Vardenafil and methylarginines in pulmonary hypertension

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Out of clutter find simplicity; from discord find harmony; in the middle of difficulty lies opportunity

*Albert Einstein (1879-1955)*
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Abstract

Background Pulmonary hypertension (PH) is a life threatening condition associated with endothelial dysfunction and vascular remodelling, leading to increased pulmonary vascular resistance (PVR) and right ventricular heart failure. Pulmonary arterial hypertension (PAH) is characterized as a mean pulmonary artery pressure (mPAP) ≥25 mmHg at rest, mean pulmonary artery wedge pressure (mPAWP) ≤15 mmHg and a normal or reduced cardiac output (CO). The pathogenesis includes increased production of vasoconstrictor compounds such as endothelin and thromboxane A2, and decreased production of vasodilator compounds, prostacyclin and nitric oxide (NO). Asymmetric dimethylarginine (ADMA), a methyldeprivate of the amino acid L-arginine, inhibits synthesis of NO, a molecule with important anti-atherosclerotic properties.

During the last decade drug therapy for PAH has undergone a fast evolution, leading to newly approved treatments. Approved PAH-specific therapy acts through three different pathways; the endothelin-, NO/cGMP- and prostacyclin pathways. Vardenafil, a phosphodiesterase type 5 inhibitor (PDE5-inhibitors), causes vasodilation through the NO/cGMP pathway.

The aim of this thesis was to investigate the clinical pharmacological and diagnostic properties of vardenafil in patients with PH and to evaluate L-arginine and methylarginines at diagnosis and during PAH-specific treatment in PAH-patients.

Methods The pharmacodynamic and pharmacokinetic effects of vardenafil were examined during right heart catheterization (RHC) in 16 individuals diagnosed with PH. Vardenafil plasma concentrations were monitored up to nine hours after vardenafil administration. In 20 patients with PH, acute vasoreactivity testing with vardenafil compared to adenosine at RHC were performed. Hemodynamic responses were recorded and responders were identified and followed for up to seven years.

Additionally, 21 patients with PAH were evaluated for plasma concentrations of ADMA, symmetric dimethylarginine (SDMA), L-arginine, L-citrulline and L-ornithine before and after PAH-specific drug treatment. These results were compared to plasma concentrations of ADMA, SDMA and L-arginine in 14 patients with left ventricular heart failure (LVHF) and 27 healthy subjects.

Results Plasma vardenafil concentrations increased rapidly and reached maximum ($t_{max}$) plasma concentration after 1 h. The elimination half-life ($t_{1/2}$) was 3.4 h. Patients co-medicaced with bosentan had a 90 % reduction of
vardenafil plasma concentration. An acute hemodynamic response in mPAP (-20.3%; \(p<0.001\)), PVR (-28.9%; \(p<0.001\)), cardiac output (CO) (10.6%; \(p=0.015\)), cardiac index (CI) (12.2%; \(p=0.01\)), systemic vascular resistance (SVR) (-28.9%; \(p<0.001\)) and PVR/SVR (-16.9%; \(p=0.002\)) was seen after a single oral dose of vardenafil. The acute haemodynamic response was related to plasma vardenafil concentrations. Both vardenafil and adenosine significantly improved hemodynamic response during acute RHC vasoreactivity test, but vardenafil was better tolerated and identified more responders.

PAH-patients had higher ADMA and SDMA levels and lower L-arginine levels and L-arginine/ADMA ratio compared with healthy subjects (\(p<0.001\)). The L-arginine/ADMA ratio was also lower in PAH-patients compared to patients with LVHF (\(p<0.05\)). WHO functional class and six minutes walking distance (6MWD) correlated to L-arginine and L-arginine/ADMA ratio in PAH at baseline (\(p<0.05\)). At follow-up, patients on mono- or combination therapy with endothelin receptor antagonists (ERA) had lower ADMA levels than patients without ERA (\(p<0.05\)). In contrast, patients treated with PDE5-inhibitors had higher ADMA levels compared to patients without PDE5-inhibitors (\(p<0.05\)).

**Conclusion** Vardenafil may be safely used in patients with PH as an acute vasodilator agent during RHC. Vardenafil rapidly improved cardiopulmonary haemodynamics in relation to plasma drug concentrations. A single oral dose of vardenafil was better tolerated than i.v. adenosine and identified more acute responders who might benefit from long-term vasodilator treatment. There was a high inter-individual variability of vardenafil pharmacokinetics in patients with PH and co-medication with bosentan lead to a pharmacokinetic drug interaction. Therapeutic drug monitoring for optimized individual dose may be warranted.

ADMA and SDMA levels were higher and L-arginine along with L-arginine/ADMA ratio were lower in PAH patients compared to healthy subjects. L-arginine was decreased in PAH compared to LVHF. L-arginine/ADMA ratio correlated to WHO functional class and L-arginine and L-arginine/ADMA ratio correlated to 6MWD. PAH-specific treatment influenced L-arginine and methylarginines. Our data suggest that L-arginine levels were useful in differentiating patients with PAH from LVHF, and that L-arginine and L-arginine/ADMA ratios were related to the severity of PAH and might be useful for follow-up evaluations of PAH patients.
Original papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals as assigned below.


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## Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ACEI</td>
<td>angiotensin-converting enzyme inhibitors</td>
</tr>
<tr>
<td>ADMA</td>
<td>asymmetric dimethylarginine</td>
</tr>
<tr>
<td>APAH</td>
<td>associated pulmonary arterial hypertension</td>
</tr>
<tr>
<td>ARB</td>
<td>angiotensin receptor blockers</td>
</tr>
<tr>
<td>AUC</td>
<td>area under curve</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;i&lt;/sub&gt;</td>
<td>AUC extrapolated to infinity</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;norm&lt;/sub&gt;</td>
<td>AUC normalised for dose and weight</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;i, norm&lt;/sub&gt;</td>
<td>AUC&lt;sub&gt;i&lt;/sub&gt; normalised for dose and weight extrapolated to infinity</td>
</tr>
<tr>
<td>BH&lt;sub&gt;4&lt;/sub&gt;</td>
<td>tetrahydrobiopterin</td>
</tr>
<tr>
<td>BNP</td>
<td>brain natriuretic peptide</td>
</tr>
<tr>
<td>CCB</td>
<td>calcium channel blocker</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CI</td>
<td>cardiac index</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>maximal plasma concentration</td>
</tr>
<tr>
<td>C&lt;sub&gt;max, norm&lt;/sub&gt;</td>
<td>C&lt;sub&gt;max&lt;/sub&gt; normalised for dose and weight</td>
</tr>
<tr>
<td>CO</td>
<td>cardiac output</td>
</tr>
<tr>
<td>CTEPH</td>
<td>chronic thromboembolic pulmonary hypertension</td>
</tr>
<tr>
<td>EMA</td>
<td>European Medicines Agency</td>
</tr>
<tr>
<td>ETA</td>
<td>endothelin-A</td>
</tr>
<tr>
<td>ETB</td>
<td>endothelin-B</td>
</tr>
<tr>
<td>eGFR</td>
<td>estimated glomerular filtration rate</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthases</td>
</tr>
<tr>
<td>FDA</td>
<td>US Food and Drug Administration</td>
</tr>
<tr>
<td>GABR</td>
<td>global arginine bioavailability ratio</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthases</td>
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<tr>
<td>IPAH</td>
<td>idiopathic pulmonary arterial hypertension</td>
</tr>
<tr>
<td>LVHF</td>
<td>left ventricular heart failure</td>
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<tr>
<td>6MWD</td>
<td>six minutes walk distance</td>
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<tr>
<td>mPAP</td>
<td>mean pulmonary arterial pressure</td>
</tr>
<tr>
<td>mPAWP</td>
<td>mean pulmonary artery wedge pressure</td>
</tr>
<tr>
<td>mRAP</td>
<td>mean right atrial pressure</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthases</td>
</tr>
<tr>
<td>nNOS</td>
<td>neuronal nitric oxide synthases</td>
</tr>
<tr>
<td>NT-proBNP</td>
<td>N-terminal pro B-type natriuretic peptide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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<tr>
<td>LC-MS/MS</td>
<td>liquid chromatography – tandem mass spectrometry</td>
</tr>
<tr>
<td>PA</td>
<td>pulmonary artery</td>
</tr>
<tr>
<td>PAH</td>
<td>pulmonary arterial hypertension</td>
</tr>
<tr>
<td>PDE5</td>
<td>phosphodiesterase type 5</td>
</tr>
<tr>
<td>PDE5-inhibitors</td>
<td>phosphodiesterase type 5 inhibitor</td>
</tr>
<tr>
<td>PH</td>
<td>pulmonary hypertension</td>
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<tr>
<td>PPH</td>
<td>primary pulmonary hypertension</td>
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<tr>
<td>PVR</td>
<td>pulmonary vascular resistance</td>
</tr>
<tr>
<td>RAP</td>
<td>right atrial pressure</td>
</tr>
<tr>
<td>RHC</td>
<td>right heart catheterisation</td>
</tr>
<tr>
<td>RV</td>
<td>right ventricular</td>
</tr>
<tr>
<td>sGC</td>
<td>soluble guanylate cyclase</td>
</tr>
<tr>
<td>SDMA</td>
<td>symmetric dimethylarginine</td>
</tr>
<tr>
<td>SVA</td>
<td>Swedish National Veterinary Institute</td>
</tr>
<tr>
<td>SVR</td>
<td>systemic vascular resistance</td>
</tr>
<tr>
<td>$t_{1/2}$</td>
<td>elimination half-life</td>
</tr>
<tr>
<td>$T_{\text{max}}$</td>
<td>time to maximum concentration</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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</table>
Svensk sammanfattning

Bakgrund

En av anledningarna till att PAH uppstår är en rubbning i kväveoxid (NO)-systemet i lungkärlen. NO är en molekyl som motverkar åderförkalkning och har kärlvidgande egenskaper. När NO-halten minskar ökar åderförkalkning och kärlen drar sig samman. Två aminosyror, asymmetrisk dimetylarginin (ADMA) och L-arginin är inblandade i bildningen av NO. En låg kvot av L-arginin/ADMA leder till minskade nivåer av NO, vilket kan leda till kärlskador. Det är känt att patienter med PAH har ökade nivåer av ADMA och att förhöjda ADMA-nivåer är associerade med en högre dödlighet hos patienter med kardiovaskulär sjukdom.


Syfte
Denna avhandling har i huvudsak två syften. Det ena är att hos patienter med PH studera hur läkemedelssubstansen vardenafil påverkar kroppen, d.v.s. hur läkemedlet åstadkommer sin effekt, och hur kroppen påverkar vardenafil, d.v.s. hur det tas upp, tas om hand, bryts ner och utsöndras. Vi har också studerat vardenafils effekt vid akut en vasoreaktivitetstest jämfört
med ett annat kärlvidgande medel, adenosin. Det andra syftet är att undersöka hur några nya biomarkörer (ADMA, symmetrisk dimetylarginin (SDMA), L-arginin, L-citrullin och L-ornitin) påverkas av PAH-specifisk läkemedelsbehandling hos patienter med PAH.

**Metod**
I de första två studierna genomfördes diagnostisk hjärtkateterisering på 16 patienter med PH, då mättades blodtrycket i hjärtats högra kammare och förmak samt trycket i lungkretsloppet. Efter administration av en tablett vardenafil mättes trycket återigen i hjärta och lungartär och blodflödet i kroppen registrerades. För att följa hur kroppen påverkade läkemedlet togs blodprov för att mäta koncentrationerna av vardenafil vid o, 15, 30, 45, 60, 120, 300 och 540 minuter efter administrering.

I den tredje studien utfördes akuta vasoreaktivitetstest med adenosin och vardenafil under hjärtkateterisering hos 20 patienter med PH. De två läkemedlens förmåga att identifiera responders jämfördes. En responder svarar på läkemedlet genom att vidga blodkärlen och blir därmed kandidat för behandling med ett specifikt kärlvidgande läkemedel, kalciumkanalblockerare. Patienter som var responders till vardenafil identifierades och samtliga patienter i studien följdes upp till sju år efteråt.


**Resultat**

PAH patienter hade högre ADMA och SDMA nivåer och lägre L-argininnivåer samt L-arginin/ADMA-kvot jämfört med friska personer, men lägre L-arginin jämfört med patienter med hjärtsvikt. L-arginin/ADMA-kvoten korrelerade med WHO funktionsklass och L-arginin samt L-arginin/ADMA-
kvoten korrelerade med gångtest hos patienter med PAH. Patienter som behandlades med endotelinreceptorantagonister hade generellt lägre ADMA nivåer än patienter som behandlades med andra PAH-specifika läkemedel. Vidare hade patienter som behandlades med PDE5-hämmare högre ADMA nivåer än de som inte behandlades med PDE5-hämmare.

**Diskussion**

Vardenafil var säkert att använda hos patienter med PH både som PAH-spezifikt läkemedel och vid diagnostik med vasoreaktivitetstest under hjärtkateterisering. En tablett vardenafil gav en snabb och kraftig effekt i lungkärlen och effekten var relaterad till läkemedelskonzentrationen i blod.

Vardenafil identifierade fler responders än adenosin. Det var en stor individuell skillnad i hur kroppen påverkade vardenafil hos patienter med PAH och vardenafilkonzentrationen i blodet minskade vid samtidig användning av endotelinreceptorantagonisten bosentan. Att följa läkemedelskonzentrationer av vardenafil eller andra PDE5-hämmare skulle kunna vara gynnsamt för att optimera den individuella behandlingen.

ADMA och SDMA var högre samt L-arginin och L-arginin/ADMA kvoten lägre hos PAH patienter jämfört med friska kontroller. L-arginin var även lägre vid PAH jämfört med patienter med hjärtsvikt. Utöver detta sägs också en korrelation mellan L-arginin och L-arginin/ADMA kvoten med WHO funktionsklass och gångtest. Dessa biomarkörer skulle kunna vara viktiga verktyg för att följa sjukdomsprogression och läkemedelsbehandling hos PAH patienter. Ändringarna av L-arginin och dimetylargininer visar indirekt att NO-systemet är påverkat hos hjärt- och kärlsjuka patienter och särskilt vid PAH.
Introduction

The history of pulmonary hypertension

Pulmonary hypertension (PH) is a newly discovered disease, although the awareness of it has existed for centuries. In the late 19th century pulmonary arteriosclerosis was widely accepted as the morphologic signature of chronic PH (1). The German physician Klob made the first known case-report in 1865. He described a patient with ankle oedema, dyspnoea and cyanosis shortly before the patient deceased. At autopsy Klob detected an impressive narrowing of the finer branches of the pulmonary artery (2). In 1891 the German physician Ernst von Romberg described the intrinsic pulmonary vascular disease as “pulmonary vascular sclerosis” (1–3). The Argentinean physician Arriilaga described the first series of patients with PH in 1913. This syndrome with cyanosis and death by right heart failure was subsequently designated as Ayerza´ s disease, and syphilis was proposed as an etiological factor. Despite some misconception about the syphilitic etiology of PH, the belief remained popular until the 1940s.

In the middle of the 20th century the histopathology of the disease was further clarified and the relationship between pulmonary vascular lesions and right ventricular (RV) hypertrophy was understood (1, 3, 4). In the 1950s, Dresdale and co-workers demonstrated that pulmonary vasoconstriction was involved in the pathogenesis of the disease and the term primary pulmonary hypertension (PPH) was conceived. They treated a patient with PPH with the systemic and pulmonary vasodilator tolazoline.

