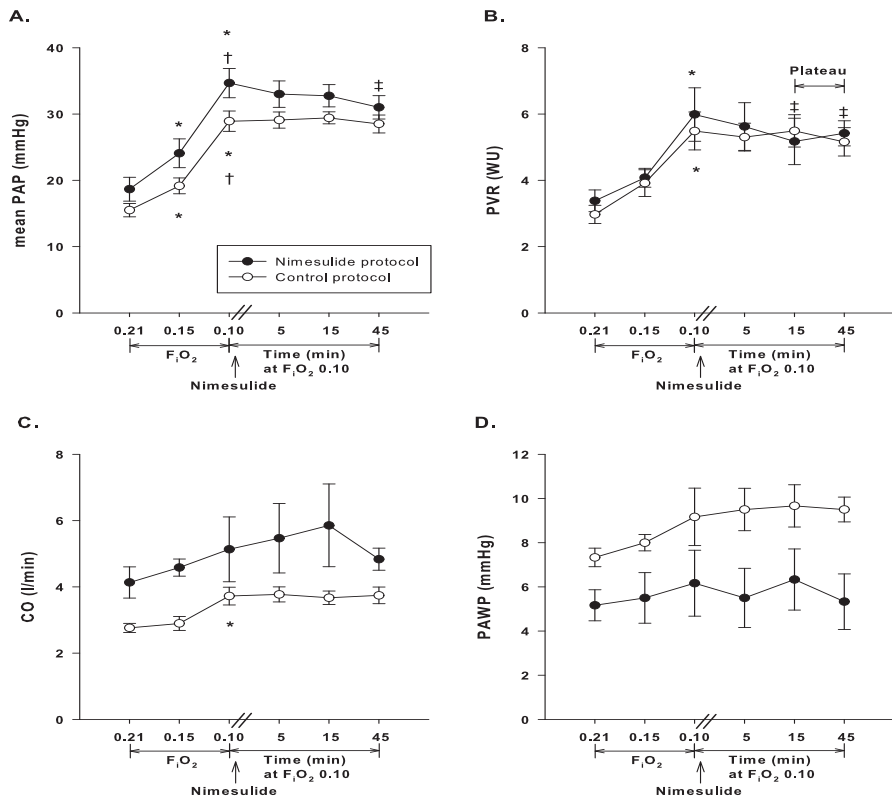


Figure 10. Effects of the COX-2 inhibitor Nimesulide on Pulmonary Haemodynamics During Acute Hypoxia. Hypoxia increased (A) mean PAP ($p < 0.001$) and (B) PVR ($p < 0.05$). Nimesulide decreased (A) mean PAP ($p < 0.013$) within 45 min and (B) PVR ($p < 0.05$) to stabilize within 15 min. (C) CO and (D) PAWP were unaltered by nimesulide. A * indicates statistical significance, as compared to F_{iO_2} 0.21; a † indicates statistical significance, as compared to F_{iO_2} 0.15; and a ‡ indicates statistical significance, as compared to hypoxia baseline at F_{iO_2} 0.10.

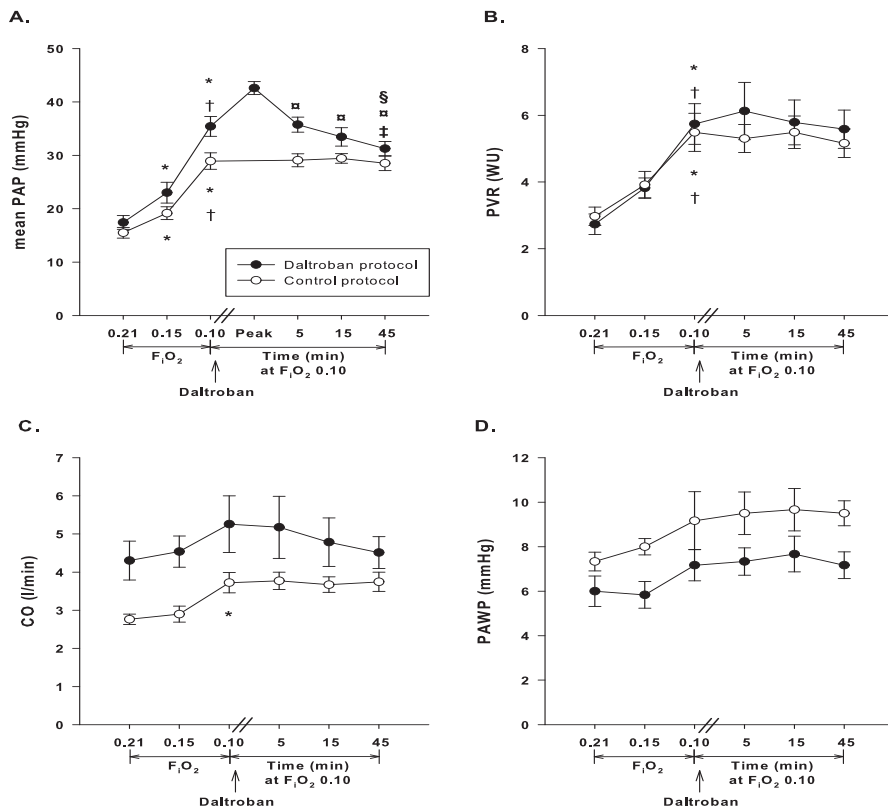


Figure 11. Effects of the TXA₂ Receptor Antagonist Daltroban on Pulmonary Haemodynamics During Acte Hypoxia.

Hypoxia increased (A) mean PAP ($p < 0.001$) and (B) PVR ($p < 0.05$). Daltroban first transiently increased (A) mean PAP ($p < 0.001$), but within 45 min mean PAP decreased to a level below the hypoxia baseline at F_iO₂ 0.10. Daltroban did not alter (B) PVR, (C) CO or (D) PAWP. A * indicates statistical significance, as compared to F_iO₂ 0.21; a † indicates statistical significance, as compared to F_iO₂ 0.15; a ‡ indicates statistical significance, as compared to hypoxia baseline at F_iO₂ 0.10; a § indicates statistical significance, as compared to the peak; and a # indicates statistical significance, as compared to the 5 min measurement.

The major COX-derived prostaglandin produced in the pulmonary circulation during acute hypoxia is prostacyclin (135). A role for prostacyclin in alleviating the hypoxic pulmonary vasopressor response is underlined by previous studies demonstrating that non-selective COX inhibition may in a variety of species potentiate both the acute and sustained phases of the HPV response *in vivo* (25, 44, 45, 48, 49, 136-139). Non-selective COX inhibition did, however, not always result in an amplified HPV response (44, 45, 140-143) and there is evidence that differences with respect to prostacyclin-induced pulmonary vasodilatation during acute hypoxia may explain intra-individual variations in the degree of HPV (25, 44, 45). Prior to our work, the contribution of COX-2-derived vasoactive agents to the modulation of HPV had, to our knowledge, not been assessed. A potential enhancing role for TXA₂ in the modulation of HPV *in vivo* was additionally debated, as TXA₂ inhibition had been demonstrated to inhibit the acute phase of the HPV response in lambs (144), whereas it had no effect on the acute phase of HPV in piglets or the sustained phase of HPV in sheep (140, 145).

In our pig model, selective inhibition of COX-2 by nimesulide attenuated the hypoxia-induced increases in mean PAP and PVR, which indicate that a COX-2-derived vasoconstrictor potentiates the hypoxic pulmonary vasopressor response. We hypothesized that this vasoconstrictor was TXA₂ and administered the TXA₂ receptor antagonist daltroban to investigate whether TXA₂-induced pulmonary vasoconstriction contributed to the potentiation of HPV. To our surprise, the administration of daltroban induced rapid, but transient pulmonary vasoconstriction, as was illustrated by an increase in mean PAP when daltroban was given. The mean PAP increase was, however, rapidly restored and mean PAP then decreased to a level approximately 11 % below the hypoxia baseline assessed before daltroban administration. These findings are indicative of TXA₂ as a potentiating modulator of HPV, but also suggests that daltroban has intrinsic activity at the TXA₂ receptor.

Concomitant with our publication, Su et al. (146) reported that the selective COX-2 inhibitor celecoxib reduced the acute phase of HPV and TXA₂ production *in situ* in the rat. This argues that our findings may be relevant also for other species. Further, reports of increased TXA₂ production during acute hypoxia *in vivo* support our results (144, 147, 148). Our findings primarily indicate that COX-2 and TXA₂ contribute to modulate the sustained phase of the HPV response in pigs, but further investigations using a TXA₂ receptor antagonist devoid of intrinsic activity are needed to definitely clarify the modulation of HPV by TXA₂.

Daltroban has previously been demonstrated to fully inhibit the pulmonary vasoconstriction to a TXA₂ analogue *in vitro* in humans (149) and *in vivo* in the cat (150) without any intrinsic activity being reported. Further literature review, however, revealed that Bertolino et al. (151) had described partial agonistic properties of daltroban at the TXA₂ receptor in the rat pulmonary circulation, which could explain the initial, transient increase in mean PAP upon daltroban administration observed in our study. Importantly, after initial, transient intrinsic

activity, daltroban did also in the study by Bertolino et al. (151) effectively inhibit pulmonary vasoconstriction induced by a TXA₂ analogue.

Paper V

Results

The P2Y₁ receptor antagonist MRS2500, administered intravenously, was demonstrated to within 15 min lower ($p<0.013$) mean PAP by 4.3 ± 1.2 mmHg, whereas PVR only showed a non-significant tendency to decrease with the treatment (Figure 12). Infusion of sodium chloride at the same infusion rate as MRS2500 did not affect mean PAP.

Intravenous administration of the P2Y₁₂ receptor antagonist cangrelor furthermore decreased mean PAP by 3.3 ± 0.4 mmHg ($p<0.036$) and 3.6 ± 0.6 mmHg ($p<0.018$) within 10 and 30 min, respectively. The effect was, however, transient and mean PAP had returned to a level not different from the hypoxia baseline within 60 min. Cangrelor did not significantly affect PVR (Figure 13).

In the control protocol, all haemodynamic variables remained at a stable level, as compared to the hypoxia baseline, over the corresponding time-periods (Figures 11-12).

Comments

Erythrocytes have been demonstrated to aggravate HPV and this has primarily been suggested to be due to the inactivation of NO by haemoglobin (32). However, erythrocytes are also recognized to release ATP in response to physiological stimuli, including a low oxygen environment, and to thereby contribute to the modulation of vascular tone (152). Extracellular adenine nucleotides affect vascular tone by activation of P1 and P2 receptors in the vessel wall. In skeletal muscle, for instance, the release of ATP from erythrocytes in response to a low oxygen content is thought to induce vasodilatation to secure tissue oxygen delivery (153). In the normoxic pulmonary circulation, ATP released from mechanically deformed erythrocytes have additionally been suggested to contribute to the low pulmonary vasomotor tone by stimulating NO production (154). Of importance, extracellular ATP is rapidly degraded by ectonucleotidases to ADP, adenosine monophosphate and finally adenosine (155), which may in a sequential manner contribute to the regulation of vascular tone (156, 157).

