Pulmonary Hypertension and the Nitric Oxide System

DAN HENROHN
Pulmonary hypertension (PH) is a pathophysiological state associated with several medical conditions, leading to progressive rise in pulmonary vascular resistance (PVR) and right ventricular failure. The clinical PH classification encompasses five main World Health Organization (WHO) groups; pulmonary arterial hypertension (PAH), PH due to left heart disease, PH due to lung diseases and/or hypoxia, chronic thromboembolic PH, and PH with unclear multifactorial mechanisms. Nitric oxide (NO) is a potent vasodilator. Impaired NO production via the classical L-arginine-NO synthase (NOS) pathway has been implicated in PH. Phosphodiesterase-5 (PDE5) inhibitors augment NO signalling, and are considered as one of the cornerstone treatments in PAH. The studies in this thesis aim at to explore and expand the understanding of the NO system in patients with PH.

In paper I, we found that PAH patients (WHO group 1) have lower bronchial NO flux compared to healthy controls and patients with PH (WHO group 2-4). This implies reduced bronchial NO formation in PAH. Compared to healthy controls, increased alveolar NO levels were found in patients with PH (WHO group 1-4) and patients with PAH. This may reflect NO diffusion disturbances in the alveoli. PAH patients had higher plasma and salivary levels of nitrite than healthy controls, which may reflect a compensatory upregulation of NOS-independent NO generating pathways.

In paper II, we observed that a single oral dose of vardenafil (a PDE5 inhibitor) causes rapid changes in cardiopulmonary haemodynamics in PH patients with PH (WHO group 1-4). We found a correlation between plasma vardenafil concentrations and the changes in mean pulmonary arterial pressure as well as PVR.

In paper III, we show that a single oral dose of vardenafil to patients with PH (WHO group 1-4) alter the plasma levels of arginine, asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA), and the arginine/ADMA ratio in a favourable manner. The increase in arginine and the arginine/ADMA ratio were associated with improved cardiac index, and the increase in the arginine/ADMA ratio at 540 min correlated with the exposure to vardenafil. Higher baseline plasma levels of ADMA and SDMA and a low arginine/ADMA ratio was associated with a more severe pulmonary haemodynamic disease state in patients with PH.

In paper IV, we found that ingestion of beetroot juice, containing inorganic nitrate, increased plasma and salivary levels of nitrate and nitrite, increased exhaled NO, decreased plasma ornithine levels and increased relative arginine availability in patients with PAH compared to placebo. Higher plasma levels of nitrite after the placebo period, reflecting basal conditions, were associated with a more severe PAH phenotype. Our findings indicate that the nitrate-nitrite-NO pathway is active and upregulated in PAH patients.

Keywords: Pulmonary hypertension, nitric oxide, ADMA, arginine, nitrate, nitrite, vardenafil, beetroot juice

© Dan Henrohn 2018

ISSN 1651-6206
urn:nbn:se:uu:diva-347767 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-347767)
OMNIA MIRARI ETIAM TRITISSIMA

Carl von Linné
List of Papers

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


*Equal contributors


Reprints were made with permission from the respective publishers.
Contents

Introduction ................................................................................................... 11
Pulmonary Hypertension .......................................................................... 11
  Definitions and clinical classification ................................................. 11
  Epidemiology ....................................................................................... 13
The nitric oxide system ............................................................................ 17
  Vascular nitric oxide ......................................................................... 17
  NO production and regulation in PH ...................................................... 21
Present investigation ..................................................................................... 25
  Aims of the thesis .................................................................................. 25
Patients and methods ................................................................................ 26
  Ethics ................................................................................................... 26
  Paper I .................................................................................................. 26
  Paper II and III ..................................................................................... 27
  Paper IV ............................................................................................... 32
Results ...................................................................................................... 37
  Paper I .................................................................................................. 37
  Paper II ................................................................................................ 40
  Paper III ............................................................................................... 43
  Paper IV ............................................................................................... 44
Discussion ................................................................................................. 51
  Exhaled NO, nitrate, and nitrite in PH ................................................. 51
  Vardenafil in PH – acute haemodynamic response and
  pharmacokinetics ................................................................................. 54
  Vardenafil in PH – acute changes in plasma arginine and
  dimethylarginines ................................................................................ 57
  The nitrate-nitrite-NO pathway in PAH – exploring the effects of
  inorganic nitrate ................................................................................... 59
Conclusions .............................................................................................. 63
  General discussion and future perspectives .............................................. 64
Acknowledgements ....................................................................................... 67
References ..................................................................................................... 69
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTPO</td>
<td>acceleration time of the pulmonary outflow tract</td>
</tr>
<tr>
<td>ADMA</td>
<td>asymmetric dimethylarginine</td>
</tr>
<tr>
<td>AT</td>
<td>anaerobic threshold</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>BALF</td>
<td>bronchoalveolar lavage fluid</td>
</tr>
<tr>
<td>BF</td>
<td>breathing frequency</td>
</tr>
<tr>
<td>Calv</td>
<td>alveolar concentration</td>
</tr>
<tr>
<td>CI</td>
<td>cardiac index</td>
</tr>
<tr>
<td>CO</td>
<td>cardiac output</td>
</tr>
<tr>
<td>CVP</td>
<td>central venous pressure</td>
</tr>
<tr>
<td>CTEPH</td>
<td>chronic thromboembolic pulmonary hypertension</td>
</tr>
<tr>
<td>CHD</td>
<td>congenital heart disease</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>DDAH</td>
<td>dimethylarginine dimethylaminohydrolase</td>
</tr>
<tr>
<td>DLCO</td>
<td>diffusing capacity of the lung for carbon monoxide</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>EBC</td>
<td>exhaled breath condensate</td>
</tr>
<tr>
<td>FeNO</td>
<td>fractional exhaled nitric oxide</td>
</tr>
<tr>
<td>GABR</td>
<td>global arginine bioavailability ratio</td>
</tr>
<tr>
<td>HR</td>
<td>heart rate</td>
</tr>
<tr>
<td>J’aw</td>
<td>bronchial flux</td>
</tr>
<tr>
<td>LAA</td>
<td>left atrial area</td>
</tr>
<tr>
<td>LVEF</td>
<td>left ventricular ejection fraction</td>
</tr>
<tr>
<td>mPAP</td>
<td>mean pulmonary artery pressure</td>
</tr>
<tr>
<td>mRAP</td>
<td>mean right atrial pressure</td>
</tr>
<tr>
<td>MCTD</td>
<td>mixed connective tissue disease</td>
</tr>
<tr>
<td>NYHA</td>
<td>New York Heart Association</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>PDE</td>
<td>phosphodiesterase</td>
</tr>
<tr>
<td>PRMT</td>
<td>protein arginine methyltransferase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PAH</td>
<td>pulmonary arterial hypertension</td>
</tr>
<tr>
<td>PAP</td>
<td>pulmonary artery pressure</td>
</tr>
<tr>
<td>PCWP</td>
<td>pulmonary capillary wedge pressure</td>
</tr>
<tr>
<td>PH</td>
<td>pulmonary hypertension</td>
</tr>
<tr>
<td>PVR</td>
<td>pulmonary vascular resistance</td>
</tr>
<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
</tr>
<tr>
<td>RAA</td>
<td>right atrial area</td>
</tr>
<tr>
<td>RER</td>
<td>respiratory exchange ratio</td>
</tr>
<tr>
<td>RHC</td>
<td>right heart catheterisation</td>
</tr>
<tr>
<td>RV</td>
<td>right ventricular</td>
</tr>
<tr>
<td>RVFAC</td>
<td>right ventricular fractional area change</td>
</tr>
<tr>
<td>RV GLPS</td>
<td>right ventricular global longitudinal peak strain</td>
</tr>
<tr>
<td>6-MWD</td>
<td>six-minute walking distance</td>
</tr>
<tr>
<td>sGC</td>
<td>soluble guanylate cyclase</td>
</tr>
<tr>
<td>SDMA</td>
<td>symmetric dimethylarginine</td>
</tr>
<tr>
<td>SLE</td>
<td>systemic lupus erythematosus</td>
</tr>
<tr>
<td>SPAP</td>
<td>systolic pulmonary artery pressure</td>
</tr>
<tr>
<td>SSc</td>
<td>systemic sclerosis</td>
</tr>
<tr>
<td>SVR</td>
<td>systemic vascular resistance</td>
</tr>
<tr>
<td>TAPSE</td>
<td>tricuspid annular plane systolic excursion</td>
</tr>
<tr>
<td>TA S’</td>
<td>tricuspid lateral annular systolic velocity wave</td>
</tr>
<tr>
<td>VE</td>
<td>minute ventilation</td>
</tr>
<tr>
<td>VO2</td>
<td>oxygen uptake</td>
</tr>
<tr>
<td>W</td>
<td>work rate</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
Introduction

Pulmonary Hypertension

Definitions and clinical classification

Pulmonary hypertension (PH) is a haemodynamic and pathophysiological state associated with a number of clinical conditions, and the disease was first identified by Ernst von Romberg in 1891 (1).

The first attempt for classification of PH was made in 1973 at a meeting in Geneva, Switzerland, organised by the World Health Organization (WHO) (2). At this meeting PH was classified into two categories; primary PH or secondary PH depending on identified causes and risk factors. Based on a detailed pathological description from 1970, three major different pathological types of primary pulmonary hypertension were described in the WHO report (arterial plexiform, veno-occlusive, and thromboembolic) (3). At that time, the primary PH group encompassed what today is termed as idiopathic, heritable and drug-induced PAH.

An extensive revision of the classification of PH was presented 25 years later at the second World Symposium on PH held in Evian, France, in 1998 (4). The Evian diagnostic classification of PH aimed at describing and individualizing different categories of PH sharing similarities in pathophysiological mechanisms, clinical features, and therapeutic options. Five groups (WHO groups 1-5) of PH were proposed (with subsets of diseases for each group); Pulmonary arterial hypertension (PAH), Pulmonary venous hypertension, PH associated with disorders of the respiratory system and/or hypoxia, PH caused by chronic thrombotic or embolic disease, and PH caused by disorders directly affecting the pulmonary vasculature (4). Since then, although maintaining the general architecture and philosophy of the Evian classification, the clinical classification of PH have been updated three times reflecting the progress in the understanding of the disease at the third, fourth and fifth World Symposium on PH (Venice 2003, Dana Point 2008, and Nice 2013) (5-7). The latest updated clinical classification of PH is shown in Table 1 (7).

In addition to the clinical classification, haemodynamic definitions of PH exist in aid for diagnosis and to differentiate between pre-capillary PH and post-capillary PH (8, 9). The general haemodynamic definition of PH is currently a mean pulmonary artery pressure (mPAP) equal to or above (≥) 25
mmHg at rest measured by right heart catheterization (RHC). The haemodynamic variable pulmonary capillary wedge pressure (PCWP) help to differentiate between pre-capillary and post-capillary PH. Pre-capillary PH is haemodynamically defined as the combination of a mPAP ≥25 mmHg and a PCWP equal to or less than (≤) 15 mmHg, whereas post-capillary PH is defined as mPAP ≥25 mmHg and a PCWP above (> ) 15 mmHg. Included in the haemodynamic definition of pre- and post-capillary PH is the presence of normal or reduced cardiac output (CO). The current haemodynamic definition of PAH as assessed by RHC at rest is the combination of; a mPAP ≥25 mmHg with a PCWP ≤15 mmHg, and elevated pulmonary vascular resistance (PVR) >3 Wood units (ie, >240 dyn.s.cm⁻⁵) (8). PAH is further defined as a clinical condition characterized by the presence of pre-capillary PH in the absence of other causes of pre-capillary PH (9). Post-capillary PH include one clinical group, PH due to left heart disease (WHO group 2) whereas pre-capillary PH includes the rest of the main clinical groups; PAH (WHO group 1), PH due to lung diseases and/or hypoxia (WHO group 3), chronic thromboembolic pulmonary hypertension (CTEPH) (WHO group 4), and PH with unclear multifactorial mechanisms (WHO group 5) (9).

Table 1. Clinical classification of PH, from the 5th World Symposium on PH held in Nice, France, 2013.

1. Pulmonary arterial hypertension
   1.1 Idiopathic PAH
   1.2 Heritable PAH
     1.2.1 BMPR2
     1.2.2 ALK-1, ENG, SMAD9, CAV1, KCNK3
     1.2.3 Unknown
   1.3 Drug and toxin induced
   1.4 Associated with:
     1.4.1 Connective tissue disease
     1.4.2 HIV infection
     1.4.3 Portal hypertension
     1.4.4 Congenital heart diseases
     1.4.5 Schistosomiasis
1’ Pulmonary veno-occlusive disease and/or pulmonary capillary hemangiomatosi
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

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 Developmental lung diseases

4. Chronic thromboembolic pulmonary hypertension

5. Pulmonary hypertension with unclear multifactorial mechanisms
   5.1 Hematologic disorders: chronic hemolytic anemia, myeloproliferative disorders, splenectomy
   5.2 Systemic disorders: sarcoidosis, pulmonary histiocytosis, lymphangioleiomyomatosis
   5.3 Metabolic disorders: glycogen storage disease, Gaucher disease, thyroid disorders
   5.4 Others: tumoral obstruction, fibrosing mediastinitis, chronic renal failure, segmental PH

Abbreviations; BMPR, bone morphogenic protein receptor type II; ALK, activating receptor-like kinase; ENG, endoglin; SMAD, mothers against decapentaplegic homolog; CAV, caveolin; KCNK, potassium channel subfamily K; HIV, human immunodeficiency virus.

Epidemiology

Epidemiologic data enabling estimation and comparison of the prevalence of the different groups of PH (WHO groups 1-5) in the general population are limited. Data from a retrospective echocardiographic database search between 2003 and 2009, including 10,314 individuals (representing 6.2% of the population in Armadale, Australia) with an estimated pulmonary artery systolic pressure $\geq 40$ mmHg, have been collected and analyzed (10). Data from this study provide information on community prevalence of PH and its subtypes. Echocardiographic evidence of PH was present in 9.1% of the cohort, and 67.9% had PH due to left heart disease (WHO group 2), 9.3% had PH due to lung diseases and/or hypoxia (WHO group 3), 2.7% had PAH (WHO group 1), 2.0% had CTEPH (WHO group 4), 2.7% had other miscellaneous diseases causing PH, and 15% had PH of unknown cause (10).

A number of registries exist for PAH, which has accumulated important information throughout the years enabling understanding of the natural history of the disease and providing information regarding survival, prognostication,
treatment, demographics and epidemiological characterization of subsets of PAH included in WHO group 1 (11).

The estimated incidence described in these registries range from 2.0-7.6 cases per million adult inhabitants per year with regard to PAH and an estimated incidence in the range of 0.9-2.6 cases per million adult inhabitants per year for idiopathic PAH (11). The estimated prevalence of PAH and idiopathic PAH range from 10.6-26 cases per million adult inhabitants and 4.6-9 cases per million adult inhabitants respectively (11). The earliest register, including patients with idiopathic and heritable PAH during 1981-1985, is the United States National Institutes of Health (NIH) Registry of primary PH (12, 13). The current world’s largest PAH register is a more contemporary register including patients with PAH (WHO group 1) from the United States, the Registry to evaluate early and long-term PAH (REVEAL), which includes PAH patients from 2006 to 2009 with a planned minimum follow-up time of five years for all patients after inclusion (14, 15). Data from this register show that the patients with associated PAH (defined in accordance with the Venice 2003 clinical classification) comprise the largest subpopulation (approximately 50%), followed by idiopathic PAH (approximately 46%) and heritable PAH (approximately 3%) as number three (15). The distribution of subgroups within the subpopulation associated PAH, in this register, show that the largest subgroup is PAH associated with connective tissue disease (equal to collagen vascular disease in the Venice 2003 classification) which comprise approximately 50% of the patients followed by PAH associated with congenital heart diseases (approximately 20%), PAH associated with portal hypertension (approximately 11%), PAH associated with drug/toxins (approximately 10%), PAH associated with HIV (4%) (15). Systemic sclerosis (SSc) is the most common diagnose in the subgroup PAH associated with connective tissue disease in the REVEAL register (68% of the patients with a known diagnosis) and in several other PAH registries (11, 15).

A comparison between REVEAL patients matched to NIH registry patients (including idiopathic PAH and heritable PAH) show that patients in the REVEAL cohort were older at diagnosis, with a mean age of 44.9 years as compared to a mean age of 36.4 years in the NIH cohort (16). A similar trend with an upward shift in mean age at diagnosis is seen also in other contemporary PH registries outside the United States, and potential explanations for the change are an increased awareness for PAH nowadays, as compared to earlier when primary PH was considered a rare disease affecting young women primarily and older patients and men were often not considered for the diagnosis, in conjunction with a greater availability of widespread screening tools such as Doppler echocardiography (11).

Despite an increased awareness of PAH little has changed with regard to functional class at diagnosis, when comparison is made between the historic NIH registry and more contemporary registries, and the majority of the PAH patients are still in functional class III or IV (71% of the cohort in the NIH
register, 73% of the NIH matched cohort in the REVEAL register, 75% in the French national PAH register) when diagnosis is established by RHC (16, 17).

The registries indicate that there is an improvement in survival in the modern treatment era (after availability of prostacyclin and its analogues and oral PAH-specific drugs) as compared with that observed previously in the NIH registry (11). An evaluation of long-term survival from time of diagnosis in PAH from the REVEAL registry (full cohort) show that the one-, three-, five- and seven-year survival rates from time of diagnostic RHC were approximately 85%, 68%, 57%, and 49% respectively (18). The survival rates (one-, three-, five-, and seven-year survival rates from the time of diagnosis) for patients with idiopathic or heritable PAH weighted to match the NIH cohort (in age, sex, and mPAP distribution) who initiated PH specific treatment within six months of diagnosis in the REVEAL registry were approximately 93%, 78%, 70%, and 66%, which is a considerable improvement when comparison is made to the corresponding estimated survival rates of 67.6%, 46.2%, 35.0%, and 31.5% using the NIH equation (18). The data from the REVEAL register suggest that patients with PAH with a profile similar to those in the NIH registry can now expect a median survival time of more than seven years as compared to 2.8 years documented for patients in the NIH registry (18).