Pulmonary blood flow measurements began already in 1912 using nitrous oxide, and hemodynamic outflow for calculation of pulmonary vascular resistance (PVR) became available in the middle of the 20th century (3) Dr. Werner Forssmann showed in 1929, in heroic experiments on himself, that it was safe to insert a catheter through a peripheral vein into the right side of the heart. In 1956 he shared the Nobel Prize with André F. Cournand and Dickinson W. Richards for their respective role in introducing and standardising cardiac catheterisation. Hellems and colleagues modified the technique to measure pulmonary pressure. In 1970, William Ganz and Harold J. Swan introduced a multilumen, balloon-tipped catheter for hemodynamic monitoring (5). The catheter made it possible to record the pressures in the pulmonary artery simultaneously.

The interest for PH increased in the late 1960s, when there was an epidemic of PH associated to the use of an appetite suppressant, aminorex fumarate
(2-amino-5-phenyl-2-oxazoline) (1, 3). The pharmacodynamic actions of aminorex included release of norepinephrine at nerve endings and an increase of serotonin levels in the circulation. Because of the aminorex epidemic, the first World Health Organisation (WHO) meeting to assess the state of knowledge about PPH and to standardise clinical and pathological nomenclature was held in Geneva 1973 (6). During the second WHO meeting in Evian, France in 1998, the first clinical classification of PH was undertaken (7). The effectiveness of the Evian classification was evaluated on the 3rd WHO meeting in Venice, Italy in 2003, where the term PPH was abandoned in favour of pulmonary arterial hypertension (PAH) including idiopathic pulmonary arterial hypertension (IPAH), familial pulmonary arterial hypertension (PAH) or associated PAH (APAH) (8). During the 5th WHO-meeting in Nice, France in 2013, it was decided to withdraw persistent PH of the newborn from group 1 (PAH) (9). It is now designated with number 1′′ (table 1).

**Diagnosis of pulmonary hypertension**

The diagnostic procedure of a patient with suspected PH requires a series of investigations to confirm the diagnosis and to confirm the clinical group of PH (10–12). The most common symptoms of PH are non-specific and include dyspnea, weakness, fatigue, chest pain, syncope, palpitations, lower extremity edema and abdominal distention. Patients with PH have many diagnostic procedures to go through before diagnosed, for example echocardiography, chest X-ray, and lung functions tests. In particular, the following diagnostic procedures were used in our studies; biochemical markers, six minutes walk distance (6MWD) and right heart catheterisation (RHC) with acute vasodilator testing.

**Biochemical markers**

Use of biochemical markers is an attractive non-invasive tool for diagnosing and monitoring patients with PH (10). There is still no specific marker for PAH, but brain natriuretic peptide (BNP) and especially its less active form N-terminal prohormone brain natriuretic peptide (NT-proBNP) are used routinely in PAH clinics and clinical trials. NT-proBNP levels correlate with myocardial dysfunction and give prognostic information at the time of diagnosis and during follow-up.

**Exercise capacity**

The exercise capacity is measured with 6MWD (13). It is a technically easy, inexpensive, reproducible and standardized test that is well tolerated by patients with PAH. 6MWD requires a flat, straight corridor that is at least 30
meters long. The length of the corridor is marked every third meter and a cone identified the turnaround points. Before start a baseline heart rate and oxygen saturation are measured and the Borg Scale is used to grade dyspnea and fatigue. The patients then walk the 30 meters back and forth during six minutes. Patients are instructed to walk as fast as they can but in a pace that allows to continue the exercise for a period of 6 minutes. The patient is allowed to use oxygen supplement during the test if needed. After the exercise heart rate and oxygen saturation are measured and the Borg Scale is assessed again. The total walk distance during six minutes is recorded.

There is a significant correlation between the baseline 6MWD and hemodynamic parameters and survival (11). Walking distances below 332 m (14), 250 m (15) or 165 m (16) are related to worse outcome in PAH. On the other hand, walking distances over 380 m (15) or 440 m (16) are associated with improved survival in PAH. Furthermore, 6MWD is a commonly used primary endpoint for evaluating treatment effects in PAH (11, 12). The current treatment goal for 6MWD is >400 m (17). The 6MWD is not adequately validated in PAH subgroups and is influenced by gender, age, body weight, height and patient motivation.

**Right heart catheterization**

RHC and hemodynamic measurements are required to confirm the diagnosis of PAH (10, 11, 18). Furthermore, it is also important to evaluate the severity of the disease and to test the vasoreactivity in the pulmonary circulation. During RHC the following hemodynamic parameters should be recorded: pulmonary artery pressure (systolic, diastolic and mean; mPAP), pulmonary artery wedge pressure (PAWP), right atrial pressure (RAP) and RV pressure. Cardiac output (CO) is measured by either thermodilution or by the Fick method, if oxygen consumption is assessed and cardiac index (CI) is subsequently calculated. Systemic blood pressure, heart rate, pulmonary artery saturation and arterial saturation should also be determined. Calculation of PVR and systemic vascular resistance (SVR) are made.

\[
PVR = \frac{mPAP - mPAWP}{CO}
\]

\[
SVR = \frac{mAP - mRAP}{CO}
\]

\[mPAP = \text{mean pulmonary artery pressure},\ mPAWP = \text{mean pulmonary artery wedge pressure},\ CO = \text{cardiac output},\ mAP = \text{mean aorta pressure},\ mRAP = \text{mean right atrial pressure}\]
Further, the PVR/SVR ratio is calculated, as a measure of pulmonary selectivity of the vasodilatory effect.

**Acute vasodilator testing**

To assess the vasoreactivity of the pulmonary circulation in patients with PAH, an acute vasoreactivity test is performed during diagnostic RHC (10). The pulmonary vasoreactive test identifies patients who may benefit from long-term therapy with calcium channel blockers (CCB). Acute vasodilator test should be performed with short acting drugs that are easy to administer and with no or limited systemic effect. Currently, the most used agent for vasodilator testing is inhaled nitric oxide (NO). Intravenous epoprostenol and adenosine can also be used, but with higher risk for systemic vasodilation.

A positive vasoreactivity response is defined as a reduction of mPAP of ≥10 mmHg to a mPAP <40 mmHg, with unchanged or increased CO (10).

**Clinical classification of pulmonary hypertension**

During the 5th WHO symposium on PH held in Nice, France in 2013, there was a consensus to update the clinical classification of PH (table 1) (10). The clinical conditions of PH are divided into five groups according to pathological and pathophysiological characteristics. PH in general is defined as a persistent increase in mPAP to ≥ 25 mmHg at rest measured by RHC (17). Patients in group 1, 3, 4 and 5 have pre-capillary PH defined as persistent increase in mPAP to ≥ 25 mmHg, a PAWP below 15 mmHg and normal or reduced CO. Patients in group 2 have post-capillary PH defined as persistent increase in mPAP to ≥ 25 mmHg, PAWP above 15 mmHg with a normal or reduced CO.

In Europe, the incidence of PAH is about 5-10 cases per million population and the prevalence 15-60 patients per million population (19). Since the introduction of PAH-specific drugs, the median survival time of PAH-patients have increased considerably, from 2.8 years if untreated (20) to 7 years with PAH-specific treatment (21).

**Pathobiology of pulmonary hypertension**

Different PH groups show different pathology and pathobiology. PH may involve multiple clinical conditions such as cardiovascular and respiratory diseases (22). PAH is a rare and progressive disease characterized by endothelial dysfunction and vascular remodelling, leading to elevated PVR and mPAP, often resulting in RV failure and premature death (17,23).
Table 1. Clinical classification of pulmonary hypertension (10)

1. **Pulmonary arterial hypertension (PAH)**
   1.1 Idiopathic
   1.2 Heritable
   1.3 Drugs and toxins induced
   1.4 Associated with:
      1.4.1 Connective tissue disease
      1.4.2 Human immunodeficiency virus (HIV) infections
      1.4.3 Portal hypertension
      1.4.4 Congenital heart disease
      1.4.5 Schistosomiasis

1’. **Pulmonary veno-occlusive disease and/or pulmonary capillary haemangiomatosis**

1”’. **Persistent pulmonary hypertension of the newborn**

2. **Pulmonary hypertension due to left heart disease**
   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**
   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 Development lung diseases

4. **Pulmonary hypertension due to chronic pulmonary artery obstruction**
   4.1 Chronic thromboembolic pulmonary hypertension (CTEPH)
   4.2 Other pulmonary artery obstructions

5. **Pulmonary hypertension with unclear and/or multifactorial mechanisms**
   5.1 Hematologic disorders
   5.2 Systemic disorders
   5.3 Metabolic disorders
   5.4 Others
Although, the exact mechanism of the onset and progression of PH is still unknown, many predisposing and/or contributing factors have been identified (24). This includes endothelial dysfunction, inflammation, aberrant vascular wall cell proliferation and genetic factors such as mutations in the bone morphogenetic protein receptor type 2 (BMPR2) gene.

The inflammatory processes are associated with activation of signalling pathways within different cells (e.g. endothelial cells and smooth muscle cells) and increased production of inflammatory-derived mediators (e.g. reactive oxygen species (ROS), interleukin-6 and tumor growth factor-β) (25). These processes can be stimulated by genetic and/or environmental factors, including hypoxia. It is well known that the endothelial dysfunction involves increased production of vasoconstrictor compounds such as endothelin and thromboxane A2, as well as decreased production of vasodilating and antiproliferative compounds such as prostacyclin and NO (17, 23). All these processes are linked together and abnormal function can lead to the development of PH. Disruption of the NO pathway with decreased levels plays an important role in the pathogenesis of PAH (26).

**Nitric oxide in pulmonary hypertension**

NO is an endogenously, synthesized, diffusible and lipophilic gas with antihypertensive, antithrombotic and antiatherosclerotic properties (27, 28). Endothelium-derived NO is essential for vascular homeostasis (27). NO is a potent pulmonary artery vasodilator via the activation of cyclic guanosine monophosphate (cGMP) and has its effects on smooth muscle relaxation and proliferation. NO and L-citrulline are produced by nitric oxide synthases (NOS) enzymes from L-arginine, presented in fig. 1 (26, 27). There are three NOS isoforms identified; neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) (27). In general, nNOS is expressed in neuronal cells and skeletal muscle and acts as an inhibitor of non-adrenergic non-cholinergic neurons and mediates neural bronchodilation. iNOS is present in epithelial and smooth muscle cells, as well as in neutrophils, macrophages and fibroblasts and regulate vascular flow. The third isoform, eNOS, is found in endothelial cells throughout the body and is the predominant source of NO production in the pulmonary circulation. Decreased NO is related to dysfunction of the eNOS enzyme and a reduced eNOS activity (28).

**NOS dysfunction**

Under conditions of cardiovascular risk factors or vascular disease, NO in the vasculature is reduced, due to decreased NO production and enhanced NO inactivation by superoxide, eNOS uncoupling (28). Several mechanisms are
implicated in eNOS uncoupling, including reduced levels of L-arginine, accumulation of asymmetric dimethylarginine (ADMA) deficiency of tetrahydrobiopterin (BH₄), and eNOS S-glutathionylation. NO produced under oxidative stress conditions may induce specific tissue injury.

NOS requires L-arginine to function and produce NO, and an abnormal arginine metabolism plays an important role in NOS dysfunction (29). Two pathways are central in this process; (1) conversion of L-arginine to ADMA by protein-arginine methyl transferase (PRMT) and (2) catabolism from L-arginine to urea and L-ornithin by arginase I and/or II. Both reduce the levels of L-arginine, and in case of ADMA, further inhibit the NOS activity. Arginase is activated by oxidative stress and is up-regulated with aging, ischemic heart disease, hypertension and heart failure. Arginase II expression is enhanced in endothelial cells of patients with PAH (26).

**L-arginine and methylated arginine derivatives**

Methylated arginines are released to the cytoplasm from protein degradation by PRMT I and II (figure 1 and 2) (30). Both types of PRMT catalyse the formation of monomethylarginine (MMA) from L-arginine. Further, PRMT I catalyses ADMA and PRMT II produces symmetric dimethylarginine (SDMA). PRMT expression is upregulated in chronic hypoxia, resulting in increased ADMA levels (31).

---

**Figure 1.** The PRMT catalysed methylation of arginine residues to generate monomethylarginine (MMA) from L-arginine. PRMT I catalyses ADMA and PRMT II produce SDMA.
MMA is metabolized by the dimethylarginine dimethylaminoxydrolase (DDAH) to L-citrulline and dimethylarginine (DMA) (27, 30). Decreased activity of DDAH increases the values of ADMA (27, 30, 32).

**Asymmetric dimethylarginine**

The most important NOS inhibitor is ADMA (27, 32). ADMA is proposed to be a pathophysiologic factor of cardiovascular and kidney diseases. By inhibiting NOS and cationic amino acid transport (CAT), ADMA decreases NO production and inhibits cellular L-arginine uptake, generating oxidative stress and endothelial dysfunction (25, 32, 33). ADMA is accumulated in different disease states and the ADMA concentration is strongly predictive of endothelial dysfunction, cardiovascular diseases and mortality (29, 30, 32, 34). Significantly elevated ADMA levels has been reported in patients with IPAH (35), PH related to sickle cell disease or systemic sclerosis (36, 37), PH associated with HIV (38), PH associated with congenital heart disease (39–41) and CTEPH (42). Together, these findings strongly indicate an association between ADMA plasma levels and PAH/PH.

Pathogenesis (43). As mediator of endothelial dysfunction and damage, ADMA is of interest as a potential biomarker for the development of PAH (39, 44, 45).

![Figure 2](image_url)

**Figure 2.** Synthesis and metabolism of ADMA through the PRMT-DDAH-ADMA axis. Methylation of arginine residues to ADMA occurs through PRMT I. ADMA is an inhibitor of NOS, leading to decreased NO production. ADMA is mainly metabolised by DDAH to L-citrulline and dimethylamine and partly via urinary excretion.
ADMA levels may also predict cardiovascular outcome and mortality in PAH (35). PRMT, DDAH and CAT activities play a critical role in determining cellular levels of ADMA (32). The elevated ADMA levels enhance the inhibition of eNOS, which may lead to the uncoupling of eNOS, and result in a shift from NO production to production of superoxides (26). ADMA, NO as well as eNOS are novel targets in the treatment of PH and other cardiovascular diseases (30,33). Plasma ADMA levels in healthy subjects are in the range 0.35 – 0.70 µmol/l (30, 33).

Symmetric dimethylarginine (SDMA)
SDMA is similar to ADMA, identified as a potential biomarker for cardiovascular disease (46). Furthermore, SDMA is mainly excreted from the kidneys and renal function and SDMA correlate (47–50). Elevated SDMA levels have been associated with cardiovascular diseases such as dilated cardiomyopathy, stroke, peripheral arterial disease as well as worse outcome after coronary angiography and all-cause and cardiovascular mortality, but the specific role of SDMA in cardiovascular disease is still unclear (46, 50). SDMA interferes with NO production by uncoupling eNOS, leading to oxidative stress, and by inhibition of cellular uptake of L-arginine by CAT (49, 50). It is also believed that SDMA indirectly may be linked to inflammation (51).