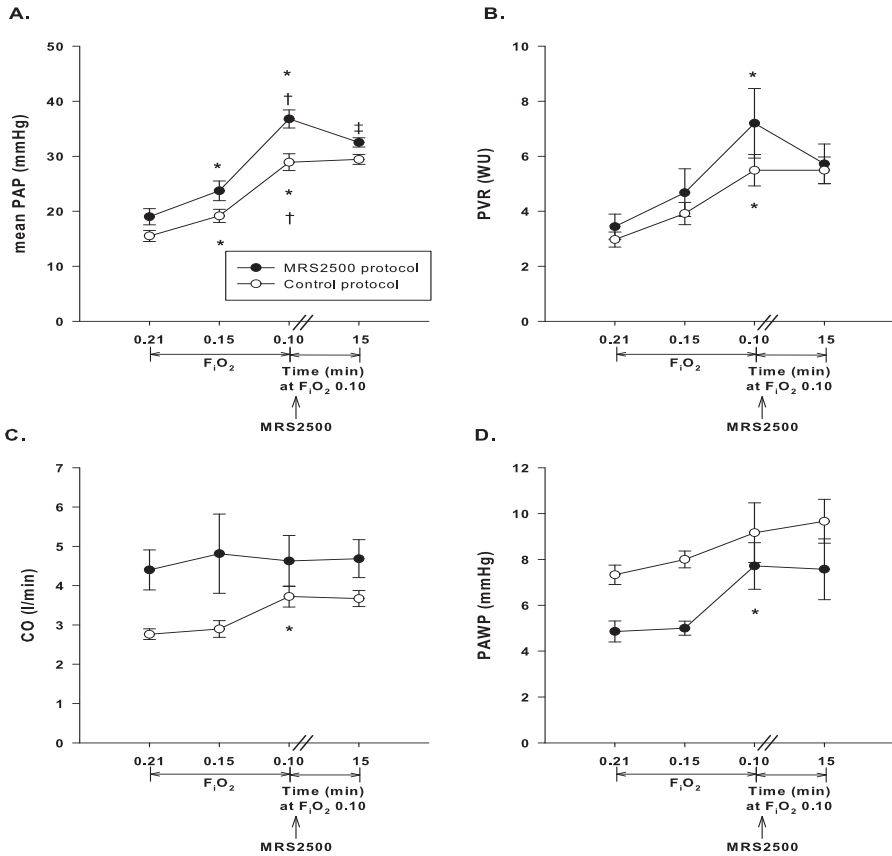


Figure 12. Effects of the P2Y₁ Receptor Antagonist MRS2500 on Pulmonary Haemodynamics During Acute Hypoxia.

Hypoxia increased (A) mean PAP ($p < 0.001$) and (B) PVR ($p < 0.05$). MRS2500 decreased (A) mean PAP ($p < 0.013$) and there was a non-significant decrease in (B) PVR after MRS2500 treatment. MRS2500 did not alter (C) CO or (D) PAWP. A * indicates statistical significance, as compared to F_{iO_2} 0.21; a † indicates statistical significance, as compared to F_{iO_2} 0.15; and a ‡ indicates statistical significance, as compared to hypoxia baseline at F_{iO_2} 0.10.

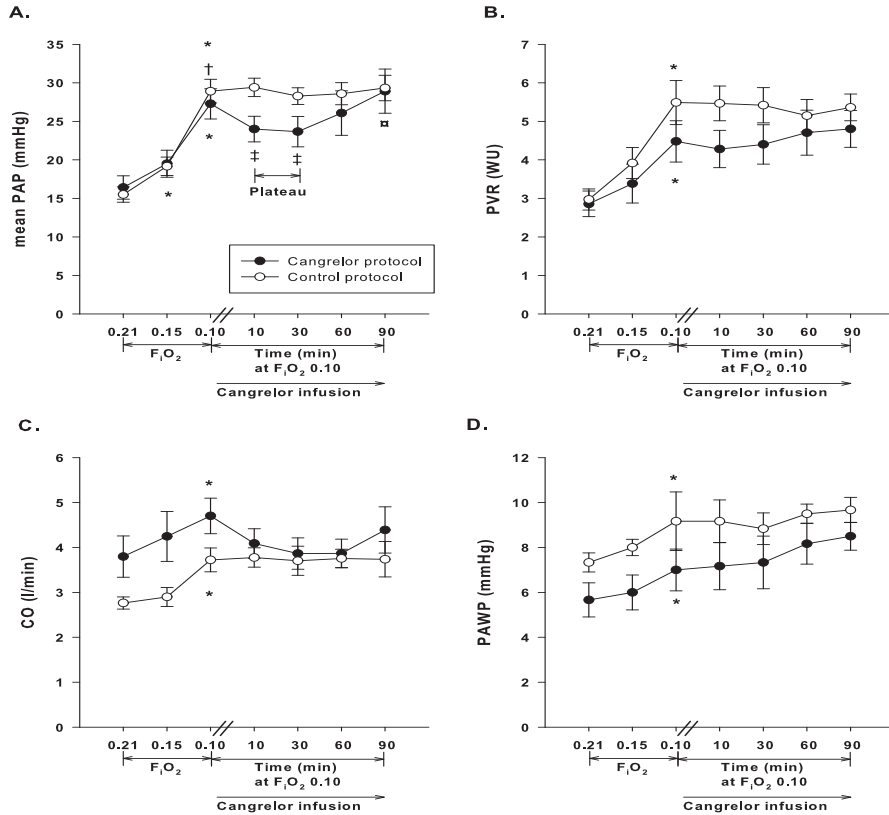


Figure 13. Effects of the P2Y₁₂ Receptor Antagonist Cangrelor on Pulmonary Haemodynamics During Acute Hypoxia. Hypoxia increased (A) mean PAP ($p < 0.05$) and (B) PVR ($p < 0.05$). Cangrelor transiently decreased (A) mean PAP ($p < 0.036$). Cangrelor did not alter (B) PVR, (C) CO or (D) PAWP. A * indicates statistical significance, as compared to F_IO₂ 0.21; a † indicates statistical significance, as compared to F_IO₂ 0.15; a ‡ indicates statistical significance, as compared to hypoxia baseline at F_IO₂ 0.10; and a α indicates statistical significance, as compared to the 10 and 30 min measurements.

We wanted to test the hypothesis if ADP, through activation of its receptors P2Y₁ and P2Y₁₂, potentiated the hypoxic pulmonary vasoconstrictor response, as contractile P2Y₁ and P2Y₁₂ receptors were recently revealed in intrapulmonary arteries of rats (157) and infusion of ADP *in vivo* increases PAP and PVR (158, 159). Of note, ADP can *in vivo* also induce vasoconstriction by activation of platelets with subsequent release of vasoconstrictor substances, such as TXA₂ (160), and whether the increase in right ventricular afterload following ADP-infusion is primarily due to pulmonary vasoconstriction or thrombus formation has been debated (158, 159).

Only four studies had prior to our work addressed the contribution of adenine nucleotides to the modulation of HPV. It was demonstrated that P2X receptor desensitization by α,β -meATP did not affect the hypoxic pulmonary vasopressor response in the isolated perfused rat pulmonary circulation (161), but that non-selective P2 receptor antagonism with suramin partially inhibited HPV in the isolated perfused rabbit pulmonary circulation (162). In the lamb, non-specific adenosine receptor blockade with aminophylline had no effect on the hypoxic pulmonary vasopressor response *in vivo* (163), whereas the PAP increase to acute hypoxia was fully inhibited by the non-selective adenosine receptor antagonist 8-phenyltheophylline *in vivo* in the rat (164).

We found that P2Y₁ receptor antagonism with MRS2500 during hypoxia decreased mean PAP by approximately 10 % within 15 min. P2Y₁₂ receptor antagonism with cangrelor during hypoxia also decreased mean PAP after 10-30 min, although the response was transient. The hypothesis that ADP potentiates HPV via P2Y₁ and P2Y₁₂ receptor activation was, however, not definitely proven as PVR was not significantly decreased by the treatments. However, there was a non-significant trend for PVR to decrease with MRS2500 treatment and CO as well as PAWP were not significantly altered by the treatments. It is thus possible that ADP is a non-mandatory modulator of HPV where the effect of the P2Y₁ and P2Y₁₂ receptor antagonists are concealed by redundant mechanisms.

The doses of MRS2500 and cangrelor that were given in our study were sufficient to inhibit increases in mean PAP and PVR to an intravenous dose of ADP that increased mean PAP to the same magnitude as acute hypoxia at F_iO₂ 0.10. This indicates sufficient dosing of the P2Y receptor antagonists in our study. Furthermore, it was demonstrated that the increases in mean PAP and PVR to the infusion of ADP were most likely due to pulmonary vasoconstriction, as both the increase in right ventricular afterload and the decrease in arterial oxygenation that followed infusion of ADP were immediately reversible. Whether this ADP-induced pulmonary vasoconstrictor response is primarily due to activation of contractile P2Y₁ and P2Y₁₂ receptors in the vascular wall, by indirect vasoconstriction due to release of vasoconstrictor substances following platelet activation or a combination of these mechanisms was not demonstrated and neither the aim of the present study.

General Comments

The present studies were performed in an invasive *in vivo* pig model of HPV. This allowed for hypotheses testing in an intact physiological system and increase the probability that the results are of relevance and applicable to a real world setting. Nonetheless, such a model is complex and demands for a broad perspective and caution when interpreting the results and putting them into a mechanistic perspective. For instance, when pharmacologically antagonizing or stimulating a biological system *in vivo* one has to take into account the possibility of redundancy by other systems. Such redundancy may fully or partially conceal the effects of an intervention. Moreover, effects unrelated to the mechanism studied, but which may affect the end-point of interest has to be considered. With respect to the study of HPV in an intact animal model, PVR is the primary end-point of choice (29), but alterations in PVR does not necessarily reflect changes in vascular diameter, as isolated variations in CO may also affect PVR. In addition, alterations in left atrial pressure, as reflected by changes in PAWP, could also change PVR. Accordingly, a full reading of the pulmonary haemodynamic system, incorporating PVR, mean PAP, PAWP and CO, has to be made when interpreting the effects of an intervention with potential effect on HPV *in vivo*. Additionally, other factors, which may affect the HPV response, such as F_iO_2 , pulmonary arterial O_2 saturations and pH has to be regularly monitored and taken into account, as was done in our studies.

Our studies were performed in an *in vivo* model using pigs as the laboratory animal. Importantly, the degree of HPV is greatly variable between different individuals and species. Pigs have a high magnitude of the hypoxic pulmonary vasopressor response (38, 39), probably making them suitable to the study of potentiating modulators of HPV. Moreover, the HPV response in anaesthetized pigs has been proven stable over a time-period of at least three hours (165). The stability of the HPV response in intact pigs was verified over a time-period of two hours in our control experiments and support the notion that alterations in pulmonary haemodynamics after the pharmacological interventions were indeed due to the treatments. As illustrated by our different protocols, there are, however, inter-individual variations with respect to the degree of the HPV response of pigs, making it hard to directly compare small groups of animals. Furthermore, invasive measurements of haemodynamics are feasible in pigs and the pig and human show high resemblance with regards to their haemodynamic parameters (166).

The current findings emphasise that the modulation of HPV *in vivo* is complex and dependent on a numerous modulatory pathways, which act together to decide the final response. We demonstrate that ET-1 is an important, mandatory modulator of the sustained HPV response *in vivo* in pigs and that COX-2-derived TXA_2 adds to amplify the response. Additionally, ADP could be a non-obligatory potentiating modulator of the porcine HPV response *in vivo*. From a therapeutic perspective, our

findings indicate that intravenous tezosentan as well as levosimendan could be potentially useful as complementary treatments for acute hypoxia-induced PH.

