Dissertation

SCREENING FOR PAH AND ANALYSIS OF PROGNOSTIC MARKERS IN PULMONARY HYPERTENSION

submitted by

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Statutory Declaration

I hereby declare that this dissertation is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this dissertation. Due acknowledgement has been made in the text to all other material used. Throughout this dissertation and in all related publications I followed the guidelines of “Good Scientific Practice”

Date: 01.03.2016
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Abbreviations

6MWD = six minute walking distance
AF488= Alexa Fluor 488
AF700=Alexa Fluor 700
APC= allophycocyanine
APC-Cy7= allophycocyanine-cyanine7
APJ=apelin receptor
BMPRII= bone morphogenic protein receptor II
BNP= brain natriuretic peptide
BSA= bovine serum albumine
$C_2 = \alpha_x V_t$ Constant to account for the disappearance of soluble gas into lung tissue
COPD=chronic obstructive pulmonary disease
CRP= C-reactive protein
CT=computer tomography
CTEPH = chronic thromboembolic pulmonary hypertension
DLCO= diffusing capacity of the lung for carbon monoxide
ECFC= endothelial colony forming cells
ELISA= Enzyme-linked Immuno Sorbent Assay
EPC = endothelial progenitor cell
$E_{TA} =$ endothelin A receptor
$E_{TB} =$ endothelin B receptor
F=female,
FDA= Food and Drug Administration

FGF= fibroblast growth factor

GDF-15= growth differentiation factor 15

IPAH = idiopathic pulmonary arterial hypertension

M=male,

miRNA= micro RNA

mPAP= mean pulmonary arterial pressure,

MPs= microparticles

MRI=magnetic resonance imaging

mRNA= messenger ribonucleic acid

NA=not available

NO= nitric oxide

NT-proBNP = N-terminal fragment of pro-brain natriuretic peptide

NYHA = New York Heart Association

PAH= pulmonary arterial hypertension

PASMC= pulmonary arterial smooth muscle cell

PAWP= pulmonary arterial wedge pressure,

P_b = ambient pressure in mmHg.

PBF = pulmonary blood flow

PBS= phosphate buffered saline

pCO_{2}= partial pressure of carbon dioxide

PE= phycoerythrin
PECy7=phycoerythrin-cyanine7

PerCP= Peridinin chlorophyll

PPARγ = Peroxisome proliferator-activated receptor gamma

PVR= pulmonary vascular resistance,

RAP=right atrial pressure,

RHC= right heart catheterization

TWEAK= TNF-like weak inducer of apoptosis

$V_{s,tot} = \text{total systemic volume}$

$V_t = \text{Lung tissue volume}$

WU= Wood units

$\alpha_b = \text{Bunsen solubility coefficient in blood}$

$\alpha_t = \text{Bunsen solubility coefficient in tissue}$
Abstract in German

Die verfügbaren Screening-Methoden für die pulmonal arterielle Hypertonie (PAH) sind nicht ausreichend sensitiv und spezifisch. Daher gibt es einen Bedarf, weitere Methoden zu entwickeln, die die Dunkelziffer senken soll. Biomarker können wichtige Informationen liefern, die in Screening-Verfahren umgesetzt werden könnten. Hier präsentieren wir vier Ansätze, die verschiedene Merkmale der Krankheit bewerten und als Werkzeuge für die weitere Kontrolle oder Risikoevaluierung dienen könnten.

Erstens wurde die Inert Gas Rückatmungsmethode für die Beurteilung des Herzminutenvolumens bei PatientInnen mit pulmonaler Hypertonie (PH) während Rechtsherzkatheter eingesetzt. Die Methode ist relativ akkurat, hat aber eine geringe Präzision. Daher gelingt die Beurteilung der akuten Veränderungen des Herzzeitvolumens nicht zuverlässig genug.

Zweitens wurden Biomarker aus dem zirkulierenden Blut bei verschiedenen Formen der pulmonalen Hypertonie gemessen und ihre Bedeutung für die Diagnose von PH beurteilt. Apelin-17 und GDF-15 wurden als vielversprechende zirkulierende Biomarker für die idiopathische pulmonal arterielle Hypertonie (PAH) und die chronisch thromboembolische pulmonale Hypertonie identifiziert.

Drittens wurde das Niveau und die Aktivität von endothelialen Mikropartikeln bei PAH-PatientInnen beurteilt, die eine Rechtsherzkatheteruntersuchung erhalten hatten. Endotheliale Mikropartikel waren bei PAH erhöht. Ob sie allerdings eine Rolle in der Pathogenese der PH spielen oder ob ihre erhöhte Anzahl die Folge der Krankheit ist, muss weiter untersucht werden.

Viertens wurden zirkulierende mononukleäre Zellen und deren Subpopulationen bei PAH untersucht. Die relative Anzahl von zirkulierenden CD133 Progenitorzellen, die aus dem Knochenmark stammen, war bei PAH erhöht. Überraschenderweise entsprechen die im Lungengewebe nachweisbaren CD133 positiven Zellen phänotypisch verschiedenen Populationen, wobei Typ II Pneumozyten überwiegen.
Abstract in English

Available screening tools for pulmonary arterial hypertension (PAH) are not sufficiently sensitive and specific, hence there is an unmet need to further develop tools that may increase the number of detected PAH patients. Biomarkers may deliver important information that could be implemented in screening processes. Here we present four approaches that assess different characteristics of the disease and may serve as tools for further screening or risk stratification.

First, inert gas rebreathing method was used for assessing cardiac output in patients with pulmonary hypertension (PH) undergoing right heart catheterization. It may represent a promising method for non-invasive assessment of cardiac output in pulmonary hypertension, although assessment of acute changes may not be reliable.

Second, blood-derived markers were measured in different forms of PH and their relevance for diagnosis of PH was assessed. Apelin-17 and GDF-15 were identified as promising circulating serum derived biomarkers for idiopathic pulmonary arterial hypertension and chronic thromboembolic pulmonary hypertension.

Third, the level and activity of endothelial microparticles in PAH patients undergoing diagnostic or follow-up right heart catheterization was assessed. Endothelial microparticles are elevated in the circulation of PAH. Whether they play a role in the pathogenesis of PH or their elevation is a result of the disease remains to be determined.

Fourth, circulating mononuclear cells and their subpopulations were investigated in PAH. The relative number of bone marrow-derived circulating CD133 positive progenitor cells was found to be elevated in PAH patients. In the lung tissue, CD133 positive cells consist of phenotypically different populations including pneumocytes type II.
1. Introduction

1.1 Screening for pulmonary arterial hypertension

Although pulmonary arterial hypertension (PAH) is acknowledged as an orphan disease, accumulating evidence suggests that the number of affected patients is increasing. Current registries and WHO reports have shown that this number is not as low as previously reported (1–14). This is probably due to increased awareness in the community and better diagnostic approaches but not to the development of effective screening tools. There is a consensus that available screening tools are not sufficiently sensitive and specific, hence there is an unmet need to further develop tools that may increase the number of detected PAH patients (15). Biomarkers may deliver important information that could be implemented in screening processes. According to the definition of the National Institute of Health, a biomarker „is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.“ (16).

Taken into account that patients with PAH present with very unspecific signs and symptoms, the need for objectively measurable markers is unquestionable. In addition to these clinical considerations, biomarker research represents an effort to describe links between blood derived biomarkers and the pathology of PAH as it has been very successfully done in the case of BNP and NT-proBNP (17,18). The importance of non-invasive assessment of PAH has been emphasized at the recent world conference (15). Like in the case of many other diseases, the search for biomarkers in PAH focuses for markers for diagnosis, prognosis and response to therapy.