L-arginine
Patients with PH have a decreased L-arginine bioavailability as a result of increased catabolism by arginase and higher plasma ADMA levels (26). The global arginine bioavailability ratio (GABR; the ratio of L-arginine to L-citrulline plus L-ornithine) is associated with mortality in PH-patients with sickle cell disease (52). PAH-patients also have a lower L-arginine/L-ornithine ratio, indicating a high arginase activity (26). Furthermore, PAH-patients with a high arginase activity had lower plasma ADMA levels than patients with low arginase activity. ADMA may compete with L-arginine as a substrate to NOS (53) and thus might affect the endothelial dysfunction by L-arginine antagonizing the eNOS inhibitor ADMA (54).

L-arginine/ADMA ratio
The L-arginine/ADMA ratio gives a greater insight into NOS activity than either component individually (48). Endothelium-dependent vasodilation may be dependent on the plasma L-arginine/ADMA ratio (53, 55). This ratio is a good predictor of the risk of arteriosclerosis and cardiovascular diseases. Several studies indicate that a low L-arginine/ADMA ratio is associated with impaired organ function, cardio- and cerebrovascular diseases and mortality.
Reference intervals for L-arginine/ADMA ratio ratio in healthy subjects are 74.3 (95% CI = 71.1-77.3) and 225 (95 % CI = 222-228), with a mean L-arginine/ADMA ratio of 150 ± 38 (47).

L-citrulline
Endothelial and vascular smooth muscle cells have the ability to regenerate L-arginine from citrulline and aspartate by synthesizing argininosuccinate. That is the reason why exogenous citrulline administration stimulates NO production in vascular endothelial cells (27).

Dimethylarginine dimethylamino hydrolase (DDAH)
Intracellular ADMA is metabolised to L-citrulline and dimethylamine by the enzyme DDAH1 and DDAH2 (48, 60, 61). DDAH1 is mainly expressed in the kidney and the liver and DDAH2 is mainly expressed in the vascular endothelium and the heart, placenta and kidney (48, 61). Furthermore, compared to healthy controls, patients with IPAH have significantly reduced DDAH2 in lung tissue (62). DDAH1 and DDAH2 isoforms show distinct tissue distribution in relationship with NOS isoforms. DDAH1 is predominatly found in tissues expressing nNOS, whereas DDAH2 predominate in tissues that express eNOS (63). DDAH is an important regulator of NO bioavailability as well as renal and vascular function. It has been reported that oxidized stress in endothelial cells, such as inflammatory cytokines, hyperhomocysteinemia, hyperglycemia and oxidized low-density lipoprotein (LDL), may reduce the DDAH activity, leading to increased levels of ADMA (54, 64). Enhancing DDAH activity, leads to decreased ADMA production and increased NO-mediated vasodilation. This may be a potential pharmacological target in cardiovascular disease (63, 65, 66). Reduced DDAH activity will result in an increased ADMA/SDMA ratio (32).

Pharmacological treatment for PAH
Over the last 20 years, treatment of PAH has undergone a fast evolution, which has led to newly approved drug treatments (figure 3) (67). Moreover, additional drugs are in the pipeline and will be available in the near future (68,69). These new PAH-specific drug therapies have lead to remarkable improvements in patients’ symptoms and extended their life-span (67–69). A meta-analysis including 23 randomised controlled studies in PAH patients reported a 43 % decrease in mortality and a 61 % reduction in hospitalizations for patients treated with PAH-specific therapy compared to placebo (70). In addition to specific PAH-drug therapy, patients are commonly treated with oral anticoagulants, diuretics, oxygen and/or digoxin (10).
**Calcium channel blockers**
Patients who show a response to acute vasodilator testing (RHC), responders, will normally start high dose CCB therapy (10). The choice of CCB is based upon the patient’s heart rate at baseline. Patients with normofrequent heart rate or bradycardia will get nifedipine or amlodipine and patients with tachycardia will get diltiazem. The most studied CCB for PAH is nifedipine. Favourable daily dose for different CCB are; nifedipine 120-240 mg, diltiazem 240-270 mg and amlodipine up to 20 mg. It is advisable to start with a low dose and increase slowly to the maximum tolerated dose. Non-responders to the acute vasodilator test should not be treated with CCB therapy because of potential adverse side effects and no effect on PAH.

**Prostacyclin analogues**
The prostanoid epoprostenol was the first available PAH-specific therapy for treatment in patients with PAH (figure 3) (10). Prostacyclin is an endogenous compound predominantly produced by endothelial cells that induces vasodilation of all vascular beds. It is an inhibitor of platelet aggregation and has cytoprotective and antiproliferative properties (71). In PAH the prostacyclin metabolic pathways are dysregulated, with reduced prostacyclin synthase expression in the pulmonary arteries and in prostacyclin urinary metabolites (72). The clinical use of prostanoids is cumbersome due to the unstable pharmacokinetics of the compounds. Today, there are four prostanoids approved by the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA); epoprostenol, iloprost, treprostinil and beraprost. In Sweden, epoprostenol, iloprost and treprostinil are available. They share the same pharmacodynamic effects. The first oral long-term therapy acting on the prostacyclin pathway, selexipag, is expected to be approved in 2016.

**Endothelin receptor antagonists**
In PAH the endothelial system is activated with increased endothelin-1 concentration in both plasma and lung tissue, which supports a prominent role in the pathogenesis of PAH (10). Endothelin-1 exerts vasoconstrictor and mitogenic effects when binding to the two endothelin receptors in pulmonary vascular smooth muscle cells, the endothelin-A (ETA) and endothelin-B receptors (ETB). Endothelin-B receptors are also present in endothelial cells, leading to release of vasodilators and antiproliferative substances, for example NO and prostacyclin (figure 4).
**Figure 3.** Evolution of PAH-specific drug therapies and the year of approval by EMA. Vardenafil is not approved for PAH treatment (yet). The numbers of the therapeutic strategies correspond to pathways depicted in figure 4.
Figure 4. Schematic presentation of the four different pathways targeted by approved PAH therapy. Endothelial dysfunction is supposed to be the result of an imbalance between the availability of endogenous vasodilatory mediators (nitric oxide and prostacyclin) and increased presence of endothelin-1, one of the most potent vasoconstrictor compounds and an inductor of smooth muscle cell proliferation. The endothelin-1 pathway with the endothelin receptors ET\(_A\) and ET\(_B\) can be blocked by the different endothelin-1 receptor antagonists; the nitric oxide pathway can be enhanced by stimulation of soluble guanylate cyclase or by constraining the breakdown of cGMP through phosphodiesterase type-5 inhibitors. Finally, the prostacyclin pathway can be increased by the administration of prostanoid analogues or non-prostanoid prostacyclin receptor agonists. ADMA can inhibit eNOS.
At present, there are three endothelin receptor antagonists (ERA) available; ambrisentan, bosentan and macitentan. Ambrisentan binds selectively to endothelin receptor type A while bosentan and macitentan binds to both endothelin-A and endothelin-B receptors. The three ERAs have different pharmacokinetic properties and their chemical structures differ markedly. Peak concentrations are reached first for ambrisentan (73), then bosentan (74) and last for macitentan (75). The major elimination route is hepatic metabolism for all ERAs. Both bosentan and macitentan are mainly metabolized by CYP3A4 and to a lesser extent CYP2C (74, 75). Bosentan is also an inducer of CYP3A4, CYP2C9 and possibly CYP2C19 (74). Ambrisentan is metabolized by uridine 5' diphosphate glucuronosyltransferases (UGTs), CYP3A4, and CYP2C19 (73). The elimination half-lives for the three compounds are; bosentan 5-8 h, ambrisentan 9-15 h and macitentan 14-16 h (73–75). Macitentan has an active metabolite with a half-life of 48 h (75).

**Soluble guanylate cyclase stimulators**

Riociguat is a soluble guanylate cyclase stimulator (sGC), which enhances cGMP production (76, 77). In contrast to phosphodiesterase type 5 inhibitors (PDE5-inhibitors) who slow down the cGMP degradation (figure 4). Riociguat also acts in synergy with endogenous NO. It is potentially more effective in improving the NO/cGMP pathway than PDE5-inhibitors (78). Pre-clinical studies have shown that riociguat has anti-remodelling and anti-proliferative properties. Riociguat is approved by EMA and FDA for treatment of PAH and CTEPH.

**Phosphodiesterase type 5 inhibitors**

Inhibition of phosphodiesterase-5 (PDE5) results in vasodilation through the NO/cGMP pathway (figure 4). PDE5 inhibitors prevent the breakdown of cGMP, leading to increased cGMP kinase activity, an enzyme that activates potassium and inhibits calcium channels (79, 80). Decreased intracellular calcium concentrations lead to smooth muscle relaxation and vasodilation as well as exerting antiproliferative effects.

The pulmonary vasculature contains substantial amounts of PDE5 and thus, PDE5 inhibitors are effective in PAH. There are three different PDE5 inhibitors available on the market; sildenafil, vardenafil and tadalafil. However, only sildenafil and tadalafil are approved for PAH treatment. Sildenafil is a potent and selective PDE5 inhibitor approved in both oral and i.v. formulations with favourable results on 6MWD, clinical symptoms and/or haemodynamics (81–83). Tadalafil is also a potent and selective oral
PDE5 inhibitor with improved results on 6MWD, symptoms, haemodynamics and time to clinical worsening at the highest dose (84–86). The three PDE5-inhibitors have different selectivities to the various PDE enzymes (table 2) (87).

**Table 2.** Selectivity to different PDE enzymes compared to each substances selectivity to PDE5 (87).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Sildenafil</th>
<th>Vardenafil</th>
<th>Tadalafil</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDE1</td>
<td>80</td>
<td>130</td>
<td>10 000</td>
</tr>
<tr>
<td>PDE2</td>
<td>700</td>
<td>1 000</td>
<td>10 000</td>
</tr>
<tr>
<td>PDE3</td>
<td>4 000</td>
<td>1 000</td>
<td>10 000</td>
</tr>
<tr>
<td>PDE4</td>
<td>700</td>
<td>1 000</td>
<td>10 000</td>
</tr>
<tr>
<td>PDE5</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>PDE6</td>
<td>10</td>
<td>15</td>
<td>700</td>
</tr>
<tr>
<td>PDE7</td>
<td>700</td>
<td>1 000</td>
<td>10 000</td>
</tr>
<tr>
<td>PDE8</td>
<td>700</td>
<td>1 000</td>
<td>9 000</td>
</tr>
<tr>
<td>PDE9</td>
<td>700</td>
<td>1 000</td>
<td>9 000</td>
</tr>
<tr>
<td>PDE10</td>
<td>700</td>
<td>1 000</td>
<td>9 000</td>
</tr>
<tr>
<td>PDE11</td>
<td>700</td>
<td>300</td>
<td>14</td>
</tr>
</tbody>
</table>

Sildenafil and vardenafil have similar chemical structure and pharmacokinetic properties, whereas tadalafil differs markedly from the others (table 3) (88). All three are absorbed rapidly after oral administration, they are lipophilic and highly protein bound. Peak concentrations are reached first for vardenafil, then sildenafil and finally tadalafil. The major route of elimination for all PDE5 inhibitors is hepatic metabolism. CYP3A is the major metabolizing enzyme, but both vardenafil and sildenafil are also metabolized through other CYPs. PDE5 inhibitors also have active metabolites. Sildenafil and vardenafil have a shorter elimination half-life compared to tadalafil. Plasma concentration of tadalafil has been detected in plasma up to 5 days after oral administration, suggesting that accumulation can occur if taken regularly, leading to increased risk of side effects.

**Vardenafil**

Vardenafil appears to be an attractive alternative for PAH treatment (89). It is a potent and selective PDE5 inhibitor, exhibiting pulmonary selectivity leading to rapid vasodilation. Administration of oral film-coated tablets of vardenafil is rapidly absorbed with an absolute bioavailability of 15 % in healthy male subjects or men with erectile dysfunction (90). Both vardenafil and its major metabolite (M1) are highly bound to plasma proteins (95 %), and the binding is reversible and independent of total drug levels.
Table 3. Pharmacological comparison of the three PDE5 inhibitors, sildenafil, vardenafil and tadalafil (88).

<table>
<thead>
<tr>
<th></th>
<th>Sildenafil</th>
<th>Vardenafil</th>
<th>Tadalafil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Available doses (mg)</td>
<td>25, 50, 100</td>
<td>5, 10, 20</td>
<td>5, 10, 20</td>
</tr>
<tr>
<td>T_{max} (h)</td>
<td>1 (0.5 - 2)</td>
<td>0.7 (0.25 - 3)</td>
<td>2 (0.5 - 6)</td>
</tr>
<tr>
<td>Duration of action (h)</td>
<td>4</td>
<td>-</td>
<td>36</td>
</tr>
<tr>
<td>Oral absorption (%)</td>
<td>92</td>
<td>-</td>
<td>36</td>
</tr>
<tr>
<td>Bioavailability (%)</td>
<td>41</td>
<td>15</td>
<td>-</td>
</tr>
<tr>
<td>t_{1/2} (h)</td>
<td>3-5 h</td>
<td>4-5 h</td>
<td>17.5 h</td>
</tr>
<tr>
<td>Excretion</td>
<td>Feces – 80 %, Urine – 13 %</td>
<td>Feces – 91-95 %, Urine – 2-6 %</td>
<td>Feces – 61 %, Urine – 36 %</td>
</tr>
<tr>
<td>CYP450-system</td>
<td>CYP3A4, CYP2C isoenzymes</td>
<td>CYP3A4, CYP3A5, CYP2C isoenzymes</td>
<td>CYP3A4</td>
</tr>
<tr>
<td>Active metabolite</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Common adverse effects</td>
<td>Headache, flushing, dyspepsia, diarrhea, blurred vision etc</td>
<td>Headache, dizziness, flushing, nasal congestion and dyspepsia etc</td>
<td>Headache, nausea, back pain, dyspepsia, myalgia, pain in extremity etc</td>
</tr>
<tr>
<td>Contra-indications</td>
<td>Concomitant use of nitrates and α-blockers</td>
<td>Concomitant use of nitrates</td>
<td>Concomitant use of nitrates</td>
</tr>
<tr>
<td>IC_{50} for PDE5, (nm)</td>
<td>3.9</td>
<td>0.1-0.7</td>
<td>0.94</td>
</tr>
<tr>
<td>Recommended dose in PAH (adults)</td>
<td>20 mg x3</td>
<td>-</td>
<td>40 mg x1</td>
</tr>
<tr>
<td>Approved for PAH</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

T_{max} = time to maximum concentration, t_{1/2} = elimination half-life, CYP450 = cytochrome P450, IC_{50} = half maximal inhibitory concentration.

The mean steady-state volume of distribution is 208 L, indicating distribution into the tissues. Time to maximum plasma concentration (t_{max}) was reached at 30 minutes to 2h (median 1h). Vardenafil is predominantly metabolized in the liver (>90 %), mainly by cytochrome P450 3A4 (CYP3A4) and to a lesser extent, by CYP3A5 and CYP2C isoenzymes and shows first-passage effects. Plasma levels of M1 are 28 % of vardenafil levels and it accounts for approximately 7 % of the total pharmacological activity.
Further, the kidneys excrete only 2-6 % of vardenafil. The elimination half-life of vardenafil is between 4-6 h and M1 is between 3-5 h (90).

Vardenafil is not yet approved for PAH-treatment, but recent studies have shown that vardenafil is an effective and well-tolerated treatment for PAH (89, 91–94). One randomized controlled trial showed favourable results for vardenafil 5 mg b.i.d. on 6MWD, haemodynamics and time to clinical worsening (89). Thus, vardenafil is an interesting compound for treatment of PAH, but further studies are needed.
Aims

The overall aim of this thesis was to investigate the utility of vardenafil and L-arginine and dimethylarginines in patients with PH.