Paper VI

Results

Within the group of SSc patients who did not develop PAH, there were no significant alterations in the levels of angiogenic or inflammatory biomarkers during the follow-up period of 12 (10-13) years (Figure 14). Plasma PIGF ($p<0.001$), sVEGFR-1 ($p=0.045$) and TNF- α ($p=0.004$) were higher in SSc patients about to develop PAH within 2 (0.8-3) years, as compared to SSc patients who did not develop PAH during a follow-up of 12 (10-13) years (Figure 14A, 14C and 14D). Plasma VEGF-D was higher ($p=0.013$) in SSc patients about to develop PAH, as compared to the first blood sampling from SSc patients who did not develop PAH, but did not significantly differ from these patients at their second blood sampling after approximately 12 years (Figure 14B). Within the group of SSc patients who developed PAH, plasma VEGF-D increased ($p=0.039$) from the blood sampling before the diagnosis of PAH to the blood sampling at PAH diagnosis 2 (0.8-3) years later (Figure 14B).

For the pooled group of iPAH and SSc-PAH patients, plasma PIGF ($p<0.001$), VEGF-A ($p=0.009$), sVEGFR-1 ($p=0.001$), IL-6 ($p=0.001$) and TNF- α ($p<0.001$) were higher, as compared to controls without PAH and SSc. Plasma sVEGFR-1 decreased ($p=0.008$) by 19 (1-42) pg/ml after initiation of PH-targeted treatments within the pooled group of iPAH and SSc-PAH patients (Figure 15).

Plasma PIGF inversely correlated (Spearman's correlation coefficient (r_s) -0.571, $p=0.039$) with 6MWD at treatment follow-up (Figure 16A). Plasma VEGF-D positively correlated with mean RAP (r_s 0.601, $p=0.014$) and NT-pro-BNP (r_s 0.769, $p=0.001$) at the treatment follow-up (Figure 16B and 16C). Plasma IL-6 inversely correlated (r_s -0.718, $p=0.005$) with 6MWD and positively correlated with mean RAP (r_s 0.542, $p=0.030$) and NT-pro-BNP (r_s 0.768, $p=0.001$) at the treatment follow-up (Figure 16D, 16E and 16F). Plasma TNF- α inversely correlated (r_s -0.622, $p=0.029$) with 6MWD at the treatment follow-up (Figure 16G).

Higher plasma PIGF ($p=0.017$) and TNF- α ($p=0.036$) at treatment follow-up were associated with increased mortality risk, exhibiting hazard ratios (95 % confidence intervals) of 1.081 (1.014-1.152) and 1.216 (1.013-1.459), respectively, in univariate COX proportional hazards models.

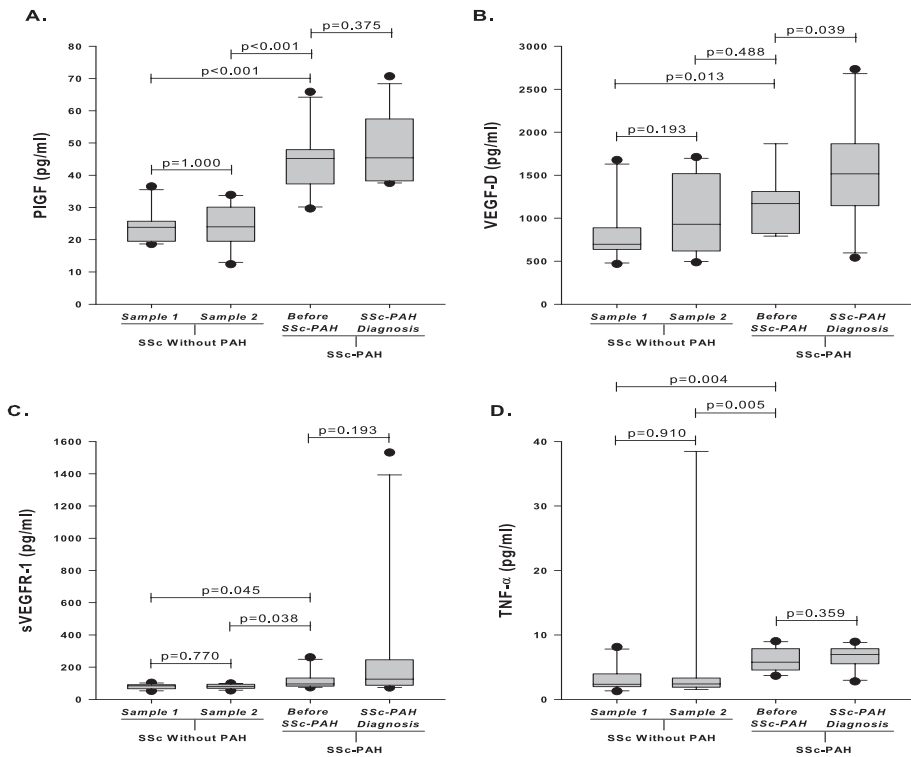


Figure 14. Potential Circulating Biomarkers for Screening of PAH Among Patients with SSc.

Plasma PIGF ($p < 0.001$), VEGF-D ($p = 0.013$), sVEGFR-1 ($p = 0.046$) and TNF- α ($p = 0.004$) were higher in SSc patients who would be diagnosed with PAH within 2 (0.8-3) years, as compared to SSc patients who during a 12 (10-13) year follow-up did not develop PAH.

Comments

The most important findings of the present study were that plasma levels of PIGF, sVEGFR-1 and TNF- α were higher in SSc patients who would eventually develop PAH, as compared to those who did not during a follow-up of over a decade. We also showed that plasma VEGF-D increased as SSc patients developed PAH. The findings suggest that these circulating angiogenic and inflammatory biomarkers could be used in the screening for PAH among SSc patients. The importance of screening programs for SSc-PAH are underlined by the often late diagnosis (3, 5, 167) and poor survival rates even compared to other forms of PAH (64, 167). Indeed, screening for SSc-PAH is currently recommended by means of echocardiography, pulmonary function testing with measurements of diffusion capacity for carbon monoxide as well as plasma NT-proBNP levels (65). A SSc-PAH screening program has additionally been demonstrated to improve survival (168). Circulating biomarkers, as those described in the present study, could contribute to simplify and to increase the sensitivity as well as specificity of PAH screening programs for patients with SSc.

During our work with the present study, another study was published, which had investigated the potential of plasma angiogenic and inflammatory biomarkers for SSc-PAH screening. In that study, plasma IL-12 and sVEGFR-1 were identified as potential circulating biomarkers for SSc-PAH screening (66). Our results complement these findings as also plasma PIGF and TNF- α were higher in SSc patients about to develop PAH, as compared to those who did not, in our study. There was in the previous study (66) similarly a trend for higher plasma PIGF levels among SSc patients who would develop PAH, supporting the validity of our findings. However, in contrast to us, they found no difference in plasma TNF- α levels for SSc patients who would or did not develop PAH. In this context, it should be noted that the follow-up time for the SSc patients who did not develop PAH in our study was approximately 10 years longer. We encourage the incorporation of these angiogenic and inflammatory biomarkers in future studies on SSc-PAH screening programs.

In the present study, we also examined plasma levels of circulating angiogenic and inflammatory biomarkers at PAH diagnosis in a cohort of patients with either SSc-PAH or iPAH. It was confirmed that levels of PIGF (169, 170), VEGF-A (67, 171, 172), sVEGFR-1 (169, 173), IL-6 (171, 174-176) and TNF- α (174, 177) are elevated in blood of PAH patients. Additionally, we found elevated plasma levels of VEGF-D at time of PAH diagnosis. These circulating biomarkers could add to the diagnostic algorithm for PH. Actually, Tiede et al. (169) recently showed that the combination of plasma PIGF and sVEGFR-1 could with acceptable accuracy be used to identify patients with PAH in a cohort referred for RHC.

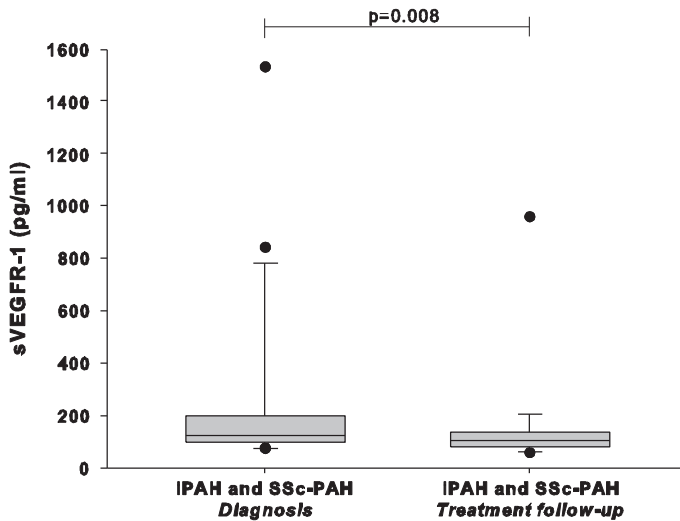


Figure 15. Plasma sVEGFR-1 at PAH Diagnosis and Follow-up after Initiation of PAH-targeted Treatments. Plasma sVEGFR-1 decreased ($p=0.008$) from time of PAH diagnosis to treatment follow-up in a pooled group of iPAH and SSc-PAH patients.

As there are only few reports of alterations in plasma angiogenic or inflammatory biomarkers after initiation of PH-targeted treatments (67, 68), but a need for new biomarkers of treatment response in PAH, we analysed plasma samples that had been gathered at treatment follow-up. It was found that plasma sVEGFR-1 levels decreased after initiation of PH-targeted treatments. Malhotra et al. (173) showed previously in a larger, prevalent cohort of PAH patients that plasma sVEGFR-1 levels were lower among patients receiving prostanoid treatment and that higher plasma sVEGFR-1 levels were associated with higher New York Heart Association functional class and increased mortality risk. This was not verified in our longitudinal study of newly diagnosed PAH patients, but there was a non-significant trend ($p=0.068$) for higher plasma sVEGFR-1 at treatment follow-up to be associated with increased mortality risk.

Additionally, we found that plasma PIGF, IL-6 and TNF- α at the treatment follow-up inversely correlated with the 6MWD and that plasma VEGF-D and IL-6 at the treatment follow-up positively correlated with mean RAP and NT-proBNP level. These are all important clinical parameters of prognostic impact, which are included in the risk stratification score of the current PH guidelines (1). Our findings therefore indicate that these circulating angiogenic and inflammatory biomarkers could potentially be used to predict prognosis in patients with PAH. For PIGF and TNF- α , this was supported by the univariate COX proportional hazards model in which higher levels of these circulating biomarkers at treatment follow-up were shown to be associated with an increased mortality risk. Our discoveries are

important as the current treatment strategy for patients with PAH is based on risk stratification at regular follow-up assessments (1) and warrants for further investigations in larger populations.

Overall, it seems that circulating angiogenic and inflammatory biomarkers may conform to several characteristics of the optimal PAH biomarker, as defined by Pezzuto et al. (63) First, these biomarkers are observer independent and non-invasive. Second, analysis by commercially available ELISA kits are feasible, although the analyses are not already introduced clinically. Third, it is indicated that higher plasma PIGF, sVEGFR-1 and TNF- α may be a specific sign of future PAH development in patients with SSc. Cut-off values were, however, not determined. The ability of circulating PIGF and sVEGFR-1 to identify patients with PAH at time of diagnosis has previously been demonstrated (169). Investigations with respect to the specificity for PAH regarding the other circulating angiogenic and inflammatory biomarkers deranged at PAH diagnosis in the present study is currently undergoing in our laboratory. Fourth, our findings suggest that several of the identified biomarkers may reflect disease activity, as there were significant correlations with important clinical variables of prognostic impact and relationships with mortality risk. Fifth, it is theoretically plausible that these angiogenic and inflammatory substances are involved in the pathogenesis of PAH and therefore could be potential therapeutic targets.