1.2 Diagnostic markers

Despite all efforts to develop non-invasive diagnostic tools, right heart catheterization has remained the gold standard method to diagnose pulmonary hypertension and precisely assess pulmonary hemodynamics. Nevertheless, there have been promising studies suggesting that non-invasive methods and their appropriate combination may play an increasing role in the diagnostic and prognostic assessment as well as follow-up of the disease and may aid clinical decisions.
An interesting approach is the imaging of the pulmonary hypertensive lung and the consecutively changed right heart using echocardiography, CT, and MRI. Several studies have attempted to design a technique or method that can replace right heart catheterization. Some of them show impressively good results, however, the studies are limited by highly selected populations, and a low number of investigated patients. Additionally, validation studies are still warranted. As an example, dynamic contrast-enhanced computer tomography was used in a pilot project aiming to detect pulmonary hypertension in a non-invasive manner. Contrast material propagation time was measured and based on these, patients with PH could be identified (19).

Systemic sclerosis is a disase where pulmonary hypertension is not only common but its presence has a negative impact on survival and quality of life. There were several attempts to describe algorithms that would reliably predict the presence of pulmonary vasculopathy in this group. A collaborative effort has made possible that 62 centers participated in a study aiming to develop an evidence-based algorithm for the presence of PAH (20).

1.3 Prognostic markers

There were several modalities as well as concepts that were applied in search for markers that would be used for prognosis. Clinical as well as blood-derived markers have been applied in several studies. The markers were recently reviewed by several authors (21–26).

In the follow-up of PAH patients it is important to repeatedly assess parameters which are prognostically relevant like cardiac output (CO). Dynamic contrast-enhanced computer tomography may be a promising tool for non-invasive CO assessment. Attenuation time curves served as measures for cardiac output. The results correlated well with CO values obtained by thermodilution (27). Impedance cardiography is another non-invasive tool for monitoring pulmonary hemodynamics. A prospective study found that there is a strong correlation between invasive hemodynamics and values obtained with impedance cardiography based on a cohort that included PH patients who underwent right heart catheterization (28).
1.4 Markers related to therapy response

Little is known about markers in PAH that would reliably predict response to treatment. In fact, the only determinant of a long-term prognosis in PAH is a positive vasoreactive response to inhaled NO during right heart catheterization which predicts a long-term response to therapy with high-dose calcium channel blockers (29–31).

1.5. Inert gas rebreathing for non-invasive pulmonary blood flow measurement using the Innocor system.

In addition to PH, there may be other indications to assess cardiac output and pulmonary blood flow where inert gas rebreathing may represent a promising new tool. The inert gas rebreathing method was evaluated in 28 consecutive patients with pulmonary fibrosis (32) and compared to the thermodilution method. According to the authors, both methods showed a high correlation and the Bland-Altman analysis revealed a mean difference of only -0.32 L/min and limits of agreement of -2.10 to +1.45. As a control, the authors also performed the measurements in 23 healthy subjects and found a similarly good agreement between the thermodilution method and the inert gas rebreathing.

In patients with heart failure, besides the assessment of cardiac output, Innocor was also used to determine oxygen uptake ($VO_2$) at rest and during exercise. As a result, a linear correlation could be identified between peak $VO_2$ and maximal cardiac output (33). The authors conclude that Innocor may be a reliable tool for $VO_2$ assessment. Although measurements during exercise may be challenging, in 86% of patients successful measurements could be performed. This was a promising result.

The determination of cardiac output during exercise is essential to characterize the physiologic properties of the pulmonary circulation and to recognize early hemodynamic changes. Inert gas rebreathing may be an alternative for invasive methods during exercise examinations. In early studies, during dobutamine stress challenge in mechanically ventilated dogs, the inert gas rebreathing system was shown to be a promising method to assess cardiac output during exercise as the obtained values strongly correlated with the values obtained by the thermodilution method ($r=0.9$) (34). The Innocor device was tested in a physiological study on 16
male cyclists who underwent standard graded exercise tests. It has been shown that the rebreathing method was suitable to assess cardiac output during exercise (35,36).

In a small swiss cohort, four methods were compared to assess cardiac output during exercise: the Fick method, the inert gas rebreathing method, impedance cardiography and pulse contour analysis (37). Twelve male subjects were included and measurements were performed simultaneously. All methods detected an increase in cardiac output during exercise, however, as compared to the other three methods, the inert gas rebreathing technique underestimated the individual values. A potential limitation was the recirculation of the gas into the pulmonary circulation resulting in incorrect cardiac output values.

1.6. Apelin and its role in the pathobiology of pulmonary hypertension

The following table summarizes the studies where apelin and its isoforms were assessed in different conditions.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Apelin level change</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early stage heart failure</td>
<td>↑</td>
<td>Kadoglou NP et al (38)</td>
</tr>
<tr>
<td>Late stage heart failure</td>
<td>↓</td>
<td>Kadoglou NP et al (38)</td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td>↓</td>
<td>Akboga MK et al (39)</td>
</tr>
<tr>
<td>Acute myocardial infarction</td>
<td>↓</td>
<td>Akboga MK et al (39)</td>
</tr>
<tr>
<td>IPAH</td>
<td></td>
<td>Goetze JP et al (40)</td>
</tr>
<tr>
<td>Type-2 diabetes mellitus</td>
<td>↑</td>
<td>Cavallo MG et al (41)</td>
</tr>
<tr>
<td>Childhood obesity</td>
<td></td>
<td>Machura E et al. (42)</td>
</tr>
<tr>
<td>COPD, fibrosis, emphysema</td>
<td>↑</td>
<td>Goetze JP et al (40)</td>
</tr>
<tr>
<td>Systemic sclerosis</td>
<td>↑</td>
<td>Aozasa N et al (43)</td>
</tr>
<tr>
<td>Gastroesophageal cancer</td>
<td>↑</td>
<td>Diakowska D et al (44)</td>
</tr>
</tbody>
</table>

The apelin receptor has a high sequence similarity to the angiotensin receptor AT1. Hence it is not surprising that the APJ/apelin axis is involved in several cardiologic conditions. It has been recently shown that apelin levels are elevated in
early-stage heart failure and decrease in a later stage. On the contrary, apelin levels were lower in patients with coronary artery disease as compared to age- and sex-matched healthy controls (38). Patients with unstable angina and acute myocardial infarction had even lower levels of apelin and logistic regression analysis showed an independent association between low apelin levels and the presence of coronary artery disease. Interestingly, in the subgroup of patients with stable coronary artery disease and a preserved collateral circulation, apelin levels were elevated (39).

Besides the coronary circulation, the APJ/apelin axis may play a relevant role in the pulmonary circulation. Apelin is expressed by pulmonary vascular endothelial cells whereas its receptor is present in both endothelial and smooth muscle cells. In a nitrofen-induced congenital diaphragmatic hernia model, apelin and its receptor were both decreased on the mRNA and protein level. This suggests that apelin may play a role already in the early development of vascular abnormalities (45).

The origin of apelin in the circulation has been discussed at length. Andersen CU et al. elegantly showed that levels of apelin did not change in the lung tissue exposed to hypoxia as compared to normoxic lung tissue. The increase of right ventricular pressure under hypoxia leads to proportional increase on apelin content of the heart. These changes were, however, not associated with plasma apelin levels. In wire myograph experiments, apelin was able to dilate endothelin-1 and angiotensin-II preconstricted arteries from normoxic rats. When these arteries were isolated from hypoxic rats, the vasodilator effect of apelin was not present anymore. This suggests that apelin has an important role in mediating vasodilation in normoxia and provides a proof for the blunted apelin signaling in the setting of pulmonary hypertension (46). Based on these results it may be concluded that the main source of apelin is the right heart.