- To evaluate the acute hemodynamic effects of a single oral dose of vardenafil in patients with PH and to study the hemodynamic effects in relation to the vardenafil drug concentration.
- To investigate the pharmacokinetic profile of a single oral dose of vardenafil in patients with PH.
- To examine the acute haemodynamic response of vardenafil compared to adenosine in patients with PH.
- To predict the prognostic value of acute vasoreactivity testing with vardenafil for long-term clinical outcome in patients with PH.
- To evaluate plasma concentrations of L-arginine and dimethylarginines in patients with PAH compared to patients with left ventricular heart failure (LVHF) and healthy subjects.
- To study the effects of PAH-specific treatment on concentrations of L-arginine and methylated arginine derivatives in patients with PAH.
Subjects and methods

Subjects
In total, 41 patients with PH were recruited. All patients were enrolled and investigated at Uppsala University Hospital in Uppsala or Skåne University Hospital in Lund (both in Sweden) from January 2006 to May 2015. Echocardiography, chest X-ray, lung function tests, 6MWD, NT-proBNP, RHC were documented in all patients and in selected cases additional computed tomography scan of the lung and pulmonary angiography were performed. The estimated glomerular filtration rate (eGFR) was calculated using the Cockcroft-Gault formula.

Study design
Three open-label acute pharmacokinetic and haemodynamic studies investigating vardenafil were performed at the regional PAH-centre in Uppsala University Hospital, Uppsala, Sweden during 2006-2007 (paper I – III). The inclusion criteria were; age over 18 years and a resting mPAP ≥ 25 mmHg measured during RHC. Exclusion criteria were hypotension (<90/50 mmHg) and severe liver dysfunction (Child-Pugh class C). Each patient received one single oral dose of either 5 mg (n = 1), 10 mg (n = 2) or 20 mg (n = 17) vardenafil depending on age and liver function. Repeated blood samples were collected for pharmacokinetic profiling and RHC was performed for haemodynamic measurements.

Study IV was an observational, prospective multi-center study performed at two regional PAH-centres located in Uppsala and in Lund, Sweden from 2010 through 2015 (paper IV). The inclusion criteria were a resting mPAP ≥ 25 mmHg, PAWP ≤ 15 mmHg with a normal or reduced CO measured during RHC and age above 18 years. Exclusion criteria were patients in PH group 2-5. Detailed medical histories and physical examinations included age, gender, weight, body surface area (BSA), height, electrocardiography, echocardiography, six minutes walking distance (6MWD), NT-proBNP and RHC. The individuals included were screened for high blood pressure, diabetes mellitus, ischemic heart disease, stroke, renal failure and thyroid disease. Blood samples for determination of ADMA, SDMA, L-arginine, L-citrulline and L-ornithine were collected from 21 PAH-patients to monitor biomarker levels before start of PAH-specific treatment. Individual PAH treatment strategies were decided by cardiologists with vast experience of PAH. For baseline comparison, 14 patients with left ventricular heart failure (LVHF) and 27 healthy subjects were examined for ADMA, SDMA and L-
arginine. All healthy subjects were non-smokers and had no symptoms or signs of common cold.

**Pharmacokinetic evaluations**
Repeated blood samples were drawn at baseline and at 15, 30, 45, 60, 120, 300 and 540 minutes after peroral administration of vardenafil to measure pharmacokinetic parameters (paper I and II). The specific pharmacokinetic values analyzed were: maximum observed plasma concentration (C\(_{\text{max}}\)), time to median maximum plasma concentration (t\(_{\text{max}}\)), area under the plasma drug concentration-time curve (AUC), total AUC extrapolated to infinity (AUC\(_{\infty}\)) and half-life (t\(_{\frac{1}{2}}\)). C\(_{\text{max}}\), AUC and AUC\(_{\infty}\) were also normalized for dose and body weight (C\(_{\text{max, norm}}\), AUC\(_{\text{norm}}\) and AUC\(_{\infty, norm}\)). C\(_{\text{max}}\) and t\(_{\text{max}}\) were directly estimated from the original data. The AUC was calculated by using the linear/logarithmic trapezoidal rule. The remaining pharmacokinetic values were calculated using a model-independent analysis in the program PK Solver (95).

**Haemodynamic measurements**
The indication for RHC was either a clinically warranted haemodynamic follow-up of known PAH or for diagnostic purposes of new PH/PAH. RHC was performed in all 41 patients at rest after an overnight fast and no medication was given prior to the intervention (paper I – IV). A fiberoptic thermodilution pulmonary artery balloon catheter was inserted through the right internal jugular vein under fluoroscopic guidance (Uppsala; Becton Dickinson Criticath SP5 107 HTD catheter Lund; Swan-Ganz catheter 7.5 F Edward Lifescience LLC, Irvine, CA, USA). All patients rested in the supine position during the examination. The following variables were measured at baseline and after 60 minutes: central venous pressure, mPAP, PAWP, RAP and cardiac output (CO). Pressures were registred with a Cathcor® system (Siemens) and CO was determined with thermodilution technique. Patients with tricuspid valve regurgitation above grade 1 were evaluated according to Fick’s principle due to poor reproducibility with the thermodilution technique. CI was derived from CO divided by the body surface area. Pulmonary artery and venous oxygen saturations were measured at baseline and during RHC. When the initial arterial oxygen saturation was below 90 %, the patients received continuous 100 % O\(_2\) for 10 minutes. PVR, SVR and PVR/SVR were calculated.

During RHC a left arterial catheter was used to measure systemic blood pressure, systemic arterial saturation and for blood sampling. Due to either technical or procedural reasons three patients did not receive an arterial line in Uppsala and the following data are missing (arterial saturation, aortic
pressure and SVR). No arterial line was used during routine RHC in Lund. Experienced cardiologists did all RHC. The patients were hospitalized one day before RHC intervention.

**Vasoreactivity testing**

Acute pulmonary vasoreactivity testing was performed in 20 patients during RHC (paper III). A positive acute vasoreactivity response was defined in accordance with the consensus statement of the European Society of Cardiology and American College of Chest Physicians as a reduction in mPAP of \( \geq 10 \) mmHg to an absolute value of mPAP \( \leq 40 \) mmHg, with an increased or unchanged CO (10). First, 18 patients received adenosine infusions with a start dose of 70 µg·kg\(^{-1}\)·min\(^{-1}\). Every two minutes the dose was gradually increased to a maximum dose of 210 µg·kg\(^{-1}\)·min\(^{-1}\) or until a positive response was recorded or the patient got an adverse reaction. Haemodynamic measurements and blood gases were monitored at baseline and during the adenosine administration. Two patients refused vasoreactivity testing with adenosine due to earlier adverse effects, but they consented to oral vardenafil testing.

When all haemodynamic variables had returned to baseline levels all 20 patients received one single oral dose of either 5 mg (\(n = 1\)), 10 mg (\(n = 2\)) or 20 mg (\(n = 17\)) vardenafil. The doses depended on age and liver function. Haemodynamic parameters and blood gases were monitored at baseline and 60 minutes after vardenafil administration.

**Long-term follow up**

After the vasoreactivity test with vardenafil the patients were followed for up to seven years in order to evaluate the long-term outcome (paper III). Clinical variables as WHO functional class, 6MWD, NT-proBNP and survival were recorded.

In paper IV a follow-up visit was performed 1-12 months (median 4 months) after the initial visit. Patient characteristics, clinical examination, NT-proBNP and 6MWD were recorded and blood samples for determination of ADMA, SDMA, L-arginine, L-citrulline and L-ornithine were collected. In addition, follow-up RHC were performed in 11 patients.

**Bioanalytical analysis**

Blood samples were obtained from patients and controls to determine plasma vardenafil, ADMA, SDMA, L-arginine, L-citrulline and L-ornithine concentrations. Blood samples were collected in EDTA-tubes (BD Diagnostics, Burlington, NC, USA) and were processed immediately in a thermostated centrifuge (Jouan BR 3.11; Thermo Fisher Scientific, Waltham,
MA, USA) for 10 minutes at 3,000 rpm. Plasma was separated and stored at -20 °C (vardenafil) or at -70 °C (methylarginines) until analysis.

**Vardenafil**

Repeated blood samples for determination of vardenafil concentration were collected from the pulmonary artery in 16 patients at baseline and 15, 30, 45 and 60 minutes, as well as from venous blood, 120, 300 and 540 minutes after peroral administration of vardenafil (paper I and II). During the blood sampling the patients were resting in a supine position.

Determination of plasma vardenafil concentrations was performed at Swedish National Veterinary Institute (SVA) in Uppsala. The samples were analyzed with liquid chromatography-tandem mass spectrometry (LC-MS/MS). Vardenafil dihydrochloride and the internal standard pentadeuterated vardenafil (vardenafil-d$_5$) were supplied by Toronto Research Chemicals (North York, ON, Canada). All other solvents and reagents were of analytical grade or higher and used without further purification. The sample pre-treatment was prepared as follows; 50 µL aliquot of internal standard solution (vardenafil-d$_5$ at 250 ng/mL in methanol) and 50 µL of methanol were added to 1.0 mL of plasma, followed by 50 µL of 2.0 M NaOH (aquesos) and 5.0 mL of ethyl acetate. The samples were mixed by mechanical inversion for 20 minutes and then centrifuged at 1,000 g for 10 minutes. The organic layer was transferred to new tubes and evaporated to dryness under a gentle stream of nitrogen at 60 °C. The samples were reconstituted in a mixture of mobile phase including 50 µL of water/methanol 80:20 (v/v) and were then transferred to vials for LC-MS/MS analysis.

The HPLC-system constituted of a Surveyor MS pump with a TSQ Quantum Ultra-tandem quadrupole mass spectrometer, with an electrospray interface operating in the positive mode (Thermo Fischer Scientific, San Jose, CA, USA). Chromotographic separation was reached on a Luna C8 (2) model (50 x 2.0 mm; Phenomenex, Torrence, CA, USA) column. Gradient elution was achieved using a mobile phase using 0.1 % acetic acid in water (solvent A) and acetonitrile (solvent B). The gradient was run as follows: 10 % B for 1.0 minute, linear increase from 10 to 90 % B during 4.0 minutes. Then there was a reduction to 10 % B for 0.10 min and then re-equilibration at 10 % B for 2.9 minutes. The total run time was 8.0 minutes, the injection volume was 10 µL and the flow rate was 200 µL/min.

The mass transition used in selected reaction monitoring (SRM) for vardenafil and the internal standard vardenafil-d$_5$ were m/z 489 [M+H]$^+$ → 151 (collision energy 40 V), and m/z 495 [M+H]$^+$ → 151, respectively. The dwell time was 0.1 s.
Stock solutions of vardenafil dihydrochloride and the internal standard vardenafil-d₃ were prepared in methanol at a concentration of approximately 0.1 mg/mL. These solutions were then further diluted and used to spike (50 µL) blank plasma to obtain calibration samples. Calibration was performed by linear curve fit (no weighting) of the peak area ratio (analyte/internal standard) as a function of the concentration. The calibration curve interval was 0.35-35 ng/mL. Quality control samples of vardenafil were prepared by adding 50 µL of separately prepared working solutions. The lower limit of quantification was 0.35 ng/mL, and the precision expressed as relative standard deviation was <2.2 % at 0.95 ng/mL.

**ADMA, SDMA, L-arginine, L-citrulline and L-ornithine**

Blood samples were collected from 21 patients with PAH, 14 patients with HF and 27 healthy subjects (paper IV). The PAH patients were in a resting supine position during the blood-sampling period.

The determination of ADMA, SDMA, arginine, ornithine and citrulline were performed at SVA in Uppsala. The sample pretreatment was as follows: to 100 µL of plasma, 50 µL of water and 50 µL of the internal standard solution containing ²H₇-ADMA, ²H₆-SDMA, ¹³C₆-arginine, ²H₆-ornithine and ²H₇-citrulline and were added followed by addition of 400 µL of acetonitrile/trifluoroacetic acid/propionic acid (1000/0.25/10 v/v/v). The samples were vortex-mixed for 5 min followed by centrifugation for 5 min at 10 000g. The supernatants were transferred vials for analysis with ultra-high performance liquid chromatography – tandem mass spectrometry (UHPLC-MS/MS). A Waters Acquity UPLC system was coupled to a Quattro Ultima Pt tandem quadrupole mass spectrometer with an electrospray interface operating in the positive mode (Waters Corporation, Milford, MA). The column was an Indra Almtakt Amino Acid (length 100 mm, I.D. 2.0 mm, particle size 3 µm) kept at 25 °C. The mobile phase consisted of (A) 100 mM ammonium formate in water and (B) 0.1 % formic acid in acetonitrile. The injection volume was 10 µL. The elution was carried out as follows: isocratic at 80% A for 1 min, increase to 95 % A during 1 min, constant at 95 % A for 5.5 min, decrease to 80 % A during 0.1 min, constant at 80 % A for 2.4 min. The flow-rate was 200 µL/min. The five analytes were quantified simultaneously in the same chromatographic run using a positive capillary voltage of 0.50 kV and a cone voltage of 40 V. The desolvation and source block temperatures were 350 °C and 120 °C, respectively, and the cone and desolvation gas flows were 120 and 924 L/h, respectively. The quantifications were performed in the selected reaction monitoring (SRM) mode with the collision cell filled with argon gas at a pressure of 1.9x10⁻³ mBar. The mass transitions used in SRM were m/z 203 → 46 for ADMA.
(collision energy 18 eV), \(m/z\ 210 \rightarrow 77\) for \([\text{H}_7]\)-ADMA (collision energy 23 eV), \(m/z\ 203 \rightarrow 172\) for SDMA (collision energy 16 eV), \(m/z\ 209 \rightarrow 116\) for \([\text{H}_6]\)-SDMA (19 eV), \(m/z\ 175 \rightarrow 70\) for arginine (collision energy 18 eV), \(m/z\ 181 \rightarrow 74\) for \([\text{C}_6]\)-arginine (collision energy 18 eV), \(m/z\ 133 \rightarrow 70\) for ornithine (collision energy 13 eV), \(m/z\ 139 \rightarrow 76\) for \([\text{C}_6]\)-ornithine (collision energy 14 eV), \(m/z\ 176 \rightarrow 113\) for citrulline (collision energy 18 eV), \(m/z\ 183 \rightarrow 120\) for \([\text{C}_7]\)-citrulline (collision energy 16 eV). The dwell time was 0.010 sec. Stock solutions of ADMA, SDMA, arginine, ornithine, citrulline and the internal standards were prepared in water at approximately 0.1–0.3 mg/mL. The reference standards and the internal standards were obtained from Toronto Research Chemicals (North York, Ontario, Canada). In order to check for matrix effects and to compensate for endogenous levels of the analytes in the spiked plasma, calibration samples were constructed for all three analytes in both water and in control plasma. The calibration curves were constructed using the chromatographic peak area ratio (analyte/internal standard) as a function of analyte concentration. The calibration functions were calculated by linear curve fit using a weighting factor of \(1/x^2\) for all three analytes. The calibration range for ADMA was 0.090–3.4 µM and the precision was in the range of 5.3–7.3 %. The calibration range for SDMA was 0.38–3.0 µM and the precision was in the range of 5.8–9.3 %. The calibration range for arginine was 4.5–150 µM and the precision was in the range of 3.5–6.2 %. The calibration range for ornithine was 4.5–151 µM and the precision was in the range of 4.1–5.2 %. The calibration range for citrulline was 4.4–150 µM and the precision was in the range of 2.5–4.8 %.