As for many studies in cohorts of newly diagnosed PAH patients, the results of the present study are limited by the small number of patients, which may have affected statistical power and restricted us from performing multivariate analyses. Nevertheless, the present study is of specific interest not least due to its longitudinal design and the identification of potential screening biomarkers for SSc-PAH using blood samples collected *before* the diagnosis of PAH was set. Therefore, we urge for future large, collaborative, prospective studies to further investigate the present findings.

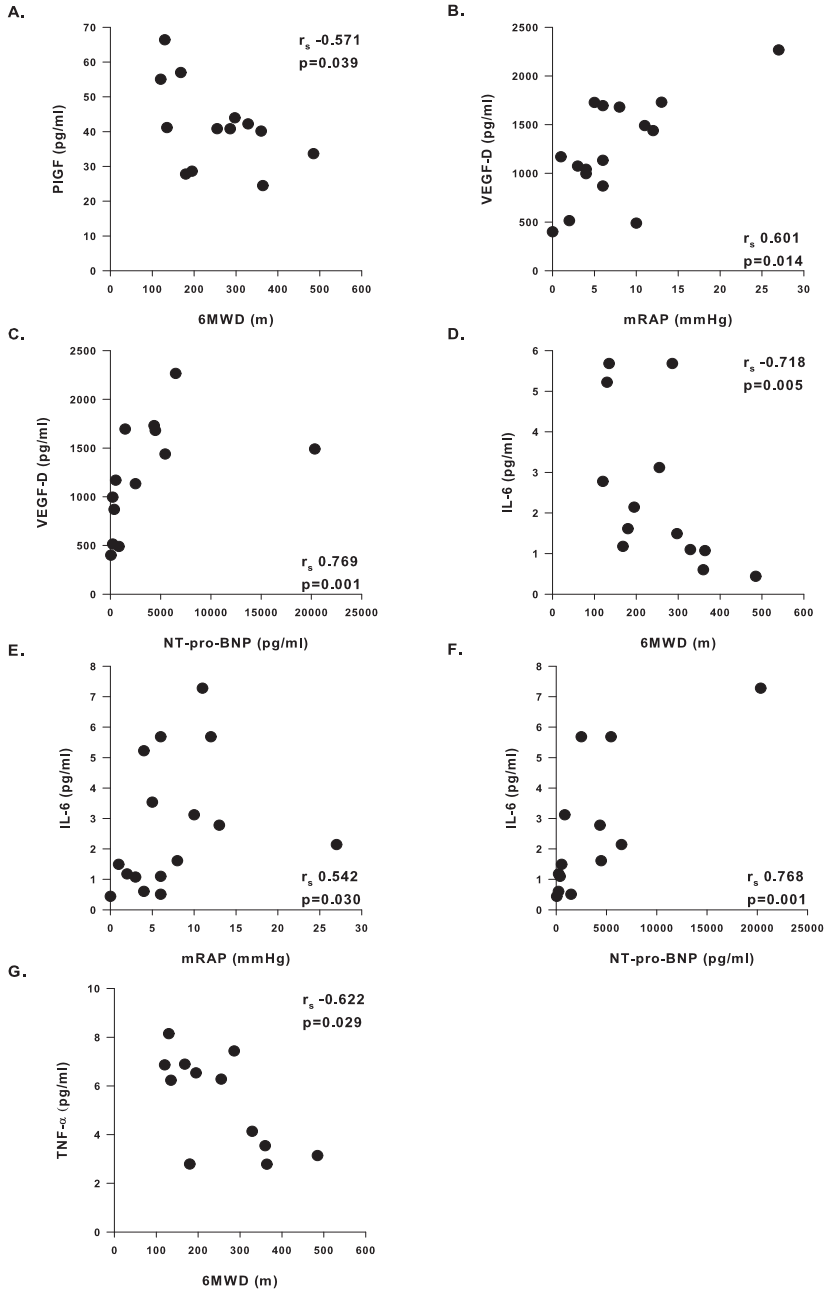


Figure 16. Correlations of Circulating Biomarkers with Clinical Parameters.

Plasma PIGF ($p=0.039$), IL-6 ($p=0.005$) and TNF- α ($p=0.029$) all negatively correlated with 6MWD. Plasma VEGF-D ($p=0.014$ and 0.001) and IL-6 ($p=0.030$ and 0.001) both positively correlated with mean RAP and NT-proBNP.

Future perspectives

Two decades have not yet passed since the first specific drug treatment for PAH was launched. Today there are five types of PH-targeted drugs with different pharmacodynamic profiles (1). The prognosis for patients with PAH is consequently better now, as compared to historic controls (178). Nevertheless, even with modern treatments and treatment strategies morbidity and mortality are high (178).

It is yet to be clarified in a randomized controlled trial if first-line triple therapy is superior to first-line dual therapy and what combinations of PH-targeted drugs should preferentially be used. A trial comparing first-line triple therapy to first-line double therapy is currently planned (ClinicalTrial.gov Identifier: NCT02558231). With respect to the development of new treatments, anti-remodelling properties are desired since the potential for vasodilatory treatments are limited as the disease progresses. Such a treatment could potentially cure PAH.

Treatment decisions regarding patients with PAH should according to current guidelines be steered by regular risk stratification based on anamnesis, clinical examination, functional capacity as well as imaging and RHC (1). This strategy is mainly supported by expert opinion (1) and only incompletely verified scientifically (20). There is a need for future studies to evaluate how to best assess prognosis at follow-up and to identify valid treatment goals. We are currently performing a nation-wide register study using the SPAHR to investigate this.

In order to earlier diagnose patients, and to thereby enable treatment start before vascular lesions are advanced, the awareness of the disease has to be further raised and new diagnostic approaches needs to be evolved. Such approaches may involve imaging or circulating biomarkers. With respect to circulating biomarkers, we identified a number of angiogenic and inflammatory biomarkers that could have potential in the screening process for SSc-PAH. Large multi-centre trials are nevertheless needed to verify their utility. New circulating biomarkers, such as those described by us, could also be of use for better prognostication, but this likewise warrants larger collaborative studies to definitely elucidate. A common problem in PAH research is indeed the small number of patients available for study. It would be valuable if the PAH community could in the future become even better at conducting collaborative studies also outside randomized controlled trials.

With respect to the modulation of HPV, it is emphasised that future studies should preferentially be performed *in vivo* to test hypotheses for relevance (29). It also appears to be important to better clarify the potentially different modulation of

the acute and sustained phases of the HPV response, respectively, and to perform investigations in various species and at different ages. This could aid in better understanding previously superficially contradictory results, but is likewise important with reference to investigations regarding novel modulatory pathways. A greater understanding of the interplay between different regulatory pathways in the modulation of HPV is also warranted.

With special reference to our findings, the potential contribution of TXA₂ for the sustained HPV response in humans, the development of high altitude pulmonary oedema and hypoxic exercise limitation is yet to be explored. As COX-2 is in chronic hypoxia primarily involved in prostacyclin synthesis (179, 180), investigations into the timing for a shift from a primarily vasoconstrictor to a vasodilator role is essential in order to understand its contributions during a continuous spectrum of hypoxic exposure. Additionally, the potential contribution of ADP and other adenine nucleotides to the HPV response needs to be better explored both in animal models and humans. This is emphasised by the important role of nucleotides in the systemic vascular response to acute hypoxia (153). In order to do so, further *in vitro* studies investigating pulmonary arterial P1 and P2 receptor expression as well as adenine nucleotide responses are probably also necessary. The development of additional specific P1 and P2 receptor antagonists is furthermore essential.

As for the treatment of acute hypoxia-induced PH, our findings in pigs indicate that intravenous tezosentan and levosimendan could be efficient in alleviating right ventricular afterload and improving cardiac function. Such treatments could be of use as complementary treatments to oxygen and treatment of the underlying cause. The results need, however, to be verified in humans. Additionally, it would be of interest to investigate the utility of these drugs in additional clinical settings where HPV may substantially contribute to the presence of PH, such as acute respiratory distress syndrome, acute worsening of chronic lung diseases, sepsis or exacerbations of PAH. Of note, one must in such cases be observant of the potential inhibition of beneficial HPV that may be important for an adequate pulmonary oxygen uptake. It could also be of interest to investigate the feasibility of replacing per oral endothelin receptor antagonist treatment with intravenous tezosentan in intensive care settings.

Conclusions

The major conclusions of each study are:

I. Survival among PAH patients in Lund between 2000 and 2011 was poor, but comparable to that in other European countries. Monotherapy is most often not sufficient in PAH, as was illustrated by the low proportion of patients that survived on monotherapy only. Initial combination therapy may more potently and rapidly than initial monotherapy improve pulmonary haemodynamics and cardiac performance. Higher PVR index or mean RAP/SV index as well as a lower 6MWD or SV index at treatment follow-up are factors associated with worse outcome in PAH and potential variables to be used as treatment goals in PAH. SV index may be a better prognostic marker than CI.

II. ET-1 contributes to potentiate the sustained phase of the HPV response *in vivo* in pigs. The effect of a single dose of intravenous tezosentan on pulmonary haemodynamics during acute hypoxia *in vivo* in the pig normalizes PVR within 30 min and the effect lasts for at least 2 hours. When administered prior to acute hypoxia, intravenous tezosentan prevents any increase in PVR above that observed in normoxia without tezosentan. Intravenous tezosentan is therefore a promising drug for complementary treatment of acute hypoxic PH, for instance in an intensive care setting.

III. Administration of levosimendan during acute hypoxia *in vivo* in the pig reduces right ventricular afterload and may prevent a cardio-depressive effect of hypoxia. Levosimendan could accordingly be beneficial as a complementary treatment for acute hypoxic PH.

IV. A COX-2-derived pulmonary vasoconstrictor potentiates the HPV response *in vivo* in pigs. It is indicated that TXA₂ may be this COX-2-derived vasoconstrictor.

V. Activation of P2Y₁ or P2Y₁₂ receptors by ADP do not appear to be mandatory for a full HPV response *in vivo* in pigs. It is indicated that ADP may be a non-obligatory modulator of HPV. Infusion of ADP in the right atrium induces pulmonary vasoconstriction through activation of P2Y₁ and P2Y₁₂ receptors. The mechanisms by which ADP induced pulmonary vasoconstriction *in vivo* remain to be fully elucidated.

VI. Plasma PIGF, sVEGFR-1, TNF- α and VEGF-D have potential as screening biomarkers for PAH among SSc patients. Plasma sVEGFR-1 could be a biomarker of response to PH-targeted treatments. Plasma PIGF, VEGF-D, IL-6 and TNF- α at treatment follow-up correlated with clinical parameters of prognostic impact. Higher plasma PIGF as well as TNF- α at treatment follow-up were associated with increased mortality risk. These circulating angiogenic and inflammatory biomarkers may consequently have potential for prognostication in PAH.