However there are still considerable differences in survival rates within subgroups of PAH. In the REVEAL register, the highest Kaplan-Meier estimates of seven-year survival from time of diagnostic RHC were observed in the subgroups PAH associated with congenital heart disease (CHD) (67%) and PAH associated with HIV (64%) whereas the lowest estimates of seven-year survival were observed in the subgroups of PAH associated with portal hypertension (29%) and PAH associated with connective tissue disease (35%) (18).

Patients with SSc and PAH (PAH-SSc), belonging to the WHO group 1 subgroup PAH associated with connective tissue disease, seem to have worse prognosis as compared with other patient groups (patients with systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA)) belonging to the same PAH subgroup. The one-year survival for the PAH-SSc patients in the REVEAL register was 82% as compared with 88%, 94%, and 96% for the PAH patients with mixed connective tissue disease (MCTD), SLE, and RA (19). The one-year survival rates for the PAH-SSc patients in this analysis of the REVEAL cohort were significantly lower than the corresponding survival rates for patients with PAH associated with SLE and RA but not significantly lower compared with the survival rates for patients with PAH associated with MCTD (19). Data from Europe (the United Kingdom), collected in the modern treatment era, show that the one- and three-year survival rates for patients with PAH-SSc were 78% and 47% as compared with 78% and 74% for patients with PAH-SLE, 89% and 63% for patients with PAH-MCTD and 83% and 66% for patients with PAH-RA (20). Due to too few numbers of patients with different connective tissue disease in this cohort study it was difficult to make definite conclusions, however the three-year survival rates for PAH-SSc was
significantly lower than that for patients with PAH-SLE (20). Although higher survival rates at one (90%) and two (78%) years from PAH diagnosis have been reported in a French cohort including SSc patients in the most modern treatment era, the three-year survival rate of 56% still indicate a very poor prognosis similar to other reports for patients with PAH-SSc (21). It thus seems as if survival has improved also for patients with PAH-SSc since historic data report PAH-SSc survival rates of 45% at one year and 40% at two years (22, 23). However data exist that indicate that survival has not improved for patients with PAH-SSc after availability to the latest advances in PAH treatment with oral endothelin receptor antagonists, and phosphodiesterase-5 (PDE5) inhibitors in addition to prostacyclin or prostacyclin analogues as compared to the treatment era which included the option for parenteral treatment with parenteral prostacyclin (epoprostenol) as the only PAH specific treatment (24).

Comparative data on survival for the different groups of PH (WHO groups 1-5), which include PH patients diagnosed between 2001 and 2010 at a specialist PH centre (Sheffield, United Kingdom), have been reported (25). Three-year survival data from this registry show that patients with PH due to left heart disease (WHO group 2) had the highest survival rate (73%) followed by patients with CTEPH (WHO group 4) (71%), PAH (WHO group 1) (68%), miscellaneous PH (PH with unclear multifactorial mechanisms, WHO group 5) (59%), and PH due to lung diseases and/or hypoxia (WHO group 3) (44%) (25). However a considerable difference in survival was noted for the different PH subgroups within each PH group (25). In WHO group 1 (PAH) the three-year survival in PAH-CHD was 85% which was significantly better than corresponding survival rate for idiopathic PAH (63%) and PAH-SSc (52%). In WHO group 2 (PH due to left heart disease) the survival was significantly worse for patients with PH secondary to valvular disease than in PH secondary to diastolic dysfunction. The lowest three-year survival rate in all WHO groups was observed in WHO group 2 (PH due to lung diseases and/or hypoxia) in PH associated with interstitial lung disease (16%), as compared to a 90% three-year survival rate in PH associated with sleep-disordered breathing/alveolar hypoventilation and 41% in PH associated with chronic obstructive pulmonary disease. Survival differed also within WHO group 4 (CTEPH), with a 83% three-year survival for patients with CTEPH undergoing pulmonary endarterectomy as compared with 37% for patients with CTEPH who were not candidates for pulmonary endarterectomy due to the presence of significant comorbidities.
The nitric oxide system

Vascular nitric oxide

Nitric oxide (NO), a small molecule and a free radical, was originally identified by Furchgott and Zawadzki as an endothelium-derived relaxing factor (EDRF) in 1980 (26). Within the same decade it was later demonstrated that EDRF produced and released from artery and vein is in fact endothelial-derived NO, and furthermore that the NO release accounts for the biological activity of EDRF (27, 28). Earlier experiments in the 1970s by Ferid Murad had demonstrated that nitrates (nitroglycerin) release NO, which can cause vasodilatation as an effect of smooth muscle cell relaxation (29-31). In 1992 NO was proclaimed “Molecule of the Year” by the journal Science (32), and in 1998 the three scientists Robert F. Furchgott, Louis J. Ignarro and Ferid Murad were awarded the Nobel Prize for discovering the role of NO as a cardiovascular signaling molecule.

NO is now recognized as the major mediator regulating vascular tone and as such NO is playing a crucial role in vascular homeostasis. NO exerts pleiotropic effects within the vasculature, and in addition to its vasorelaxing effects NO has been shown to inhibit platelet adhesion and aggregation, modulate platelet production, inhibit vascular smooth muscle cell proliferation, modulate vascular permeability, and to exert atheroprotective as well as antithrombotic and anti-inflammatory effects (33-36).

Vascular NO production occurs in several cell types including platelets, macrophages, eosinophils, neutrophils, natural killer cells, vascular smooth muscle cells and in the vascular endothelium where the production is most pronounced in medium- and large sized arteries and arterioles (35,37,38).

A family of enzymes consisting of three well characterized isoforms of NO synthase (NOS) has been described which catalyze the production of NO along with formation of L-citrulline from the aminoacid L-arginine (39). A number of cofactors (flavin mononucleotide, flavin adenine dinucleotide, tetrahydrobiopterin, Ca^{2+}-calmodulin, and heme) are required for enzyme activity and molecular oxygen and nicotinamide adenine dinucleotide phosphate serve as cosubstrates whereas L-arginine is required as substrate (40,41). The three distinct isoforms are neuronal NOS (NOS I, constitutively expressed in neuronal cells, certain epithelial cells, kidney macula dense cells, pancreatic islet cells, skeletal muscle cells, and endothelial cells), inducible NOS (NOS II, expressed in several types of immune-cells, vascular smooth muscle cells, renal tubular epithelium, airway epithelial cells, hepatocytes, and mesengial cells) and the endothelial NOS (eNOS, NOS III, constitutively expressed in endothelial cells) (37, 39, 42, 43).
Vascular vasorelaxation as an effect of the activation of the L-arginine-NO-cyclic guanosine monophosphate (cGMP) pathway starts with the generation of NO by eNOS in the endothelium. NO then diffuses freely across the cell membrane to the nearlylying vascular smooth muscle cells where NO activates intracellular soluble guanylate cyclase (sGC) to produce cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (44). cGMP then activates protein kinase G (PKG), and through a series of actions mediated by the activated PKG the result is a decrease in the cytosolic Ca\(^{2+}\) concentration within the vascular smooth muscle cell which inhibits the calcium-calmodulin myosin light chain kinase complex formation ultimately resulting in vasorelaxation (45, 46). Degradation of cGMP in the vascular smooth muscle cells is catalyzed by cyclic nucleotide phosphodiesterases (PDEs), and in the pulmonary vasculature the levels of cGMP is regulated mainly by PDE5 (47-49).

Activation of eNOS and regulation of eNOS enzyme activity is complex and involves several mechanisms (50-52). Calcium-dependent or calcium-independent activation of eNOS to produce NO has been described after stimuli by acetylcholine, bradykinin, histamine, estradiol, vascular endothelial growth factor, and fluid shear stress (52, 53). In order for NOS to generate NO instead of superoxide anions (O\(_2^-\)) and hydrogen peroxide (H\(_2\)O\(_2\)) the three NOS isoforms, which are synthesized as monomers, need to form dimers in order to bind BH4 and the substrate L-arginine (51, 53). Sufficient levels of BH4 and L-arginine are needed to ensure coupled NOS and generation of NO, whereas suboptimal concentrations of BH4 or L-arginine reduce production of NO and favor NOS uncoupling leading to production of O\(_2^-\) and H\(_2\)O\(_2\) (51, 54, 55). Reactive oxygen species (ROS) as well as cGMP can regulate eNOS expression. It has been demonstrated that an increase in ROS activity upregulates eNOS in endothelial cells (56), whereas downregulation of eNOS expression by NO has been demonstrated to be mediated by cGMP (57). Furthermore it has been demonstrated that hypoxia inhibits expression of eNOS leading to suppressed production of NO and cGMP (58). Interestingly data from human studies indicate that exercise training up-regulate eNOS protein expression and phosphorylation (59).

The substrate L-arginine is essential for NOS dependent NO biosynthesis, and L-arginine bioavailability is ensured through dietary protein intake, body protein breakdown or endogenous de novo arginine production (60). The enzymatic breakdown of L-arginine to ornithine and urea by arginase limits the bioavailability of L-arginine as substrate for eNOS in the production of NO (60, 61). Two arginase isoforms, arginase I (cytosolic) and II (mitochondrial), exist and these are expressed at various levels in several different tissues (62). In the vasculature most of the arginases are present in the endothelium (predominantly arginase II), and only low levels are expressed in vascular smooth muscle cells (63). While arginase in the endothelium may modulate NO for-
mation it has been demonstrated that arginase and in particular elevated arginase expression in vascular smooth muscle cells primarily function to enhance the production of polyamines and proline, which in turn stimulate vascular smooth muscle cell growth and collagen synthesis potentially promoting intimal hyperplasia and vascular stiffness (64-66).

The endogenous de novo production of L-arginine encompasses two pathways, the urea cycle and the citrulline-NO cycle, both of which involves citrulline and the enzymes argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL) (60, 61). Thus, decreased bioavailability of L-arginine and citrulline can contribute to deficient NO production. In the citrulline-NO cycle, citrulline is formed as a by-product of NOS activity in the production of NO and citrulline is then recycled to L-arginine via the enzymes ASS and ASL in two steps. The arginine that is produced in the liver urea cycle is not, however, released to plasma in healthy conditions (67). Furthermore it has been shown in pulmonary artery endothelial cells that hypoxia can inhibit L-arginine bioavailability of the eNOS by inhibiting the transport of L-arginine into the endothelial cells and also that hypoxia inhibits L-arginine synthesis from citrulline in these cells (68, 69).

Vascular NO synthesis can be impaired by asymmetric dimethylarginine (ADMA) and monomethylarginine (L-NMMA), endogenous analogs of L-arginine that compete with L-arginine for the active site of eNOS (70, 71). ADMA and L-NMMA can in addition to blocking NO synthesis uncouple eNOS to generate O2·- (72, 73). ADMA and L-NMMA are derived from proteolysis of methylated arginine residues on proteins, and the methylation is mediated through a group of enzymes the protein-arginine methyl transferases (PRMTs) (70). The PRMTs can be activated by LDL cholesterol and shear stress (74, 75). The clearance of ADMA and L-NMMA is mediated mainly (80%) through enzymatic metabolism by dimethylarginine dimethylaminohydrolase (DDAH I and II) to produce citrulline and methylamines, with DDAH-II being expressed predominately in tissues were eNOS is active (76-78). Another endogenous analog of L-arginine is symmetrical dimethylarginine (SDMA) which is produced in the same manner as the other methylarginines, but SDMA is unlike ADMA and L-NMMA eliminated mainly unchanged by renal excretion and is not an inhibitor of NOS (70, 79, 80). SDMA may however contribute to decreased NO production from NOS indirectly by limiting the intracellular availability of L-arginine through its action as a competitor with L-arginine for the human cationic amino acid transporter hCAT-2B (81).

Other sources of vascular NO have been described in addition to the well characterized L-arginine-NOS pathway. The other sources of vascular NO include S-nitrosothiols (such as S-nitroso-hemoglobin, S-nitroso-glutathione, S-nitroso-cystein, and S-nitroso-albumin) and formation of NO from nitrite and nitrate. The S-nitrosothiols are endogenous metabolites of NO, they are more
stable compared to NO and may serve as storage pool for bioavailable NO in the vasculature (82-85).

The NOS-independent generation of NO through the nitrate-nitrite-NO pathway provides an important and complementary way to transduce NO bioactivity (86). Nitrate and nitrite is formed as NO undergoes rapid oxidative inactivation (37, 87). It has been demonstrated in fasted humans that the major contribution to plasma nitrite comes from the L-arginine-NO pathway (88). Other sources contributing to circulating levels of nitrite come from dietary intake of food containing nitrite and generation of nitrite from the conversion of dietary nitrate by commensal oral bacteria (89). Thus dietary nitrate intake affects the circulating levels of nitrite, and it has been demonstrated that nitrate is concentrated in the saliva through enterosalivary circulation of nitrate whereby ingested nitrate actively is absorbed by the salivary glands and secreted into saliva in the mouth (89-92).

In comparison to NO both nitrate and nitrite have much longer circulating half-life in human vasculature. The half-life in human blood of NO has been estimated to 0.05-1 second whereas the half-life for nitrite and nitrate has been estimated to 110 seconds and 5-8 hours respectively (93, 94). As a consequence of the rapid oxidation of nitrite to nitrate by oxygenated hemoglobin, basal plasma nitrate levels are much higher than plasma nitrite levels (94). Due to the longer half-life of nitrate and nitrite these substances may, in similar with the S-nitrosothiols, transfer NO bioactivity in an endocrine fashion as opposed to the paracrine and autocrine effects that NO generated from the L-arginine-NOS pathway mediate.

Although the vasodilating effects of inorganic nitrite have been known and described as early as in the later parts of the 19th century and the early part of the 20th century (95-99), it is not until more recent days that mechanisms of NO generation from nitrite have been understood and described. In 1994 it was reported that nitrite can be a substrate for NOS-independent in vivo generation of NO resulting from the acidic reduction of inorganic nitrite (100, 101). Several pathways involving both enzymatic and non-enzymatic mechanisms have been described with regard to nitrite reduction to NO. Nitric reductase activity have been described and linked to deoxyhemoglobin (102, 103), deoxymyoglobin (104), xanthine oxidase (105, 106), aldehyde oxidase (107), and respiring mitochondria (108, 109). Nitrite reduction to NO is thus an important alternative NOS-independent mechanism ensuring maintenance of NO homeostasis and NO bioavailability. This mechanism is proposed to be of particular importance in ischemic and hypoxic conditions since
the classical L-arginine-NO-pathway depends upon sufficient oxygen availability and substrate and cofactor delivery whereas in comparison most of the nitrite reductive pathways are augmented in hypoxic conditions (110).

NO production and regulation in PH

Pulmonary vasoconstriction, vascular smooth muscle cell proliferation, and in situ thrombosis are pathological features that characterize PH. Endothelial dysfunction with an imbalance in pulmonary vasodilators and vasoconstrictors in PH, in particular NO, prostacyclin (also called prostaglandin I₂), and endothelin-1 contribute to these pathological characteristic features (111, 112). Data indicating impairment in the biosynthesis of prostacyclin and increased expression of endothelin-1 in PH exist and reviews have been published describing the role of prostacyclin and endothelin-1 in the pathogenesis of PH (113-120).

Data indicating reduced NO production and disturbances in the NO pathway in PH are accumulating. Studies examining eNOS expression in the lungs of patients with PH are present in the literature. One of the most cited studies is the study from Giaid and Saleh presented in 1995 (121). In this study patients with plexogenic pulmonary arteriopathy (a mix of patients with PAH, corresponding to WHO group 1 in the current clinical classification system) and patients with secondary PH (a mix of patients with PAH and PH, corresponding to WHO; group 1.4.4, group 2, and group 3 in the current clinical classification system) were included and compared to controls. The expression of eNOS in the vascular endothelium of pulmonary arteries was reduced in both of the PH groups compared to the control group. The intensity of the eNOS immunoreactivity correlated inversely with the severity of histologic changes, with presence of a rather strong staining for eNOS in pulmonary arteries with the least severe histologic abnormality grade. Expression of eNOS in the pulmonary epithelium was reported as strong and in the endothelium of pulmonary veins the immunostaining was reported as relatively unchanged.

Moreover an inverse correlation between arterial expression of eNOS and total pulmonary resistance in patients with plexogenic pulmonary arteriopathy was demonstrated.

Corroborating evidence has been reported demonstrating reduced eNOS expression in small pulmonary arterioles in patients with primary PH (corresponding to PAH WHO group 1.1, 1.2, and 1.3 in the current clinical classification system) and in patients with secondary PH compared to controls (122). In the same study however, strong and consistent expression of eNOS was reported in the plexiform lesions, which are complex vascular formations originating from remodeled pulmonary arteries, in the patients with primary and secondary PH (122). Strong immunostaining not only for eNOS but also for
iNOS has also been reported in plexiform lesions in another study on patients with PAH associated with congenital heart disease (WHO group 1.4.4) (123). Contradictory results with regard to eNOS expression in lung tissue in patients with PH exist. Zhao et al. found eNOS expression in lung tissue to be similar in four patients with idiopathic PAH (WHO group 1.1) and normal control lungs (124). However, in this study lung tissue, which includes several histologic structures, was examined with no information specifically of the level of eNOS expression in pulmonary arteries.

Data demonstrating decreased NOS-dependent whole body NO production has been presented in patients with primary PH (corresponding to WHO group 1, PAH) (125). Similarly, in patients with PAH (WHO group 1.1 and 1.3) whole-body NO production, assessed by 24-hour urinary NO metabolite excretion, was found to be decreased compared to healthy control subjects (126). As an indication of deficient pulmonary NO synthesis, several studies using different methodology have demonstrated low levels of NO from the airways in patients with PAH (WHO group 1) (126-129). A decrease in exhaled NO has also been demonstrated in patients with PH due to chronic obstructive pulmonary disease (COPD) (WHO group 3.1) compared to COPD patients without PH (120, 130). In the study by Clini et al. a negative correlation between exhaled NO and systolic PAP was observed in the patients with PH due to COPD (130).