**Statistical analysis**

A p-value < 0.05 was considered significant for all tests and 95 % confidence intervals were used.

**Paper I**

Baseline data, haemodynamic parameters and vardenafil concentration are expressed as median and range (minimum to maximum). Statistical analyses were performed with SAS 9.2 (SAS Institute Inc, Cary, NC). Differences between baseline data and post treatment values were calculated by Wilcoxon’s signed rank test. The Mann Whitney U test was performed to evaluate the differences between groups. The association between plasma vardenafil exposure (concentration or AUC) and changes in haemodynamic parameters were calculated by the Spearman rank correlation test. Non-parametric test was used due to non-normal data distributions and small sample sizes.
**Paper II**
Baseline data are given as mean ± standard deviation (SD). Pharmacokinetic parameters are expressed as geometric mean ± SD, except for $t_{\text{max}}$, which is presented as median and range (minimum to maximum). The unpaired $t$-test was used based on log-transformed data to compare groups. Statistical analysis was calculated with the SPSS software (version 19; SPSS, Chicago, IL, USA).

**Paper III**
The Wilcoxon signed rank test was used to evaluate the effect of vardenafil and adenosine on haemodynamic variables and to calculate the comparison in percentage change from baseline between vardenafil and adenosine. Differences between responders and non-responders were evaluated by the Mann Whitney U test. Non-parametric test was used due to non-normal data distributions and small sample sizes.

**Paper IV**
Clinical characteristics, biochemical indicators and haemodynamic parameters were summarized as frequencies for categorical variables and as median and range (minimum to maximum) for continuous variables. Non-parametric tests were used due to non-normal data distributions and small sample size.

The analysis of change was based on the percentage change from baseline to follow-up and we used the Wilcoxon signed rank test for evaluation of statistical significance. For test of differences between two groups the Mann Whitney U test was used. For test of differences between three groups (patients with PAH, patients with HF and healthy subjects) the Kruskal-Wallis test was used. In case the Kruskal–Wallis test was significant ($p<0.05$) Mann Whitney U tests were applied to compare the PAH patients with HF patients and with the healthy subjects.

Spearman rank-order correlations were used to evaluate the association between haemodynamic variables and L-arginine and methylarginines. Adjustment for possible confounders was performed using partial Spearman’s correlation. A p-value <0.05 was considered statistically significant. Because analyses were exploratory, no adjustments for multiple comparisons were made. Statistical analyses were performed with SAS 9.3 (SAS Institute Inc, Cary, NC).

**Ethics**
All studies of this thesis were approved by the Independent Ethics Committee, Uppsala, Sweden (Dnr 2006/275, 2010/343) and in Lund (Dnr
and conducted in accordance with the Helsinki Declaration and the International Conference on Harmonization Good Clinical Practice guidelines. The studies including vardenafil (paper I-III) were also approved as clinical trial (2006-005239-32) by Medical Products Agency. All the patients gave their informed consent.
Results

All statistical analyses are reported as presented in the original papers (I-IV). However, in the following summary of results all data are presented as median and range, except for the pharmacokinetic study (paper II), where data are presented as geometric mean and geometric standard deviation (SD).

Subjects
A total of 41 PAH-patients were included in this thesis. Sixteen of the patients participated in three of the studies (paper I, II and III) and an additional four patients were included in the study presented in paper III. These patients received one single oral dose of vardenafil. Due to high age or liver failure one patient received 5 mg and two patients received 10 mg vardenafil, all other patients received 20 mg per oral vardenafil. All patients were on at least one of the following background therapies (angiotensin converting enzyme inhibitors, angiotensin receptor blockers, β-adrenoreceptor blockers, diuretics, warfarin, digoxin or CCB). The study presented in paper IV included 21 patients, 10 patients from Lund and 11 patients from Uppsala, Sweden.

Paper I and II
In these papers 16 patients diagnosed with IPAH (WHO group 1, n=7), PH due to left heart disease (group 2, n=5), PAH associated with connective tissue disease (group 1, n=2), PH due to lung disease (group 3, n=1) and CTEPH (group 4, n=1) were included. The patient group consisted of 10 women and 6 men, median age 63 years (range 29-85) and in WHO functional class III (n=12) and II (n=4). Four of the patients had an earlier diagnosis of PAH and subsequently were on PAH-specific treatment before the RHC (bosentan n=3, sildenafil n=1). The remaining 12 patients were newly diagnosed with PH or PAH and had not started PAH-specific treatment yet.

Paper III
In this paper 20 patients (13 women and 7 men) with a median age of 63 years (range 29-85) were included. Sixteen of the twenty patients had also been included in paper I and II. The patients were diagnosed with IPAH (WHO group 1, n=7), APAH (group 1, n=5), PH due to left heart disease (group 2, n=5), PH due to lung disease (group 3, n=2) and CTEPH (group 4, n=1) and were in WHO functional class III (n=16) and II (n=4). At baseline the median 6MWD was 331 (range 60-658) m and the median NT-proBNP
was 1027 (range 4-7927) ng/L. Six of the patients had an earlier diagnose of PAH and subsequently were on PAH-specific treatment before the RHC (bosentan n=3, sildenafil n=1 and sildenafil + epoprostenol n=1). The remaining 14 patients were diagnosed with PH or PAH had not started PAH-specific treatmet yet.

**Paper IV**

The patient group consisted of 21 treatment naïve PAH-patients (13 women and 8 men), with a median age of 73 years (range 45-85 years). According to clinical criteria, the patients were diagnosed with IPAH (n=17) and APAH (n=4) and were in WHO functional class II (n=5), III (n=15) or IV (n=1). The median 6MWD was 245 m (range 0–450 m) and NT-proBNP was 2276.5 ng/L (range 148–5413 ng/L) at baseline. After the diagnostic RHC all patients were started on PAH-specific therapy. Three responders to acute vasodilator testing received CCB (nifedipin). The rest of the patients started mono therapy with PDE5-inhibitors (n=8) or ERA (n=5) or combination therapy with PDE5-inhibitors + ERA (n=4) or ERA + prostacyclin (n=1).

This study included two control groups, one consisted of 14 patients with LVHF (8 women, 6 men) with a median age of 67 years (range 48-82 years). The median 6MWD was 415 m (range 120-530 m) and the median NT-proBNP was 755.5 ng/L (range 194–6172 ng/L). The other control group consisted of 27 healthy subjects (19 women, 8 men) with a median age of 61 years (range 30–77 years).

![Figure 5](image)

**Figure 5.** Time-course of the vardenafil plasma concentration in 16 PH patients after administration of one single dose of oral 5 (n=1), 10 (n=2) or 20 (n=13) mg.
Pharmacokinetic data of vardenafil in PH-patients

Pharmacokinetic parameters of vardenafil in patients with PH are depicted in table 4 (Paper II). Individual vardenafil plasma concentrations are summarized in figure 5.

After oral vardenafil administration a rapid increase was observed in vardenafil plasma concentrations for all doses (5, 10 and 20 mg). After 15 minutes, 13 of 16 patients showed quantifiable levels of vardenafil in plasma. In the three remaining patients quantifiable levels were observed after 45 minutes (patient no. 2) and 60 minutes (patients no. 4 and 13). The median $t_{\text{max}}$ was 1 h (range 15 min to 5 h). The geometric mean of $C_{\text{max}}$ is shown in table 4. The geometric coefficient of variation (CV) for $C_{\text{max}}$ was 25 %. For AUC the CV was 7.3 %. The CV for $C_{\text{max}, \text{norm}}$ and $\text{AUC}_{\text{norm}}$ were 5.6 and 1.4 %, respectively. The elimination geometric mean half-life ($t_{\frac{1}{2}}$) for vardenafil was 3.4 h with a CV of 45 %.

<table>
<thead>
<tr>
<th>Parameter \hspace{1cm}</th>
<th>Units \hspace{1cm}</th>
<th>n \hspace{1cm}</th>
<th>Mean and SD \hspace{1cm}</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}^a$ \hspace{1cm}</td>
<td>µg/L \hspace{1cm}</td>
<td>13 \hspace{1cm}</td>
<td>11.8 ± 2.9 \hspace{1cm}</td>
<td>1.5 to 39.6</td>
</tr>
<tr>
<td>$C_{\text{max}, \text{norm}}$ \hspace{1cm}</td>
<td>g/L \hspace{1cm}</td>
<td>16 \hspace{1cm}</td>
<td>47.9 ± 2.7 \hspace{1cm}</td>
<td>4.3 to 144.6</td>
</tr>
<tr>
<td>$t_{\text{max}}^b$ \hspace{1cm}</td>
<td>h \hspace{1cm}</td>
<td>16 \hspace{1cm}</td>
<td>1.0 \hspace{1cm}</td>
<td>0.25 to 5.0</td>
</tr>
<tr>
<td>$\text{AUC}^a$ \hspace{1cm}</td>
<td>(µg · h/L) \hspace{1cm}</td>
<td>16 \hspace{1cm}</td>
<td>39.6 ± 2.9 \hspace{1cm}</td>
<td>6.5 to 149.6</td>
</tr>
<tr>
<td>$\text{AUC}_{\text{norm}}$ \hspace{1cm}</td>
<td>(g · h/L) \hspace{1cm}</td>
<td>16 \hspace{1cm}</td>
<td>159.8 ± 2.8 \hspace{1cm}</td>
<td>17.6 to 774.8</td>
</tr>
<tr>
<td>$\text{AUC}_{l}$ \hspace{1cm}</td>
<td>(µg · h/L) \hspace{1cm}</td>
<td>10 \hspace{1cm}</td>
<td>47.2 ± 2.5 \hspace{1cm}</td>
<td>9.9 to 131.3</td>
</tr>
<tr>
<td>$\text{AUC}_{l, \text{norm}}$ \hspace{1cm}</td>
<td>(g · h/L) \hspace{1cm}</td>
<td>10 \hspace{1cm}</td>
<td>202.6 ± 2.4 \hspace{1cm}</td>
<td>28.1 to 643.7</td>
</tr>
<tr>
<td>$t_{\frac{1}{2}}$ \hspace{1cm}</td>
<td>h \hspace{1cm}</td>
<td>10 \hspace{1cm}</td>
<td>3.4 ± 1.5 \hspace{1cm}</td>
<td>2.0 to 7.2</td>
</tr>
</tbody>
</table>

Data are shown in geometric mean, geometric standard deviation (SD) and total range. $^a$20 mg dose; $^b$median

When comparing measurements in men and women, there was a tendency for differences, however they did not reach statistically significance. Men had lower geometric mean values for $C_{\text{max}}$ (17.0 ± 1.7 vs. 25.8 ± 1.6 µg/L) and $C_{\text{max}, \text{norm}}$ (73.2 ± 1.5 vs. 83.5 ± 1.6 g/L) than women. On the other hand, men had higher geometric mean values for AUC (76.5 ± 2.0 vs 67.7 ± 1.4 µg · h/L) and $\text{AUC}_{\text{norm}}$ (279.2 ± 1.9 vs. 249.7 ± 1.7 g · h/L). The geometric mean value for $t_{\frac{1}{2}}$ was 3.2 h in men and 3.4 h in women. There were no differences in median $t_{\text{max}}$ between genders.

Pharmacokinetic drug-drug interaction

Four patients had considerably lower plasma concentrations than the rest of the patients. These patients were targets for drug interaction. Three of them
were on bosentan treatment and they had the lowest Cmax in the study group; 1.5, 2.6 and 4.5 μg/L, respectively. These patients also had delayed tmax (median 2 h) and a significantly reduced Cmax, norm 9.1 g/L (range 4.3-14.6 g/L) and AUC norm 27.6 g·h/L (range 18.4-36.0 g·h/L) (p<0.05) compared to the patients without drug interactions Cmax, norm 79.1 g/L (range 34.2-144.6 g/L) and AUC norm 261.6 g·h/L (range 145.2-714.1 g·h/L) (table 5). AUCI, norm was 287.4 ± 1.6 g·h/L in the patients without drug interactions. The fourth patient was on karbamazepin treatment and had also lower Cmax (5.0 μg/L) and AUC (24.2 μg·h/L).

Table 5. Pharmacokinetic parameters in PH patients on monotherapy with vardenafil or on combination therapy with vardenafil and bosentan.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Only vardenafil (n=12)</th>
<th>Vardenafil and bosentan (n=3)</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cmax (μg/L)a</td>
<td>21.4 ± 1.7</td>
<td>2.6 ± 1.7</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Cmax, norm (g/L)</td>
<td>79.1 ± 1.6</td>
<td>9.1 ± 1.9</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>AUC (μg · h/L)a</td>
<td>71.5 ± 1.6</td>
<td>8.0 ± 1.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>AUC norm (g · h/L)</td>
<td>261.6 ± 1.7</td>
<td>27.6 ± 1.4</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>tmax (h)</td>
<td>1.0 (0.5 - 5.0)</td>
<td>2.0 (0.3 - 3.0)</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Data are shown in geometric mean and geometric standard deviation, except tmax who is shown in median (minimum - maximum). a means are only given for patients who received 20 mg vardenafil (n=9). *p-value<0.05 was considered statistical significant

Effects of vardenafil on hemodynamic parameters

In paper I, 60 minutes after vardenafil administration a significant reduction in mPAP (-20.3 %; range -48.3 to 3.0; p<0.001) and PVR (-28.9 %; range -61.5 to -5.9, p<0.001) was observed compared to baseline. At the same time, there was a significant increase in CO (10.6 %; range -25.0 to 88.1, p=0.015) and CI (12.1 %; range -24.0 to 94.4; p=0.01). Both SVR (-28.9 %; range -61.5 to -5.9; p<0.001) and PVR/SVR (-16.9 %, range -49.0 to 16.5; p=0.002) were significantly reduced. Heart rate, aortic pressure, mPAWP, mRAP, pulmonary artery (PA) saturation and arterial saturation did not show significant changes after vardenafil administration.