Bibliography

1. Galiè N, Humbert M, Vachiéry JL, Gibbs S, Lang I, Torbicki A, et al. 2015 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension: The Joint Task Force for the Diagnosis and Treatment of Pulmonary Hypertension of the European Society of Cardiology (ESC) and the European Respiratory Society (ERS): Endorsed by: Association for European Paediatric and Congenital Cardiology (AEPC), International Society for Heart and Lung Transplantation (ISHLT). *Eur Heart J*. 2016;37(1):67-119.
2. Simonneau G, Gatzoulis MA, Adatia I, Celermajer D, Denton C, Ghofrani A, et al. Updated clinical classification of pulmonary hypertension. *J Am Coll Cardiol*. 2013;62(25 (Suppl D)):D34-D41.
3. Humbert M, Sitbon O, Chaouat A, Bertocchi M, Habib G, Gressin V, et al. Pulmonary arterial hypertension in France: results from a national registry. *Am J Respir Crit Care Med*. 2006;173(9):1023-30.
4. Peacock AJ, Murphy NF, McMurray JJV, Caballero L, Stewart S. An epidemiological study of pulmonary arterial hypertension. *Eur Respir J*. 2007;30(1):104-9.
5. Escribano-Subias P, Blanco I, López-Meseguer M, Lopez-Guarch CJ, Roman A, Morales P, et al. Survival in pulmonary hypertension in Spain: insights from the Spanish Registry. *Eur Respir J*. 2012;40:596-603.
6. Badesch DB, Raskob GE, Elliott CG, Krichman AM, Farber HW, Frost A, et al. Pulmonary arterial hypertension: baseline characteristics from the REVEAL registry. *Chest*. 2010;137(2):376-87.
7. Pietra GG, Capron F, Stewart S, Leone O, Humbert M, Robbins IM, et al. Pathologic assessment of vasculopathies in pulmonary hypertension. *J Am Coll Cardiol*. 2004;43:25S-32S.
8. Tuder RM, Archer SL, Dorfmueller P, Erzurum SC, Guignabert C, Michelakis E, et al. Relevant issues in the pathology and pathobiology of pulmonary hypertension. *J Am Coll Cardiol*. 2013;62(25 (Suppl D)):D4-D12.
9. Vonk Noordegraaf A, Haddad F, Chin KM, Forfia PR, Kawut SM, Lumens J, et al. Right heart adaptation to pulmonary arterial hypertension: physiology and pathobiology. *J Am Coll Cardiol*. 2013;62(25 (Suppl)):D22-D33.
10. D'Alonzo GE, Barst RJ, Ayres SM, Bergofsky EH, Brundage BH, Detre KM, et al. Survival in patients with primary pulmonary hypertension. Results from a national prospective registry. *Ann Intern Med*. 1991;115:343-9.

11. Lee WT, Ling Y, Sheares KK, Pepke-Zaba J, Peacock AJ, Johnson MK. Predicting survival in pulmonary arterial hypertension in the UK. *Eur Respir J*. 2012;40(3):604-11.
12. Humbert M, Sitbon O, Yaïci A, Montani D, O'Callaghan DS, Jaïs X, et al. Survival in incident and prevalent cohorts of patients with pulmonary arterial hypertension. *Eur Respir J*. 2010;36:549-55.
13. Humbert M, Morrell NW, Archer SL, Stenmark KR, MacLean MR, Lang IM, et al. Cellular and molecular pathobiology of pulmonary arterial hypertension. *J Am Coll Cardiol*. 2004;43(12 (Suppl S)):13S-24S.
14. Sitbon O, Channick R, Chin KM, Frey A, Gaine S, Galiè N, et al. Selexipag for the treatment of pulmonary arterial hypertension. *N Engl J Med*. 2015;373(26):2522-33.
15. Rich S, Kaufmann E, Levy PS. The effect of high doses of calcium-channel blockers on survival in primary pulmonary hypertension. *N Engl J Med*. 1992;327:76-81.
16. Sitbon O, Humbert M, Jaïs X, Iqbal V, Hamid AM, Provencher S, et al. Long-term response to calcium channel blockers in idiopathic pulmonary arterial hypertension. *Circulation*. 2005;111:3105-11.
17. Galiè N, Hoeper MM, Humbert M, Torbicki A, Vachiéry J-L, Barbera JA, et al. Guidelines for the diagnosis and treatment of pulmonary hypertension. *Eur Respir J*. 2009;34:1219-63.
18. Galiè N, Barbera JA, Frost AE, Ghofrani HA, Hoeper MM, McLaughlin VV, et al. Initial use of ambrisentan plus tadalafil in pulmonary arterial hypertension. *N Engl J Med*. 2015;373(9):834-44.
19. McLaughlin VV, Gaine SP, Howard LS, Leuchte HH, Mathier MA, Mehta S, et al. Treatment goals of pulmonary hypertension. *J Am Coll Cardiol*. 2013;62(25 (Suppl D)):D73-D81.
20. Nickel N, Golpon H, Greer M, Knudsen L, Olsson K, Westerkamp V, et al. The prognostic impact of follow-up assessments in patients with idiopathic pulmonary arterial hypertension. *Eur Respir J*. 2012;39:589-96.
21. von Euler US, Liljestrand G. Observations on the pulmonary arterial blood pressure in the cat. *Acta Phys Scand*. 1946;12:301-20.
22. Motley HL, Cournand A, Werkö L, Himmelstein A, Dresdale D. The influence of short periods of induced acute anoxia upon pulmonary artery pressures in man. *Am J Physiol*. 1947;150(2):315-20.
23. Marshall C, Marshall B. Site and sensitivity for stimulation of hypoxic pulmonary vasoconstriction. *J Appl Physiol Respirat Environ Exercise Physiol*. 1983;55(3):711-6.
24. Marshall BE, Marshall C, Magno M, Lilagan P, Pietra GG. Influence of bronchial arterial PO₂ on pulmonary vascular resistance. *J Appl Physiol*. 1991;70(1):405-15.
25. Brimiouille S, Lejeune P, Vachiéry J-L, Delcroix M, Hallemans R, Leeman M, et al. Stimulus-response curve of hypoxic pulmonary vasoconstriction in intact dogs: effects of ASA. *J Appl Physiol*. 1994;77(1):476-80.

26. De Canniere D, Stefanidis C, Hallemans R, Delcroix M, Brimiouille S, Naeije R. Stimulus-response curves for hypoxic pulmonary vasoconstriction in piglets. *Cardiovasc Res.* 1992;26(10):944-9.
27. Kato M, Staub NC. Response of small pulmonary arteries to unilobar hypoxia and hypercapnia. *Circ Res.* 1966;19(2):426-40.
28. Gao Y, Raj JU. Role of veins in regulation of pulmonary circulation. *Am J Physiol Lung Cell Mol Physiol.* 2005;288(2):L213-L26.
29. Sylvester JT, Shimoda LA, Aaronson PI, Ward JP. Hypoxic pulmonary vasoconstriction. *Physiol Rev.* 2012;92(1):367-520.
30. Aaronson PI, Robertson TP, Ward JP. Endothelium-derived mediators and hypoxic pulmonary vasoconstriction. *Respir Physiol Neurobiol.* 2002;132(1):107-20.
31. Deem S, Bishop MJ, Alberts MK. Effect of anemia on intrapulmonary shunt during atelectasis in rabbits. *J Appl Physiol.* 1995;79(6):1951-7.
32. Deem S. Red blood cells and hemoglobin in hypoxic pulmonary vasoconstriction. *Adv Exp Med Biol.* 2006;588:217-31.
33. Brimiouille S, Vachiéry JL, Brichant JF, Delcroix M, Lejeune P, Naeije R. Sympathetic modulation of hypoxic pulmonary vasoconstriction in intact dogs. *Cardiovasc Res.* 1997;34(2):384-92.
34. Ketabchi F, Egemnazarov B, Schermuly RT, Ghofrani HA, Seeger W, Grimminger F, et al. Effects of hypercapnia with and without acidosis on hypoxic pulmonary vasoconstriction. *Am J Physiol Lung Cell Mol Physiol.* 2009;297(5):L977-L83.
35. Barer GR, Howard P, Shaw JW. Stimulus-response curves for the pulmonary vascular bed to hypoxia and hypercapnia. *J Physiol.* 1970;211(1):139-55.
36. Bland RD, Demling RH, Selinger SL, Staub NC. Effects of alveolar hypoxia on lung fluid and protein transport in unanesthetized sheep. *Circ Res.* 1977;40(3):269-74.
37. Dorrington KL, Clar C, Young JD, Jonas M, Tansley JG, Robbins PA. Time course of the human pulmonary vascular response to 8 hours of isocapnic hypoxia. *Am J Physiol.* 1997;273(Heart Circ Physiol 3 Pt 2):H1126-H34.
38. Elliott AR, Steffey EP, Jarvis KA, Marshall BE. Unilateral hypoxic pulmonary vasoconstriction in the dog, pony and miniature swine. *Respir Physiol.* 1991;85(3):355-69.
39. Wauthy P, Pagnamenta A, Vassalli F, Naeije R, Brimiouille S. Right ventricular adaptation to pulmonary hypertension: an interspecies comparison. *Am J Physiol Heart Circ Physiol.* 2004;286(4):H1441-H7.
40. Weissmann N, Akkayagil E, Quanz K, Schermuly RT, Ghofrani HA, Fink L, et al. Basic features of hypoxic pulmonary vasoconstriction in mice. *Respir Physiol Neurobiol.* 2004;139(2):191-202.
41. Will DH, Hicks JL, Card CS, Reeves JT, Alexander AF. Correlation of acute with chronic hypoxic pulmonary hypertension in cattle. *J Appl Physiol.* 1975;38(3):495-8.
42. Reeves JT, Leathers JE. Circulatory changes following birth of the calf and the effect of hypoxia. *Circ Res.* 1964;15:343-54.

43. Rendas A, Branthwaite M, Lennox S, Reid L. Response of the pulmonary circulation to acute hypoxia in the growing pig. *J Appl Physiol Respirat Environ Exercise Physiol.* 1982;52(4):811-4.
44. Hales CA, Rouse ET, Slate JL. Influence of aspirin and indomethacin on variability of alveolar hypoxic vasoconstriction. *J Appl Physiol Respirat Environ Exercise Physiol.* 1978;45(1):33-9.
45. Ahmed T, Oliver Jr. W, Wanner A. Variability of hypoxic pulmonary vasoconstriction in sheep. Role of Prostaglandins. *Am Rev Respir Dis.* 1983;127(1):59-62.
46. Dehnert C, Mereles D, Greiner S, Albers D, Scheurlen F, Zügel S, et al. Exaggerated hypoxic pulmonary vasoconstriction without susceptibility to high altitude pulmonary edema. *High Alt Med Biol.* 2015;16(1):11-7.
47. Tessler R, Wu S, Fiori R, MacGowan C, Belik J. Sildenafil acutely reverses the hypoxic pulmonary vasoconstriction response of the newborn pig. *Pediatr Res.* 2008;64(3):251-5.
48. Garrett RC, Thomas HM 3rd. Meclofenamate uniformly decreases shunt fraction in dogs with lobar atelectasis. *J Appl Physiol Respirat Environ Exercise Physiol.* 1983;54(1):284-9.
49. Garrett RC, Thomas HM 3rd. Relation of prostanoids to strength of hypoxic vasoconstriction in dogs with lobar atelectasis. *J Appl Physiol.* 1985;59(1):72-7.
50. Rodriguez-Roisin R, Drakulovic M, Rodríguez DA, Roca J, Barberà JA, Wagner PD. Ventilation-perfusion imbalance and chronic obstructive pulmonary disease staging severity. *J Appl Physiol.* 2009;106(6):1902-8.
51. Agustí AG, Barberà JA, Roca J, Wagner PD, Guitart R, Rodriguez-Roisin R. Hypoxic pulmonary vasoconstriction and gas exchange during exercise in chronic obstructive pulmonary disease. *Chest.* 1990;97(2):268-75.
52. Agustí AG, Roca J, Gea J, Wagner PD, Xaubet A, Rodriguez-Roisin R. Mechanisms of gas-exchange impairment in idiopathic pulmonary fibrosis. *Am Rev Respir Dis.* 1991;143(2):219-25.
53. Gao Y, Raj JU. Regulation of the pulmonary circulation in the fetus and newborn. *Physiol Rev.* 2010;90(4):1291-335.
54. Huez S, Faoro V, Vachiéry J-L, Unger P, Martinot J-B, Naeije R. Images in cardiovascular medicine. High-altitude-induced right-heart failure. *Circulation.* 2007;115(9):e308-e9.
55. Weitzenblum E, Apprill M, Oswald M, Chaouat A, Imbs J. Pulmonary hemodynamics in patients with chronic obstructive pulmonary disease before and during an episode of peripheral edema. *Chest.* 1994;105(5):1377-82.
56. Scherrer U, Rexhaj E, Jayet PY, Allemann Y, Sartori C. New insights in the pathogenesis of high-altitude pulmonary edema. *Prog Cardiovasc Dis.* 2010;52(6):485-92.
57. Faoro V, Boldingh S, Moreels M, Martinez S, Lamotte M, Unger P, et al. Bosentan decreases pulmonary vascular resistance and improves exercise capacity in acute hypoxia. *Chest.* 2009;135(5):1215-22.