There are several potential mechanisms, how apelins may be protective against PH and how the lack of apelin may lead to development of the disease. In an experimental setting using monocrotaline-induced pulmonary hypertension, injection of apelin caused a decrease in myocardial injury and improved right ventricular function in rats (47). In addition, apelin-12 increased contractility in the failing right heart in hypoxic rats in a dose dependent manner, mainly due to a positive inotropic action (48). Apelin knock-out mice developed a more severe PH compared to wild type mice when they were exposed to hypoxia (49).
Adressing an alternative mechanism, when hypoxic pulmonary smooth muscle cells were treated with apelin, the proliferation and migration of them was inhibited. This occurred under the activation of the PI3K/Akt/mTOR pathway (50). Apelin inhibited proliferation and induced apoptosis in PASMCs. When PPARγ was deleted in endothelial cells, mice were apelin-deficient. These animals developed pulmonary hypertension. Moreover, administration of apelin reversed pulmonary hypertension in these mice (51).

Apelin mRNA expression is regulated by BMPRII signaling. The presence of impaired BMPRII signaling in pulmonary hypertension is a challenging pathway for the industry that aims to develop therapeutic targets. In a recent study more that 3,000 FDA-approved bioactive compounds were tested and the strongest signal for BMPRII activation was achieved with tacrolimus. In addition, it has been shown that low-dose tacrolimus, is able to induce apelin signaling in pulmonary arterial endothelial cells derived from IPAH patients (52) and there may be clinical effects (53).

There is a difference between the molecular signature of right ventricular failure and adaptive right ventricular hypertrophy. A gene expression pattern difference was found between these two entities by microarray analysis. The most regulated genes were angiogenetic factors such as vascular endothelial growth factor, insulin-like growth factor 1, apelin, angiopoetin-1, as well as a set of glycolytic enzymes. Cell growth, angiogenesis and energy metabolism differed in both models (54). The result that the expression of the apelin gene is mainly upregulated in the failing right heart points to the hypothesis that the main source of apelin is the right ventricle.

MicroRNA network analysis based results lead to the finding that miR-130/301 modulated apelin-miR-424/503-FGF2 signaling in endothelial cells (55). In pulmonary arterial endothelial cells, there is an apelin dependent miRNA-FGF signaling which points to the importance of apelin in the pulmonary vascular bed. Apelin deficiency led to increased expression of FGF2 and its receptor FGFR1 which was mediated by two known micro RNAs already described in pulmonary hypertension, miR-424 and miR-503 (56).
1.7. *Circulating microparticles in pulmonary hypertension*

Microparticles are phospholipid vesicles derived from different types of cells such as leukocytes, platelets, endothelial cells. The surface marker expression of these vesicles is highly dependent on the cell from which they are originating. For example, CD39 is a newly identified surface marker which has been found to be present on platelet and endothelial microparticles from patients with IPAH (57).

Cell activation and apoptosis are the most important mechanisms that lead to microparticle formation. These phenomena are common in pulmonary hypertension which may explain why in a recent study pro-coagulatory microparticles were elevated in 19 patients with pulmonary hypertension as compared to 16 controls. In the analysis platelet-, leukocyte- and endothelial microparticles were included and the investigated microparticles were defined as follows: platelet (CD31+CD61+), leukocyte- (CD11b+) and endothelial- (CD62E+) (58). The study also revealed a correlation of the levels of these microparticles with each other.

Microparticles might also have a pathophysiologic role in the development of PH (59) or, according to another hypothesis they may represent a response to vascular changes (57). There is evidence that the prostacyclin analogue epoprostenol inhibits platelet microparticle formation (60,61). In the monocrotalin-induced pulmonary hypertension rat model it has been shown that microparticles may induce changes which are characteristic for pulmonary hypertension (59). In a recent report, in rats with experimental pulmonary embolism followed by pulmonary hypertension, isolated microparticles from the blood were subject to proteomic analysis. Mass spectrometry revealed that proteins involved in clotting and thrombus formation were elevated suggesting a possible involvement of endothelial microparticles in the pathogenesis of pulmonary embolism followed by chronic thromboembolic pulmonary hypertension (62). A similar study was performed on rats exposed to hypoxia and it could be shown that microparticles originating from hypoxic rats may induce endothelial dysfunction.

In vitro, microparticles increased oxidative stress in pulmonary endothelial cells but not in aortic endothelial cells (63) suggesting that they are biologically and selectively active. This is supported by the finding that microparticles can transfer
receptors or antigens to such cells which are not specific to their origin. Their involvement in inflammation, coagulation and cardiovascular diseases, including PAH (64) and their potential use as treatment tools (65) has been reviewed (66). In the end, however, it is still uncertain, whether circulating microparticles in the lung milieu are bystanders of the disease or if they play an active role in the pathogenesis of pulmonary hypertension.

The idea that circulating microparticles can be used as biomarkers for pulmonary hypertension has been addressed in recent investigations. A report proposed that endothelial microparticles might be early markers of pulmonary hypertension in Eisenmenger Syndrome, as CD144 and CD146 positive endothelial microparticles were elevated in Eisenmenger patients (n=16) as compared to controls (n=37), and pulmonary artery intima/media thickness was positively correlated with CD144 microparticle level (67).

1.8. Circulating progenitor cells in pulmonary arterial hypertension

Circulating cell populations may play an important role among the pathologic mechanisms in pulmonary hypertension (68,69). According to recent studies, circulating mesenchymal precursors of monocyte/macrophage lineage, as well as c-kit positive cells accumulate in the remodelled arteries of patients and contribute to the thickening of the vessel wall (70–72). In addition, circulating endothelial progenitor cells (EPCs), fibrocytes and mast cells may also contribute to the development of the disease (73–75). In contrast, a recent investigation has shown that circulating cells may be protective against the development of PH, as regulatory T cells attenuated pulmonary vascular injury and endothelial dysfunction (76).

Due to the differences in the number of circulating cells between PAH and controls, circulating cells were discussed as possible biomarkers for the disease (77). Elevated levels of circulating EPCs have been reported in pulmonary arterial hypertension (PAH) patients (75,78). However, in other studies, idiopathic PAH, Eisenmenger and sickle cell disease related PAH patients had lower levels of EPCs than controls (79–81). A potential explanation for conflicting results is that there is no
generally accepted standard for the phenotypic characterization and definition of these cell populations (82). Missing standards for cell isolation as well as differences in the pathologic mechanisms of the investigated patients may also have contributed to these discrepancies. There is a consensus that further investigations are necessary to answer the question if the number of circulating progenitors is a suitable marker of PAH and what role these cells may play in the pathology of the disease (82).
Aims

1. To test the inert gas rebreathing method for assessing cardiac output in patients with pulmonary hypertension undergoing right heart catheterization

2. To search for blood-derived markers in different forms of pulmonary hypertension and to assess their relevance for diagnosis

3. To assess the level and activity of endothelial microparticles in pulmonary arterial hypertension patients undergoing diagnostic or follow-up right heart catheterization

4. To characterize circulating progenitor cells in pulmonary arterial hypertension
2. Material and methods

This thesis describes the results of five studies performed in the field of biomarkers for pulmonary vascular remodelling and hypertension (indicated below from 2.1 to 2.5). The methods used in the particular studies are listed consecutively.

2.1 Inert gas rebreathing for non-invasive pulmonary blood flow measurement using the Innocor System

The inert gas rebreathing method has been previously described on small patients cohorts with pulmonary hypertension as a novel non-invasive technique to measure cardiac output (CO) using the single rebreathing method (83,84). Assuming there is no intracardiac shunt, pulmonary blood flow (PBF) refers to the blood volume which flows from the right ventricle through the lung vessels and reflects the cardiac output (Peacock A et. al, Pulmonary Circulation 2011, third edition).