There were no statistically significant haemodynamic differences between patients on bosentan therapy (n = 3) compared with PAH patients (n = 7) or the whole group of PH patients (n = 13). However, patients treated with bosentan tended to have a lower decrease in mPAP (-10.8 %; range -20.0 to 3.0) compared to other PAH patients (-23.8 %; -34.1 to 0) (p=0.095). Furthermore, there were no statistically significant haemodynamic
Table 6. Haemodynamic parameters at baseline and after intravenous adenosine or peroral vardenafil in PH-patients.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Adenosine</th>
<th></th>
<th>Vardenafil</th>
<th></th>
<th>p-value*</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (bpm)</td>
<td>n=18</td>
<td>73 (56-94)</td>
<td>18</td>
<td>74 (58-96)</td>
<td>0.7 (-7.9-25.7)</td>
<td>0.45</td>
</tr>
<tr>
<td>Mean aortic pressure</td>
<td>n=17</td>
<td>93 (79-139)</td>
<td>16</td>
<td>86 (65-120)</td>
<td>-10.8 (-35.0-22.2)</td>
<td>0.072</td>
</tr>
<tr>
<td>mPAP (mmHg)</td>
<td>n=18</td>
<td>43 (26-65)</td>
<td>18</td>
<td>37 (24-72)</td>
<td>-6.4 (-40.0-10.8)</td>
<td>0.13</td>
</tr>
<tr>
<td>mPAWP (mmHg)</td>
<td>n=13</td>
<td>10 (2-28)</td>
<td>13</td>
<td>12 (6-33)</td>
<td>0.0 (-11-100)</td>
<td>0.13</td>
</tr>
<tr>
<td>mRAP (mmHg)</td>
<td>n=18</td>
<td>7 (2-28)</td>
<td>18</td>
<td>7 (2-28)</td>
<td>0.0 (-44-78)</td>
<td>1.00</td>
</tr>
<tr>
<td>CO (l min⁻¹)</td>
<td>n=18</td>
<td>4.1 (2.1-10.0)</td>
<td>18</td>
<td>5.7 (2.3-13.1)</td>
<td>20.3 (-36.8-56.1)</td>
<td>0.049</td>
</tr>
<tr>
<td>CI (l min⁻¹ m⁻²)</td>
<td>n=18</td>
<td>2.5 (1.4-5.1)</td>
<td>18</td>
<td>3.0 (1.5-6.6)</td>
<td>21.8 (5.9-104.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PVR (dynes s cm⁻³)</td>
<td>n=18</td>
<td>652 (208-1144)</td>
<td>18</td>
<td>491 (49-870)</td>
<td>-25.5 (-78.4-4.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SVR (dynes s cm⁻³)</td>
<td>n=17</td>
<td>1584 (680-2968)</td>
<td>16</td>
<td>1241 (452-3300)</td>
<td>-26.3 (-46.3-53.9)</td>
<td>0.061</td>
</tr>
<tr>
<td>PVR/SVR</td>
<td>n=17</td>
<td>0.4 (0.1-0.6)</td>
<td>16</td>
<td>0.4 (0.0-1.0)</td>
<td>-5.2 (-86.4-50.0)</td>
<td>1.00</td>
</tr>
<tr>
<td>PA sat (%)</td>
<td>n=18</td>
<td>63.8 (45.9-78.0)</td>
<td>17</td>
<td>70.4 (48.6-81.1)</td>
<td>9.9 (-6.7-21.4)</td>
<td>0.018</td>
</tr>
<tr>
<td>Art sat (%)</td>
<td>n=17</td>
<td>91.2 (85.0-93.5)</td>
<td>16</td>
<td>92.3 (83.4-96.0)</td>
<td>0.7 (-2.3-5.3)</td>
<td>0.129</td>
</tr>
</tbody>
</table>

Data are presented as median (range). Observed hemodynamic parameters in the adenosine group refer to measurements at maximu tolerated dose and in the vardenafil group 60 minutes after peroral administration. *Wilcoxon signed rank test p<0.05
differences between patients with PAH (WHO group 1) and patients with PH (WHO group 2-4) either.

Observed changes in haemodynamic parameters during intravenous adenosine and oral vardenafil vasodilator testing are shown in table 6 (Paper III). During adenosine infusion mPAP, PVR, SVR and mSAP were significantly reduced. There was a significant increase in CO, CI, PA- and arterial oxygen saturation. No statistical changes were seen for PVR/SVR. After Bonferroni-Holm correction the associations for mSAP, mPAP, SVR and arterial saturation was non-significant, however, remained significant for PVR, CO, CI and PA saturation.

Sixty minutes after vardenafil administration, mPAP, PVR, SVR, PVR/SVR and mSAP had decreased significantly and there was a significant increase in CO and CI. The association became non-significant for PVR/SVR after Bonferroni-Holm correction, and remained significant for mSAP, mPAP, PVR, SVR, CO and CI.

**Vardenafil plasma concentration and haemodynamic response**

Plasma concentrations of vardenafil were obtained at baseline and 15, 30, 45 and 60 minutes after administration of the drug (paper I). At the same timepoints, haemodynamic measurements of mPAP were registered. A significant reduction of median mPAP was observed already after 15 minutes (table 7). The relation between median mPAP and vardenafil plasma concentration was significant at 30, 45 and 60 minutes. In addition, there was a significant correlation between vardenafil concentration and percentage change in mPAP at 60 minutes after vardenafil administration.

**Table 7.** Differences between mPAP at baseline and at time-points 15, 30, 45 and 60 minutes after vardenafil administration.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Vardenafil concentration (µM)</th>
<th>mPAP (mmHg)</th>
<th>%change</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.00</td>
<td>40.0</td>
<td>0.00</td>
<td>0.013</td>
</tr>
<tr>
<td>15</td>
<td>1.01</td>
<td>36.5</td>
<td>-10.5</td>
<td>0.002</td>
</tr>
<tr>
<td>30</td>
<td>5.99</td>
<td>35.5</td>
<td>-16.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>45</td>
<td>5.43</td>
<td>34.5</td>
<td>-17.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>60</td>
<td>6.30</td>
<td>34.5</td>
<td>-20.3</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are presented as median. *Wilcoxon’s signed rank test.

The relationship between haemodynamic response at 60 minutes after vardenafil administration and plasma vardenafil concentration is shown in table 8. There was a significant correlation between plasma vardenafil
concentration and change in mPAP \((r=-0.579, p=0.019)\), PVR \((r=-0.662, p=0.005)\). There was also a significant correlation between vardenafil AUC (0-45 min) and mPAP \((r=-0.668, p=0.007)\) and PVR \((r=-0.540, p=0.038)\) as well as AUC (0-60 min) and mPAP \((r=-0.744, p=0.001)\) and PVR \((r=-0.588, p=0.021)\).

**Table 8.** Relationships between haemodynamic parameters and vardenafil concentration at 60 minutes after vardenafil concentration.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Vardenafil concentration</th>
<th></th>
<th></th>
<th>Vardenafil AUC (0 to 60 min)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Spearman’s rho</td>
<td>P-value</td>
<td>n</td>
<td>Spearman’s rho</td>
<td>P-value</td>
</tr>
<tr>
<td>Heart rate (beats · min(^{-1}))</td>
<td>16</td>
<td>0.291</td>
<td>0.274</td>
<td>15</td>
<td>0.054</td>
<td>0.850</td>
</tr>
<tr>
<td>Aortic Pressure (mmHg)</td>
<td>14</td>
<td>-0.552</td>
<td>0.041</td>
<td>14</td>
<td>-0.270</td>
<td>0.350</td>
</tr>
<tr>
<td>Mean aortic pressure sPAP (mmHg)</td>
<td>14</td>
<td>-0.582</td>
<td>0.029</td>
<td>14</td>
<td>-0.481</td>
<td>0.081</td>
</tr>
<tr>
<td>mPAP (mmHg)</td>
<td>16</td>
<td>-0.397</td>
<td>0.128</td>
<td>15</td>
<td>-0.504</td>
<td>0.056</td>
</tr>
<tr>
<td>mPAWP (mmHg)</td>
<td>16</td>
<td>-0.148</td>
<td>0.585</td>
<td>15</td>
<td>-0.087</td>
<td>0.757</td>
</tr>
<tr>
<td>mRAP (mmHg)</td>
<td>16</td>
<td>-0.397</td>
<td>0.128</td>
<td>15</td>
<td>-0.362</td>
<td>0.184</td>
</tr>
<tr>
<td>CO (l · min(^{-1}))</td>
<td>16</td>
<td>0.282</td>
<td>0.289</td>
<td>15</td>
<td>0.021</td>
<td>0.940</td>
</tr>
<tr>
<td>CI (l · min(^{-1}) · m(^{-2}))</td>
<td>16</td>
<td>0.308</td>
<td>0.247</td>
<td>15</td>
<td>-0.129</td>
<td>0.648</td>
</tr>
<tr>
<td>PVR (dynes · s · cm(^{-5}))</td>
<td>16</td>
<td>-0.662</td>
<td>0.005</td>
<td>15</td>
<td>-0.588</td>
<td>0.021</td>
</tr>
<tr>
<td>SVR (dynes · s · cm(^{-5}))</td>
<td>14</td>
<td>-0.486</td>
<td>0.078</td>
<td>14</td>
<td>-0.222</td>
<td>0.446</td>
</tr>
<tr>
<td>PVR/SVR</td>
<td>14</td>
<td>-0.191</td>
<td>0.513</td>
<td>14</td>
<td>-0.433</td>
<td>0.122</td>
</tr>
<tr>
<td>PA sat (%)</td>
<td>13</td>
<td>-0.137</td>
<td>0.655</td>
<td>12</td>
<td>0.077</td>
<td>0.812</td>
</tr>
<tr>
<td>Art sat (%)</td>
<td>11</td>
<td>-0.264</td>
<td>0.433</td>
<td>11</td>
<td>-0.191</td>
<td>0.574</td>
</tr>
</tbody>
</table>

The median plasma concentration of vardenafil, 60 minutes after a single oral dose of vardenafil in 16 patients, was 5.1 (0.3-39.6) µg/L. The median AUC (0-45 min) and AUC (0-60 min) were 2.5 (0.0-17.9) µg·h/L and 3.9 (0.04-23.1) µg·h/L, respectively. Three patients on bosentan treatment had significantly lower plasma vardenafil concentration at 60 minutes compared
to patients without bosentan treatment (see also table 5). No significant correlation with haemodynamic parameters was seen.

**Positive response to acute vasodilator test**
In accordance with the criteria for positive response in acute vasoreactivity testing (10), three of 18 patients (17 %) were considered responders after adenosine infusion and five of 20 (25 %) after vardenafil administration, shown in supplementary table 1 (paper III). Three patients responded to both adenosine and vardenafil and of the two additional vardenafil responders one was not tested with adenosine. Responders to adenosine had diagnosis in the following WHO groups according to the Nice classification; APAH (n=2) and IPAH (n=1) and responders to vardenafil in APAH (n=3), IPAH (n=1) and PH due to left heart disease (n=1).

**Baseline characteristics in responders and non-responders**
Clinical characteristics and baseline haemodynamics between responders and non-responders were compared (paper III). The median values of NT-proBNP was lower for responders compared with non-responders 129 vs. 1522 ng/L, respectively (p=0.019). The median values of S-creatinine were lower 58 vs. 88 µM (p=0.021) and eGFR were higher 113 vs. 67 mL/min (p=0.019) in responders compared to non-responders.

**Safety and tolerability of vardenafil compared to adenosine**
Administration of a single oral dose of vardeanfil was well tolerated and no adverse side effects were observed in our studies. Neither hypotension nor clinically significant changes in haemodynamic or biochemical parameters were seen (paper I-III). Adenosine infusion, on the other hand, caused headache and flush, both well-known adverse effects, and only 6 out of 18 patients tolerated the targeted maximum infusion dose of 210 µg · kg⁻¹ · min⁻¹ (paper III).

**Long-term follow-up and survival after vardenafil vasoreactivity testing**
After adenosine and vardenafil vasoreactivity testing, all patients received standard PAH-specific treatment according to the outcome of RHC (paper III). The five responders to vardenafil received sildenafil and CCB (n=2), sildenafil and bosentan (n=1) and CCB (n=1) therapy. One patient diagnosed with PH due to left heart disease was not eligible to receive PAH-treatment. The fifteen vardenafil non-responders received sildenafil (n=7), bosentan (n=3) and iloprost (n=1) therapy. Four patients were not eligible to receive PAH-specific treatment based on clinical PH-classification.
Survival data for all patients are shown in table 9. One responder was lost to follow-up, still, survival data of this patient was included in the analysis. All patients, both responders and non-responders, were re-evaluated for WHO functional class, 6MWD and NT-proBNP 1, 3, 5 and 7 years after vasoreactivity testing (table 9). After comparison with Mann-Whitney U test, there were statistically significant differences in NT-proBNP levels between responder and non-responders at baseline ($p=0.019$) and after 3 years ($p=0.049$).

**Table 9.** Long-term follow-up of responders and non-responders to vardenafil.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Responder</th>
<th>n</th>
<th>Non-Responder</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WHO functional class (II/III)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>5</td>
<td>0/5</td>
<td>15</td>
<td>3/12</td>
</tr>
<tr>
<td>1 year</td>
<td>3</td>
<td>2/1</td>
<td>14</td>
<td>3/11</td>
</tr>
<tr>
<td>3 years</td>
<td>3</td>
<td>2/1</td>
<td>7</td>
<td>3/4</td>
</tr>
<tr>
<td>5 years</td>
<td>3</td>
<td>1/2</td>
<td>6</td>
<td>3/3</td>
</tr>
<tr>
<td>7 years</td>
<td>1</td>
<td>1/0</td>
<td>5</td>
<td>2/3</td>
</tr>
<tr>
<td><strong>6MWD (m)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>4</td>
<td>324 (300-450)</td>
<td>14</td>
<td>333 (60-658)</td>
</tr>
<tr>
<td>1 year</td>
<td>3</td>
<td>450 (310-460)</td>
<td>13</td>
<td>390 (0-630)</td>
</tr>
<tr>
<td>3 years</td>
<td>3</td>
<td>300 (285-500)</td>
<td>6</td>
<td>475 (360-590)</td>
</tr>
<tr>
<td>5 years</td>
<td>2</td>
<td>353 (180-525)</td>
<td>4</td>
<td>475 (440-590)</td>
</tr>
<tr>
<td>7 years</td>
<td>1</td>
<td>550</td>
<td>3</td>
<td>465 (410-490)</td>
</tr>
<tr>
<td><strong>NT-proBNP (ng/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>5</td>
<td>129 (97-1027)</td>
<td>14</td>
<td>1522 (4-7927)*</td>
</tr>
<tr>
<td>1 year</td>
<td>3</td>
<td>183 (86-413)</td>
<td>13</td>
<td>1395 (15-12361)</td>
</tr>
<tr>
<td>3 years</td>
<td>3</td>
<td>149 (129-210)</td>
<td>7</td>
<td>759 (11-4278)*</td>
</tr>
<tr>
<td>5 years</td>
<td>3</td>
<td>57 (38-2181)</td>
<td>6</td>
<td>1019 (47-3415)</td>
</tr>
<tr>
<td>7 years</td>
<td>1</td>
<td>108</td>
<td>5</td>
<td>693 (96-8545)</td>
</tr>
</tbody>
</table>

Data are presented as median (range). $P$ value for comparison between groups was based on Mann-Whitney U test ($p<0.05$).

**Baseline plasma concentrations of L-arginine, ADMA and SDMA**

In paper IV, patients with PAH had significantly higher baseline plasma ADMA and SDMA levels and significantly lower baseline plasma L-arginine levels and L-arginine/ADMA ratio compared to healthy controls ($p<0.001$) (table 10). Furthermore, L-arginine was significantly lower in patients with PAH compared to patients with LVHF ($p<0.05$).
<table>
<thead>
<tr>
<th></th>
<th>PAH (n=21)</th>
<th>LVHF (n=14)</th>
<th>Healthy subjects (n=27)</th>
<th>PAH vs LVHF p-value</th>
<th>PAH vs healthy subjects p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADMA (µM)</td>
<td>0.50 (0.34-0.91)</td>
<td>0.56 (0.50-0.74)</td>
<td>0.36 (0.23-0.44)</td>
<td>0.245</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SDMA (µM)</td>
<td>0.83 (0.47-2.43)</td>
<td>0.74 (0.50-1.08)</td>
<td>0.42 (0.32-0.59)</td>
<td>0.225</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>L-arginine (µM)</td>
<td>55.1 (30.0-116.0)</td>
<td>81.8 (43.8-113.8)</td>
<td>85.8 (58.2-132.9)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>L-arginine/ADMA</td>
<td>102.2 (59.3-230.2)</td>
<td>140.4 (81.2-214.8)</td>
<td>237.8 (176.5-365.7)</td>
<td>0.074</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are presented as median and range. P-value for comparison between groups are based on Mann-Whitney U test (P-value=0.05).