58. Naeije R, Huez S, Lamotte M, Retailleau K, Neupane S, Abramowicz D, et al. Pulmonary artery pressure limits exercise capacity at high altitude. *Eur Respir J*. 2010;36(5):1049-55.
59. Biomarkers Definitions Working Group. Biomarkers and surrogate end-points: preferred definitions and conceptual framework. *Clin Pharmacol Ther*. 2001;69(3):89-95.
60. Nagaya N, Nishikimi T, Uematsu M, Satoh T, Kyotani S, Sakamaki F, et al. Plasma brain natriuretic peptide as a prognostic indicator in patients with primary pulmonary hypertension. *Circulation*. 2000;102(8):865-70.
61. Fijalkowska A, Kurzyna M, Torbicki A, Szewczyk G, Florczyk M, Pruszczyk P, et al. Serum N-terminal brain natriuretic peptide as a prognostic parameter in patients with pulmonary hypertension. *Chest*. 2006;129(5):1313-21.
62. Andreassen AK, Wergeland R, Simonsen S, Geiran O, Guevara C, Ueland T. N-terminal pro-B-type natriuretic peptide as an indicator of disease severity in a heterogenous group of patients with chronic precapillary pulmonary hypertension. *Am J Cardiol*. 2006;98(4):525-9.
63. Pezzuto B, Badagliacca R, Poscia R, Ghio S, D'Alto M, Vitulo P, et al. Circulating biomarkers in pulmonary arterial hypertension: update and future direction. *J Heart Lung Transplant*. 2015;34(3):282-305.
64. Hesselstrand R, Wildt M, Ekmeahag B, Wuttge DM, Scheja A. Survival in patients with pulmonary arterial hypertension associated with systemic sclerosis from a Swedish single centre: prognosis still poor and prediction difficult. *Scand J Rheumatol*. 2011;40:127-32.
65. Khanna D, Gladue H, Channick R, Chung L, Distler O, Furst DE, et al. Recommendations for screening and detection of connective tissue disease-associated pulmonary arterial hypertension. *Arthritis Rheum*. 2013;65(12):3194-201.
66. McMahan Z, Schoenhoff F, Van Eyk JE, Wigley FM, Hummers LK. Biomarkers of pulmonary hypertension in patients with scleroderma: a case-control study. *Arthritis Res Ther*. 2015;17(1):201.
67. Kümpers P, Nickel N, Lukasz A, Golpon H, Westerkamp V, Olsson KM, et al. Circulating angiopoietins in idiopathic pulmonary arterial hypertension. *Eur Heart J*. 2010;31(18):2291-300.
68. Karavolias GK, Georgiadou P, Gkouziouta A, Kariofillis P, Karabela G, Tsiapras D, et al. Short and long term anti-inflammatory effects of bosentan therapy in patients with pulmonary arterial hypertension: relation to clinical and hemodynamic responses. *Expert Opin Ther Targets*. 2010;14(12):1283-9.
69. Cracowski JL, Leuchte HH. The potential of biomarkers in pulmonary arterial hypertension. *Am J Cardiol*. 2012;110(6 Suppl):32S-8S.
70. Pàvek K, Boska D, Selecky FV. Measurement of cardiac output by thermodilution with constant rate injection of indicator. *Circ Res*. 1964;15:311-9.
71. Mortensen OH, Nielsen AR, Erikstrup C, Plomgaard P, Fischer CP, Krogh-Madsen R, et al. Calprotectin--a novel marker of obesity. *PLoS One*. 2009;4(10):e7419.

72. Humbert M, Barst RJ, Robbins IM, Channick RN, Galiè N, Boonstra A, et al. Combination of bosentan with epoprostenol in pulmonary arterial hypertension: BREATHE-2. *Eur Respir J*. 2004;24:353-9.
73. Kemp K, Savale L, O'Callaghan DS, Jaïs X, Montani D, Humbert M, et al. Usefulness of first-line combination therapy with epoprostenol and bosentan in pulmonary arterial hypertension: an observational study. *J Heart Lung Transplant*. 2012;31:150-8.
74. McLaughlin VV, Shillington A, Rich S. Survival in primary pulmonary hypertension: the impact of epoprostenol therapy. *Circulation*. 2002;106:1477-82.
75. Sitbon O, Humbert M, Nunes H, Parent F, Garcia G, Hervé P, et al. Long-term intravenous epoprostenol infusion in primary pulmonary hypertension: prognostic factors and survival. *J Am Coll Cardiol*. 2002;40:780-8.
76. Provencher S, Sitbon O, Humbert M, Cabrol S, Jaïs X, Simonneau G. Long-term outcome with first-line bosentan therapy in idiopathic pulmonary arterial hypertension. *Eur Heart J*. 2006;27:589-95.
77. Humbert M, Sitbon O, Chaouat A, Bertocchi M, Habib G, Gressin V, et al. Survival in patients with idiopathic, familial, and anorexigen-associated pulmonary arterial hypertension in the modern management era. *Circulation*. 2010;122:156-63.
78. Benza RL, Miller DP, Gomberg-Maitland M, Frantz RP, Foreman AJ, Coffey CS, et al. Predicting survival in pulmonary arterial hypertension: insights from the registry to evaluate early and long-term pulmonary arterial hypertension disease management (REVEAL). *Circulation*. 2010;122:164-72.
79. Kane GC, Maradit-Kremers H, Slusser JP, Scott CG, Frantz RP, McGoon MD. Integration of clinical and hemodynamic parameters in the prediction of long-term survival in patients with pulmonary arterial hypertension. *Chest*. 2011;139:1285-93.
80. Yanagisawa M, Kurihara H, Kimura S, Tomobe, Kobayashi M, Mitsui Y, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature*. 1988;332(6163):411-5.
81. Inoue A, Yanagisawa M, Kimura S, Kasuya Y, Miyauchi T, Goto K, et al. The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. *Proc Natl Acad Sci U S A*. 1989;86(8):2863-3867.
82. Ortega Mateo A, de Artiñano AA. Highlights on endothelins: a review. *Pharmacol Res*. 1997;36(5):339-51.
83. Cargill RI, Kiely DG, Clark RA, Lipworth BJ. Hypoxaemia and release of endothelin-1. *Thorax*. 1995;50(12):1308-10.
84. Coe Y, Haleen SJ, Welch KM, Liu Y-A, Cocceani F. The endothelin A receptor antagonists PD 156707 (CI-1020) and PD 180988 (CI-1034) reverse the hypoxic pulmonary vasoconstriction in the perinatal lamb. *J Pharmacol Exp Ther*. 2002;302(2):672-80.
85. Earley S, Nelin LD, Chicoine LG, Walker BR. Hypoxia-induced pulmonary endothelin-1 expression is unaltered by nitric oxide. *J Appl Physiol*. 2002;92(3):1152-8.

86. Elton TS, Oparil S, Taylor GR, Hicks PH, Yang RH, Jin H, et al. Normobaric hypoxia stimulates endothelin-1 gene expression in the rat. *Am J Physiol.* 1992;263(6 Pt 2):R1260-R4.
87. Li H, Chen SJ, Chen YF, Meng QC, Durand J, Oparil S, et al. Enhanced endothelin-1 and endothelin receptor gene expression in chronic hypoxia. *J Appl Physiol.* 1994;77(3):1451-9.
88. Shirakami G, Nakao K, Saito Y, Magaribuchi T, Jougasaki M, Mukoyama M, et al. Acute pulmonary alveolar hypoxia increases lung and plasma endothelin-1 levels in conscious rats. *Life Sci.* 1991;48(10):969-76.
89. Wagner OF, Christ G, Wojta J, Vierhapper H, Parzer S, Nowotny PJ, et al. Polar secretion of endothelin-1 by cultured endothelial cells. *J Biol Chem.* 1992;267(23):16066-8.
90. Bialecki RA, Fisher CS, Murdoch WW, Barthlow HG, Bertelsen DL. Functional comparison of endothelin receptors in human and rat pulmonary artery smooth muscle. *Am J Physiol.* 1997;272(2 Pt 1):L211-L8.
91. Hirata Y, Emori T, Eguchi S, Kanno K, Imai T, Ohta K, et al. Endothelin receptor subtype B mediates synthesis of nitric oxide by cultured bovine endothelial cells. *J Clin Invest.* 1993;91(4):1367-73.
92. Berti F, Rossoni G, Della Bella D, Villa LM, Buschi A, Trento F, et al. Nitric oxide and prostacyclin influence coronary vasomotor tone in perfused rabbit heart and modulate endothelin-1 activity. *J Cardiovasc Pharmacol.* 1993;22(2):321-6.
93. Fukuroda T, Fujikawa T, Ozaki S, Ishikawa K, Yano M, Nishikibe M. Clearance of circulating endothelin-1 by ETB receptors in rats. *Biochem Biophys Res Commun.* 1994;199(3):1461-5.
94. Chen SJ, Chen Y-F, Opgenorth TJ, Wessale JL, Meng QC, Durand J, et al. The orally active nonpeptide endothelin A-receptor antagonist A-127722 prevents and reverses hypoxia induced pulmonary hypertension and pulmonary vascular remodeling in Sprague-Dawley rats. *J Cardiovasc Pharmacol.* 1997;29(6):713-25.
95. Sato K, Morio Y, Morris Jr. KG, Rodman DM, McMurtry IF. Mechanism of hypoxic pulmonary vasoconstriction involves ET(A) receptor inhibition of K(ATP) channel. *Am J Physiol Lung Cell Mol Physiol.* 2000;278(3):L434-L42.
96. Tilton RG, Munsch CL, Sherwood SJ, Chen S-J, Chen Y-F, Wu C, et al. Attenuation of pulmonary vascular hypertension and cardiac hypertrophy with sitaxsentan sodium, an orally active ET(A) receptor antagonist. *Pulm Pharmacol Ther.* 2000;13(2):87-97.
97. Franco-Cereceda A, Holm P. Selective or nonselective endothelin antagonists in porcine hypoxic pulmonary hypertension? *J Cardiovasc Pharmacol.* 1998;31(Suppl 1):S447-S52.
98. Holm P, Liska J, Franco-Cereceda A. The ETA receptor antagonist, BMS-182874, reduces acute hypoxic pulmonary hypertension in pigs in vivo. *Cardiovasc Res.* 1998;37(3):765-71.
99. Hubloue I, Biarent D, Abdel Kafi S, Bejjani G, Kerbaul F, Naeije R, et al. Endogenous endothelins and nitric oxide in hypoxic pulmonary vasoconstriction. *Eur Respir J.* 2003;21(1):19-24.