Recruitment of the patients

In our prospective observational study we included n=35 patients undergoing diagnostic or follow-up right heart catheterization (RHC) with strong suspicion for pulmonary hypertension. The study was approved by the local ethics committee (24-079 ex 11/12) and was registered on the ClinicalTrials.gov (Identifier: NCT01606839). All patients who participated in the study gave their written informed consent. Inclusion criteria were written informed consent, diagnostic or follow-up right heart catheterisation. If the diagnosis of pulmonary hypertension was not known, patients with known comorbidities (significant heart, lung, liver, kidney and blood diseases) were also included. The diagnosis of pulmonary hypertension was excluded if mean pulmonary arterial pressure (mPAP) was < 25mmHg as assessed during right heart catheterization. Patients with newly diagnosed pulmonary hypertension underwent the diagnostic algorithm according to the recent guidelines (85). Patients´characteristics are shown in Table 1.
Table 1. Patients’ characteristics that underwent simultaneous cardiac output measurements during right heart catheterization using the thermodilution and the inert gas rebreathing method. Values are expressed as mean ± standard deviation

<table>
<thead>
<tr>
<th></th>
<th>No PH (n=16)</th>
<th>PH (n=19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years) (mean ± SD)</td>
<td>57 ± 13.4</td>
<td>62 ± 12.4</td>
</tr>
<tr>
<td>Sex (F/M)</td>
<td>13/3</td>
<td>12/7</td>
</tr>
<tr>
<td>mPAP (mmHg) (mean ± SD)</td>
<td>16.8 ± 3.9</td>
<td>38.6 ± 11.6</td>
</tr>
<tr>
<td>RAP (mmHg) (mean ± SD)</td>
<td>5.9 ± 2.1</td>
<td>9.7 ± 5.2</td>
</tr>
<tr>
<td>PAWP (mmHg) (mean ± SD)</td>
<td>8.6 ± 3</td>
<td>11.4 ± 5.8</td>
</tr>
<tr>
<td>PVR (WU) (mean ± SD)</td>
<td>1.8 ± 0.9</td>
<td>7.3 ± 4.5</td>
</tr>
<tr>
<td>6MWD (m) (mean ± SD)</td>
<td>466 ± 75</td>
<td>360 ± 130</td>
</tr>
<tr>
<td>WHO class (I/II/III/IV)</td>
<td>3/11/2/0</td>
<td>0/11/8/0</td>
</tr>
<tr>
<td>NT-proBNP (pg/mL) (mean ± SD)</td>
<td>590 ± 1532</td>
<td>2637 ± 3847</td>
</tr>
</tbody>
</table>

**Abbreviations:** F=female, M=male, mPAP= mean pulmonary arterial pressure, RAP=right atrial pressure, PAWP= pulmonary arterial wedge pressure, PVR= pulmonary vascular resistance, WU= Wood units, 6MWD= six-minute walk distance, WHO class= World Health Organization functional class, NT-proBNP= N-terminal pro-brain natriuretic peptide
Pulmonary blood flow (PBF) measurements were carried out using the inert gas rebreathing system (Innocor®, Innovision, Kaltenkirchnen, Germany). A mixture of blood soluble (nitrous oxide (N₂O)) and blood insoluble gas (sulphur hexafluoride (SF₆)) and environmental air is inhaled and the concentration of N₂O, SF₆, O₂ and CO₂ is measured by a photoacoustic analysor. The duration of a measurement is usually about one minute. Figure 1 demonstrates the volumes used for PBF calculation.

**Figure 1.** Systemic volumes used for pulmonary blood flow calculation using the inert gas rebreathing method.

The total systemic volume is defined as: \( V_{s,tot} = V_L + V_{ds} + V_{ds,rb} + V_{rb} \), where \( V_L \) = Lung volume at the end of an expiration, \( V_{ds} \) = dead space volume of rebreathing valve, \( V_{ds,rb} \) = residual volume of bag when empty, \( V_{rb} \) = volume of rebreathing bag. The inert gas rebreathing system is based on the principle that a known concentration/volume
of soluble and an insoluble gas are being rebreathed where the soluble gas is a nitrous oxide (N$_2$O) and the insoluble gas is sulfur hexafluoride (SF$_6$). The pulmonary blood flow can be calculated using the following formula:

$$PBF = -\beta \cdot \frac{V_{s,tot} \cdot C_1 + C_2}{\alpha_b}$$

where $PBF =$ pulmonary blood flow, $V_{s,tot} =$ total systemic volume, $C_1 = 760/(P_B-47)$, $C_2 = \alpha_t \times V_t$. Constant to account for the disappearance of soluble gas into lung tissue, $\alpha_b =$ Bunsen solubility coefficient in blood (for N$_2$O = 0.412), $\alpha_t =$ Bunsen solubility coefficient in tissue (for N$_2$O = 0.407), $V_t =$ Lung tissue volume (default 600 ml), $P_B =$ ambient pressure in mmHg.

If there is no hemodynamically relevant intrapulmonary shunt, the pulmonary blood flow represents the cardiac output. Figure 2 represents an example of a healthy subject for oxygen uptake measurement:

![Figure 2. Measurement diagram for oxygen. X-axis depicts the percentage of normalized oxygen concentration. Y-axis represents time expressed in seconds. Blue regression line through expiratory points rebreathing curve duration and timepoint of expiration](image-url)
bars represent the duration and timepoint of expiration when the measurements were taken. Green line depicts the regression line through the expiratory points.

Figure 3 depicts a rebreathing diagram for soluble gas from the same healthy subject showing decreasing concentration of oxygen within three breathing cycles (blue bars).

**Figure 3.** Semilogarithmic plot for soluble gas (N₂O) X-axis depicts the decreasing concentrations of the soluble gas. Y-axis represents time expressed in seconds. Blue bars represent the duration and timepoint of expiration when the measurements were taken. Green line depicts the regression line through the expiratory points.

Figure 4 represents the diagram for insoluble gas from the same healthy subject. The concentration of the insoluble gas remains constant during the measurements and its concentration serves as a reference. Hence, due to no concentration loss, the regression line remains horizontal as depicted in Figure 4.
**Figure 4. Semilogarithmic plot for insoluble gas (SF₆)** X-axis depicts the concentrations of the insoluble gas. Y-axis represents time expressed in seconds. Blue bars represent the duration and timepoint of expiration when the measurements were taken. Green line depicts the regression line through the expiratory points.

**Study procedure**

The patients were instructed to breath with a mean rate of 20 breaths/minute during SF₆ and N₂O rebreathing in a closed system. In order to monitor for acute hemodynamic changes after start of a PAH-specific targeted therapy and to compare the results with the thermodilution method (RHC), the measurements using the Innocor system were carried out during diagnostic or follow-up right heart catheterizations and combined with pharmacological testing. One baseline measurement was carried out after introducing the catheter and further measurements after sildenafil and iloprost inhalation. During RHC the thermodilution method (which is considered a gold standard) was used for CO assessment and direct comparison with inert gas rebreathing. During a pharmacologic testing,
measurements were performed at baseline, 35 min after sildenafil, 65 min after sildenafil, 14 min after iloprost and 35 min after iloprost application (Figure 5).

Pharmacologic testing protocol

In some cases, the patients underwent an exercise testing with ergometer. Here, the PBF was measured at baseline. By exercise testing with spiroergometry PBF was measured at baseline and in the supine position. Additionally, 5 healthy controls were tested in different settings (bag volume, gas bolus, body position) in order to test the inert gas rebreathing system for relevant measurement variables.