Reduced levels of biochemical oxidation products of NO in bronchoalveolar lavage fluid (BALF) and in exhaled breath condensate (EBC) has been shown in patients with PAH (WHO group 1) compared to healthy control subjects (127, 131). Further analysis of the BALF and the EBC in these studies revealed decreased levels of nitrate (nitrate plus S-nitrosothiol in the study by Kaneko et al.) and similar levels of nitrite in airways of PAH patients compared to control (127, 131). In the study by Kaneko et al. total NO reaction products in BALF were inversely correlated with PAP and years of diagnosis (127). Treatment with PAH-specific therapy results in an increase of levels of exhaled NO in patients with PAH. This has been demonstrated in patients with PAH at follow-up after initiating treatment with prostacyclin or a prostacyclin analogue (129, 131, 132) or bosentan (126). In the study by Ozkan et al., levels of exhaled NO was lower in patients with primary PH (corresponding to PAH WHO group 1.1, 1.2 and 1.3 in the current clinical classification system) not receiving prostacyclin compared to healthy control, whereas the patients with primary PH or secondary PH (a mix of patients corresponding to WHO group; 1.4, 3.1, and 4) that were receiving prostacyclin had higher levels of exhaled NO compared to healthy control and initiation of prostacyclin treatment to four PH patients (three primary PH patients and one secondary PH patient) led to an increase in exhaled NO after 24 hours (129). Interestingly, in the study by Machado et al. the increase over time in exhaled NO was noted in the patients who survived to complete the study (131).
Further evidence on disturbances in the L-arginine-NO-cGMP pathway in patients with PH has been reported. Elevated levels of ADMA, the endogenous inhibitor of NOS, have been reported over a wide range of patients in the PH clinical classification system including patients with: idiopathic PAH (WHO group 1.1), persistent PH of the newborn (WHO group 1’’), PAH associated with systemic sclerosis (WHO group 1.4.1), PAH associated with congenital heart disease (WHO group 1.4.4), CTEPH (WHO group 4), and PAH associated with chronic hemolytic anemia (WHO group 5.1) (133-140). It has been shown that elevated levels of ADMA, in patients with idiopathic PAH and CTEPH, correlate with severity of disease and is associated with worse survival (134, 139). Moreover, in patients with PAH-SSc (WHO group 1.4.1) a negative correlation between ADMA serum levels and six-minute walking distance (6-MWD) has been demonstrated (136). Analysis of lung tissue in patients with idiopathic PAH has demonstrated that the ADMA degrading enzyme DDAH-II is reduced (133). SDMA which unlike ADMA does not inhibit NOS directly has been reported to be elevated in patients with PH. SDMA may however reduce NO production from NOS indirectly by limiting bioavailability of L-arginine (81). Plasma and lung tissue levels of SDMA are increased in patients with idiopathic PAH (133). Elevated levels of SDMA, in the urine, have been reported also in infants with persistent PH of the newborn (WHO group 1’’) (135).

L-arginine, the substrate for eNOS, is also a substrate for arginase which produces ornithine and urea. Arginase II, the predominant isoform in the endothelium, thus competes with eNOS for the common substrate L-arginine. Decreased plasma levels of L-arginine have been reported in patients with PAH associated with congenital heart disease (WHO group 1.4.4) and in patients with CTEPH (WHO group 4) (138, 139). Moreover, it has been demonstrated in patients with idiopathic PAH (WHO group 1.1) that plasma levels of L-arginine correlate with mean right atrial pressure (mRAP), CO, CI, mixed-venous oxygen saturation and New York Heart Association (NYHA) functional class with an association between low levels of L-arginine and increased severity of the disease (141). A significant negative correlation has been reported between the level of arginine in lung epithelial cells and systolic PAP in patients with PAH (WHO group 1) (142). In the study by Cua et al. the arginine/ornithine ratio was lower in the patients with PH indicating increased arginase activity (138). Similarly, high plasma arginase activity has been reported in patients with hemolysis-associated PH (WHO group 5.1), and a low arginine/ornithine ratio was associated with greater severity of PH (143). High arginase activity in serum as well as a low arginine/ornithine ratio has also been reported in patients with PAH (WHO group 1) compared to healthy controls, and in the same study arginase II protein expression and arginase activity was reported as increased in pulmonary artery endothelial cells derived from PAH lungs compared to control and the cells from PAH patients produced
lower NO (142). In addition to disturbances in the NO generating pathway there is data to support that PDE5 expression is increased in patients with PH. PDE5 is considered as the main regulator of the NO effector molecule cGMP in pulmonary artery smooth muscle cells, responsible for the enzymatic breakdown of cGMP (48, 49, 144). An increase in PDE5 expression in pulmonary tissue has been described in patients with PAH (WHO group 1.1 and 1.4.4) and in patients with PH due to lung disease (WHO group 3) (145, 146).

The presence of PDE5 in pulmonary vascular tissue constitutes the basis for treatment of PAH patients with PDE5 inhibitors which lead to an augmentation of NO signaling by inhibiting the breakdown of NO generated cGMP (147, 148, 149).

As of today two PDE5 inhibitors, sildenafil and tadalafil, are registered and approved for treatment of PAH based on data from phase 3 double-blind, placebo-controlled clinical trials (150, 151). Vardenafil is a third commercially available PDE5 inhibitor, which along with the two other PDE5 inhibitors currently is approved for the treatment of erectile dysfunction but not yet for PAH treatment. However vardenafil is the most potent PDE5 inhibitor (152, 153), and data in support of beneficial treatment effects of vardenafil in PH patients have been reported from a randomised, double-blind, placebo-controlled study (PAH; WHO group 1.1, 1.4.1, and 1.4.4), from a long term open label study (PAH; WHO group 1.1, 1.4.1, and 1.4.4), and from several case reports in patients with PH (WHO group 1, 3, and 4) (154-158).

The therapeutic portfolio of drugs in PH that uses augmentation of NO signaling as the primary mode of action has recently been expanded to include riociguat, a sGC stimulator. Two phase 3 studies provided data in support for the approval of riociguat for the treatment of PAH (WHO group 1) and CTEPH (WHO group 4) (159, 160).
Present investigation

Aims of the thesis

The general aim of the thesis was to explore and expand the understanding of the NO system in patients with PH.

The specific aims were;

- Paper I - To compare patients with PH, with regard to PH aetiology, and healthy controls in terms of exhaled NO measured at different exhalation flow-rates and estimated NO flow-independent exchange parameters as well as levels of salivary and plasma nitrite and nitrate.

- Paper II - To evaluate the acute haemodynamic effects of a single oral dose of vardenafil, and study the drug concentration in relation to haemodynamic effects in patients with PH.

- Paper III – To study the effects of a single oral dose of vardenafil on plasma levels of dimethylarginines (ADMA and SDMA) and arginine in patients with PH.

- Paper IV – To study the effects of nitrate-rich beetroot juice in patients with PAH, and explore the nitrate-nitrite-NO pathway in patients with PAH.
Patients and methods

Ethics

All studies were approved by the regional Ethical review board in Uppsala, Sweden. All the study subjects gave their written informed consent for participation and for the use of their data in the studies. The study presented in paper IV is listed on ClinicalTrials.gov (NCT02000856).

Paper I

Patients and controls

The patients participating in the study were patients with PH of different aetiologies, representing WHO group 1-4, and they were all recruited through the regional PAH centre at Uppsala University Hospital. Separate analysis of the results were made based on PH aetiology, subdividing patients with PAH (WHO group 1) into one group and the other PH patients into another group (WHO group 2-4), in accordance with the WHO clinical classification system (5). The patients were in NYHA/WHO functional class II or III. Twenty patients were treated with PH-specific treatment (sildenafil, bosentan) either as monotherapy or as a combination therapy. Six patients were treated with a Ca²⁺ antagonist either as monotherapy or in combination with a PH-specific therapy (sildenafil and/or bosentan). All patients were on a stable treatment regimen, prior to inclusion. The study included 24 patients (17 females and seven males, 31-77 years of age (median 60.5)). However, two patients were excluded from the experiments, one due to asthma and one due to current smoking. Thus, 22 PH patients (13 with PAH; WHO group 1 and 9 with PH; WHO group 2-4) were included in total for assessment of exhaled NO levels. Eighteen of these patients (ten with PAH; WHO group 1 and eight with PH; WHO group 2-4) could perform the exhaled NO measurements required to assess the NO flow-independent parameters. These 18 patients are characterized as per below. The PAH patients (WHO group 1) consisted of eight females and 2 males (median age 42 years) in NYHA functional class II (6 patients) or III (4 patients) and 70% of the patients were on treatment with PH-specific therapy. The PH patients (WHO group 2-4) consisted of five females and three males (median age 66 years) in NYHA functional class II (2 patients) and III (6 patients) and 87.5% of the patients were treated with PH-specific therapy. The PAH patient group (WHO group 1) was significantly younger than the PH patient group (WHO group 2-4) however sex distribution, NYHA class and use of PH-specific therapy did not differ significantly between groups. The control group consisted of 21 non-smoking healthy individuals with a median age of 61 years of which 20 individuals were included in the assessment of NO flow-independent parameters. All subjects (patients and
controls) were instructed to avoid physical exercise and food containing high levels of nitrate the evening before and on the morning of the assessments.

**Exhaled NO**
The chemiluminescence analyzer, NIOX Flex (Aerocrine AB, Solna, Sweden) was used for exhaled NO measurements at flow rates of 20, 50, 100, 200, and 300 mL/s (duplicate in a random order). A third measurement was obtained if two values for a specific flow rate differed by more than 10%. Assessment of exhaled NO at 100, 200, and 300 mL/s and the slope-intercept model (161) were used for estimation of alveolar NO concentration (CalvNO) and bronchial NO flux (J’aw NO).

**Nitrate and nitrite measurements**
Saliva and plasma were collected from both patients and healthy controls. Two mL of unstimulated saliva was collected at each time and 10 mL of venous blood were collected in EDTA tubes with centrifugation of blood at 800 g for 12 min. All samples (plasma/saliva) were immediately frozen at -80 °C for later analysis. Salivary nitrate/nitrite were measured after methanol precipitation of proteins (1:1 v/v) by use of a high-performance liquid chromatography system (ENO-20; EiCom, Kyoto, Japan). Plasma nitrate/nitrite concentrations were determined by gas-phase chemiluminescence with a NO analyzer (Eco Physics AL77, Dürnten, Switzerland) following reductive cleavage and subsequent measurement of the NO released into the gaseous phase.

**Statistical analysis**
GRAPHPAD Prism, version 4 (GraphPAD Inc, San Diego, CA, USA) was used for statistical analyses. The Mann-Whitney test was used for analysis of group comparisons involving patients with PH and healthy controls. The Fisher’s exact test was used to compare the PH groups for differences in NYHA class, gender, and use of PH specific therapy. The Pearson correlation test was used to test the validity of the slope-intercept model.

**Paper II and III**

**Patients**
All patients participating in the study (Paper II and III) were patients with PH of different aetiologies, representing WHO group 1-4, according to the clinical classification of PH (6). The patients were in WHO functional class II or III. All patients were recruited from the regional PAH centre at Uppsala University Hospital. All patients were receiving at least one of the conventional background therapies (angiotensin-converting-enzyme inhibitor, angiotensin receptor blocker, β-adrenoceptor blocker, Ca2+ antagonist, digoxin, diuretics, or warfarin).
The study population presented in Paper II consisted of 16 PH patients (ten females and 6 males, 29-85 years of age (median 63)). Nine patients with PAH (WHO group 1) and seven patients with PH (WHO group 2-4) were included. Four patients with a previous diagnosis of PAH were treated with a PH-specific therapy at stable doses (one with sildenafil and three with bosentan). The other twelve patients were diagnosed with PH or PAH at the time of enrolment after confirmation at RHC.

The inclusion criteria were PH with a resting mPAP ≥ 25 mmHg measured by RHC and an age over 18 years. The exclusion criteria were; severe liver dysfunction (Child-Pugh class C), or hypotension, (<90/50 mmHg).

Baseline plasma data for arginine, ADMA, and SDMA were collected for twelve of the 16 PH patients (eight females, four males, 29-85 years of age (median 54.5)), in which the effects of vardenafil on plasma levels arginine and the dimethylarginines were studied up to 540 min (presented in Paper III). The study population presented in Paper III consisted of six patients with PAH (WHO group 1) and 6 with PH (WHO group 2-4).

**Study design**

The study was an open-label trial with the primary aims to investigate the acute effects of an oral single dose of vardenafil on changes in haemodynamics and pharmacokinetics (Paper II) and changes in plasma levels of arginine and dimethylarginines (Paper III). Depending on age and liver function, each patient received a single oral dose of either 5 mg (n=1), 10 mg (n=2), or 20 mg (n=13) vardenafil (Levitra®, Bayer Schering Pharma). Haemodynamic and mixed venous oxygen saturation measurements were performed during RHC at baseline and 15, 30, 45, and 60 min after vardenafil administration. Blood samples for vardenafil concentration measurements were taken from the pulmonary artery at baseline, and at 15, 30, 45, and 60 min after vardenafil administration (Paper II). Blood samples for determination of arginine, ADMA, and SDMA plasma concentrations were collected at baseline, and 15, 30, 45, 60, 120, 300, and 540 min after administration of vardenafil (Paper III). All blood samples were collected into EDTA-tubes (BD Diagnostics) and were processed immediately in a thermostatic (20°C) centrifuge (Jouan BR 3.11) for ten min at 3000 rpm. Plasma was separated and stored at –20°C until analysis.

**Haemodynamic assessment**

Haemodynamic data were collected from all patients that were included in the study (Paper II and Paper III). The indication for RHC was either haemodynamic follow-up of known PAH (n=4) or for diagnostic confirmation of suspected PAH/PH (n=12). Patients were hospitalized one day before and one day after the RHC, and all patients were fasting 24 hours before the procedure.
The RHC procedure included insertion of a fiberoptic thermodilution pulmonary artery catheter (Becton Dickinson Criticath SP5 107 HTD catheter) through the right internal jugular vein into the pulmonary artery, and correct position was verified by fluoroscopy. The patients were in a resting supine position during the examination. Pulmonary artery pressure (PAP) was measured at baseline and 15, 30, 45 and 60 min, whereas central venous pressure (CVP), pulmonary artery wedge pressure (PAWP), CO and cardiac index (CI) were measured at baseline and 60 min. The pressures were registered with a Cathcor® system (Siemens) and the flow was calculated with thermodilution technique or with Fick’s principle. Patients with tricuspid valve regurgitation > grade 1 were evaluated with Fick’s principle due to greater variation with the thermodilution technique. Calculation was made of PVR, systemic vascular resistance (SVR) and the PVR/SVR ratio. During RHC some patients did not receive an arterial line because of either technical or procedural reasons or an arterial sampling was missed, resulting in missing data for the following set of haemodynamic variables (arterial saturation, aorta pressure, SVR, and pulmonary arterial oxygen saturation).

Bioanalytical method for determination of vardenafil concentration

Patient plasma samples were sent to the Swedish National Veterinary Institute (SVA) in Uppsala for bioanalysis of vardenafil concentration (results presented in Paper II). The determination of vardenafil concentration was performed by use of liquid chromatography – tandem mass spectrometry (LC-MS/MS).

Vardenafil dihydrochloride and the internal standard pentadeuterated vardenafil (vardenafil-d₅) were purchased from Toronto Research Chemicals (North York, ON, Canada), and the water was purified using a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other chemicals were of analytical grade or better and used without further purification.

Sample pretreatment was carried out as follows. To 1.00 mL of plasma, 50 µL of internal standard solution (vardenafil-d₅ at 250 ng/mL in methanol) and 50 µL of methanol were added followed by 50 µL of 2.0 M NaOH (aq) and 5.0 mL of ethyl acetate. The samples were then shaken for 20 min and centrifuged at 1000 g for 10 min. The organic phases were transferred to new tubes and were evaporated to dryness under a gentle stream of nitrogen at 60 °C. The samples were then reconstituted in 50 µL of water/methanol 80:20 (v/v) where after they were transferred to vials for LC-MS/MS analysis.

A Surveyor MS pump was hyphenated to a TSQ Quantum Ultra tandem quadrupole mass spectrometer, with an electrospray interface operating in the positive mode (Thermo Fischer Scientific, San José, CA, USA). The column was a Luna C8(2) with the dimensions: length 50 mm, I.D. 2.0 mm (Phenomenex, Torrence, CA, USA). The mobile phase consisted of (A) 0.1 % acetic acid in
water and (B) acetonitrile. A gradient was run as follows: 10 % B for 1 min, increase from 10 % to 90 % B in 4 min, reduction to 10 % B in 0.10 min and constantly at 10 % B, for 2.90 min. The total run time was 8.00 min, the flow rate was 200 µL/min and the injection volume was 10 µL.

The data acquisition mode was Selected Reaction Monitoring (SRM) and the transitions were \( m/z \ 489 \ [M+H]^+ \rightarrow 151 \) for vardenafil (collision energy 40 V), and \( m/z \ 495 \ [M+H]^+ \rightarrow 151 \) for the internal standard vardenafil-d₅ (collision energy 40 V). The dwell time was 0.10 sec.

Stock solutions of vardenafil dihydrochloride and the internal standard were prepared in methanol at approximately 0.1 mg/ml. These solutions were 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 were prepared by adding 50 µL of separately prepared working solutions. The Lowest Limit of Quantification (LLOQ) was 0.35 ng/mL and the precision expressed as relative standard deviation was < 2.2 %.