100. Johnson W, Nohria A, Garrett L, Fang JC, Igo J, Katai M, et al. Contribution of endothelin to pulmonary vascular tone under normoxic and hypoxic conditions. *Am J Physiol Heart Circ Physiol.* 2002;283(2):H568-H75.
101. Wong J, Vanderford PA, Winters JW, Chang R, Soifer SJ, Fineman JR. Endothelin-1 does not mediate acute hypoxic pulmonary vasoconstriction in the intact newborn lamb. *J Cardiovasc Pharmacol.* 1993;22(Suppl 8):S262-S6.
102. Wang Y, Coe Y, Toyoda O, Coceani F. Involvement of endothelin-1 in hypoxic pulmonary vasoconstriction in the lamb. *J Physiol.* 1995;482(2):421-34.
103. Ambalavanan N, Philips JBr, Bulger A, Oparil S, Chen YF. Endothelin-A receptor blockade in porcine pulmonary hypertension. *Pediatr Res.* 2002;52(6):913-21.
104. Petersen B, Deja M, Bartholdy R, Donaubaauer B, Laudi S, Francis RCE, et al. Inhalation of the ETA receptor antagonist LU-135252 selectively attenuates hypoxic pulmonary vasoconstriction. *Am J Physiol Regul Integr Comp Physiol.* 2008;294(2):R601-R5.
105. Geiger R, Trembl B, Kleinsasser A, Neu N, Fischer V, Stein JI, et al. Intravenous tezosentan and vardenafil attenuate acute hypoxic pulmonary hypertension. *High Alt Med Biol.* 2008;9(3):223-7.
106. Pham I, Wuerzner G, Richalet J-P, Peyrard S, Azizi M. Endothelin receptors blockade blunts hypoxia-induced increase in PAP in humans. *Eur J Clin Invest.* 2010;40(3):195-202.
107. Pham I, Wuerzner G, Richalet J-P, Peyrard S, Azizi M. Bosentan effects in hypoxic pulmonary vasoconstriction: Preliminary study in subjects with or without high altitude pulmonary edema-history. *Pulm Circ.* 2012;2(1):28-33.
108. Willette RN, Ohlstein EH, Mitchell MP, Sauermelch CF, Beck GR, Luttmann MA, et al. Nonpeptide endothelin receptor antagonists. VIII: Attenuation of acute hypoxia-induced pulmonary hypertension in the dog. *J Pharmacol Exp Ther.* 1997;280(2):695-701.
109. Modesti PA, Vanni S, Morabito M, Modesti A, Marchetta M, Gamberi T, et al. Role of endothelin-1 in exposure to high altitude. Acute mountains sickness and endothelin-1 (ACME-1) study. *Circulation.* 2006;114(13):1410-6.
110. Clozel M, Ramuz H, Clozel JP, Breu V, Hess P, Loffler BM, et al. Pharmacology of tezosentan, new endothelin receptor antagonist designed for parenteral use. *J Pharmacol Exp Ther.* 1999;290(2):840-6.
111. Fitzgerald RK, Oishi P, Ovadia B, Ross GA, Reinhartz O, Johengen MJ, et al. Tezosentan, a combined parenteral endothelin receptor antagonist, produces pulmonary vasodilation in lambs with acute and chronic pulmonary hypertension. *Pediatr Crit Care Med.* 2004;5(6):571-7.
112. Geiger R, Kleinsasser A, Meier S, Neu N, Pajk W, Fischer V, et al. Intravenous tezosentan improves gas exchange and hemodynamics in acute lung injury secondary to meconium aspiration. *Intensive Care Med.* 2008;34(2):368-76.
113. Konrad D, Oldner A, Rossi P, Wanecek M, Rudehill A, Weitzberg E. Differentiated and dose-related cardiovascular effects of a dual endothelin receptor antagonist in endotoxin shock. *Crit Care Med.* 2004;32(5):1192-9.

114. Wang L, Zhu DM, Su X, Bai CX, Ware LB, Matthay MA. Acute cardiopulmonary effects of a dual-endothelin receptor antagonist on oleic acid-induced pulmonary arterial hypertension in dogs. *Exp Lung Res.* 2004;30(1):31-42.
115. Lourenço AP, Vasques-Nóvoa F, Oliveira-Pinto J, Fontoura D, Roncon-Albuquerque RJ, Leite-Moreira AF. Haemodynamic and neuroendocrine effects of tezosentan in chronic experimental pulmonary hypertension. *Intensive Care Med.* 2012;38(6):1050-60.
116. Denault AY, Pearl RG, Michler RE, Rao V, Tsui SSL, Seitelberger R, et al. Tezosentan and right ventricular failure in patients with pulmonary hypertension undergoing cardiac surgery: the TACTICS trial. *J Cardiothorac Vasc Anesth.* 2013;27(6):1212-7.
117. Kota B, Prasad AS, Economides C, Singh BN. Levosimendan and calcium sensitization of the contractile proteins in cardiac muscle: impact on heart failure. *J Cardiovasc Pharmacol Ther.* 2008;13(4):269-78.
118. Antoniadis C, Tousoulis D, Koumallos N, Marinou K, Stefanadis C. Levosimendan: beyond its simple inotropic effect in the heart. *Pharmacol Ther.* 2007;114(2):184-97.
119. De Witt BJ, Ibrahim IN, Bayer E, Fields AM, Richards TA, Banister RE, et al. An analysis of responses to levosimendan in the pulmonary vascular bed of the cat. *Anesth Analg.* 2002;94(6):1427-33.
120. Kivikko M, Antila S, Eha J, Lehtonen L, Pentikäinen PJ. Pharmacodynamics and safety of a new calcium sensitizer, levosimendan, and its metabolites during an extended infusion in patients with severe heart failure. *J Clin Pharmacol.* 2002;42(1):43-51.
121. Kivikko M, Lehtonen L, Colucci WS. Sustained hemodynamic effects of intravenous levosimendan. *Circulation.* 2003;107(1):81-6.
122. Siebenmann C, Lundby C. Regulation of cardiac output in hypoxia. *Scand J Med Sci Sports.* 2015;25(Suppl 4):53-9.
123. Amà R, Leather HA, Segers P, Vandermeersch E, Wouters PF. Acute pulmonary hypertension causes depression of left ventricular contractility and relaxation. *Eur J Anaesthesiol.* 2006;23(10):824-31.
124. Kerbaul F, Rondelet B, Demester JP, Fesler P, Huez S, Naeije R, et al. Effects of levosimendan versus dobutamin on pressure load-induced right ventricular failure. *Crit Care Med.* 2006;34(11):2814-1819.
125. Kerbaul F, Gariboldi V, Giorgi R, Mekkaoui C, Guieu R, Fesler P, et al. Effects of levosimendan on acute pulmonary embolism-induced right ventricular failure. *Crit Care Med.* 2007;35(8):1948-54.
126. Oldner A, Konrad D, Weitzberg E, Rudehill A, Rossi P, Wanecek M. Effects of levosimendan, a novel inotropic calcium-sensitizing drug, in experimental septic shock. *Crit Care Med.* 2001;29(11):2185-93.
127. Chew MS, Hawthorne WJ, Bendall J, Whereat S, Huang S, Ting I, et al. No beneficial effects of levosimendan in acute porcine endotoxaemia. *Acta Anaesthesiol Scand.* 2011;55(7):851-61.

128. Cunha-Goncalves D, Perez-de-Sa V, Dahm P, Grins E, Thörne J, Blomquist S. Cardiovascular effects of levosimendan in the early stages of endotoxemia. *Shock*. 2007;28(1):71-7.
129. Kleber FX, Bollmann T, Borst MM, Costard-Jäckle A, Ewert R, Kivikko M, et al. Repetitive dosing of intravenous levosimendan improves pulmonary hemodynamics in patients with pulmonary hypertension: results of a pilot study. *J Clin Pharmacol*. 2009;49(1):109-15.
130. Morelli A, Teboul JL, Maggiore SM, Vieillard-Baron A, Rocco M, Conti G, et al. Effects of levosimendan on right ventricular afterload in patients with acute respiratory distress syndrome: a pilot study. *Crit Care Med*. 2006;34(9):2287-93.
131. Félétou M, Huang Y, Vanhoutte PM. Endothelium-mediated control of vascular tone: COX-1 and COX-2 products. *Br J Pharmacol*. 2011;164(3):894-912.
132. Schmedtje JFJ, Ji YS, Liu WL, DuBois RN, Runge MS. Hypoxia induces cyclooxygenase-2 via the NF-kappaB p65 transcription factor in human vascular endothelial cells. *J Biol Chem*. 1997;272(1):601-8.
133. Nakahata N. Thromboxane A2: Physiology/pathophysiology, cellular signal transduction and pharmacology. *Pharmacol Ther*. 2008;118(1):18-35.
134. Fetalvero KM, Martin KA, Hwa J. Cardioprotective prostacyclin signaling in vascular smooth muscle. *Prostaglandins Other Lipid Mediat*. 2007;82(1-4):109-18.
135. Gerber JG, Voelkel N, Nies AS, McMurtry IF, Reeves JT. Moderation of hypoxic vasoconstriction by infused arachidonic acid: role of PGI₂. *J Appl Physiol Respir Environ Exerc Physiol*. 1980;49(1):107-12.
136. Leeman M, Naeije R, Lejeune P, Mélot C. Influence of cyclo-oxygenase inhibition and leukotriene receptor blockade on pulmonary vascular pressure/cardiac index relationships in hyperoxic and in hypoxic dogs. *Clin Sci*. 1987;72(6):717-24.
137. Leeman M, Zegers de Beyl V, Biarent D, Maggiorini M, Mélot C, Naeije R. Inhibition of cyclooxygenase and nitric oxide synthase in hypoxic vasoconstriction and oleic acid-induced lung injury. *Am J Respir Crit Care Med*. 1999;159(5 Pt 1):1383-90.
138. Lennon PF, Murray PA. Attenuated hypoxic pulmonary vasoconstriction during isoflurane anesthesia is abolished by cyclooxygenase inhibition in chronically instrumented dogs. *Anesthesiology*. 1996;84(2):404-14.
139. Weir EK, McMurtry IF, Tucker A, Reeves JT, Grover RF. Prostaglandin synthetase inhibitors do not decrease hypoxic pulmonary vasoconstriction. *J Appl Physiol*. 1976;41(5):714-8.
140. Yoshimura K, Kobayashi T, Kusama S, Sakai A, Ueda G. Thromboxane synthetase inhibition and pulmonary response to hypoxia in conscious adult sheep. *Jpn Circ J*. 1988;52(1):66-71.
141. Shams H, Schulz H, Mohr M, Kobayashi H, Mesch U, Das A, et al. Cyclooxygenase inhibition and effects of hypoxia on pulmonary circulation and gas exchange in anesthetized dogs. *Respir Physiol*. 1989;75(1):39-49.
142. Rubin LJ, Hughes JD, Lazar JD. The effects of eicosanoid synthesis inhibitors on normoxic and hypoxic pulmonary vascular tone in dogs. *Am Rev Respir Dis*. 1985;132(1):93-8.