2.2 Pilot studies for blood-derived biomarker assessment

Based on a literature search, candidate molecules as listed below were selected for the pilot studies which were then assessed in a prospective manner based on a power analysis.

Recruitment of the patients for the pilot study

In order to assess the blood levels of candidate biomarkers for pulmonary hypertension, randomly selected patients of our Centre for Pulmonary Hypertension (patients with IPAH, CTEPH) as well as sex- and age-matched healthy volunteers participated in the pilot study. Inclusion criteria for IPAH: signed informed consent,
mPAP≥25mmHg, PAWP≤15mmHg with no significant heart, lung, liver, kidney and blood diseases. Inclusion criteria for CTEPH: signed informed consent, mPAP≥25mmHg, PAWP≤15mmHg with no significant heart, lung, liver, kidney and blood diseases and the presence of pulmonary chronic thromboembolism assessed by ventilation perfusion lung scan. Excluded were all patients with mPAP<25mmHg, PAWP>15mmHg and the presence of significant heart, lung, liver, kidney and blood diseases. All study participants gave their written informed consent. The study was approved by the local ethics committee (23-408 ex 10/11). Patients´ characteristics are shown in Table 2.

**Table 2.** Patients´ characteristics that participated in the pilot study for serum biomarkers. Values are expressed as mean ± standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>Control (n=10)</th>
<th>CTEPH (n=10)</th>
<th>IPAH (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years) (mean ± SD)</td>
<td>57.5 ± 16.4</td>
<td>69.2 ± 14.9</td>
<td>57.5 ± 16.4</td>
</tr>
<tr>
<td>Sex (F/M)</td>
<td>8/2</td>
<td>4/6</td>
<td>8/2</td>
</tr>
<tr>
<td>mPAP (mmHg) (mean ± SD)</td>
<td>NA</td>
<td>52.2 ± 8.4</td>
<td>40.6 ± 10.9</td>
</tr>
<tr>
<td>RAP (mmHg) (mean ± SD)</td>
<td>NA</td>
<td>10.8 ± 3.3</td>
<td>5.5 ± 5.2</td>
</tr>
<tr>
<td>PAWP (mmHg) (mean ± SD)</td>
<td>NA</td>
<td>9.5 ± 2.7</td>
<td>7.4 ± 3</td>
</tr>
<tr>
<td>PVR (WU) (mean ± SD)</td>
<td>NA</td>
<td>10.9 ± 2.5</td>
<td>5.4 ± 2.7</td>
</tr>
<tr>
<td>6MWD (m) (mean ± SD)</td>
<td>NA</td>
<td>297.3 ± 154.2</td>
<td>389.4 ± 102</td>
</tr>
<tr>
<td>WHO class (I/II/III/IV)</td>
<td>NA</td>
<td>0/3/7/0</td>
<td>0/4/6/0</td>
</tr>
<tr>
<td>NT-proBNP (pg/mL) (mean ± SD)</td>
<td>NA</td>
<td>2662.4 ± 2205.7</td>
<td>554.3 ± 553.8</td>
</tr>
</tbody>
</table>
**Abbreviations:** F=female, M=male, mPAP= mean pulmonary arterial pressure, RAP= right atrial pressure, PAWP= pulmonary arterial wedge pressure, PVR= pulmonary vascular resistance, WU= Wood units, 6MWD= six-minute walk distance, WHO class= World Health Organization functional class, NT-proBNP= N-terminal pro-brain natriuretic peptide, CTEPH= chronic thromboembolic pulmonary hypertension, IPAH= idiopathic pulmonary arterial hypertension, NA= not available

**Procedure of blood sampling**

Peripheral blood was taken from an antecubital vein in 9 mL separation gel containing Vacutainer® tubes. Serum samples were kept for 30 minutes on room temperature, then centrifuged on 1500 G for 10 minutes and aliquoted to 250 µL. They were stored at -80°C in Biobank of Medical University of Graz until used.

Commercially available enzyme like immunosorbent assays (ELISA) were used to determine the concentrations of apelin-12 (antibodies-online, Cat. No: ABIN366820), apelin-13 (Biotrend Chemikalien, Cat. No: CSB-E13072h), apelin-17 (USCN Life Sciences, Cat. No:CED065Hu), apelin-36 (Biotrend Chemikalien, Cat. No: CSB-E13566h), GDF-15 (R&D Systems, Cat. No.: DGD150) and TWEAK (EIAab, Cat. No.: E1883h). Based on a power calculation (see below), four targets were selected and the study population was extended as given below.

**2.3. Prospective study for apelin isoforms and GDF-15**

**Recruitment of the patients**

Randomly selected patients of our Centre for Pulmonary Hypertension (patients with IPAH, CTEPH) as well as sex- and age-matched healthy donors participated in the prospective study. Inclusion criteria for IPAH: signed informed consent, mPAP≥25mmHg, PAWP≤15mmHg with no significant heart, lung, liver, kidney and blood diseases. Inclusion criteria for CTEPH: signed informed consent, mPAP≥25mmHg, PAWP≤15mmHg with no significant heart, lung, liver, kidney and blood diseases and the presence of pulmonary chronic thromboembolism assessed by ventilation perfusion lung scan. Excluded were all patients with mPAP<25mmHg, PAWP>15mmHg and the presence of significant heart, lung, liver, kidney and blood
diseases. All study participants gave their written informed consent. The study was approved by the local ethics committee (23-408 ex 10/11).

The cohort was built up based on the results of the pilot study using the power analysis with a power of 80%. Accordingly, thirty-one IPAH patients and their age- and sex matched healthy controls as well as twenty-four CTEPH patients were included. Patients’ characteristics are shown in Table 3.

**Table 3.** Patients’ characteristics that participated in the prospective study for serum biomarkers. Values are expressed as mean ± standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>Control (n=31)</th>
<th>CTEPH (n=24)</th>
<th>IPAH (n=31)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years) (mean ± SD)</strong></td>
<td>55.9 ± 17.1</td>
<td>65 ± 14.6</td>
<td>56.8 ± 17</td>
</tr>
<tr>
<td><strong>Sex (F/M)</strong></td>
<td>23/8</td>
<td>15/9</td>
<td>23/8</td>
</tr>
<tr>
<td><strong>mPAP (mmHg) (mean ± SD)</strong></td>
<td>NA</td>
<td>41.3 ± 12.8</td>
<td>49.2 ± 18.8</td>
</tr>
<tr>
<td><strong>RAP (mmHg) (mean ± SD)</strong></td>
<td>NA</td>
<td>8 ± 4</td>
<td>7 ± 4</td>
</tr>
<tr>
<td><strong>PAWP (mmHg) (mean ± SD )</strong></td>
<td>NA</td>
<td>9 ± 3</td>
<td>9 ± 4</td>
</tr>
<tr>
<td><strong>PVR (WU) (mean ± SD)</strong></td>
<td>NA</td>
<td>8.3 ± 4.2</td>
<td>10 ± 7.3</td>
</tr>
<tr>
<td><strong>6MWD (m) (mean ± SD)</strong></td>
<td>NA</td>
<td>353 ± 133</td>
<td>378 ± 129</td>
</tr>
<tr>
<td><strong>WHO class (I/II/III/IV)</strong></td>
<td>NA</td>
<td>1/11/12/0</td>
<td>0/13/15/3</td>
</tr>
<tr>
<td><strong>NT-proBNP (pg/mL) (mean ± SD)</strong></td>
<td>NA</td>
<td>2009 ± 2688</td>
<td>1452 ± 785</td>
</tr>
</tbody>
</table>