Women with PAH had significantly lower median plasma ADMA levels at baseline compared to men with PAH, 0.50 (range 0.34 to 0.70) µM vs 0.61 (range 0.39 to 0.91) µM, (p<0.05). After PAH-specific treatment women had a median increase in ADMA of 7.1 % (range -21.5 to 67.7), as opposed to men, whose median plasma ADMA concentrations decreased by -13.9 % (range -38.3 to 30.8). No statistically significant differences in ADMA between baseline and follow-up, neither in women nor men, were seen (data not shown).

Significantly lower L-arginine/ADMA ratios were observed at baseline in WHO functional class 3, as compared to WHO functional class 2 -50.1 (range -92.5 to -7.7) (p<0.05). Furthermore, a positive correlation between 6MWD and L-arginine 7.9 (range 3.5 to 12.3), L-arginine/ADMA 17.32 (6.3 to 28.3) and L-ornithine 7.6 (1.1 to 14.2) was found (p<0.05) (figure 6). Moreover, a statistically significant relationship between creatinine clearance (eGFR) and L-arginine and L-citrulline (p<0.05), respectively, was seen.
Figure 6. The association between 6MWD and L-arginine and L-arginine/ADMA at baseline in 21 patients with PAH. Multiple regression analysis was used for statistical analysis.

Effect of PAH-specific therapy on clinical outcome and methylarginines

In the 21 PAH-patients there was a significant improvement in 6MWD, with a median increase of 20.13 % (range -71.4 – 100.0, p=0.008) between the first visit and the follow-up (paper IV). In 11 PAH-patients who underwent RHC both at baseline and at follow-up a statistically significant improvement in haemodynamic response was seen, measured as percent change in sPAP, mPAP, CI, PVR and SVR (table 11). At follow-up, the 10 patients on mono- (n=5) and combination therapy (n=5) on ERA had significantly lower plasma ADMA levels than the 11 patients without ERA (p<0.05) (Table 12). In contrast, the 12 patients on mono- (n=8) and combination therapy (n=4) on PDE5-inhibitors had significantly higher plasma ADMA (p<0.05) and ornithine levels (p<0.05) compared to the 9 patients without PDE5-inhibitor treatment but on other PAH-specific therapy. For statistical reasons due to small sample sizes we did not compare monotherapy on ERA with monotherapy on PDE5-inhibitors.

The median time from the initial visit to the follow-up visit for patients on ERA or PDE5-inhibitors after initial visit was 4 (range 2-12) and 5 (range 2-12) months, respectively. Moreover, during routine follow-up with RHC the six patients on ERA had significantly reduced sPAP -12.8 (range -21.4 to -2.4) (p<0.05) mmHg, mPAP -18.6 (range -25.0 to -3.64) (p<0.05), PVR -32.2 (-50.7 to -15.2) (p<0.05) and SVR -23.5 (-60.8 to -15.6) (p<0.05). The six patients on PDE5-inhibitors showed no significant differences in haemodynamic parameters between baseline and follow-up. There was a trend towards a larger reduction in SDMA levels in patients on combination
therapy with PAH-specific agents as compared to monotherapy, but this did not reach statistical significance.

Table 11. Haemodynamic parameters in 11 patients with PAH shown at baseline and after treatment with PAH-specific therapy.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>Follow-up</th>
<th>%change</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate</td>
<td>79</td>
<td>74</td>
<td>-2.2</td>
<td>0.286</td>
</tr>
<tr>
<td>(beats×min⁻¹)</td>
<td>(65 – 100)</td>
<td>(54-99)</td>
<td>(-20.6-10.8)</td>
<td></td>
</tr>
<tr>
<td>SAP (mmHg)</td>
<td>137</td>
<td>125</td>
<td>-8.4</td>
<td><strong>0.033</strong></td>
</tr>
<tr>
<td>(111 – 146)</td>
<td>(93 – 157)</td>
<td>(-36.3-22.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mPAP (mmHg)</td>
<td>49</td>
<td>41</td>
<td>-15.8</td>
<td><strong>0.040</strong></td>
</tr>
<tr>
<td>(28 – 59)</td>
<td>(27 – 53)</td>
<td>(-38.2-42.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mPAWP (mmHg)</td>
<td>6</td>
<td>6</td>
<td>0</td>
<td>0.677</td>
</tr>
<tr>
<td>(2 – 11)</td>
<td>(2 – 11)</td>
<td>(-60-200)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mRAP (mmHg)</td>
<td>7</td>
<td>4</td>
<td>-20</td>
<td>0.239</td>
</tr>
<tr>
<td>(2 – 14)</td>
<td>(0 – 12)</td>
<td>(-100-100)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CO (l×min⁻¹)</td>
<td>3.6</td>
<td>4.0</td>
<td>11.1</td>
<td>0.092</td>
</tr>
<tr>
<td>(2.5 – 6.5)</td>
<td>(2.9 – 7.1)</td>
<td>(-20.6-77.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CI (l×min⁻¹×m⁻²)</td>
<td>2.1</td>
<td>2.5</td>
<td>10.5</td>
<td><strong>0.050</strong></td>
</tr>
<tr>
<td>(1.6 – 2.8)</td>
<td>(1.8 – 3.2)</td>
<td>(-10.8-77.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PVR (dynes×s×cm⁻⁵)</td>
<td>808</td>
<td>600</td>
<td>-29.0</td>
<td><strong>0.009</strong></td>
</tr>
<tr>
<td>(480 – 1496)</td>
<td>(328 – 984)</td>
<td>(-66.7-19.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SVR (dynes×s×cm⁻⁵)</td>
<td>1880</td>
<td>1512 (1040 – 1512)</td>
<td>-19.6</td>
<td><strong>0.018</strong></td>
</tr>
<tr>
<td>(1120 – 3072)</td>
<td>2374</td>
<td>(-60.8-12.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PVR/SVR</td>
<td>0.4</td>
<td>0.4</td>
<td>-7.0</td>
<td>0.126</td>
</tr>
<tr>
<td></td>
<td>(0.3 – 0.7)</td>
<td>(0.3 – 0.6)</td>
<td>(-47.2-26.8)</td>
<td></td>
</tr>
<tr>
<td>PA sat (%)</td>
<td>59.2</td>
<td>60.6</td>
<td>-0.7</td>
<td>0.689</td>
</tr>
<tr>
<td></td>
<td>(50.9 – 66.1)</td>
<td>(47.2 – 75.0)</td>
<td>(-14.5-30.7)</td>
<td></td>
</tr>
<tr>
<td>Art sat (%)</td>
<td>88.8</td>
<td>88.0</td>
<td>0.2</td>
<td>0.656</td>
</tr>
<tr>
<td></td>
<td>(81.1 – 96.0)</td>
<td>(78.1 – 97.6)</td>
<td>(-6.3-4.6)</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as median (range). *Wilcoxon’s signed rank test.

**Relationship between haemodynamic response and methylarginines during PAH specific treatment**

The relationship between haemodynamic parameters and plasma ADMA, SDMA, L-arginine, L-citrulline and L-ornithine levels, as well as, the L-arginine/ADMA ratio, the L-arginine/(L-ornithine + L-citrulline) ratio and the L-arginine/L-ornithine ratio at baseline and follow-up were explored (paper IV). The relationship between haemodynamic variables and methylarginines at follow-up and percentage change between baseline and follow-up were investigated. Spearman rank correlation test showed a positive correlation between SDMA and mPAWP (n=11, 0.739, p<0.05) and mRAP (n=11, 0.782, p<0.05) and a negative correlation with pulmonary
artery saturation (n=11, -0.765, p<0.05). No relationship between ADMA and haemodynamic parameters was seen in this study.

**Table 12.** Plasma ADMA concentrations in PAH-patients on mono- or combination therapy with ERA or PDE5 inhibitors (PDE5i) compared to patients on other PAH-specific treatment.

<table>
<thead>
<tr>
<th></th>
<th>+</th>
<th>-</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>n=10</td>
<td>n=11</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>0.50 (0.36-0.91)</td>
<td>0.58 (0.34-0.75)</td>
<td></td>
</tr>
<tr>
<td>Follow-up</td>
<td>n=10</td>
<td>n=11</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>0.48 (0.34-0.59)</td>
<td>0.55 (0.44-0.69)</td>
<td></td>
</tr>
<tr>
<td>PDE5i</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>n=12</td>
<td>n=9</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>0.56 (0.34-0.71)</td>
<td>0.48 (0.36-0.91)</td>
<td></td>
</tr>
<tr>
<td>Follow-up</td>
<td>n=12</td>
<td>n=9</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>0.56 (0.46-0.69)</td>
<td>0.47 (0.34-0.56)</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as median and range. +, patients who received this therapy; -, patients who did not receive this therapy. P-value for comparison between groups, based on Mann-Whitney U test (P-value=0.05).
Discussion

PAH is a disease characterised by endothelial dysfunction and vascular remodelling, leading to increased PVR and right ventricle HF. The pathogenesis behind PAH is still unknown, but many predisposing and/or contributing factors have been identified, the most important being the disruption of the NO pathway. Current routine diagnostic strategies include invasive haemodynamic measurements during RHC, as well as blood tests such as NT-proBNP and standardized exercise test. Despite considerable progress in classification, diagnose and treatment of PAH, there is still no tests available that could serve as a supplement, or evenmore as a substitute, for invasive diagnostic procedures in this patient group. Thus, a sophisticated, non-invasive test for sensitive and specific diagnosis of PAH is warranted. Moreover, the tests should ideally aid choice of treatment strategy and be useful for short- and long-term prognosis and therapeutic outcome.

Specific drugs for PAH treatment have been developed during the last two decades. The common goal of all PAH-specific treatment, vasodilation, is mainly achieved through interference with the endothelin-, NO- or prostacyclin pathways. Unfortunately, as the exact pathogenesis of PAH still is uncertain, the current treatment strategies are by necessity focused on symptom relief and not causal. Considering the severity and poor prognosis of PAH, it appears that intensive treatment at an early stage is crucial (96, 97). In the future, better therapeutic strategies with individualized pharmacotherapy and optimal drug combinations are needed.

The present thesis investigated the diagnostic and therapeutic utility of vardenafil in acute vasodilation testing and its potential for short-term effects on the hemodynamics. It also evaluated the usefulness of L-arginine and methylarginines in diagnosis and follow-up of PAH and how these changes in the dysfunctional NO-pathways present in patients with PAH.

Why we chose to investigate vardenafil

When we started our studies in 2006, only epoprostenol, bosentan, iloprost and sildenafil were approved for treatment of PAH (figure 3). We wanted to do an experimental study during RHC using a peroral vasodilator drug with fast acute effects. The choice fell on a PDE5-inhibitor, vardenafil, that due to its rapid onset and its potent inhibitory activity on PDE5, showed the most promising pharmacological profile among PDE5-inhibitors for treatment of PH-patients (87, 88).
**Vardenafil in patients with PH**
We have demonstrated that a single oral dose of vardenafil is a safe, potent and selective PDE5-inhibitor in patients with PH. Vardenafil had a fast onset of action and rapid effects on cardiopulmonary haemodynamic variables and there was a strong plasma drug concentration-response-relationship. Just 15 minutes after the vardenafil intake, the correlation between mPAP and vardenafil plasma concentration became significant. mPAP decreased further as vardenafil concentration increased, continuing throughout the study period of 60 minutes. Vardenafil plasma concentrations measured during RHC were strongly correlated with the acute haemodynamic changes in mPAP, PVR and mean aortic pressure. Further, we only investigated the effects of one single dose of vardenafil and did not test the effects of repeated doses or long-term treatment. In one randomized, double-blind and placebo-controlled study, treatment during 24 weeks with 5 mg vardenafil twice daily was evaluated in 66 patients with PAH (89). In this study, long-term treatment with vardenafil was well tolerated with only mild and transient adverse effects. The haemodynamic response, exercise capacity and clinical outcome improved significantly. Apparently, the effectiveness of single-dose vardenafil shown in the present thesis is maintained during long-term therapy. Furthermore, vardenafil is considered to be the PDE5-inhibitor with the highest absolute affinity to PDE5 (87). This, together with the relatively low cost of vardenafil makes this drug an attractive candidate also for long-term treatment of patients with PAH.

**Vardenafil versus adenosine in vasoreactivity testing**
Vardenafil may be safely used as a vasodilator agent in the acute vasoreactivity test that identifies responders during RHC in patients with PAH. Acute haemodynamic responses to vardenafil were comparable to adenosine, but vardenafil resulted in greater reductions of mPAP, and identified additional responders who might benefit for long-term vasodilator treatment. Moreover, vardenafil had fewer adverse effects. However, the clinical significance of the higher response rate to vardenafil remains unclear and can still be a chance finding in our study. On the other hand, there are some pharmacological characteristics of vardenafil that may serve as a support of its superiority. Vardenafil has the potential to block calcium fluxes in the PA in rabbits and rats in addition to PDE5 inhibition and this could explain the higher response rate to vardenafil compared to adenosine (98–100) in the acute setting. Earlier studies have shown that only vardenafil, not sildenafil and tadalafil, has calcium channel blocking activity demonstrated studies with rat aorta, rabbit pulmonary artery and human washed platelets (94, 98, 99).
Two recently published studies compared sildenafil to NO or iloprost in acute vasoreactivity test and they confirmed our results that a PDE5-inhibitors may be a useful alternative as a vasoreactivity agent during RHC in patients with PAH (101, 102).

**Vardenafil pharmacokinetics in PH**

Overall, the pharmacokinetics of vardenafil in patients with PH reported in this thesis were in line with earlier published data (103–107). The haemodynamic effects after one single dose of vardenafil could only be monitored during 60 minutes. While the effect on mPAP was still improving and the median plasma vardenafil concentrations were still increasing at 60 minutes, the RHC had to be stopped due to practical reasons. The elimination half-life of vardenafil in our study was relatively short (3.35 h). Ideally, a once-daily dose of vardenafil would be preferable for long-term treatment, but the short half-life indicate that repeated intake will be necessary during long-term treatment. This is in line with the practice used in the study on long-term treatment with vardenafil discussed earlier, where twice daily administration was practiced (89).

An interesting observation of vardenafil pharmacokinetics in patients with PH was the considerably higher inter-individual variability compared to earlier studies in healthy men or patients with erectile dysfunction (103–105, 108). There may be several explanations for the large inter-individual variability in our material. Our study group was rather inhomogeneous. This, applies to age, gender, type of PH, disease stage, cardiac function, WHO functional class, comorbidity and use of other drug treatments (108–113). In addition, the patients received vardenafil while in a supine position during on-going RHC, which may have influenced the absorption and time to maximum concentration (114). Considering the large intra-individual variability, therapeutic drug monitoring for individual dose optimization may be warranted in PAH patient.