143. Naeije R, Hallemans R, Melot C, Boeynaems J, Mols P, Lejeune P, et al. Eicosanoids and hypoxic pulmonary vasoconstriction in normal man. *Bull Eur Physiopathol Respir.* 1987;23(6):613-7.
144. Alexander F, Manny J, Lelcuk S, Shepro D, Hechtman HB. Cyclo-oxygenase products mediate hypoxic pulmonary hypertension. *J Pediatr Surg.* 1986;21(12):1101-7.
145. Bradley LM, Stambouly JJ, Czaja JF, Goldstein RE. Influence of thromboxane A2 receptor antagonism on pulmonary vasoconstrictor responses. *Pediatr Res.* 1989;26(3):175-9.
146. Su CL, Yuan DW, Chiang LL, Lee HL, Chen KH, Wang D. Inducible cyclooxygenase expression mediating hypoxia/reoxygenation-induced pulmonary vasoconstriction is attenuated by a cyclooxygenase inhibitor in rats. *Transplant Proc.* 2012;44(4):929-32.
147. Arima T, Matsuura M, Shiramatsu T, Gennda T, Matsumoto I, Hori T. Synthesis of prostaglandins, TXA₂, and PGI₂, during one lung asesthesia. *Prostaglandins.* 1987;34(5):668-84.
148. Richalet J-P, Hornych A, Rathat C, Aumont J, Larmignat P, Rémy P. Plasma prostaglandins, leukotrienes and thromboxane in acute high altitude hypoxia. *Respir Physiol.* 1991;85:205-15.
149. Sjöberg T, Steen S. The strong contractile effect of the thromboxane receptor agonist U-46619 in isolated human pulmonary arteries and its competitive antagonism by BM-13,505. *Acta Physiol Scand.* 1989;136(2):161-5.
150. Hood JS, Nossaman BD, Ibrahim IN, McMahon TJ, Babykos CR, Kadowitz PJ. Daltroban blocks thromboxane responses in the pulmonary vascular bed of the cat. *J Appl Physiol.* 1992;72(6):2305-10.
151. Bertolino F, Valentin J-P, Patoiseau J-F, Rieu J-P, Colpaert FC, John GW. Evidence for partial agonist properties of daltroban (BM 13,505) at TP receptors in the anaesthetized open-chest rat. *Naunyn-Schmiedeberg's Arch Pharmacol.* 1997;356:462-6.
152. Ellsworth ML. The red blood cell as an oxygen sensor: what is the evidence? *Acta Physiol Scand.* 2000;168(4):551-9.
153. Mortensen SP, Saltin B. Regulation of the skeletal muscle blood flow in humans. *Exp Physiol* 2014;99(12):1552-8.
154. Sprague RS, Ellsworth ML, Stephenson AH, Lonigro AJ. ATP: the red blood cell link to NO and local control of the pulmonary circulation. *Am J Physiol.* 1996;271(Heart Circ Physiol 40):H2717-H22.
155. Gordon JL. Extracellular ATP: effects, sources and fate. *Biochem J.* 1986;233(2):309-19.
156. Olivecrona GK, Göteborg M, Harnek J, Wang L, Jacobson KA, Erlinge D. Coronary artery reperfusion: The ADP receptor P2Y₁ mediates early reactive hyperemia *in vivo* in pigs. *Purinergic Signal.* 2004;1(1):59-65.

157. Mitchell C, Syed NI, Tengah A, Gurney AM, Kennedy C. Identification of contractile P2Y1, P2Y6, and P2Y12 receptors in rat intrapulmonary artery using selective ligands. *J Pharmacol Exp Ther.* 2012;343(3):755-62.
158. Hyman AL, Woolverton WC, Pennington DG, Jaques WE. Pulmonary vascular responses to adenosine diphosphate. *J Pharmacol Exp Ther.* 1971;178(3):549-61.
159. Silove ED. Effects of haemolysed blood and adenosine diphosphate on the pulmonary vascular resistance in calves. *Cardiovasc Res.* 1971;5(3):313-8.
160. Kahner BN, Shankar H, Murugappan S, Prasad GL, Kunapuli SP. Nucleotide receptor signaling in platelets. *J Thromb Haemost.* 2006;4(11):2317-26.
161. McCormack DG, Barnes PJ, Evans TW. Purinoceptors in the pulmonary circulation of the rat and their role in hypoxic vasoconstriction. *Br J Pharmacol.* 1989;98(2):367-72.
162. Baek EB, Yoo HY, Park SJ, Kim HS, Kim SD, Earm YE, et al. Luminal ATP-induced contraction of rabbit pulmonary arteries and role of purinoceptors in the regulation of pulmonary arterial pressure. *Pflugers Arch.* 2008;457(2):281-91.
163. Konduri GG, Woodard LL, Mukhopadhyay A, Deshmukh R. Adenosine is a pulmonary vasodilator in newborn lambs. *Am Rev Respir Dis.* 1992;146(3):670-6.
164. Thomas T, Marshall JM. The role of adenosine in hypoxic pulmonary vasoconstriction in the anaesthetized rat. *Exp Physiol.* 1993;78(4):541-3.
165. Lamm WJ, Neradilek B, Polissar NL, Hlastala MP. Pulmonary response to 3 h of hypoxia in prone pigs. *Respir Physiol Neurobiol.* 2007;159(1):76-84.
166. Lelovas PP, Kostomitsopoulos NG, Xanthos TT. A comparative anatomic and physiologic overview of the porcine heart. *J Am Assoc Lab Anim Sci.* 2014;53(5):432-8.
167. Kylhammar D, Persson L, Hesselstrand R, Rådegran G. Prognosis and response to first-line single and combination therapy in pulmonary arterial hypertension. *Scand Cardiovasc J.* 2014;48(4):223-33.
168. Humbert M, Yaïci A, de Groote P, Montani D, Sitbon O, Launay D, et al. Screening for pulmonary arterial hypertension in patients with systemic sclerosis: clinical characteristics at diagnosis and long-term survival. *Arthritis Rheum.* 2011;63(11):3522-30.
169. Tiede SL, Gall H, Dörr O, Troidl C, Liebetrau C, Voss S, et al. New potential diagnostic biomarkers for pulmonary hypertension. *Eur Respir J.* 2015;46(5):1390-6.
170. Smadja DM, Gaussem P, Mauge L, Lacroix R, Gandrille S, Remones V, et al. Comparison of endothelial biomarkers according to reversibility of pulmonary hypertension secondary to congenital heart disease. *Pediatr Cardiol.* 2010;31:657-62.
171. Selimovic N, Bergh C-H, Andersson B, Sakiniene E, Carlsten H, Rundqvist B. Growth factors and interleukin-6 across the lung circulation in pulmonary hypertension. *Eur Respir J.* 2009;34:662-8.
172. Pendergrass SA, Hayes E, Farina G, Lemaire R, Farber HW, Whitfield ML, et al. Limited systemic sclerosis patients with pulmonary arterial hypertension show biomarkers of inflammation and vascular injury. *PLoS One.* 2010;5(8):e12106.

173. Malhotra R, Paskin-Flerlage S, Zamanian RT, Zimmerman P, Schmidt JW, Deng DY, et al. Circulating angiogenic modulatory factors predict survival and functional class in pulmonary arterial hypertension. *Pulm Circ.* 2013;3(2):369-80.
174. Soon E, Holmes AM, Treacy CM, Doughty NJ, Southgate L, Machado RD, et al. Elevated levels of inflammatory cytokines predict survival in idiopathic and familial pulmonary arterial hypertension. *Circulation.* 2010;122:920-7.
175. Itoh T, Nagaya N, Ishibashi-Ueda H, Kyotani S, Oya H, Sakamaki F, et al. Increased plasma monocyte chemoattractant protein-1 level in idiopathic pulmonary arterial hypertension. *Respirology.* 2006;11(2):158-63.
176. Humbert M, Monti G, Brenot F, Sitbon O, Portier A, Grangeot-Keros L, et al. Increased interleukin-1 and interleukin-6 serum concentrations in severe primary pulmonary hypertension. *Am J Respir Crit Care Med.* 1995;151:1628-31.
177. Hoeper MM, Welte T. Systemic inflammation, COPD, and pulmonary hypertension. *Chest.* 2007;131(2):634-5.
178. McGoon MD, Benza RL, Escribano-Subias P, Jiang X, Miller DP, Peacock AJ, et al. Pulmonary arterial hypertension: epidemiology and registries. *J Am Coll Cardiol.* 2013;62(25 Suppl):D51-D9.
179. Pidgeon GP, Tamosiuniene R, Chen G, Leonard I, Belton O, Bradford A, et al. Intravascular thrombosis after hypoxia-induced pulmonary hypertension: Regulation by cyclooxygenase-2. *Circulation.* 2004;110(17):2701-7.
180. Fredenburgh LE, Ma J, Perrella MA. Cyclooxygenase-2 inhibition and hypoxia-induced pulmonary hypertension: Effects on pulmonary vascular remodeling and contractility. *TCM.* 2009;19(2):31-7.

Acknowledgements

This dissertation would never have been initiated, nor completed without the enthusiastic support of my supervisor **Göran Rådegran**. Thank you, Göran, for introducing me into the exciting field of hypoxia and pulmonary hypertension research, for always being available, for support and for encouraging me. I especially appreciate that you constantly put us younger researchers forward, but even more that you introduced me to the beautiful and exciting city of Copenhagen. You should know that I remember your mentor **Bengt Saltin** with warmth and I am very grateful that he was so generous as to support the establishment of your research group, which I had the pleasure to be part of for quite a few years. I hope that we can continue to collaborate in the future!

I also want to direct my sincerest gratitude to my co-supervisor **Roger Hesselstrand** who has likewise been very positive and supportive. You have continuously contributed with new, important perspectives and I much appreciate your unpretentiousness and easy-going attitude towards the collaborative work between the Departments of Cardiology and Rheumatology.

A special thanks goes out to my fellow PhD candidates and other students in the pulmonary hypertension and heart transplantation research group at the Department of Cardiology. To **Jakob Lundgren, Carl Söderlund, Anna Werther-Evaldsson, Annika Ingvarsson, Annaeva Wiklund, Petter Hedelin, Habib Bouzina** and **Eveline Löfdahl** – thank you all for providing new perspectives and insights through your respective work and for being good colleagues and friends.

I also wish to acknowledge **Liselotte Persson, Anneli Ahlqvist, Karin Paulsson, Laurids T. Bune, Camilla Scheele, Søren Nielsen, Ruth Rosving, Hanne Villumsen** and **Marie Wildt** who in different ways and during different periods contributed to the completion of this dissertation. The essential work in the clinic by **Björn Kornhall, Øyvind Reitan, Johan Holm, Carl-Johan Höijer** and the staff at the ward and outpatient clinic for patients with PAH was equally crucial and I direct to you my genuine appraisal.

In addition, I am very grateful that **Patrik Gilje** and **Oscar Braun** sometimes popped up at their desks to say a few words about anything. Had I not looked away from the screen for a chat every now and then I possibly would have gone mad.

To **Monica Magnusson** and **Lena Lindén** – you both played important roles during the work with this dissertation. The Department of Cardiology would not have been what it is without either one of you and I mean this in the most positive

way. Thank you for your hard work, for laughter and lots of coffee. For her regular contribution of laughter and coffee, I also wish to thank **Ewa Löfvendahl**.

Finally, I want to thank my family. My father **Martin** and my mother **Birgitta** for always having been there and for making me curious and appreciative of education and science. My sister **Stina** and my brother **Axel** for friendship and fun. My grandmother **Margareta** for being proud of me and for being the greatest grandmother one could ever have wished for. My mother-in-law **Ingrid** for caring, emotionally as well as in action.

Most of all, however, I wish to thank my wise and beautiful love and life companion **Marie** and my very cute and brilliant daughter **Ellen**. You constantly remind me what is most important in life and I do not know what I would have done without you. I love you!



David Kylhammar was born 17 March 1985 in Linköping. He studied medicine in Lund and graduated in 2012. Thereafter he completed his internship at Skåne University Hospital in 2015. Together with Marie, he will soon have two children.