**Abbreviations:** F=female, M= male, mPAP= mean pulmonary arterial pressure, RAP= right atrial pressure, PAWP= pulmonary arterial wedge pressure, PVR= pulmonary vascular resistance, WU= Wood units, 6MWD= six-minute walk distance, WHO class= World Health Organization functional class, NT-proBNP= N-
terminal pro-brain natriuretic peptide, CTEPH= chronic thromboembolic pulmonary hypertension, IPAH= idiopathic pulmonary arterial hypertension, NA= not available

2.4. Assessment of circulating endothelial microparticles in PH

Recruitment of the patients

All patients undergoing diagnostic or follow-up right heart catheterization at our Centre for Pulmonary Hypertension were included in a prospective manner. Inclusion criteria for PAH: signed informed consent, mPAP≥25mmHg, PAWP≤15mmHg with no significant lung, liver, kidney and blood diseases. Inclusion criteria for CTEPH: signed informed consent, mPAP≥25mmHg, PAWP≤15mmHg with no significant heart, lung, liver, kidney and blood diseases and the presence of pulmonary chronic thromboembolism assessed by ventilation perfusion lung scan. Excluded were all patients with mPAP<25mmHg, PAWP>15mmHg and the presence of significant lung, liver, kidney and blood diseases. Healthy volunteers served as controls. All study participants gave their written informed consent for the participation in the study which was approved by the local ethics committee (23-408 ex 10/11). Patients’ characteristics are shown in Table 4.

Study procedure

During the RHC, six milliliter of central venous blood was taken into sodium cytrate tubes. The samples were ultracentrifuged and aliquoted within one hour and stored in aliquots for the further procedure. The level of endothelial microparticles was assessed using Innovaence ETP kit (Siemens Healthcare Diagnostics, Cat No.: OPGA035). Endogenous thrombin potential was determined using commercially available ELISA kit (Zymuphyen, Coachrom Diagnostica, Cat. No.: 521096).
Table 4. Patients’ characteristics that participated in the prospective study for assessment of circulating endothelial microparticles. Values are expressed as mean ± standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>Control (n=15)</th>
<th>CTEPH (n=16)</th>
<th>PAH (n=36)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years) (mean ± SD)</td>
<td>57.5 ± 16.4</td>
<td>69.2 ± 14.9</td>
<td>57.5 ± 16.4</td>
</tr>
<tr>
<td>Sex (F/M)</td>
<td>8/2</td>
<td>4/6</td>
<td>8/2</td>
</tr>
<tr>
<td>mPAP (mmHg) (mean ± SD)</td>
<td>NA</td>
<td>52.2 ± 8.4</td>
<td>40.6 ± 10.9</td>
</tr>
<tr>
<td>RAP (mmHg) (mean ± SD)</td>
<td>NA</td>
<td>10.8 ± 3.3</td>
<td>5.5 ± 5.2</td>
</tr>
<tr>
<td>PAWP (mmHg) (mean ± SD )</td>
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</tr>
<tr>
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<td>0/4/6/0</td>
</tr>
<tr>
<td>NT-proBNP (pg/mL) (mean ± SD)</td>
<td>NA</td>
<td>2662.4 ± 2205.7</td>
<td>554.3 ± 553.8</td>
</tr>
</tbody>
</table>

**Abbreviations:** F=female, M=male, mPAP= mean pulmonary arterial pressure, RAP=right atrial pressure, PAWP= pulmonary arterial wedge pressure, PVR= pulmonary vascular resistance, WU= Wood units, 6MWD= six-minute walk distance, WHO class= World Health Organization functional class, NT-proBNP= N-terminal pro-brain natriuretic peptide, CTEPH= chronic thromboembolic pulmonary hypertension, IPAH= idiopathic pulmonary arterial hypertension, NA= not available
2.5. Characterization of circulating progenitor cells

Recruitment of the patients

Patients with PAH of our Centre for Pulmonary Hypertension (mPAP≥25mmHg, PAWP≤15mmHg with no significant heart, lung, liver, kidney and blood diseases) undergoing diagnostic or follow-up right heart catheterization were included in a prospective manner. Age- and sex-matched healthy volunteers selected on a 1:1 basis served as controls. This explorative study was approved by the ethics committee of the Medical University of Graz (23-408 ex 10/11). Written informed consent was obtained from all study participants. Patients’ characteristics are shown in Table 5.

Study procedure

Blood (3mL) was taken from PAH patients within 30 days of right heart catheterization and from their individual controls from an antecubital vein in EDTA containing Vacutainer® tubes. The samples were processed within one hour after the blood was drawn as follows: Peripheral blood mononuclear cells (PBMNCs) were isolated via Ficoll-Paque PLUS (GE Healthcare Life Sciences, Vienna, Austria) density gradient centrifugation. 3 mL of blood was diluted in 1:1 ratio with sterile phosphate buffered saline (PBS). The obtained solution was then carefully transferred onto Ficoll-Paque PLUS solution. The tubes were centrifuged for 30 min at 400 G. The obtained mononuclear cells were washed twice in PBS and resuspended in PBS containing 1% bovine serum albumin (BSA).

Fluorescence activated cell sorting

Prior to staining, non-specific antibody binding was blocked via incubation with Fc Receptor binding inhibitor (eBioscience, Vienna, Austria) for 20 minutes on ice. Cells were stained simultaneously with fluorescent conjugated antibodies against the following cell surface markers or their respective isotype controls: CD117 (c-kit), CXCR2, CD309, CD34, CD14, CD31, CD133, CD16, CD45 (Table 6).
Table 5. Patients’ characteristics that participated in the prospective study for assessment of circulating progenitor cells. Values are expressed as mean ± standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=20)</th>
<th>PAH (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years) (mean ± SD)</td>
<td>56.3 ± 13.4</td>
<td>59.3 ± 17.9</td>
</tr>
<tr>
<td>Sex (F/M)</td>
<td>15/5</td>
<td>15/5</td>
</tr>
<tr>
<td>mPAP (mmHg) (mean ± SD)</td>
<td>NA</td>
<td>45.1 ± 16.4</td>
</tr>
<tr>
<td>RAP (mmHg) (mean ± SD)</td>
<td>NA</td>
<td>7.7 ± 4.3</td>
</tr>
<tr>
<td>PAWP (mmHg) (mean ± SD)</td>
<td>NA</td>
<td>9.5 ± 3.7</td>
</tr>
<tr>
<td>PVR (WU) (mean ± SD)</td>
<td>NA</td>
<td>9.9 ± 6.4</td>
</tr>
<tr>
<td>6MWD (m) (mean ± SD)</td>
<td>NA</td>
<td>378.2 ± 111.3</td>
</tr>
<tr>
<td>WHO class (I/II/III/IV)</td>
<td>20/0/0/0</td>
<td>1/6/13/0</td>
</tr>
<tr>
<td>NT-proBNP (pg/mL) (mean ± SD)</td>
<td>NA</td>
<td>899.5 ± 941.4</td>
</tr>
</tbody>
</table>

**Abbreviations:**  F=female, M=male, mPAP= mean pulmonary arterial pressure, RAP=right atrial pressure, PAWP= pulmonary arterial wedge pressure, PVR= pulmonary vascular resistance, WU= Wood units, 6MWD= six-minute walk distance, WHO class= World Health Organization functional class, NT-proBNP= N-terminal pro-brain natriuretic peptide, CTEPH= chronic thromboembolic pulmonary hypertension, IPAH= idiopathic pulmonary arterial hypertension, NA= not available
Table 6. Antibody-fluorochrome combinations