**Bioanalytical method for determination of arginine, ADMA, and SDMA in plasma**

Blood samples for chemical analysis of arginine, ADMA, and SDMA were collected at baseline and at the following time intervals thereafter; 15, 30, 45, 60, 120, 300, and 540 min (results presented in Paper III). The bioanalysis was performed at the National Veterinary Institute (SVA) in Uppsala, Sweden. The sample pretreatment for plasma was as follows. To 100 µL of plasma, 100 µL of water and 50 µL of the internal standard solution containing \(^2\)H-ADMA, \(^{13}\)C₆-arginine and \(^2\)H₆-SDMA were added followed by addition of 900 µL of mobile phase B. The samples were vortex-mixed for 30 sec followed by centrifugation for 5 min (10 000 g). The supernatants were transferred to new tubes and were evaporated under a stream of nitrogen at 55 °C. The samples were reconstituted in 75 µL of mobile phase A/mobile phase B (70:30 v/v). The samples were analyzed 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 EC 250/3 Nucleosil 100-5 (length 250 mm, I.D. 3.0 mm, particle size 5 µm) kept at ambient temperature. The mobile phase consisted of (A) water/propionic acid/trifluoroacetic acid (1000:10:0.25) and (B) acetonitrile/propionic acid/trifluoroacetic acid (1000:10:0.25; v/v/v). The injection volume was 10 µL. The elution was carried out isocratically with 80 % A and 20 % B at a flow-rate of 900 µL/min. The three analytes were analyzed simultaneously in the same chromatographic
run using a positive capillary voltage of 1.50 kV. The desolvation and source block temperatures were 350°C and 120°C, respectively, and the cone and desolvation gas flows were 110 and 1019 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 25 eV), m/z 210 → 77 for [2H7]-ADMA (collision energy 26 eV), m/z 203 → 172 for SDMA (collision energy 28 eV), m/z 210 → 116 for [3H6]-SDMA (20 eV), m/z 175 → 70 for arginine (collision energy 22 eV), and m/z 181 → 74 for [13C6]-arginine (collision energy 25 eV). The dwell time was 0.010 sec. Stock solutions of ADMA, SDMA, arginine 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 0.090-3.0 µM and the precision calculated from quality control (QC) samples were in the range of 5.3-7.3 %. The calibration range for SDMA was 0.090-3.0 µM and the precision calculated from QC samples was in the range of 3.2–6.9 %. The calibration range for arginine was 4.5-150 µM and the precision calculated from QC samples were in the range of 0.9–1.6 %.

**Statistical analysis**

Paper II: Data are presented with median and range (minimum to maximum) values. Differences in study variables between baseline and posttreatment values were evaluated by Wilcoxon’s signed-rank test and differences between groups were evaluated by the Mann-Whitney U test. The Spearman rank correlation test was used to evaluate the relationship between vardenafil exposure (plasma concentration or AUC) and change in haemodynamic variables. Statistical significance was defined as p < 0.05.

Paper III: Data are presented with median and range (minimum to maximum) values. The non-parametric Friedman test was used to assess potential changes in arginine, ADMA, SDMA or the arginine/ADMA ratio over the study period. The Wilcoxon signed-rank test was performed for pair-wise comparisons with baseline if the Friedman test indicated significant differences. The Wilcoxon signed-rank test was based on the % change from baseline to the studied time-point. The Mann-Whitney U test was used to assess differences between groups (PAH WHO group 1 versus PH WHO group 2-4). The Spearman rank correlation test was used to evaluate the relationship between variables. Due to non-normal data distributions and small sample size
non-parametric tests were applied. Statistical significance was defined as $p < 0.05$.

Paper IV

Patients

All patients participating in the study were patients with PAH, representing WHO group 1, according to the clinical classification of PH (6). The patients were in WHO functional class II or III. All patients were recruited from the regional PAH centre at Uppsala University Hospital.

The study population consisted of 15 patients (eight females and seven males, 31-82 years of age (median 57)). All patients were receiving at least one of the conventional background therapies (angiotensin-converting enzyme inhibitor, angiotensin receptor blocker, β-adrenoceptor blocker, Ca$^{2+}$ antagonist, digoxin, diuretics). Prior to inclusion eleven patients was on stable treatment with a PAH-specific therapy either in monotherapy or as combination therapy (endothelin receptor antagonist, prostacyclin analogue, PDE5 inhibitor), and four patients were not on treatment with a PAH-specific drug at inclusion as they were newly diagnosed at the time of inclusion.

Study design

The study was a randomised, double-blind, placebo-controlled, crossover study (see Figure 1 for a schematic representation of the study design). All patients signed written informed consent prior to initiation of study procedures. The inclusion criteria were a diagnosis of PAH (WHO group 1) in stable functional class II or III, age over 18 years. Stable treatment with a PAH-specific therapy was allowed and patients with a new diagnosis at the time of screening were allowed to participate if a delay in initiating PAH-specific therapy was deemed as safe during the course of the study (three weeks). The exclusion criteria were WHO functional class I or IV, pregnancy systolic blood pressure < 95 mmHg, intolerance or allergy to beetroot, treatment with allopurinol, and treatment with prostacyclin analogue inhalations (iloprost). After informed consent had been obtained, the patients were randomised in blocks of four to receive study treatment, and to start with either nitrate-rich beetroot juice or nitrate-depleted beetroot juice (serving as placebo) twice a day (70 ml morning and evening) for seven consecutive days after which they switched to the alternative treatment in the crossover trial. Both of the beetroot juices (70 ml each) was produced by the same manufacturer (James White Drinks Ltd, Ipswich, UK) and the nitrate-rich beetroot juice contained ~8 mmol nitrate per bottle. The juices (nitrate-rich and placebo) were indistinguishable with regard to taste, colour and appearance of the bottles. Before switching to the alternative treatment in the crossover trial a washout period of four to nine days was applied. During the course of the study, the patients
received detailed instructions to avoid consumption of nitrate-rich food and they were also instructed to avoid anti-bacterial mouth washing as nitrate is converted to nitrite in the oral cavity by commensal bacteria. The patients were instructed not to administer any oral PAH-specific treatment in the morning at the day of study assessments after treatment period one and two. On the day of the assessments (after treatment period one and two), the patients were instructed to eat a standardized nitrate-low breakfast at home and during the day at the hospital they were offered a standardized nitrate-low lunch. The study assessments after treatment period one and two were performed in the same and following order during the study; laboratory blood tests, ergospirometry, systemic blood pressure measurement, transthoracic echocardiography, exhaled NO measurements, and finally a 6-minute walking test (6MWT). The laboratory blood tests, the ergospirometry, and the measurement of systemic blood pressure, were performed in the morning approximately 2.5-3 hours after ingestion of the beetroot juice and the other assessments (echocardiography, exhaled NO, and the 6MWT) were performed in the afternoon approximately 5-6 hours after administration of the beetroot juice. To ensure compliance, the patients received regular telephone calls to remind them to administer the study treatments during the course of the study and they were also instructed to bring the empty bottlers with them to the hospital at the day of the assessments after treatment period one and two.

Figure 1. Schematic overview of the study design. Abbreviations; R, randomisation; BRJ, nitrate-rich beetroot juice.
Laboratory analysis
We analyzed circulatory levels of ADMA, arginine, citrulline, hemoglobin, nitrate, nitrite, N-terminal pro-brain natriuretic peptide (NT-pro BNP), ornithine, SDMA and salivary levels of nitrate and nitrite. Measurements of nitrate and nitrite in saliva and in plasma were analyzed by use of a high-performance liquid chromatography system as described for analysis of saliva nitrate/nitrite in Paper I.

The nitrate content in the nitrate-rich beetroot juice and the nitrate-depleted beetroot juice (placebo) was determined by chemiluminescence, as described for analysis of plasma nitrate in Paper I. The nitrate content was ~8 mmol per 70 ml in the nitrate-rich beetroot juice, whereas almost complete depletion of nitrate was confirmed in the placebo beetroot juice (~0.059 mmol per 70 ml).

NT-proBNP, hemoglobin, and creatinine were analyzed at the department of Clinical Chemistry and Pharmacology, Uppsala University Hospital, Sweden.

ADMA, SDMA, arginine, ornithine and citrulline were quantitatively analyzed at the National Veterinary Institute (SVA) in Uppsala, Sweden. To the plasma samples (100 µL), water (50 µL) and internal standard solution (50 µL) were added. The internal standard solution consisted of $^2$H$_7$-ADMA, $^2$H$_6$-SDMA, $^{13}$C$_6$-arginine, $^3$H$_6$-ornithine and $^2$H$_7$-citrulline. The precipitation liquid acetonitrile/trifluoroacetic acid/propionic acid (1000/0.25/10 v/v/v) was subsequently added (400 µL) followed by vortex-mixing (5 min) and centrifugation (5 min) at 10 000 g. The supernatants were analyzed using ultra-high performance liquid chromatography – tandem mass spectrometry (UHPLC-MS/MS). The chromatographic system was an Acquity UPLC hyphenated to a Quattro Ultima Pt tandem quadrupole mass spectrometer with an electrospray interface operating in the positive mode (Waters Corporation, Milford, MA). For analytical separation, an Indra Almtakt Amino Acid column (length 100 mm, I.D. 2.0 mm, particle size 3 µm) kept at 25 ºC was used. The composition of the mobile phase was (A) 100 mM ammonium formate in water and (B) 0.1 % formic acid in acetonitrile. A 10 µL portion of the sample was injected. The following gradient at a flow-rate of 200 µL/min was used: 80% A for 1 min, 80-95 % A during 1 min, 95 % A for 5.5 min, 95-80 % A during 0.1 min, 80 % A for 2.4 min. The interfacial settings were as follows: capillary voltage 0.50 kV, cone voltage 40 V, desolvation and source block temperatures 350 ºC and 120 ºC, respectively, and the cone and desolvation gas flows were 120 and 924 L/h, respectively. A simultaneous quantification of the five analytes was performed in the selected reaction monitoring (SRM) mode with argon as the collision gas (1.9x10$^{-3}$ mBar). The transitions were $m/z$ 203 $\rightarrow$ 46 for ADMA (collision energy 18 eV), $m/z$ 210 $\rightarrow$ 77 for $[^2$H$_7]$-ADMA (collision energy 23 eV), $m/z$ 203 $\rightarrow$ 172 for SDMA (collision energy 16 eV), $m/z$ 209 $\rightarrow$ 116 for $[^3$H$_6$]-SDMA (19 eV), $m/z$ 175 $\rightarrow$ 70 for arginine (collision energy 18 eV), $m/z$ 181 $\rightarrow$ 74 for $[^{13}$C$_6$]-arginine (collision energy
18 eV), m/z 133 → 70 for ornithine (collision energy 13 eV), m/z 139 → 76 for [13H6]-ornithine (collision energy 14 eV), m/z 176 → 113 for citrulline (collision energy 18 eV), m/z 183 → 120 for [13C7]-citrulline (collision energy 16 eV). The dwell time was 0.010 sec. Stock solutions at 0.1 – 0.3 mg/mL of the analytes and internal standards were prepared in water. The reference compounds and the internal standards were purchased from Toronto Research Chemicals (North York, Ontario, Canada). Since the analytes are endogenous compounds, calibration samples were prepared for all analytes in both water and control plasma. The calibration curves were plotted with the chromato-graphic peak area ratio (analyte/internal standard) versus analyte concentration. Linear curve fit with a weighting factor of 1/x^2 was used for calculation of the calibration functions. The following calibration ranges were used: ADMA 0.090-3.4 µM (precision 5.3 - 7.3 %), SDMA 0.38-3.0 µM (precision 5.8 – 9.3 %), arginine 4.5 - 150 µM (3.5 – 6.2 %), ornithine 4.5 – 151 µM (precision 4.1 - 5.2 %), and citrulline 4.4 – 150 µM (precision 2.5 – 4.8 %).

**Exhaled NO**

Fractional exhaled NO (FeNO) was assessed at flow-rates of 50, 100, 200, 250 and 300 mL/s with a chemiluminescence analyzer, NIOX Flex® (Aerocrine AB, Solna, Sweden). Assessment of exhaled NO at 100, 200, and 300 mL/s and the slope-intercept model (161) were used for estimation of Calv NO and J'aw NO.

**Echocardiography**

Transthoracic 2D-echocardiography, using Philips iE Ultrasound system (Philips Medical System, Bothel, WA), was performed in the standard paras-ternal views, apical four-, three and two-chamber views, and the right ventric-ular (RV) focused view. Echocardiographic images were acquired twice, after the first and the second treatment period. The assessments were performed by two experienced echocardiographers, and with one exception the same echo-cardiographer collected images at both assessment occasions and performed the offline data analysis.

The left ventricular (LV) function assessment included measurement of LV ejection fraction (LVEF). The RV function assessment included measurements of RV end-diastolic and end-systolic area with calculation of fractional area change (RVFAC), the tricuspid annular plane systolic excursion using M-mode (TAPSE), Doppler tissue imaging derived tricuspid lateral annular systolic velocity wave (TA S’), RV Tei-index (calculated as isovolumetric contraction time and isovolumetric relaxation time divided by ejection time), and RV global longitudinal peak strain (RV GLPS). The RV GLPS was derived by speckle tracking (frame rate above 40 Hz) and calculated as the average of the RV free wall and the septal segment strains. An external software, Image Arena V 4.6 Build 4.6.4.10 (Tom Tec Imaging system, Munich, Ger-
many) was used for all speckle-tracking based analysis of RV strain. The systolic pulmonary artery pressure (SPAP) was calculated by use of the tricuspid regurgitation jet and an estimation of RA pressure based on inferior vena cava size and collapsibility. The acceleration time of pulmonary flow (ACTPO) was assessed by pulsed Doppler in the RV outflow tract close to the pulmonary valve.

The left atrial area (LAA) and the right atrial area (RAA) were estimated in the apical four chamber view. The basal (RVD1), the mid-cavity (RVD2), and the base to apex (RVD3) RV linear dimensions were measured using maximal transversal dimension in the basal, the middle one third of RV inflow and base to apex at end-diastole, respectively. The proximal RV outflow diameter (RVOT prox) was measured from the anterior RV wall to the interventricular septal-aortic junction at end-diastole.

Ergospirometry and measurements of diffusing capacity
The ergospirometry was performed on a bicycle (calibrated) in an upright position, and work load was increased gradually in steps of 10 Watts per minute with discontinuation when the patients were physically exhausted or developed dizziness or dyspnea.

Ventilation and gas exchange measurements (oxygen and carbon dioxide levels) at rest and during the exercise test was obtained by use of Ergomedic 939E (Monark Exercise AB, Vansbro, Sweden) in combination with Oxicon pro (Jaeger, Würzburg, Germany). The following variables were assessed; the oxygen consumption (VO2) and carbon dioxide output (VCO2) at rest, at the anaerobic threshold (AT) and peak, peak power output (W peak), the ratio of W peak to VO2 peak, the minute ventilation (VE) (at rest, AT, and peak), the VE/VCO2 ratio at AT, the Borg rating of perceived exertion (RPE) at peak exercise, the respiratory exchange ratio (RER) (at rest and peak), maximum breathing frequency (BF), and the breathing reserve (BR).

A 12-lead electrocardiogram continually monitored the heart rate (HR). A cuff on the left upper arm over the brachial artery and a Doppler placed over the left radial artery was used to measure the systolic blood pressure.

The single breath technique using the Masterlab Transfer (Erich Jager AG, Würzburg, Germany) was used to assess the diffusing capacity of the lung for carbon monoxide (DLCO), and corrected values for actual hemoglobin levels were used in the calculations.

The 6MWT
The following parameters were collected in the 6MWT; 6MWD, baseline HR, HR at 6 min, saturation of oxygen at rest and at 6 min, need for supplementation at rest and at 6 min, Borg dyspnea index, and the Borg fatigue index.
Statistical analysis
No sample size calculation was performed due to the exploratory nature of the study.

The crossover design allowed for assessment of treatment effects with each patient serving as his or her own control. The Koch's adaptation of the Mann-Whitney U test was used, allowing for a possible period effect, and Hodges-Lehmann 95% confidence intervals for treatment effect are also presented. The Spearman rank correlation test was used to evaluate the relationship between study variables after the placebo period (which served as a reflection of basal conditions). We studied the correlation between plasma levels of nitrate and nitrite and the NO-flow-independent parameters (Calv NO and J’aw NO) after placebo period, and the following variables assessed after the placebo period; ADMA plasma levels, NT-proBNP plasma levels, RAA, RVFAC, 6MWD, TAPSE, and Tei-index. Differences in plasma levels of nitrate and nitrite between patients naïve to PAH-specific therapy and patients on PAH-specific therapy was analyzed with the Mann-Whitney U test. Due to non-normal data distribution and small sample size non-parametric tests were used, and no adjustments for multiplicity were made as the results are to be considered exploratory. Statistical significance was defined as p < 0.05. SAS 9.4 (SAS Institute Inc, Cary, NC) was used for all statistical analyses.

Results
Paper I

*Increased plasma and salivary nitrite and decreased bronchial contribution to exhaled NO in pulmonary arterial hypertension.*

Exhaled NO and NO flow-independent parameters in patients with PH
No significant difference in FeNO levels or bronchial NO flux (J’aw NO) was detected between the total group of PH patients (WHO group 1-4) and the control group. The patients with PH (WHO group 1-4) displayed significantly higher levels of alveolar NO (Calv NO) compared to healthy controls (2.80 vs 1.9 ppb; p = 0.01). Patients with PAH (WHO group 1) had significantly lower exhaled NO levels (at flow rates in the range of 20-200 mL/s) than patients with PH (WHO group 2-4) (Fig. 2a). Similar results, with significantly lower exhaled NO levels (at flow-rates in the same range) was observed in patients with idiopathic PAH (9 patients, WHO group 1.1) compared to patients with PH (WHO group 2-4) (all p-values < 0.05). Neither patients with PAH (WHO group 1) nor patients with PH (WHO group 2-4) differed from the healthy controls with regard to FeNO levels, at any flow-rate (Fig. 2b shows patients with PAH (WHO group 1) versus healthy controls). However, patients with
idiopathic PAH (9 patients, WHO group 1.1) had significantly (p = 0.01) lower FeNO, at the 20 mL/s flow-rate, in comparison to healthy controls.

Patients with PAH (WHO group 1) had significantly lower bronchial NO flux (J’aw NO) (430 pL/s) than patients with PH (WHO group 2-4) (807 pL/s) and healthy controls (731 pL/s) (p = 0.02 for both comparisons) (Fig. 3a). The PAH patients (WHO group 1) had significantly higher alveolar NO levels (Calv NO) than healthy controls (2.61 ppb versus 1.97 ppb, p = 0.03), and similar levels as the patients with PH (WHO group 2-4) (Fig. 3b). A trend for higher alveolar NO levels (Calv NO) was seen in patients with PH (WHO group 2-4) compared to healthy controls (4.22 ppb versus 1.97 ppb, p = 0.06).