**Vardenafil drug-drug interactions with bosentan**

Pharmacokinetic drug interactions are important to consider in PH since they usually are treated with other PAH specific therapies, warfarin and a number of cardiovascular drugs. CYP enzymes metabolize many of these drugs and in particular CYP3A4 is prone to pharmacokinetic drug interactions. Vardenafil is metabolized by the CYP3A4, CYP3A5 and CYP2C isoenzymes. In our study we found four patients who were co-medicated with CYP3A4 inducing drugs, three with bosentan and one with carbamazepine. Bosentan is known to be a strong CYP3A4 and CYP2C9
inducer. The patients who co-medicated with bosentan had a large reduction in plasma vardenafil drug concentration compared to patients without bosentan co-medication. The patient with the highest dose of bosentan had the highest reduction in vardenafil concentration. Altogether, these three patients had the poorest effect of vardenafil on haemodynamic variables, even though this observation didn’t reach statistical significance, probably due to the small sample size. Earlier studies have shown that co-administration of bosentan with sildenafil or tadalafil decreased plasma concentration by about 60 and 40 %, respectively (115–117). Simultaneously, sildenafil increased plasma concentration of bosentan by about 40 % (117). On the other hand, the combination of bosentan and sildenafil was well tolerated with no serious adverse events. Still, in the COMPASS-2 study, combination therapy with bosentan and sildenafil was not superior to sildenafil monotherapy in delaying the time to the first morbidity/mortality event during a median ≥24 months of follow-up (118). Our observations altogether underline the relevance of taking into account drug to drug interactions when treating PAH patients with PDE5-inhibitors. Especially since combination therapy with ERA and PDE5-inhibitors are common and recommended by PAH guidelines (10). Individual dose adjustment according to results from therapeutic drug monitoring may be necessary to optimize the drug dose, particularly in non-responders or patients with progressing PAH. Nevertheless, further drug interaction studies between PDE5-inhibitors and ERA are warranted and while some of those combinations might not be favourable, real-life data analysed retrospectively indicate that the combination of sildenafil with bosentan works well clinically (119).

**Mono or combination therapy**

Until recently PAH-specific therapy has commonly been initiated as mono therapy. Today, combination therapy is recommended in the PAH guidelines (10). The rationale for upfront combination therapy is based on the fast progression of the disease and the high mortality. When selecting the PAH specific therapy it is important to know the pharmacological properties of the drugs. This is especially important in combination therapy in order to cope with drug-drug interactions. It is also essential to know the differences between the drugs within the groups.

Recently, the AMBITION study showed that initial combination therapy with tadalafil and ambrisentan was associated with a lower risk of clinical-failure events compared to initial mono therapy in PAH patients (120). Similarly, the COMPASS-1 study, the pharmacodynamic and pharmacokinetic effect of drug interaction between sildenafil and bosentan was explored (121). The conclusions of these two studies were in line with
our results where combination therapy tended to have a better effect in patients with PAH. Our results showed that it was a tendency for a more favourable effect on L-arginins and methylarginines with combination therapy than monotherapy. This is not surprising since both PDE5-inhibitors and ERA may have synergistic and apparently complementary effects on the L-arginine/NO pathway. Indeed, our results indicated that patients receiving mono or combination therapy with ERA had significantly lower plasma ADMA levels compared to patients on PAH-specific therapies without ERA. On the other hand, patients on mono- or combination therapy with PDE5-inhibitors surprisingly had significantly higher plasma ADMA levels compared to patients on PAH-specific therapies without PDE5-inhibitors. A possible explanation to this unexpected observation could be that ERA not only inhibits endothelin effects, but also exerts additional effects in the L-arginine/NO pathway (122–125). This might explain why ERA apparently had a bigger impact on eNOS compared to PDE5-inhibitors in patients with PAH. Further, ERA prevent the proliferation of pulmonary endothelial and smooth muscle cells, acting more as an antiproliferative agent than vasodilator agent (126). Although, ERA and PDE5-inhibitors have different metabolic drug interaction, with the right knowledge about these drugs, combination therapy with necessary dose adjustments probably is the best way to initiate initial PAH-specific therapy in patients with PAH.

**Methylarginines as potential biomarkers in PAH**

As PAH is a potentially fatal disease with an prodromal period of unknown length, it is important to develop tests or discover biochemical markers that allow for non-invasive, sensitive and specific diagnostics. The purpose of which would be optimizing choice of appropriate treatment strategies for the individual and giving fast feedback on treatment effects. Such an ideal marker has yet to be identified. L-arginine and its methylated derivatives are attractive candidates for this type of biomarkers. They play a central role in the control of NO-synthesis (45). Both extracellular and intracellular ADMA concentrations exert negative effects on vascular homeostasis by impairing endothelial function, increasing arterial stiffness and promoting vascular inflammation (127, 128). There is a strong association between high plasma ADMA levels and the pathogenesis of PAH (35). This makes ADMA a viable candidate biomarker for PAH (35,39,43,129). It is proven a useful predictor for cardiovascular outcome and mortality. Furthermore, plasma ADMA levels are significantly correlated with mixed-venous oxygen saturation, RAP, CI and survival in IPAH-patients (35). In addition, serum ADMA levels have been correlated to mPAP and PVRI in PAH-patients (45).
In our study, we found that plasma ADMA levels were significantly higher in patients with PAH compared to healthy subjects. In contrast, compared to patients with LVHF there was no difference in plasma ADMA levels and both patient groups had high levels of ADMA compared to healthy subjects. Thus, plasma ADMA levels should rather be considered a general marker of cardiovascular dysfunction than a specific marker of PAH. Interestingly, we found a statistically significant decrease in L-arginine between PAH-patients compared to both LVHF patients and healthy subjects. This may relate to a specific endothelial dysfunction in PAH-patients comprising inhibition of endothelial NO production and lower plasma arginine values due to increased consumption of L-arginine for NO-production. L-arginine is an important substrate of NOS and decreased L-arginine levels may result in a higher cardiovascular risk. Therefore, we believe that lower L-arginine levels may represent increased arginase activity and higher levels represent antagonizing effects on ADMA, contrary to what we found in patients with PAH (26, 54). ADMA is an important marker to measure in the PAH-population. Further, the L-arginine/ADMA ratio appears to be more indicative of NOS function than ADMA alone (130). Endothelium dependent vasodilation may be more dependent on the plasma L-arginine/ADMA ratio (131). L-arginine/ADMA ratio is an important and sensitive marker for atherosclerosis (55). Furthermore, GABR and L-arginine/L-ornithine ratio, represent arginase activity, as an overall measure of arginine bioavailability. These ratios may be important to monitor during follow-up of PAH patients (26). We did not measure L-citrulline or L-ornithine in LVHF-patients or healthy subjects. Further studies on these potential biomarkers in PAH and other cardiovascular diseases are warranted.

**Pharmacotherapies influencing the L-arginine/NO pathway**

L-arginine and methylarginines are centrally involved in PAH-pathogenesis and could meet the criteria of biomarkers for diagnosis, treatment and follow-up of PAH. PAH-specific therapies should be associated with fluctuations in L-arginine and methylarginines. Choice of therapy should aim at lowering ADMA as much as possible. We found no effect of PAH-specific therapy on plasma levels of ADMA, SDMA, L-arginine, L-citrulline and L-ornithine as well as L-arginine/ADMA, GABR and L-arginine/ornithine ratio. Further, no significant differences in methylarginines were observed between mono and combination therapy, even though there was a trend towards a more pronounced decrease of SDMA in patients receiving combination therapy. Interestingly, at follow-up patients on mono or combination therapy with ERA had significantly lower plasma ADMA levels than patients without ERA. Moreover, patients on
PDE5-inhibitors had significantly higher plasma ADMA levels as compared to patients not treated with PDE5-inhibitors. This observation of potential different effects of ERA and PDE5-inhibitors on ADMA should, however, be interpreted with caution. When working with small sample sizes there is always risk of chance findings. Still, two earlier published studies observed significantly decreased plasma ADMA levels after iloprost treatment for one week (65) and sildenafil treatment for six months (44).

Still, the L-arginine - NO pathway is a relevant target for the treatment of PH and other cardiovascular diseases. Methylarginines may also serve as a potential early marker of treatment response, since they are intimately involved in the regulation of endothelium-dependent vasodilation and at the same time platelet aggregation (30, 132). Infusion of ADMA has been demonstrated to impair endothelium-dependent vasodilation, to increase systemic and renal vascular resistance and to lower CO (132). Infusions with L-arginine and especially L-citrulline have an opposite effect and improve vasodilation, similar to the effect achieved by NO inhalation (133–136).

Specific ADMA-lowering therapies are not available to-date, however several drug therapies have shown the ability to decrease ADMA levels indirectly, such as statins, angiotensin-converting enzyme inhibitors (ACEI), angiotensin receptor blockers (ARB), hypoglycaemic agents and hormone replacement therapy (132). Theoretically, ADMA-levels can be reduced either by minimizing the formation of ADMA in the course of proteolysis of proteins with methylated arginine residues, or by increasing elimination of ADMA, either renally or through metabolic degradation via DDAH. The mechanism behind the ADMA-lowering effects of statins, ACEI and ARB are thought to be the result of reduced vascular oxidative stress (28). ACEIs decrease NADPH oxidase activity, superoxide and peroxynitrite production and increase vascular BH₄ levels in animal studies. ARBs induce BH₄ and reduce eNOS S-glutathionylation and eNOS enzymatic activity by restoring eNOS phosphorylation (28, 30). Statins improve BH₄ biosynthesis, inhibit NADPH oxidase activity and upregulate eNOS expression leading to stimulated eNOS activity. Increased DDAH expression and/or activity with better cardiovascular outcome has been observed in connection with the following drugs: aspirin, pravastatin, fenofibrate, nebivolol, losartan, telmisartan and pioglitazone (61).

Interestingly, one single oral dose of vardenafil induced acute decrease of ADMA levels in patients with PH (137). Furthermore, it has been demonstrated that treatment with sildenafil during six months decreased ADMA concentrations in patients with PAH due to congenital heart disease (44). Iloprost treatment during one week also resulted in decreased ADMA...
levels in patients with peripheral arterial occlusive disease (65). The mechanisms behind these PAH-specific therapies are believed to come from their effect on the NO/cGMP-pathway. A recently published study showed that vardenafil reduced oxidative stress through 8-iso-prostaglandin-F2α and 3-nitrotyrosine, leading to increased eNOS expression in PAH-patients (138). There is also emerging evidence that sildenafil and tadalafil reduce the oxidative stress in vitro and in animal studies (139–141). Further, bosentan appears to inhibited oxidative stress associated with PH in animal studies (126). Thus, reduction of oxidative stress looks to be an important goal of PAH-specific therapy.

**Limitations and possible bias of the studies**

All of our studies in patients with PH/PAH comprise a relatively small number of patients, this hamper the power of statistical analysis and stratifications. Still, considering the rareness of the disease, the sample size in our studies is substantial and comparable to other studies in the field. Most of all, our findings are of significance for treatment of PH/PAH patients. In contrast, the inhomogeneous groups of PH-patients in our material show the reality in PAH clinics today.

**Limitations regarding the vardenafil studies**

In the pharmacokinetic study when blood samples were collected to monitor plasma vardenafil concentration, nine hours observation time (2.5 half-lives) was too short to be able to follow the concentration until the drug completely had left the body (paper II). Due to procedural reasons, some blood samples were taken on non-scheduled time points, which might have influenced the pharmacokinetic estimations. Further, we were unable to measure the active metabolite, N-desethyl vardenafil (M1) of vardenafil. On the other hand, the contribution of M1 is relatively small and it is unlikely that it would have had an impact on our result.

In the pharmacodynamic studies, haemodynamic variables were not available in all patients due to logistic reasons (paper I). In the acute vasoreactivity test, adenosine was given prior to vardenafil, since adenosine was given intravenous and vardenafil was given orally (paper III). This could theoretically have influenced the impact of vardenafil response, however, priming and carry-over effects of adenosine are unlikely due to short half-life (less than 10 seconds) and pharmacodynamics action of maximum 2 minutes (17,23). Adenosine is no longer first choice for acute vasodilator testing, but at the time when the study was performed it was still the standard vasodilator agent in Uppsala.
Limitations regarding the L-arginine and methylarginines study

This study presents several limitations that deserve to be addressed (paper IV). Our patients suffered from a multifactorial disease and the majority of the patients were at an advanced age suffering from several comorbidities such as diabetes mellitus, hypercholesterolemia and hypertension that in itself could affect the methylarginines in the L-arginine - NO pathway. In addition, the patients received other drugs, which could interfere with the L-arginine/NO pathway. Furthermore, all blood samples were not taken routinely at the same time since the last dose. Methylarginines were measured extracellularly and not intracellularly, which may not reflect true intracellular events. Further, we did not measure urea or enzyme regulating oxidative stress.
Conclusion and future research

Conclusion
In conclusion, this thesis showed that one single oral dose of vardenafil is safe to use in patients with PH. We have studied the cardiopulmonary haemodynamic effects, pharmacokinetic effects, adverse effects and long-term clinical outcome in vardenafil acute responders. Vardenafil demonstrated rapid acute cardiopulmonary haemodynamic effects during one hour of RHC in patients with PH. The plasma vardenafil concentration was correlated with acute improvements in mPAP and PVR, haemodynamic variables that are strongly associated with mortality in PAH patients. The pharmacokinetic profile of vardenafil revealed a high inter-individual variability in patients with PH. Furthermore, a potential pharmacokinetic drug interaction between vardenafil and bosentan was observed when bosentan significantly decreased vardenafil plasma concentration. Furthermore, vardenafil may be safely used as an acute vasodilator agent to identify responders during RHC in patients with PH. The acute haemodynamic response to oral vardenafil is comparable to intravenous adenosine, but vardenafil had fewer adverse effects and may identify additional responders who could benefit from long-term vasodilator treatment. Furthermore, PAH specific treatments favorably influence the L-arginine and NO pathway in patients with PAH. Vardenafil is an interesting drug with important mechanisms for treating PAH, but more long-term studies are needed.

Future research
Very little is known about the pathomechanism of PAH but it is widely accepted that there is an imbalance in the endothelial function, leading to increased production of endogenous vasoconstrictors and proliferative agents (endothelin-1 and thromboxane A2) and decreased production of vasodilator and antiproliferative agents (NO and prostacyclin). However, the underlying mechanisms and factors that trigger and accelerate this imbalance are still unknown. Further basic research on pathomechanisms is urgently needed. We now know that the oxidative stress plays a central role in propagation and progression of the disease. It would appear rational to apply a multimodal approach to treatment of this disease applying both PAH-specific therapy in combination with adjuvant therapies that aim at lowering oxidative stress and increasing DDAH activity. Further, the potential role of the PRMT-enzyme for initiation and progression of PAH warrants further investigations.
PAH-specific therapy aims to reduce symptoms and is not causal. In the future we need to find the early triggers of the disease and to start pharmacological therapy in its early phase. Individualized therapy demands access to sensitive and specific diagnostic tools to test the effectiveness of the therapeutic approach and to allow adjustments to therapy if needed. It is also important to learn more about the pharmacokinetic drug to drug interactions of PAH-specific therapy in combination therapy. In the future, therapeutic drug monitoring of PAH-specific therapy may be warranted for individual dose optimization. We have used an experimental model in humans to explore vascular haemodynamics and aspects of vascular biology in relation to PDE5-inhibitors in this thesis. A study based on information from the Swedish Pulmonary Arterial Hypertension Registry (SPAHR) to observe the effects of mono- and combination therapy in patients with PAH and CTEPH would be of great interest. Observational studies with more patients, standardized treatment and homogeneous patient groups should be performed. Finally, the future of PAH specific therapy and the pathomechanism behind it is more than exciting. This is a relatively new area of research and there is much left to learn in order to improve the life of these patients. Thus, we will continue to study the effect of PAH specific therapy and other cardiovascular drugs on the L-arginine – NO pathway in larger patient materials and through that continue to learn more about vascular biology and biomarkers.
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