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fluorochrome</th>
<th>Isotype Control</th>
<th>Clone</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD117</td>
<td>PE-Cy7</td>
<td>Mouse IgG1, k</td>
<td>104D2</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CXCR2</td>
<td>PerCP</td>
<td>Mouse IgG2A</td>
<td>48311</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>CD309</td>
<td>PE</td>
<td>Mouse IgG1</td>
<td>89106</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>CD34</td>
<td>AF488</td>
<td>Mouse IgG1, k</td>
<td>581</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD14</td>
<td>APC-eFluor 780</td>
<td>Mouse IgG1, k</td>
<td>61D3</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD31</td>
<td>AF700</td>
<td>Mouse IgG1</td>
<td>MEM-05</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD133</td>
<td>APC</td>
<td>Mouse IgG12b</td>
<td>293C3</td>
<td>Milteny Biotech</td>
</tr>
<tr>
<td>CD16</td>
<td>BD Horizon V500</td>
<td>Mouse IgG1, k</td>
<td>3G8</td>
<td>BD Horizon</td>
</tr>
<tr>
<td>CD45</td>
<td>eFluor 450</td>
<td>Mouse IgG1, k</td>
<td>HI30</td>
<td>eBioscience</td>
</tr>
</tbody>
</table>

The cells were then incubated for 30 minutes at 4°C. Flow cytometric analysis was performed and analysed on a LSR II with FACS Diva Software Version 6.2 (both from Beckton Dickinson Biosciences). Automatic and manual adjusted compensation was used with single colour stained samples. Instrument configuration is given in Table 7; a compensation matrix is shown in Table 8. A minimum of 50,000 events were recorded. After exclusion of cell debris and doublets, lymphocytes and monocytic cells were gated. The regions of interest were selected according to the negative and isotype controls. A detailed gating strategy is shown in Figure 6.

Table 7. Instrument configuration

<table>
<thead>
<tr>
<th>Laser</th>
<th>PMT</th>
<th>Dichroic Mirror/ Longpass Filter (nm)</th>
<th>Bandpass Filter (nm)</th>
<th>Fluorochrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argon 488</td>
<td>A</td>
<td>735</td>
<td>780/60</td>
<td>PE-Cy7</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>635</td>
<td>670/14</td>
<td>PerCP</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>550</td>
<td>576/26</td>
<td>PE</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>505</td>
<td>530/30</td>
<td>AF488</td>
</tr>
<tr>
<td>Red Diode 633</td>
<td>A</td>
<td>755</td>
<td>780/60</td>
<td>APC-eFluor730</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>675</td>
<td>730/45</td>
<td>AF700</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>N/A</td>
<td>660/20</td>
<td>APC</td>
</tr>
<tr>
<td>Violet 405</td>
<td>A</td>
<td>505</td>
<td>525/50</td>
<td>BD V500</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>N/A</td>
<td>440/40</td>
<td>eFluor450</td>
</tr>
</tbody>
</table>
### Table 8. Compensation matrix

<table>
<thead>
<tr>
<th>Fluorochrome</th>
<th>(-)% Fluorochrome</th>
<th>Spectral Overlap</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE</td>
<td>AF488</td>
<td>17</td>
</tr>
<tr>
<td>APC</td>
<td>AF488</td>
<td>1</td>
</tr>
<tr>
<td>AF488</td>
<td>PE</td>
<td>1</td>
</tr>
<tr>
<td>PECy7</td>
<td>PerCP</td>
<td>12</td>
</tr>
<tr>
<td>APC</td>
<td>PerCP</td>
<td>7</td>
</tr>
<tr>
<td>PE</td>
<td>PE-Cy7</td>
<td>1</td>
</tr>
<tr>
<td>PerCP</td>
<td>PE-Cy7</td>
<td>2</td>
</tr>
<tr>
<td>APC-Cy7</td>
<td>PE-Cy7</td>
<td>6</td>
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<tr>
<td>AF700</td>
<td>APC</td>
<td>23</td>
</tr>
<tr>
<td>APC-Cy7</td>
<td>APC</td>
<td>5,8</td>
</tr>
<tr>
<td>APC</td>
<td>AF700</td>
<td>1</td>
</tr>
<tr>
<td>APC-Cy7</td>
<td>AF700</td>
<td>14</td>
</tr>
<tr>
<td>AF488</td>
<td>AmCyan</td>
<td>3,1</td>
</tr>
<tr>
<td>AmCyan</td>
<td>Pacific Blue</td>
<td>9</td>
</tr>
<tr>
<td>AF700</td>
<td>APC-Cy7</td>
<td>12</td>
</tr>
<tr>
<td>APC</td>
<td>APC-Cy7</td>
<td>8</td>
</tr>
<tr>
<td>PE-Cy7</td>
<td>APC-Cy7</td>
<td>10</td>
</tr>
</tbody>
</table>

**Abbreviations:**

PE = phycoerythrin

APC = allophycocyanine

AF488 = Alexa Fluor 488

PECy7 = phycoerythrin-cyanine7

PerCP = Peridinin chlorophyll

AF700 = Alexa Fluor 700

APC-Cy7 = allophycocyanine-cyanine7
Figure 6. Gating strategy for the detection of cell populations from peripheral blood mononuclear cells. A representative example of a PAH patient is shown. A, B, C, D and E show the gating for different cell types.
Gating for lymphocytic and monocytic cells is demonstrated in A (X-axis: Forward Scatter (FSC-A), Y-Axis: Side Scatter (SSC-A)). CD133 positive cells were identified from the lymphocytic gate (B). Next, double positive events were gated from the lymphocytic region. Panel C demonstrates cells expressing both CD133 (X-axis) and CD45 (Y-axis). In Panel D CD133 (X-Axis) and CD14 (Y-Axis) positive events are demonstrated. Monocytes were identified according to the expression of CD16 and CD14 (Panel E). Fibrocytes were identified from the CD45 and CD14 positive monocytes (Panel F) defining them as CD34+CD45+CD14+ subpopulation of mononuclear cells (Panel G).

**Cytospin and immunofluorescent staining**

Cells were stained with fluorescence tagged antibodies against CD34 and CD133 as given in Table 6. 20,000 cells were diluted in 200 µL of PBS containing 1% BSA and put onto microscope slides using the cytospin technique. Briefly, the chamber slides were loaded with 200 µL working solution and centrifuged with 700 RPM for 5 minutes. After fixation with 1.4% paraformaldehyde the slides were mounted with Vectashield® Mounting Medium containing 4’,6-diamidino-2-phenylindole (DAPI) (Vector laboratories). Negative controls were performed with the isotype specific antibodies. For visualization, a Zeiss LSM 510 META scanning laser confocal microscope was used.

**ELISA**

The ELISA for the soluble CD133 (prominin-1) was performed according to the manufacturer’s protocol (Biotrend Chemikalien, Germany, Cat No:CSB-EL018751HU). Plasma was isolated from blood samples obtained from the patients and controls in exactly the same way. Blood samples were kept on ice until centrifugation. Plasma was obtained within 1 hour of blood draw, aliquotted and stored at -80°C in the Biobank of the Medical University of Graz.
**Immunohistochemistry and immunofluorescence**

For immunohistochemistry studies, lung tissue was obtained from IPAH patients undergoing lung transplantation. Samples from donor lungs not used for transplantation served as controls. The study protocol for tissue donation was approved by the ethical committee of the Medical University of Vienna (976/2010).

In order to assess the localization of CD133 positive cells in the lung tissue we stained IPAH and healthy donor lung tissues for this marker using immunohistochemistry and tissue immunofluorescence. The slides were analysed under the supervision of an expert pathologist in lung cytology (Prim. Dr. Martin Tötsch).