The total group of patients with PH (WHO group 1-4) had, compared to healthy controls, significantly higher levels of plasma nitrate (median 22.8 µM
versus 16.3 µM, p = 0.047). However analysis of the two PH groups separately (WHO group 1 and WHO group 2-4) in comparison to healthy controls did not reveal any significant differences (Fig. 4a). Patients with PAH (WHO group 1) and patients with PH (WHO group 2-4) did not differ in plasma nitrate levels (22.7 µM versus 22.8 µM, p = 0.95) (Fig. 4a).

Plasma levels of nitrite were elevated in the total group of PH patients (WHO group 1-4) compared to healthy controls (median 118 nM versus 40.6 nM, p = 0.007) and in patients with PAH (WHO group 1) compared to healthy controls (median 120 nM versus 40.6 nM, p = 0.01, Fig. 4b). Plasma nitrite did not differ between patients with PAH (WHO group 1) and patients with PH (WHO group 2-4) (median 120 nM versus 78.7, p = 0.79, Fig. 4b). No significant correlations were found between plasma levels of nitrate or nitrite and NO flow-independent exchange parameters (data not shown).

Figure 4. Plasma nitrate (Fig.4a) and nitrite levels (Fig. 4b) in healthy controls, PAH patients (WHO group 1), and patients with PH (WHO group 2-4). Individual levels, with horizontal line at the median level.

Salivary nitrate and nitrite levels in patients with PH
The total group of patients with PH (WHO group 1-4) had, compared to healthy controls, significantly higher levels of salivary nitrate (median 54.1 µM versus 9.2 µM, p < 0.001). Salivary levels of nitrate were elevated in both PH groups (WHO group 1 and WHO group 2-4) compared to healthy controls (median 44.7 µM (WHO group 1), 63.6 µM (WHO group 2-4), healthy controls 9.2 µM; p < 0.01 for both comparisons) (Fig. 5a). The patients with PAH (WHO group 1) and the patients with PH (WHO group 2-4) did not differ significantly in levels of salivary nitrate (p = 0.60) (Fig. 5a).

Salivary levels of nitrite were elevated in the total group of PH patients (WHO group 1-4) compared to healthy controls (median 128 µM versus 35.8 µM, p = 0.03) and in patients with PAH (WHO group 1) compared to healthy controls (median 110 µM versus 35.8 µM, p = 0.047, Fig. 5b). The patients with PAH (WHO group 1) and the patients with PH (WHO group 2-4) did not differ significantly in levels of salivary nitrite (median 110 µM versus 146
μM, p =0.69) (Fig. 5a). No correlations were found between salivary levels of nitrate or nitrite and NO flow-independent exchange parameters (data not shown).

Figure 5. Salivary nitrate (Fig. 5a) and nitrite levels (Fig. 5b) in healthy controls, PAH patients (WHO group 1), and patients with PH (WHO group 2-4). Individual levels, with horizontal line at the median level.

Paper II

Acute hemodynamic response in relation to plasma vardenafil levels in patients with pulmonary hypertension.

Haemodynamic effects of a single oral dose of vardenafil in patients with PH

Following a single oral dose of vardenafil a favourable acute haemodynamic response was demonstrated. Significant haemodynamic changes assessed during RHC, from baseline to 60 min, were observed for mean aortic pressure, systolic PAP, mPAP, CO, CI, PVR, and SVR as shown in Table 2. Furthermore pulmonary selectivity of the vasodilatory effect was demonstrated as the PVR:SVR ratio decreased significantly.

Three of the patients with PAH (WHO group 1) were on treatment with bosentan. These patients demonstrated a -10.8% median decrease in mPAP (at 60 min) compared with -23.8% for the other PAH patients (WHO group 1) not exposed to bosentan (n =6) (p = 0.095). There were no significant differences in haemodynamic response at 60 min between these PAH patients (n = 3, WHO group 1, exposed to bosentan) compared with the rest of the PAH/PH patients (n =13, WHO group 1-4) (data not shown). Furthermore, no significant differences in haemodynamic response at 60 min were noted between patients with PAH (n = 9, WHO group 1) and the other patients with PH (n = 7, WHO group 2-4).
Table 2. Changes in haemodynamic parameters during right heart catheterization from baseline to 60 min and pharmacokinetic parameters after a single oral dose of vardenafil.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>N</th>
<th>Baseline median (min, max)</th>
<th>60 min median (min, max)</th>
<th>Change (% median (min, max)</th>
<th>*p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heart rate (beats/min)</strong></td>
<td>16</td>
<td>71 (56, 94)</td>
<td>72 (50, 102)</td>
<td>-0.9 (-19.2, 19.4)</td>
<td>0.978</td>
</tr>
<tr>
<td><strong>Aortic pressure (mmHg)</strong></td>
<td>14</td>
<td>135 (118, 224)</td>
<td>127.5 (104, 321)</td>
<td>-5.8 (-26.2, 122.9)</td>
<td>0.153</td>
</tr>
<tr>
<td><strong>Mean aortic pressure (mmHg)</strong></td>
<td>14</td>
<td>94 (81, 139)</td>
<td>88 (68, 119)</td>
<td>-6.0 (-27.0, 2.1)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><strong>Systolic PAP (mmHg)</strong></td>
<td>16</td>
<td>65.5 (41, 98)</td>
<td>52.5 (26, 89)</td>
<td>-17.2 (-36.6, 3.8)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><strong>mPAP (mmHg)</strong></td>
<td>16</td>
<td>40 (29, 65)</td>
<td>34.5 (15, 58)</td>
<td>-20.3 (-48.3, 3.0)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><strong>mPAWP (mmHg)</strong></td>
<td>16</td>
<td>11 (4, 32)</td>
<td>10.5 (3, 32)</td>
<td>0.0 (-40.0, 50.0)</td>
<td>0.977</td>
</tr>
<tr>
<td><strong>mRAP (mmHg)</strong></td>
<td>16</td>
<td>8.5 (2, 28)</td>
<td>6.5 (1, 23)</td>
<td>-13.1 (-83.3, 100.0)</td>
<td>0.720</td>
</tr>
<tr>
<td><strong>CO (L/min)</strong></td>
<td>16</td>
<td>3.95 (2.10, 5.83)</td>
<td>4.95 (2.60, 7.90)</td>
<td>10.6 (-25.0, 88.1)</td>
<td>0.015</td>
</tr>
<tr>
<td><strong>CI (L/min/m²)</strong></td>
<td>16</td>
<td>2.3 (1.40, 3.40)</td>
<td>2.75 (1.70, 4.50)</td>
<td>12.1 (-24.0, 94.4)</td>
<td>0.010</td>
</tr>
<tr>
<td><strong>PVR (dyne s cm⁻⁵)</strong></td>
<td>16</td>
<td>612 (192, 1144)</td>
<td>404 (80, 816)</td>
<td>-28.9 (-61.5, -5.9)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><strong>SVR (dyne s cm⁻⁵)</strong></td>
<td>14</td>
<td>1852 (848, 2968)</td>
<td>1444 (640, 2248)</td>
<td>-20.6 (-61.2, 1.7)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><strong>PVR/SVR</strong></td>
<td>14</td>
<td>0.28 (0.09, 0.53)</td>
<td>0.25 (0.09, 0.50)</td>
<td>-16.9 (-49.0, 16.5)</td>
<td>0.002</td>
</tr>
<tr>
<td><strong>PA sat (%)</strong></td>
<td>13</td>
<td>60.5 (45.9, 69.4)</td>
<td>62.45 (51, 76.5)</td>
<td>2.6 (-7.7, 36.1)</td>
<td>0.168</td>
</tr>
<tr>
<td><strong>Art sat (%)</strong></td>
<td>11</td>
<td>91.2 (85, 93.7)</td>
<td>90.4 (79.7, 96.55)</td>
<td>-1.3 (-6.6, 5.2)</td>
<td>0.375</td>
</tr>
<tr>
<td><strong>Vardenafil conc. (μg/L)</strong></td>
<td>16</td>
<td></td>
<td>5.07 (0.33, 39.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Vardenafil AUC(0-45) (μg h/L)</strong></td>
<td>15</td>
<td>2.53 (0.00, 17.9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Vardenafil AUC(0-60) (μg h/L)</strong></td>
<td>15</td>
<td>3.88 (0.04, 23.08)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Wilcoxon's signed-rank test. Abbreviations; m, mean; PAP, pulmonary artery pressure; PAWP, pulmonary arterial wedge pressure; RAP, right atrial pressure; CO, cardiac output; CI, cardiac index; PVR, pulmonary vascular resistance; SVR, systemic vascular resistance; PA, pulmonary artery; Art, arterial; AUC (0-45 and 0-60), area under the plasma concentration-time curve from time zero to 45 and 60 min respectively.

**Vardenafil pharmacokinetics and haemodynamic response**

The median plasma concentration of vardenafil at 60 min following a single oral dose (5, 10, or 20 mg) and the exposure presented as median area under the curve (AUC) from time zero to 45 or 60 min is presented in Table 2. The
three PAH patients (WHO group 1) exposed to bosentan displayed a significantly lower median vardenafil concentration at 60 min compared with the other PAH/PH patients (n = 13, WHO group 1-4) (1.52 μg/L versus 7.3 μg/L, p = 0.014). The bosentan dose in the three patients were; 250 mg twice daily, 125 mg twice daily, and 62.5 mg twice daily. The patient on the highest bosentan dose displayed the lowest vardenafil concentration of all patients (0.33 μg/L) at 60 min, and the patient on the lowest bosentan dose (62 mg twice daily) displayed a higher vardenafil concentration than the patient treated with bosentan at 125 mg twice daily (1.52 μg/L versus 0.88 μg/L) at 60 min. The plasma vardenafil concentrations at 60 min varied widely between individual patients also in the more uniform subgroup of PAH patients (WHO group 1) unexposed to bosentan receiving 20 mg vardenafil (n = 10, range 0.68 μg/L to 39.6 μg/L).

Assessment of the relationship between plasma vardenafil exposure and haemodynamic response at 60 min after vardenafil administration demonstrated a significant negative correlation between vardenafil concentration and change in mPAP (r = -0.579, p = 0.019) and change in PVR (r = -0.662, p =0.005). A similar relationship was demonstrated for vardenafil AUC and change in mPAP (AUC(0-45min); r = -0.668, p =0.007 and AUC(0-60min); r = -0.744, p = 0.001) and change in PVR (AUC(0-45min); r = -0.540, p = 0.038 and AUC(0-60min); r = -0.588, p= 0.021). Thus a higher vardenafil concentration or vardenafil AUC was associated with a more favourable haemodynamic response in mPAP and PVR.

Repeated haemodynamic assessment of mPAP from baseline to 15, 30, 45 and 60 min after administration of a single oral dose of vardenafil in 14 of the patients revealed that the acute effect with reduction of mPAP was very rapid. A significant decrease in mPAP compared to baseline was observed at every time-point as shown in Table 3. A clear trend with larger reduction of mPAP in parallel with increasing plasma concentration of vardenafil was noted from baseline to 60 min (Table 3).

Table 3. Vardenafil concentrations and % change in mPAP after a single oral dose of vardenafil at different time-points from baseline to 60 min. Median values are presented.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Vardenafil (μg/L)</th>
<th>mPAP (mmHg)</th>
<th>% change median</th>
<th>*p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.00</td>
<td>40.00</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>1.01</td>
<td>38.50</td>
<td>-10.54</td>
<td>0.013</td>
</tr>
<tr>
<td>30</td>
<td>5.99</td>
<td>36.50</td>
<td>-16.15</td>
<td>0.002</td>
</tr>
<tr>
<td>45</td>
<td>5.43</td>
<td>35.50</td>
<td>-17.51</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>60</td>
<td>6.30</td>
<td>34.50</td>
<td>-20.34</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

* Wilcoxon's signed-rank test.
Paper III

*Changes in plasma levels of asymmetric dimethylarginine, symmetric dimethylarginine, and arginine after a single dose of vardenafil in patients with pulmonary hypertension.*

**Effects of a single oral dose of vardenafil on arginine, ADMA, and SDMA in patients with PH**

Changes, following administration of vardenafil, in plasma levels of arginine, ADMA, SDMA as well as the arginine/ADMA ratio were explored at different time-points from baseline up to 540 min. Transient reductions in plasma levels of ADMA and SDMA was noted, whereas plasma arginine levels displayed a clear pattern of increase over time. A significant reduction in ADMA compared to baseline was observed at 30 min and 45 min after administration of vardenafil with a median decrease of -11.1% (range -24.1 to 8.0, p = 0.021) and -12.5% (range -36.8 to 6.1, p = 0.002) respectively. Plasma levels of SDMA decreased compared to baseline at 45 min with a median decrease of -5.3% (range -28.1 to 4.5, p = 0.032). Plasma levels of arginine increased significantly compared to baseline at 120 min (median 40.3%, range -1.1 to 112, p = 0.002), 300 min (median 45.0%, range -25.1 to 132.2, p = 0.010), and 540 min (median 77.1%, range -9.6 to 136.0, p = 0.008). A significant increase in the arginine/ADMA ratio was observed compared to baseline at 15 min (median 11.7%, range -5.7 to 34.7, p = 0.012), 45 min (median 32.5%, range -6.3 to 48.1, p = 0.003), 60 min (median 26.5%, range -17.5 to 71.9, p = 0.021), 120 min (median 33.0%, range -7.1 to 82.1, p = 0.007), 300 min (median 48.5%, range -17.0 to 100.5, p = 0.007), and at 540 min (median 63.1%, range -6.9 to 117.2, p = 0.008).

No significant difference was found between patients with PAH (WHO group 1, n = 6) and patients with PH (WHO group 2-4, n =6) with regard to change in plasma levels of ADMA, SDMA, arginine or the arginine/ADMA ratio from baseline to either 45 min or 540 min (data not shown).

A positive correlation between plasma vardenafil exposure (AUC\text{norm} from baseline to 540 min) and change in the arginine/ADMA ratio (from baseline to 540 min) was observed (r = 0.8, p = 0.01).

**The relationship between haemodynamic parameters and levels of arginine, ADMA, and SDMA**

High plasma levels of ADMA or SDMA or a low arginine/ADMA ratio at baseline were associated with a more haemodynamically severe disease state. At baseline, ADMA and SDMA correlated positively with baseline mRAP (ADMA; r = 0.65, p = 0.023, SDMA; r = 0.61, p = 0.035) and ADMA correlated negatively with baseline pulmonary artery oxygen saturation (r = -0.66, p = 0.020). The arginine/ADMA ratio at baseline correlated positively with
baseline CI ($r = 0.61$, $p = 0.036$) and baseline CO ($r = 0.59$, $p = 0.045$) and negatively with baseline mRAP ($r = -0.79$, $p = 0.002$).

The relationship between haemodynamic response from baseline to 60 min and change in plasma levels of arginine, ADMA, SDMA, and change of the arginine/ADMA ratio in the same time interval was explored. The change in plasma arginine or the arginine/ADMA ratio correlated positively with change in CI ($r = 0.77$, $p = 0.003$ and $r = 0.61$, $p = 0.037$). Thus, an increase in plasma arginine levels or an increase of the arginine/ADMA ratio was associated with an increase in CI.

**Paper IV**

*Effects of oral supplementation with nitrate-rich beetroot juice in patients with pulmonary arterial hypertension – results from BEET-PAH a randomised, double-blind, placebo-controlled crossover study.*

**Plasma and salivary levels of nitrate and nitrite**

Plasma and saliva nitrate and nitrite concentrations were significantly higher after ingestion of nitrate-rich beetroot juice compared to after placebo (Table 4).

<table>
<thead>
<tr>
<th></th>
<th>BRJ Median (Q1;Q3)</th>
<th>Placebo Median (Q1;Q3)</th>
<th>Median of differences (95% CI)*</th>
<th>P-value† BRJ vs Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Salivary nitrite</strong></td>
<td>1238.0 (689.9;2127.9)</td>
<td>146.3 (70.7;189.1)</td>
<td>1281.6 (622.1;2001.1)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>(µM) (N=14)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Salivary nitrate</strong></td>
<td>7179.5 (5314.9;10360.5)</td>
<td>368.9 (127.6;655.4)</td>
<td>7396.3 (4871.8;11048.2)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>(µM) (N=14)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Plasma nitrite</strong></td>
<td>0.36 (0.21;0.60)</td>
<td>0.16 (0.13;0.19)</td>
<td>0.25 (0.08;0.36)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>(µM) (N=15)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Plasma nitrate</strong></td>
<td>581.5 (476.6;835.5)</td>
<td>36.10 (19.17;48.07)</td>
<td>574.0 (449.9;815.6)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>(µM) (N=15)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations; BRJ, nitrate-rich beetroot juice; Q1, first quartile; Q3, third quartile. *Confidence interval (CI) of the Hodges-Lehmann’s median differences. †Koch's adaptation of the Mann-Whitney U test.
The plasma nitrate and nitrite levels, after the placebo period, did not differ significantly between patients that were naïve to PAH-specific treatment (N=4) in comparison to the patients that were on stable treatment with PAH-specific therapy (N=11), data not shown.

We performed a correlation analysis between plasma levels of nitrate and nitrite after the placebo period and the following study parameters after the placebo period; ADMA plasma concentrations, NT-proBNP plasma concentrations, RAA, RVFAC, 6MWD, TAPSE, Tei-index, and VO2 peak (Table 5). We observed a significant negative correlation between plasma nitrate levels and VO2 peak (r = -0.71, p = 0.004). Furthermore, we observed a significant negative correlation between plasma nitrite levels and TAPSE (r = 0.73, p = 0.002), and a significant positive correlation between plasma nitrite levels and plasma ADMA levels (r = 0.66, p = 0.014) (Fig. 6).
Table 5. Spearman correlation analysis.

<table>
<thead>
<tr>
<th></th>
<th>Plasma nitrite (µM)</th>
<th>Plasma nitrate (µM)</th>
<th>J'aw NO (pL/s)</th>
<th>Calv NO (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VO2 peak (ml/min)</td>
<td>-0.42 (-0.77 to -0.27)</td>
<td>-0.71 (-0.90 to -0.16)</td>
<td>-0.12 (-0.72 to 0.60)</td>
<td>-0.32 (-0.80 to 0.46)</td>
</tr>
<tr>
<td>P-value (N)</td>
<td>0.16 (14)</td>
<td>0.004 (14)</td>
<td>0.765 (9)</td>
<td>0.4064 (9)</td>
</tr>
<tr>
<td>ADMA (µM)</td>
<td>0.66 (0.14 to 0.64)</td>
<td>0.15 (-0.44 to 0.62)</td>
<td>0.10 (-0.61 to 0.71)</td>
<td>0.53 (-0.23 to 0.88)</td>
</tr>
<tr>
<td>P-value (N)</td>
<td>0.88 (14)</td>
<td>0.628 (14)</td>
<td>0.798 (9)</td>
<td>0.139 (9)</td>
</tr>
<tr>
<td>RAA (cm²)</td>
<td>0.40 (-0.16 to 0.52)</td>
<td>0.01 (-0.51 to 0.64)</td>
<td>-0.50 (-0.85 to 0.21)</td>
<td>0.07 (-0.59 to 0.67)</td>
</tr>
<tr>
<td>P-value (N)</td>
<td>0.140 (13)</td>
<td>0.980 (13)</td>
<td>0.138 (10)</td>
<td>0.855 (10)</td>
</tr>
<tr>
<td>RVFAC (%)</td>
<td>0.13 (-0.42 to 0.70)</td>
<td>0.30 (-0.26 to 0.70)</td>
<td>0.22 (-0.48 to 0.74)</td>
<td>0.33 (-0.39 to 0.79)</td>
</tr>
<tr>
<td>P-value (N)</td>
<td>0.657 (15)</td>
<td>0.271 (15)</td>
<td>0.533 (10)</td>
<td>0.347 (10)</td>
</tr>
<tr>
<td>TAPSE (cm)</td>
<td>-0.73 (-0.90 to 0.41)</td>
<td>-0.13 (-0.60 to 0.41)</td>
<td>0.27 (-0.45 to 0.76)</td>
<td>-0.26 (-0.76 to 0.45)</td>
</tr>
<tr>
<td>P-value (N)</td>
<td>0.002 (15)</td>
<td>0.633 (15)</td>
<td>0.454 (10)</td>
<td>0.464 (10)</td>
</tr>
<tr>
<td>Tei-index</td>
<td>0.49 (-0.07 to 0.81)</td>
<td>0.28 (-0.30 to 0.70)</td>
<td>-0.27 (-0.78 to 0.50)</td>
<td>0.50 (-0.27 to 0.87)</td>
</tr>
<tr>
<td>P-value (N)</td>
<td>0.072 (14)</td>
<td>0.334 (14)</td>
<td>0.488 (9)</td>
<td>0.137 (9)</td>
</tr>
<tr>
<td>NT-proBNP (ng/L)</td>
<td>0.45 (-0.10 to 0.64)</td>
<td>0.20 (-0.35 to 0.46)</td>
<td>-0.37 (-0.80 to 0.36)</td>
<td>0.05 (-0.60 to 0.66)</td>
</tr>
<tr>
<td>P-value (N)</td>
<td>0.78 (15)</td>
<td>0.467 (15)</td>
<td>0.293 (10)</td>
<td>0.881 (10)</td>
</tr>
<tr>
<td>6MWD (m)</td>
<td>-0.29 (-0.69 to 0.25)</td>
<td>-0.31 (-0.71 to 0.25)</td>
<td>-0.02 (-0.64 to 0.62)</td>
<td>0.36 (-0.37 to 0.80)</td>
</tr>
<tr>
<td>P-value (N)</td>
<td>0.27 (15)</td>
<td>0.254 (15)</td>
<td>0.960 (10)</td>
<td>0.310 (10)</td>
</tr>
</tbody>
</table>

Abbreviations; r, Spearman rank order correlation coefficient; CI, confidence interval of the Spearman rank order correlation coefficient.
Figure 6. Spearman correlation analysis between plasma nitrite concentrations and TAPSE (panel a), and plasma nitrite concentrations and ADMA plasma levels (panel b), after the placebo period.

Exhaled NO and NO flow-independent parameters

Significantly higher FeNO levels, at all flowrates, were observed following ingestion of nitrate-rich beetroot juice compared to placebo (Fig. 7). Alveolar NO concentrations (Calv NO median of differences; 1.35 ppb, p = 0.032) and bronchial NO flux (J’aw NO median of differences; 722.2 pL/s, p = 0.016) were significantly higher after intake of nitrate-rich beetroot juice compared to after placebo.

We did not observe any significant correlations between the NO-flow-independent parameters (J’aw NO and Calv NO) after the placebo treatment period and the following study variables assessed after the placebo period; 6MWD, VO2 peak, ADMA plasma levels, NT-proBNP plasma levels, RAA, RVFAC, TAPSE, and Tei-index (Table 5).
Figure 7. FeNO levels at flow-rates in the range of 50-300 ml/s in patients with PAH after ingestion of nitrate-rich beetroot juice (closed diamonds) and placebo (closed circles). The symbols denote median values and the ends of the whiskers represent the first and the third quartile values. Significant differences, between nitrate-rich beetroot juice and placebo, are marked with *** (p < 0.001) and ** (p < 0.01). P-values from Koch's adaptation of the Mann-Whitney U test.

**Effects on plasma levels of ADMA, arginine, citrulline, NT-proBNP, ornithine, SDMA and relative arginine availability**

Significantly lower plasma levels of ornithine was observed after ingestion of nitrate-rich beetroot juice compared to placebo (median of differences; -12.68 µM, p = 0.006).

The systemic arginine bioavailability was estimated by calculation of the global arginine bioavailability ratio (GABR, plasma arginine divided by the sum of plasma ornithine plus plasma citrulline) and the plasma arginine to plasma ornithine ratio (arginine/ornithine).

The GABR as well as the arginine/ornithine ratio were significantly higher after administration of nitrate-rich beetroot juice compared to after placebo (GABR median of differences; 0.07, p = 0.030, and the arginine/ornithine ratio median of differences; 0.11, p = 0.011). No significant differences were found in plasma levels of ADMA, arginine, citrulline, NT-proBNP, or SDMA between the two study treatments.
**Echocardiography**

We did not observe any statistically significant differences between the two interventions in any of the echocardiographic study variables, which included assessment of; ACTPO, SPAP, interventricular septum at diastole, LAA, LV end-diastolic diameter, LVEF, RAA, RVD 1, RVD 2, RVD 3, RVFAC, RV GLPS, RVOT prox, TAPSE, TA S’, and RV Tei-index.

However, as an indication of improved RV systolic function following ingestion of nitrate-rich beetroot juice we observed numerical, but non-significant, improvements in RVFAC (median of differences; 4.9%, p = 0.152), RV GLPS (median of differences; -1.4%, p = 0.097), and in the TA S’ (2 cm/s, p = 0.119). A normalization of RVFAC defined as RVFAC > 32% or > 35% was observed in five out of eleven (45%) and three out of twelve (25%) patients, respectively, after administration of nitrate-rich beetroot juice. Three out of eleven (27%) patients normalized in RV GLPS, defined as RV GLPS ≤ -17%, after the treatment with nitrate-rich beetroot juice.

**Ergospirometry, 6MWT, and DLCO**

The results from the ergospirometry are presented in Table 6. A significantly lower BF was observed after nitrate-rich beetroot juice compared to after placebo (median of differences; -2 br/min, p = 0.001). Compared to placebo, nitrate-rich beetroot juice tended to decrease VE max (median of differences; -4.25 L/min, p = 0.066) and tended to increase the W peak/VO2 peak ratio (median of differences: 0.51 watt/(mL/min), p = 0.101).

The 6MWD and the percent-predicted DLCO did not differ between the two interventions (6MWD median of differences; 3.25 m, p = 0.445, and percent predicted DLCO median of differences; 1%, p = 0.235).
Table 6. Ergospirometry after nitrate-rich beetroot juice and placebo.

<table>
<thead>
<tr>
<th>Variable</th>
<th>BRJ Median (Q1; Q3)</th>
<th>Placebo Median (Q1; Q3)</th>
<th>Median of differences (95% CI)*</th>
<th>P-Value† BRJ vs Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR rest (bpm)</td>
<td>68 (63;76)</td>
<td>75 (69;81)</td>
<td>-5 (-12;2)</td>
<td>0.191</td>
</tr>
<tr>
<td>HR max (bpm)</td>
<td>125 (100;145)</td>
<td>113 (104;140)</td>
<td>5 (-4;20)</td>
<td>0.343</td>
</tr>
<tr>
<td>Borg RPE peak</td>
<td>16.5 (15;17)</td>
<td>17.0 (15;17)</td>
<td>-0.5 (-3;3)</td>
<td>0.743</td>
</tr>
<tr>
<td>W peak (watt)</td>
<td>70 (60;80)</td>
<td>60 (54;80)</td>
<td>2 (-2;9)</td>
<td>0.379</td>
</tr>
<tr>
<td>VO2 rest (mL/min/kg)</td>
<td>4.82 (3.36;6.03)</td>
<td>4.23 (3.83;4.57)</td>
<td>0.64 (-0.51;2.08)</td>
<td>0.295</td>
</tr>
<tr>
<td>VO2 AT (mL/min/kg)</td>
<td>9.46 (7.82;11.64)</td>
<td>11.15 (7.48;13.76)</td>
<td>-0.50 (-2.14;1.42)</td>
<td>0.534</td>
</tr>
<tr>
<td>VO2 peak (mL/min/kg)</td>
<td>11.69 (9.46;16.70)</td>
<td>12.29 (11.09;14.59)</td>
<td>-0.03 (-1.46;0.86)</td>
<td>1.000</td>
</tr>
<tr>
<td>VE rest (L/min)</td>
<td>14 (10;21)</td>
<td>13 (11;17)</td>
<td>1.25 (-1.5;8)</td>
<td>0.387</td>
</tr>
<tr>
<td>VE max (L/min)</td>
<td>51 (44;61)</td>
<td>58 (52;61)</td>
<td>-4.25 (-11;0)</td>
<td>0.066</td>
</tr>
<tr>
<td>VCO2 rest (mL/min/kg)</td>
<td>3.41 (2.98;4.80)</td>
<td>3.28 (3;3.86)</td>
<td>0.57 (-0.38;2.14)</td>
<td>0.181</td>
</tr>
<tr>
<td>VCO2 AT (mL/min/kg)</td>
<td>8.93 (7.76;11.53)</td>
<td>10.85 (6.97;13.33)</td>
<td>-0.47 (-2.17;1.54)</td>
<td>0.366</td>
</tr>
<tr>
<td>VCO2 peak (mL/min/kg)</td>
<td>12.64 (8.93;20.57)</td>
<td>13.73 (11.37;17.60)</td>
<td>-0.40 (-2.80;1.04)</td>
<td>0.731</td>
</tr>
<tr>
<td>VE/VCO2 AT</td>
<td>49.60 (43.69;55.25)</td>
<td>47.48 (42.47;53.4)</td>
<td>0.57 (-2.52;2.94)</td>
<td>0.731</td>
</tr>
<tr>
<td>RER rest</td>
<td>0.82 (0.77;0.91)</td>
<td>0.81 (0.75;0.86)</td>
<td>0.01 (-0.04;0.06)</td>
<td>0.534</td>
</tr>
<tr>
<td>RER peak</td>
<td>1.05 (0.98;1.14)</td>
<td>1.11 (1.03;1.21)</td>
<td>-0.04 (-0.18;0.03)</td>
<td>0.295</td>
</tr>
<tr>
<td>W peak/VO2 peak (watt/mL/min)</td>
<td>7.70 (6.64;7.89)</td>
<td>6.67 (6.28;7.84)</td>
<td>0.51 (-0.05;1.00)</td>
<td>0.101</td>
</tr>
<tr>
<td>BF max (br/min)</td>
<td>30 (28;33)</td>
<td>34 (31;36)</td>
<td>-2 (-4;2)</td>
<td>0.001</td>
</tr>
<tr>
<td>BR max (%)</td>
<td>28 (23;33)</td>
<td>27 (15;31)</td>
<td>4.5 (-5.5;20)</td>
<td>0.311</td>
</tr>
</tbody>
</table>

Abbreviations; BRJ, nitrate-rich beetroot juice; Q1, first quartile; Q3, third quartile. *Confidence interval (CI) of the Hodges-Lehmann’s median differences. †Koch's adaptation of the Mann-Whitney U test. *N=13 for all variables except for HR max (N=12), and Borg RPE max (N=10).
Safety assessment
Administration of nitrate-rich beetroot juice and nitrate-depleted beetroot juice (placebo) was well tolerated during the study, and no patient withdrew from the study or discontinued study treatment due to adverse events. No serious adverse events were reported. The nitrate-rich beetroot juice did not alter the systemic blood pressure compared to placebo (systolic blood pressure, p = 0.482; diastolic blood pressure, p = 1.000). Five patients reported minor adverse events during treatment with the nitrate-rich beetroot juice (constipation (n = 1), loose stools (n = 3), and transient nausea (n = 1). Three patients reported minor adverse events during treatment with placebo (increase in heart palpitations (n = 1), loose stools (n = 1), and vertigo (n = 1).

Discussion
Exhaled NO, nitrate, and nitrite in PH
Assessment of exhaled NO, as an indicator of pulmonary production of NO, in PH is attractive due to its non-invasive approach. Methods have been developed to allow for estimation of the peripheral (alveolar) and central (bronchial) contribution to exhaled NO (162).

The results in our study, presented in Paper I, demonstrate that patients with PAH (WHO group 1) display lower levels of exhaled NO as compared with patients with PH (WHO group 2-4). The decrease in FeNO was evident at exhalation flow-rates in the range from 20-200 mL/s. Although neither patients with PAH (WHO group 1) or patients with PH (WHO group 2-4) differed from healthy controls with regard to FeNO levels at any of the exhalation flow-rates, it was however demonstrated that the subgroup of PAH patients with idiopathic PAH (WHO group 1.1) had significantly lower levels of FeNO at the lowest exhalation flow-rate in comparison to healthy control subjects. Furthermore patients with PAH (WHO group 1) demonstrated reduced bronchial NO flux compared with patients with PH (WHO group 2-4) and healthy controls. A significant increase in alveolar NO concentrations were demonstrated in patients with PAH (WHO group 1) compared with healthy controls.

The finding of decreased bronchial NO flux in patients with PAH (WHO group 1) along with a reduced FeNO at the lowest exhalation flow-rate in the subgroup of patients with idiopathic PAH (WHO group 1.1) compared to healthy control indicate that patients with PAH have reduced production of pulmonary derived NO in the central airways. Reduced pulmonary production of NO from the bronchial compartment may thus serve as an explanation for the decreased bronchial NO flux noted in patients with PAH. However, since bronchoconstriction has been reported in patients with PAH (WHO group 1) a decrease in the bronchial area for NO exchange may serve as an alternative explanation or contribute to the low levels of bronchial NO flux (163-166).
Previous studies on levels of NO from the airways have indicated that patients with PAH (WHO group 1) have reduced production of pulmonary NO (126-129). Our results are in corroboration with the results from another study in patients with PAH (eight patients with idiopathic PAH and two patients with anorexigen-associated PAH corresponding to WHO group 1.1 and 1.3 respectively according to the current PH classification) in which decreased FeNO was demonstrated at the lowest expiratory flow-rates compared to healthy controls and the reduced FeNO concentrations in the PAH patients were reported as due to decreased airway wall concentration of NO (126).

The decrease in exhaled NO noted in our study in patients with PAH (WHO group 1) in comparison to patients with PH (WHO group 2-4) was not due to differences in gender distribution, functional class or use of PH-specific therapy. This difference may thus represent a difference in the production of pulmonary NO between patients with PAH (WHO group 1) and patients with PH (WHO group 2-4). A similar finding with lower levels of exhaled NO in patients with primary PH (corresponding to PAH WHO group 1.1, 1.2 and 1.3 in the current clinical classification system) in comparison to patients with secondary PH (a mix of patients corresponding to WHO group; 1.4, 3.1, and 4) has been reported (129).

Differences in the expression of eNOS may serve as an explanation for the difference in exhaled NO between patients with PAH (WHO group 1) and patients with PH (WHO group 2-4) observed in our study. Data indicating reduced expression of eNOS in small- and medium-sized pulmonary arteries in patients with plexogenic pulmonary arteriopathy (corresponding to PAH WHO group 1 in the current clinical classification system) compared with patients with secondary PH (corresponding to WHO; group 1.4.4, group 2, and group 3 in the current clinical classification system) have indeed been reported (121). Furthermore as an indication of lower production of NO in patients with PAH, it has been reported that patients with primary PH (PAH WHO group 1) have significantly lower systemic levels of NO metabolites (total nitrate and nitrite) compared to patients with secondary PH (which in that study included PH patients within WHO group 2 and 4 according to the current clinical classification system) (167).

In our study significantly higher levels of alveolar NO was observed in patients with PAH (WHO group 1) compared with healthy controls, and a trend for higher alveolar NO was also noted for patients with PH (WHO group 2-4). This represents a novel finding and is not in line with the results presented by Girgis et al. in which no significant difference in alveolar NO was observed in patients with PAH (WHO group 1) compared to control subjects (126). The reason for this difference in results with regard to levels of alveolar NO in patients with PAH is unclear but may be due to methodological differences in estimation of alveolar NO. Furthermore, the patients with PAH in our study comprised a mix of patients within WHO group 1 (including PAH patients in WHO group 1.1, 1.4.1 and 1.4.4) and it cannot be excluded that differences in
levels of alveolar NO exist within the PAH WHO group 1. For instance it has been shown that alveolar NO is increased in patients with PAH-SSc (PAH WHO group 1.4.1) compared to patients with primary PH (168). In that study a negative correlation between alveolar NO concentration and the diffusing capacity of the lung for carbon monoxide (DLCO) was observed in patient with SSc lung disease, and the patients with SSc-PAH had lower DLCO than patients with primary PH (168). Furthermore they calculated the diffusing capacity of NO from the alveoli into blood (DLNO) from the DLCO and found that DLNO was particularly low in patients with PAH-SSc and concluded that a reduced diffusing capacity of NO from the alveolar space into the blood could explain the observed increase in alveolar NO (168). Thus, the increase in alveolar NO observed in our study in patients with PAH (WHO group 1) may be explained by a dysfunctional alveolocapillary membrane.

Our study reveals differences between patients with PH and healthy controls in levels of plasma and salivary nitrate and nitrite. Plasma as well as salivary levels of nitrate and nitrite were increased in the total group of PH patients (WHO group 1-4) compared to healthy controls. Patients with PAH (WHO group 1) as well as patients with PH (WHO group 2-4) had higher salivary levels of nitrate in comparison to healthy controls. The patients with PAH (WHO group 1) had higher levels of plasma and salivary nitrate than healthy controls. The levels of plasma and salivary nitrate or nitrite did not differ between patients with PAH (WHO group 1) and patients with PH (WHO group 2-4). No correlations were found between plasma or salivary levels of nitrate or nitrite and NO flow-independent exchange parameters in our study.

Previous studies have reported reduced levels of biochemical oxidation products of NO in BALF and in EBC in patients with PAH (WHO group 1) compared to healthy control subjects, and further analysis of the BALF and the EBC in these studies revealed decreased levels of nitrate (nitrate plus S-nitrosothiol in the study by Kaneko et al.) and similar levels of nitrite in airways of PAH patients compared to control (127, 131). In the study by Kaneko et al., a negative correlation between PAP and total NO reaction products in BALF was observed (127). However, none of these two studies found any systemic alterations as the serum levels of biochemical reaction products of NO was similar in patients with PAH compared to healthy controls (127, 131). Increased levels of plasma nitrate have been reported in pediatric patients with PAH-CHD (WHO group 1.4.4) compared to healthy controls (169). The plasma nitrate levels correlated positively with the pulmonary to systemic pressure ratio and plasma nitrate normalized postoperatively in patients without residual PH (169). Furthermore, it has been shown in healthy subjects that ingestion of nitrate lead to an increase in exhaled NO and that elevation of exhaled NO is paralleled by an increase in salivary nitrate and nitrite (170). Similarly it has been demonstrated that ingestion of nitrate increase bronchial NO flux (171). These two studies demonstrate examples on generation of NO through the nitrate-nitrite-NO pathway. In our study a reduced bronchial NO
flux was observed in patients with PAH (WHO group 1) compared to healthy controls despite elevated levels of salivary nitrate and elevated levels of plasma and salivary nitrite. This suggests that the deficiency in pulmonary NO production generated through NOS-dependent mechanisms in the central airways is not fully counterbalanced by the nitrate-nitrate-NO pathway in patients with PAH (WHO group 1).

Vardenafil in PH – acute haemodynamic response and pharmacokinetics

Treatment with PDE5 inhibitors is considered as one of the cornerstones in the treatment of patients with PAH today (9). However, little is known about the haemodynamic effects in relation to PDE5 inhibitor drug concentrations for the commercially available PDE5 inhibitors, and the drug concentration to obtain optimal long-term clinical effect is unknown. Pfizer has submitted pharmacodynamic data to the European Medicines Agency from study A1481024 in patients with PH were intravenous sildenafil was administered to reach different target concentrations (172). The study found that the maximum effect in reducing mPAP and PVR occurred at a plasma sildenafil concentration of 100 ng/mL, and mean absolute values of CO tended to increase across concentrations in the range of 10-100 ng/mL (172).

In our study the main finding, presented in Paper II, was the observation that plasma vardenafil drug concentration and exposure correlated with the acute responses in mPAP and PVR in patients with PH. Higher vardenafil concentrations or vardenafil exposure (expressed as AUC) were associated with larger reductions in mPAP and PVR at 60 min following an oral single dose administration of vardenafil. These haemodynamic variables are of clinical relevance as they (both or either alone) have been indicated as predictive factors for survival in patients with PAH (WHO group 1) (173-176), PH due to lung disease (WHO group 3) (177-179), and in CTEPH (WHO group 4) (180-182).

Prior to our investigations data from a single dose haemodynamic study of three PDE5 inhibitors (sildenafil, tadalafil, and vardenafil), which included patients with PAH (WHO group 1), demonstrated that oral vardenafil at 10mg and 20 mg caused significant pulmonary vasorelaxation (183). In that study, vardenafil at both doses significantly decreased mPAP and PVR index while CI increased and there were no differences between oral sildenafil, tadalafil and vardenafil with regard to change in mPAP, PVR index or CI. Evaluation of the kinetics of peak pulmonary vasodilatory response (expressed as change in PVR index) of the three PDE5 inhibitors revealed that the peak haemodynamic effect was obtained after 40 to 45 min for vardenafil, 60 min for sildenafil, and 75 to 90 min for tadalafil (183). The acute haemodynamic effects of oral vardenafil (5 mg) has been explored also in another study which consisted
of five patients with PH of different aetiology (one with primary PH, two with CTEPH and two with PAH-CHD) (157). The haemodynamic effects were monitored up to 90 min, and oral 5 mg administration of vardenafil significantly decreased PVR (a decrease was observed already at 30 min) and increased CO while no reduction in mPAP was observed (157). In our study, we evaluated the haemodynamic response at 60 min after administration of a single oral dose of vardenafil to patients with PH (WHO group 1–4) and observed a significant decrease in mPAP and PVR and an increase in CI and CO. Analysis of the patients with PAH (WHO group 1) and patients with PH (WHO group 2–4) in our study did not reveal any differences with regard to haemodynamic response at 60 min indicating similar vasorelaxing properties of vardenafil in these two groups. Furthermore we examined the kinetics of the pulmonary vasorelaxing effects of vardenafil by measuring mPAP every 15 min from baseline to 60 min and found that a significant decrease in mPAP could be observed already at 15 min after administration of a single oral dose of vardenafil. Vardenafil differs from sildenafil and tadalafil as it has been demonstrated that vardenafil in addition to its mode of action as a PDE5 inhibitor also blocks Ca²⁺ fluxes which enhance its vasorelaxant properties (19, 184, 185). The pharmacological properties of vardenafil may thus explain the rapid onset of effect seen in our study.

Three PAH patients (two in PAH WHO group 1.1 and one in PAH WHO group 1.4.1) in our study were on stable treatment with bosentan, a dual endothelin receptor antagonist. The PAH patients on bosentan displayed significantly lower plasma vardenafil concentrations in comparison to the other patients unexposed to bosentan, indicating a possible drug interaction between vardenafil and bosentan as bosentan induces cytochrome P450 3A4 and vardenafil is metabolised by this enzyme (186, 187). The haemodynamic changes at 60 min following administration of vardenafil in the PAH patients exposed to bosentan were not significantly different from the haemodynamic response observed in the other PH patients (WHO group 1–4) unexposed to bosentan. A numerically smaller median reduction in mPAP at 60 min was observed in the three PAH patients exposed to bosentan (–10.8%) in comparison to the other PAH patients unexposed to bosentan (–23.8%), however this difference did not reach statistical significance. The induction of cytochrome P450 3A4 by bosentan constitutes the mechanism for the described drug interaction between bosentan and the two other PDE5 inhibitors (sildenafil and tadalafil), and it has been demonstrated that this interaction leads to reduction of plasma exposure of sildenafil and tadalafil by approximately 60% and 40% respectively (188–190). The pharmacodynamic relevance of this drug interaction between bosentan and PDE5 inhibitors in patients with PAH was noted in the pivotal tadalafil phase 3 study. In that study addition of tadalafil to PAH patients on background bosentan therapy did not lead to a significant effect on the placebo corrected 6-MWD, and it was further reported that the effects on
all secondary end points tended to be worse in bosentan treated subjects compared with treatment-naïve patients on tadalafil monotherapy (151).

We observed a large variation between individuals in plasma vardenafil concentrations at 60 min also in the uniform subgroup of PAH patients (WHO group 1) unexposed to bosentan that received 20mg of vardenafil, with almost a 60-fold difference between the lowest and the highest concentration. Our study reveals a high inter-interindividual variability of vardenafil pharmacokinetics in patients with PH (WHO group 1-4) and by use of data from our study the pharmacokinetic profile of vardenafil have been presented in more details elsewhere (191).

In the study by Ghofrani et al. there were no significant differences in acute haemodynamic response to vardenafil (change in mPAP, PVR index, or CI) between patients receiving a single oral dose of vardenafil at 10 mg or 20 mg (183). In the same study no difference in haemodynamic response to tadalafil were observed between patients receiving single dose tadalafil at 20, 40, or 60 mg (183). A considerable inter-individual overlap in resulting plasma concentrations following administration of different doses of vardenafil and tadalafil may explain the absence of a dose-dependent haemodynamic response in the study by Ghofrani et al. (183).

The current licensed indication for sildenafil and tadalafil is PAH (WHO group 1). In the latest European PH treatment guideline, the use of drugs approved for PAH (including PDE5 inhibitors) is not recommended in patients with PH due to left heart diseases (WHO group 2) or lung diseases (WHO group 3) (9). For patients with CTEPH (WHO group 4) the same guideline state that off-label use of drugs approved for PAH may be considered in symptomatic patients who have been classified as having inoperable CTEPH (9). There are however studies that have reported beneficial treatment effects with PDE5 inhibition in patients with PH due to left heart disease (WHO group 2) (192-194), PH due to lung disease and or hypoxia (WHO group 3) (195-201), and in patients with CTEPH (WHO group 4) (202-204). The study population included in our study comprised patients with PH from WHO group 1-4, reflecting the spectra of patients with PH that currently receives treatment with PDE5 inhibitors in clinical practice. One randomised, double-blind, placebo-controlled trial investigated the effects of vardenafil monotherapy for twelve weeks (5 mg once daily for four weeks and then 5 mg twice daily for 8 weeks) in patients with PAH (WHO group 1) (154). Patients that completed the twelve week randomised part of the study were treated with open label vardenafil (5 mg twice daily) for a further twelve weeks (154). The results from that study demonstrate that vardenafil was well tolerated and improved exercise capacity (6-MWD), Borg dyspnea index, WHO functional class, haemodynamics (CI, mPAP, and PVR) and reduced clinical worsening events (154). Interestingly the study by Jing et al. demonstrate that monotherapy treatment with vardenafil to patients with PAH reduce clinical worsening events, this has not been conclusively shown for monotherapy treatment with sildenafil or
tadalafil in patients with PAH (150, 151, 154). In the phase 3 tadalafil pivotal PAH study, tadalafil at 40 mg improved clinical worsening compared to placebo but over 50% of the patients in the study were on treatment with bosentan before tadalafil or placebo was started (151).

Vardenafil has currently no regulatory approval for the treatment of PAH (WHO group 1), and in the latest European PH treatment guideline use of vardenafil as monotherapy for PAH has received a class IIb recommendation (usefulness/efficacy is less well established by evidence/opinion) based on B level of evidence (data derived from a single randomized clinical trial or large non-randomised studies) (9).

Vardenafil in PH – acute changes in plasma arginine and dimethylarginines

Disturbances in the L-arginine-NO-cGMP pathway have been reported in patients with PH, and impaired NO availability represents an important feature of endothelial dysfunction and is considered as one of the key factors in the pathophysiology of PH. The regulation of NOS dependent NO production is complex as several biochemical reactions, substrates, co-factors, and enzymes are involved. In vascular disease research, particular interest has been directed towards the endogenous NOS inhibitor ADMA, and ADMA is now recognized as marker of cardiovascular disease and mortality (205, 206). It has been demonstrated that ADMA is not merely a marker of disease and disease severity as infusion of ADMA to healthy volunteers, resulting in pathophysiologically relevant ADMA blood concentrations, has been shown to inhibit NO synthesis, reduce CO and stroke volume, increase PVR, reduce renal perfusion, and increase mean arterial blood pressure (134, 207, 208). Elevated circulatory levels of ADMA have been demonstrated in patients with PH of different aetiology (133-140). Furthermore it has been demonstrated that ADMA levels correlate with severity of disease (134, 136, 139), with elevated levels being associated with poor survival (134, 139). Less attention has been paid to SDMA, another dimethylarginine, which unlike ADMA does not inhibit NOS directly but which may impair NO production indirectly by limiting the bioavailability of L-arginine (81). SDMA has been reported to be elevated in patients with idiopathic PAH (lung tissue and plasma) and in infants with persistent PH of the newborn (urine) (133, 135). Reduced plasma levels of L-arginine, the substrate for eNOS, have been reported in patients with PAH associated with congenital heart disease (WHO group 1.4.4) and in patients with CTEPH (WHO group 4) (138, 139). In patients with idiopathic PAH (WHO group 1.1) it has been demonstrated that plasma L-arginine correlated with several important haemodynamic variables as well as NYHA functional class revealing an association between low levels of L-arginine and a more severe disease state (141). Additionally it has been demonstrated that PDE5
expression in pulmonary tissue is increased in patients with PAH (WHO group 1.1 and 1.4.4) and in patients with PH due to lung disease (WHO group 3) (145, 146).

In light of the above we wanted to explore whether a single dose administration of vardenafil, a PDE5 inhibitor, had an effect on plasma levels of ADMA, SDMA or arginine.

In Paper III, data is presented in support of an acute effect mediated by vardenafil on metabolic key determinants involved in NOS dependent production of NO in patients with PH. In our study transient reductions in plasma ADMA and SDMA levels were noted, with a significant decrease in ADMA levels at two time-points (30 and 40 min) and a significant decrease in SDMA levels at 45 min compared to baseline, after administration of a single oral dose of vardenafil. Changes in the plasma levels of arginine were also noted in our study after administration of vardenafil. A significant increase in plasma arginine levels compared to baseline was observed over several time-points (120, 300, and 540 min). The plasma levels of arginine reached the highest level at 540 min, and as we did not include further assessments it is unclear if the levels of arginine at 540 min represent a zenith or if plasma arginine continues to increase. As a consequence of the plasma changes in ADMA and arginine, significant changes in the arginine/ADMA ratio were observed. The arginine/ADMA ratio was significantly increased at 15 min and at all time-points between 45 to 540 min compared to baseline. The change in the arginine/ADMA ratio correlated positively with plasma vardenafil exposure, thus a higher vardenafil drug exposure was associated with a larger increase in the arginine/ADMA ratio. The vardenafil mediated increase in the arginine/ADMA ratio observed in our study may reflect a restoration of the physiological arginine/ADMA ratio favouring improved eNOS activity (209). However, we did not assess if endogenous NOS dependent NO production increased in parallel with the increase in plasma arginine and the increase in the arginine/ADMA ratio. Previous studies in patients with PH have demonstrated that exogenous administration of L-arginine reduces mPAP and PVR and results in increased levels of plasma citrulline indicating increased NO production via NOS (210, 211). In the study by Mehta et al. peak plasma citrulline levels correlated significantly with the reductions in mPAP and PVR (210).

In our study we also explored the relationship between the baseline arginine/ADMA ratio and baseline plasma levels of ADMA and SDMA and several baseline haemodynamic variables collected during assessment with RHC. We observed that at baseline, plasma levels of ADMA and SDMA correlated positively with baseline mRAP and ADMA correlated negatively with baseline pulmonary artery oxygen saturation. The arginine/ADMA ratio at baseline correlated positively with baseline CI and baseline CO and negatively
with baseline mRAP. Thus, a high plasma level of ADMA or SDMA or a low arginine/ADMA ratio at baseline was associated with a more haemodynamically severe disease state. Furthermore, we explored the relationship between the haemodynamic response from baseline to 60 min and the change in plasma levels of arginine, ADMA, SDMA, and change of the arginine/ADMA ratio in the same time interval. The change in plasma arginine or the arginine/ADMA ratio correlated positively with change in CI. An increase in plasma arginine levels or an increase of the arginine/ADMA ratio was thus associated with an increase in CI.

Our results are in agreement with previous studies in patients with PH in which an association between plasma levels of ADMA and severity of the disease have been reported. It has been demonstrated in patients with PAH-SSc (WHO group 1.4.1) that plasma ADMA correlate negatively with 6-MWD (136). In patients with idiopathic PAH (WHO group 1.1) and CTEPH (WHO group 4) ADMA plasma concentrations was reported to correlate positively with mRAP and negatively with CI (134, 139), and in patients with PAH-CHD (WHO group 1.4.4) plasma ADMA correlated positively with mPAP, PVR, and mean right ventricular pressure (212). Previous studies on the relationship between the arginine/ADMA ratio and haemodynamic parameters in patients with PH are lacking. However, it has been demonstrated in patients with idiopathic PAH (WHO group 1.1) that plasma levels of L-arginine correlate with mRAP, CO, CI, mixed-venous oxygen saturation and NYHA functional class with an association between low levels of L-arginine and increased severity of the disease (141). In a study that was published almost at the same time as ours, Fang et al. demonstrate that plasma ADMA levels significantly decreased after six months of treatment with sildenafil in ten patients with PAH-CHD (WHO group 1.4.4), with reductions in ADMA accompanied by reduced PAP (assessed by echocardiography) and improvement in the 6-MWD (212).

Our study is the first study to demonstrate that PH-specific therapy has an acute effect on plasma levels of arginine, SDMA and the arginine/ADMA ratio in addition to ADMA in a broad PH population. It remains to be demonstrated whether treatment with PH-specific drug therapies other than PDE5 inhibitors alter plasma levels of arginine, ADMA or SDMA in a favourable manner in patients with PH.

The nitrate-nitrite-NO pathway in PAH – exploring the effects of inorganic nitrate

The nitrate-nitrite-NO pathway provides generation of NO by NOS-independent mechanisms, and is thus a complementary pathway to the classical L-arginine-NO-pathway (86). Exogenous administration of nitrate or nitrite is already being explored as new therapeutic options in a variety of cardiovascular
and metabolic diseases were NO deficiency is considered as part of the pathophysiology (213). Clinical data on the effects of nitrite in patients with PH is now emerging, whereas no data on the effects of inorganic nitrate in patients with PH are available. Encouraging results from a phase II clinical trial in patients with PH of different etiologies have recently been published. The acute cardiopulmonary effects of inhaled nitrite administered in single doses of 45 mg and 90 mg was studied in patients with PAH (WHO group 1), PH patients with heart failure with preserved ejection fraction (PH-HFpEF) (WHO group 2), and patients with PH due to lung disease and/or hypoxia (WHO group 3) and significant acute changes in several hemodynamic variables were observed in all three study populations (214). In the PAH patients, treatment with inhaled nitrite resulted in a decrease in the right atrial pressure, the pulmonary arterial systolic pressure, and the RV diastolic pressure. Differences in response to inhaled nitrite were observed between the PH groups in the study by Simon et al. The patients with PH due to lung disease and/or hypoxia (WHO group 3) was the only group that responded with a reduction in PVR, and this group also responded with greater reductions in pulmonary artery diastolic and mean pressures compared to the PAH patients. This difference in response to therapy with inhaled nitrite may be explained, at least in part, by differences in the reductive pathways of nitrite to NO which is augmented in hypoxic conditions (110).

Furthermore, treatment with inorganic nitrate and nitrite has been explored in animal models of PH (215). Interestingly, and of interest to our study, administration of dietary nitrate resulted in significant reversal of RV systolic pressure and RV hypertrophy in mice with established PH in the hypoxia-induced PH model (215). It was shown that the effects of dietary nitrate were associated with increased plasma and lung tissue levels of nitrite and cGMP, which indicate that the nitrate-nitrite-NO pathway indeed was active in their model. The existing data thus indicate that the nitrate-nitrite-NO pathway may be a promising and potential therapeutical target in patients with PH or PAH.

We therefore sought to evaluate the effects of dietary nitrate supplementation, by use of nitrate-rich beetroot juice, to patients with PAH (WHO group 1) with the hypothesis that treatment with inorganic nitrate would result in increased NO production and bioavailability via the nitrate-nitrite-NO pathway. The study, which is presented in Paper IV, was a randomised, double-blind, placebo-controlled, crossover trial, which included assessments of exhaled NO, biomarkers associated with the regulation and production of NO, exercise capacity, and echocardiographic examinations. The patients received inorganic nitrate at a dose of ~16 mmol/day (~1000 mg) during treatment with concentrated nitrate-rich beetroot juice.

The results from our study, which is the first study to explore the effects of inorganic nitrate in patients with PH, indicate that the nitrate-nitrite-NO pathway is indeed active in patients with PAH (WHO group 1) as ingestion of
nitrate-rich beetroot juice resulted in elevated plasma and salivary levels of nitrate and nitrite and an increase in exhaled NO (FeNO, CalvNO and J’aw NO) compared to placebo. The clinical relevance of an increase in exhaled NO, as observed in our study following ingestion of nitrate-rich beetroot juice, remains to be determined. However, it has been shown that survival as an indicator of successful PAH therapy is associated with an increase in exhaled NO whereas no elevation of exhaled NO was observed in the group of patients who did not survive during follow-up (131).

We did not observe and differences between the interventions in exercise capacity assessed by 6MWT neither did we observe any statistically significant differences in the parameters assessed by echocardiography. However, the echocardiographic examination revealed a tendency for improvement in RV systolic function in three out of five measurements of RV systolic function (RVFAC, RV GLPS, and TA S’) after ingestion of nitrate-rich beetroot juice compared to placebo.

The breathing frequency at maximum work decreased significantly during ergospirometry after administration of nitrate-rich beetroot juice compared to placebo, while no significant differences were observed for the other variables. Ingestion of nitrate-rich beetroot juice thus resulted in a decrease in breathing frequency compared to placebo at similar work rate and similar oxygen consumption at peak work load as after placebo.

Plasma levels of ADMA, arginine, citrulline, and SDMA did not differ between the two interventions, whereas we observed a decrease in plasma ornithine levels and an increase in the arginine/ornithine ratio as well as an increase in GABR after administration of nitrate-rich beetroot juice. The enzyme responsible for the hydrolysis of arginine to ornithine and urea is arginase, and the changes observed in our study indicate an effect on the arginine metabolism with a decrease in the arginase enzyme activity and increased arginine bioavailability. This is of interest as an increase in arginase activity limits arginine availability for NO production in the classical L-arginine-NO-pathway (64), and patients with PAH (WHO group 1) has been shown to have increased arginase activity compared to healthy individuals (142). Of relevance to our results, it has been demonstrated in rats that dietary nitrate suppress cardiac arginase expression and activity and elevate cardiac arginine concentrations (216). The mechanism by which nitrate-rich beetroot juice decreases plasma ornithine remains to be elucidated, however it has been demonstrated in mice that sildenafil downregulate arginase expression and decrease arginase enzyme activity which indicate that the mechanism may involve cGMP (217).

We performed a correlation analysis on data retrieved after the placebo period (to reflect basal conditions). This analysis demonstrates that plasma nitrate correlate negatively with VO2 peak, which indicate that patients with high basal plasma levels of nitrate have lower exercise aerobic capacity. We also observed that plasma nitrite correlated positively with plasma ADMA
levels and negatively with TAPSE. High basal plasma levels of nitrite were associated with high plasma levels of ADMA and more pathological TAPSE values. In addition we observed a trend for a positive correlation between plasma nitrite levels and RV Tei-index ($r = 0.49, p = 0.072$), and a trend for a positive correlation between plasma nitrite levels and NT-proBNP plasma levels ($r = 0.45, p = 0.092$). ADMA as well as NT-proBNP are biomarkers with strong prognostic value in PAH (134, 218), and TAPSE and RV Tei-index are well established echocardiographic measures of RV function that also predict survival in PAH (219, 220).

In a recent study it was shown that NO plasma metabolites (NOx) are decreased in patients with PAH (WHO group 1.1) at the time of diagnosis compared to healthy controls, and plasma NOx correlated inversely with mPAP, PVR and survival at follow-up (221). In the study by Zhang et al. plasma nitrate and plasma nitrite results were not presented separately. Low levels of NOx do not preclude that patients with PAH may indeed have higher levels of plasma nitrite compared to healthy controls as observed by us in Paper I. In our studies, Paper I and Paper IV, plasma levels of nitrite were approximately 200-fold lower than plasma nitrate in patients with PAH and thus contribute very little to plasma NOx. The data presented by Zhang et al. on plasma NOx levels in PAH patients is thus not necessarily a contradicting finding to our observations presented in Paper I.

The observation from our previous study, presented in Paper I, which demonstrated that patients with PAH (WHO group 1) had higher levels of plasma and salivary nitrite than healthy controls and the observations presented in Paper IV on the relationship between plasma nitrite levels and ADMA and TAPSE collectively indicate that the nitrate-nitrite-NO pathway is active in PAH and upregulated in PAH patients with a more severe disease state. An upregulated nitrate-nitrite-NO pathway in patients with PAH may be explained as a compensatory mechanism to counterbalance a deficient production of NO from the classical L-arginine-NO-pathway.

In conclusion, our findings in Paper IV, although from a small and exploratory study, indicates that inorganic nitrate in the form of nitrate-rich beetroot juice administered to patients with PAH is safe and well tolerated and increase pulmonary NO production via the NOS-independent nitrate-nitrite-NO pathway. In addition, treatment with nitrate-rich beetroot juice decreased plasma ornithine levels and increased relative arginine availability in patients with PAH (WHO group 1) compared to placebo. Intriguingly, we also observed a tendency for improved RV systolic function. Further studies are warranted to confirm whether long-term treatment with inorganic nitrate is of clinical benefit to patients with PAH or patients with other PH etiologies.
Conclusions

Based on the findings in Paper I-IV, the following conclusions are made:

- Patients with PAH (WHO group 1) have lower bronchial NO flux compared to healthy controls and patients with PH (WHO group 2–4). This implies reduced bronchial NO formation in PAH.

- Compared to healthy controls, increased alveolar NO levels were found in subjects with PH (WHO group 1-4) and in patients with PAH (WHO group 1). This may reflect NO diffusion disturbances in the alveoli.

- Patients with PAH (WHO group 1) had higher plasma and salivary levels of nitrite than healthy controls. The exact cause for this finding is unclear but may reflect a compensatory upregulation of NOS-independent NO generating pathways.

- A single oral dose of the PDE5 inhibitor vardenafil causes rapid changes in cardiopulmonary hemodynamics in patients with PH (WHO group 1-4), and there is a correlation between plasma vardenafil drug concentration and the acute changes in mPAP as well as PVR. Furthermore, the patients in our study that were on bosentan treatment displayed very low concentrations of vardenafil, indicating a possible drug interaction between vardenafil and bosentan.

- Vardenafil administered as a single oral dose to patients with PH (WHO group 1-4) has comprehensive effects on mediators involved in the production and regulation of NO. Vardenafil acutely altered the plasma levels of arginine, ADMA, SDMA, and the arginine/ADMA ratio in a favourable manner in these PH patients. The increase in arginine and the arginine/ADMA ratio were associated with improved CI, and the increase in the arginine/ADMA ratio at 540 min strongly correlated with the exposure to vardenafil.

- We found that higher baseline plasma levels of ADMA and SDMA and a low arginine/ADMA ratio was associated with a more severe pulmonary hemodynamic disease state in patients with PH.

- Administration of inorganic nitrate, in the form of beetroot juice, increased plasma and salivary levels of nitrate and nitrite, increased exhaled NO, decreased plasma ornithine levels and increased relative arginine availability in patients with PAH (WHO group 1) compared to placebo. Higher plasma levels of nitrite after the placebo period, reflecting basal conditions, were associated with a more severe PAH phenotype. Our findings indicate that the nitrate-nitrite-NO pathway is active and upregulated in patients with PAH.
General discussion and future perspectives

Despite an increased awareness of PH and PAH these conditions remain serious and are associated with poor survival rates. The understanding of the NO system is still an evolving research field, even though three decades has passed since the discovery of NO. Vascular NO research has been a success in general and for PAH patients in particular as we now have several PAH specific treatments approved and available for this patient group which target NO pathophysiology in these patients. The PDE5 inhibitors sildenafil and tadalafil which are approved for the treatment of PAH, are now considered as cornerstone treatments in PAH, and riociguat a stimulator of sGC is the latest contribution of treatment targeting the NO system.

Exhaled NO measurements have been utilized as a simple and noninvasive method to measure and monitor pulmonary NO production. As of today it is mainly used to aid in diagnosis and monitoring of disease activity in patients with asthma. The data from others in the field of exhaled NO and our own results presented in Paper I indicate that there are disturbances in the pulmonary NO system that can be detected by exhaled NO measurements in patients with PH/PAH. Further research involving larger sets of patients is warranted with this technology to explore differences in pulmonary NO production between the different WHO PH groups. Potentially this technology may also be used as an attractive noninvasive method to monitor treatment response in PAH. However further studies are needed to confirm its potential.

In Paper II we found a clear association between vardenafil plasma concentrations and the acute hemodynamic effects on mPAP and PVR. Furthermore the resulting plasma concentrations following administration of a single oral dose of vardenafil varied substantially between patients in our study. Large interindividual variability and overlap in plasma concentrations following administration of PDE5 inhibitor treatment at different doses may explain the relatively weak association between dose and effect in the pivotal sildenafil and tadalafil phase III studies in PAH (150, 151). The pharmacokinetics in patients with PH/PAH may be altered due to heart failure. The mechanisms for altered drug metabolism in patients with congestive heart failure is multifaceted and include reduced hepatic drug elimination, impairment in drug absorption, reductions in the synthesis of plasma proteins, and decreased renal elimination of drugs (222-224). This highlights the need for a more careful evaluation of actual drug plasma concentrations in PH/PAH patients and individualised dose titration to obtain optimal drug concentrations in order to reach maximal benefit to the patients. With regard to PDE5 inhibitor treatment there is need for long-term studies to further evaluate the dose-concentration-effects relationships for sildenafil and tadalafil in particular, as the optimal
plasma concentration to be reached during chronic PDE5 inhibition with either of these two drugs has not been established in PAH.

The finding in Paper II where a drug-drug interaction between vardenafil and bosentan was demonstrated is in line with previous findings for sildenafil and tadalafil (188-190). The drug-drug interaction between bosentan and PDE5 inhibitors have clinical relevance as the current summary of product characteristics for sildenafil and tadalafil conclude that the efficacy of sildenafil or tadalafil in patients already on bosentan has not been conclusively demonstrated in patients with PAH (225, 226). This further underscores the importance of reaching optimal plasma drug concentrations. It remains to be demonstrated if higher doses of tadalafil or sildenafil, when combined with bosentan, are needed to achieve better clinical efficacy and offset the drug-drug interaction between bosentan and the two PDE5 inhibitors when they are used in combination in PAH.

Interestingly in Paper II the acute response to a single oral dose of vardenafil was similar between patients suffering from PAH (WHO group 1) and the patients with PH of other etiologies (WHO group 2-4). The current licensed indication for sildenafil and tadalafil is PAH (WHO group 1), however our results indicate that treatment with a PDE5 inhibitor may be beneficial in the other PH groups and not only in PAH. Specifically the potential of chronic PDE5 inhibition in patients with PH due to left heart disease (WHO group 2), patients with PH due to lung diseases and/or hypoxia (WHO group 3), and patients with CTEPH (WHO group 4), should be further explored.

ADMA is a potent endogenous NOS inhibitor and several studies have demonstrated that ADMA is elevated in PH/PAH, with elevated levels associated to poor prognosis (134, 139). Our findings in Paper III which indicate that the PDE5 inhibitor vardenafil, already at a single dose administration, have profound acute effects on biomarkers involved in the regulation and production of NO should be followed by further studies. In particular further studies in patients with PAH are warranted to evaluate whether chronic PDE5 inhibition is associated with stable lowering of ADMA and if this in turn is associated with improved long-term outcome for these patients.

An increasing interest to pathways involved in NO generation independent of the classical L-arginine-NO-cGMP has evolved within the cardiovascular research community as it has been demonstrated that NOS-independent generation of NO through the nitrate-nitrite-NO pathway provides an important and complementary way to transduce NO bioactivity (86). Exogenous administration of nitrate or nitrite is already being explored as new therapeutic options in a variety of cardiovascular and metabolic diseases were NO deficiency is considered as part of the pathophysiology (213). Treatment studies with nitrate and nitrite in preclinical models of PH/PAH demonstrating beneficial effects have been published and clinical data exist demonstrating benefits with
nitrite treatment in patients with PH/PAH (214, 215). The results described in Paper IV adds to the understanding of the nitrate-nitrite-pathway in PAH and indicate that there may be potential benefits associated with ingestion of inorganic nitrate, in the form of nitrate-rich beetroot juice, in patients with PAH. Further studies, with longer treatment duration and different dosing regimens, are warranted to further explore the benefits of nitrate-rich beetroot juice supplementation in patients with different PH etiologies. It would be of great importance for patients with PH/PAH if future studies with dietary supplementation of inorganic nitrate will prove to be of clinical benefit to these patients, in particular if it can be shown that inorganic nitrate in a daily amount that is readily achievable through a diet rich in fruits and vegetables is sufficient (227).

I believe that further research and exploration of the NO system in PH/PAH will uncover and reveal novel and important clues for the understanding of PH/PAH, and I am confident that patients will benefit from these efforts. I hope to continue working with academic studies concerning PH/PAH with a focus on vascular NO research and aim to broaden the research to include also preclinical PH/PAH models.
Acknowledgements

This thesis was carried out at the Department of Medical Sciences, Faculty of Medicine, Uppsala University, Sweden. I would like to express my gratitude to all of you who have supported me during these years and to all who have contributed to this thesis.

In particular I would like to acknowledge:

All the patients who participated in this research.

Gerhard Wikström, my principal supervisor, for your optimism, guidance, encouraging support and friendship. I admire your commitment to your patients and to clinical science.

Ulla Lindqvist, my co-supervisor, for your support and encouragement. You inspired me to become a rheumatologist.

The late Rolf Dahl, a great rheumatologist, for inspiring me to learn about patients with systemic sclerosis and PAH.

Jon O. Lundberg, for inspiring me to learn more about the NO system in general and the nitrate-nitrite-NO pathway in particular. “Inspiration is hard to come by. You have to take it where you find it” - according to Bob Dylan. Your lecture in Uppsala at the IMV seminar inspired me to plan and initiate the BEET-PAH study, to explore the effects of beetroot juice in patients with PAH – thank you for your inspiration, support, and collaboration.

My co-authors; Kjell Alving, Tomasz Baron, Kristoffer Björkstrand, Ulf Bondesson, Hanna Egeröd, André Eriksson, Sven-Olof Granstam, Mikael Hedeland, Hans Hedenström, Inga J. Ingimarsdóttir, Martin Jansson, Jon O. Lundberg, Andrei Malinovschi, Anna Sandqvist, and Mona-Lisa Wernroth for fruitful and valuable collaboration.

All former colleagues at the Department of Rheumatology, Uppsala University Hospital.
I am grateful for the support from my former employers (Uppsala University Hospital, Otsuka Pharma Scandinavia AB, and the Swedish Medical Products Agency) and present employer (Vifor Pharma Nordiska AB).

My parents for their never ending support.

My beloved wife Maria and my children, Julia, Alma, and Ester - the light and joy of my life - I love you with all my heart!
References


45. Francis SH, Blount MA, Zoraghi R, Corbin JD. Molecular properties of mammalian proteins that interact with cGMP: protein kinases, cation channels, phosphodiesterases, and multi-drug anion transporters. Front Biosci. 2005 Sep 1;10:2097-117.


79. McDermott JR. Studies on the catabolism of Ng-methylarginine, Ng, Ng-dimethylarginine and Ng,Ng-dimethylarginine in the rabbit. Biochem J. 1976 Jan 15;154(1):179-84.


Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 1461

Editor: The Dean of the Faculty of Medicine

A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)

Distribution: publications.uu.se
urn:nbn:se:uu:diva-347767

ACTA UNIVERSITATIS UPSALIENSIUS UPPSALA 2018