Six different antibodies with serial dilutions were used on 4 antigen retrieval systems and 3 developing kits. Additionally, 2 glioblastoma multiforme and prostate cancer tissues were used as positive controls for all antibodies. In order to further characterize the localization of CD133 positive cells, serial sections were made from IPAH patients and donors using 3 paraffin blocks from each. 5 slides/block were consecutively stained as follows: CD133, von Willebrand Factor (vWF) and α-smooth muscle actin (α-SMA), Ki67, haematoxilin&eosin (H&E). Negative controls were performed using 10% bovine serum albumin instead of the primary antibody. Paraffin embedded lung tissues were cut into 4 µm thick pieces and mounted on microscope slides. After deparaffinization with decreasing alcohol concentrations the heat induced antigen retrieval was performed with sodium citrate solution (pH=6). Dual enzyme blocking was performed with normal horse serum (DAKO). The primary antibodies against CD133 (host: rabbit), vWF (host:mouse), alpha smooth muscle actin (host:goat) were incubated for 1 hour at room temperature. After washing with PBS the secondary antibodies were applied and incubated for 1 hour at room temperature. 3,3′-Diaminobenzidine (DAB) was used for antigen detection and the slides were counterstained with methyl green.

Paraffin embedded tissues were cut into 6 µm thick pieces and mounted on microscope slides. After deparaffinization with decreasing alcohol concentrations the antigen retrieval was performed with trypsin (DAKO). Slides were incubated with CD133 antibody (Abnova, Taipei, Taiwan) for 30 minutes. After fixation with 1.4% paraformaldehyde the slides were mounted with 4′,6-diamidino-2-phenylindole
(DAPI) containing medium. The slides were analyzed with a laser-scanning confocal microscope, LSM 510 Meta (Zeiss), with the following Ex/Em settings: 405/BP420-480 (DAPI); 488/BP505-550 (Alexa Fluor 488) and 633/679-754 (Alexa Fluor 680). The 1024x1024 resolution images were taken with a Zeiss 40x oil immersion objective with 1.4 NA.

Real time polymerase chain reaction

Gene expression changes in CD133 positive cells were investigated using real time PCR using a Light Cycler 480 (Roche). Primer sequences are given in Table 4. Blood was taken from healthy subjects and PAH patients and the CD133 positive cells were sorted using MACS (AutoMACS, Milteny, Vienna, Austria). Total RNA was extracted from the isolated cells using peqGOLD Total RNA Kit (Peqlab). A preamplification kit was used for cDNA synthesis (NuGEN, Ovation PicoSL System V2, Berlin, Germany). mRNA levels of octamer binding transcription factor (Oct3/4), sex determining region Y-box 2 (SOX2), homebox transcription factor (Nanog), C-X-C chemokine receptor type 4 (CXCR4) and Ki67 were assessed by RT-PCR on a Light Cycler 480 (Roche). Primers were designed on intron spanning sequences (Table 9). For calculation of mRNA expression the delta CT method was used, using beta-2-macroglobulin as the reference gene.

Table 9. Primer sequences used for RT-PCR

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (3’-5’)</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oct 3/4</td>
<td>CTGGGGGTTCTATTTGGGAGGG</td>
<td>GTTCGCTTTTCTTTTCGGGC</td>
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</tr>
<tr>
<td>SOX2</td>
<td>GCGTCAAGCGGGCCCA</td>
<td>GCTTCTCCGTCTCCGACAAA</td>
<td>144</td>
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<tr>
<td>Nanog</td>
<td>ATGCCTCACACGGAGACTGT</td>
<td>AGGGCTGTCCTGAATAAGCA</td>
<td>65</td>
</tr>
<tr>
<td>CXCR4</td>
<td>CCAGTAGCCACCGCATCT</td>
<td>ATAGTCCCCTGAGCCCCATTT</td>
<td>99</td>
</tr>
<tr>
<td>Ki67</td>
<td>ACGAGACGCTGTTACTATC</td>
<td>GCTCATCAATAACAGACCTTTAC</td>
<td>225</td>
</tr>
<tr>
<td>β2-macroglobulin</td>
<td>CCTGGAGGCTATCCAGCGTA</td>
<td>TGTCGGATGGATGAACCACCAGACA</td>
<td>112</td>
</tr>
</tbody>
</table>
Statistical analysis applied for all studies

Data are presented as means ± standard deviation or median with interquartile range (IQR). Statistical analysis was performed using GraphPad Prism software (Version 5.04, GraphPad Software Inc., La Jolla, California). Non-parametric test (Mann Whitney U-test) was used for calculation of differences between groups. For normally distributed values Pearson correlation analysis, and for non-normal distributions Spearman correlation was used. P<0.05 was considered as statistically significant. Bland-Altman analysis was performed to compare the cardiac output values assessed by thermodilution and by inert gas rebreathing.

To calculate the best cut-off values for apelin isoforms and GDF-15, receiver operating curves were used and area under the curve was calculated.

Power analysis was used for calculation of sample size for the prospective study assessing serum levels of apelin-17 based on the results of our explorative study (see 2.2). This revealed that 31 IPAH patients and matched controls were needed to achieve statistical significance with a power of 80%.
3. Results

3.1 Inert gas rebreathing for non-invasive pulmonary blood flow measurement using Innocor System.

In order to assess if the inert gas rebreathing method using the Innocor® system is suitable for non-invasive cardiac output (CO) measurements, we compared the assessed pulmonary blood flow (PBF) values with those obtained during right heart catheterization measured by thermodilution. As in the absence of relevant shunting PBF is consistent with CO, in the following, the term CO will be used. As shown in Figure 7, in our patients, both methods showed a moderate positive correlation ($R^2=0.47$, $p<0.0001$).

![Figure 7](image_url)  
**Figure 7.** Correlation analysis between cardiac output values determined by inert gas rebreathing system and the gold standard thermodilution method in n=23 consecutive patients. Both measurements were performed during right heart catheterization. Abbreviations: $R^2=\text{Pearson correlation coefficient. CO=cardiac output.}$

In order to further analyze the association between both methods (inert gas rebreathing and thermodilution) a Bland-Altman analysis was performed in the same
This revealed no relevant systematic difference or bias between the CO values measured by inert gas rebreathing and thermodilution. However, the accuracy of the inert gas rebreathing method was only moderate and at CO values above 5 liter the methods appeared to have a larger scattering than at lower ranges of CO (Figure 8.).

Figure 8. Bland Altman analysis of CO values determined by inert gas rebreathing and thermodilution as assessed during right heart catheterization. Dashed lines indicate the confidence intervals. Each point represents the measured differences between the methods at a given CO in individual patients.

In order to test if the inert gas rebreathing method can reliably indicate acute changes in cardiac output during pharmacologic testing, we concomitantly examined a subgroup of patients (n=7) undergoing pharmacologic testing with both methods. Individual changes in CO measured by thermodilution and Innocor of these patients are shown in Figure 9. Generally, an increase of the CO could be observed by thermodilution after the application of sildenafil and after iloprost inhalation. This was also indicated by the rebreathing device in most cases (Fig. 9). However, the quantity
of acute changes showed large individual differences (in some cases > 1 L/min) in several patients, without showing a general pattern for these discrepancies (Figure 9). Figure 10 summarizes the results of individual CO measurements by thermodilution and Innocor.

**Figure 9.** Individual changes of cardiac output measured by Innocor and the thermodilution method during the pharmacologic testing in seven patients. On the X-Axis the time points of the measurements are represented as follows: Base1=measurement at baseline, Sil1=measurement at 30 minutes after sildenafil application, Sil2=measurement at 60 minutes after sildenafil application, Ilo1=measurement at 15 minutes after iloprost inhalation, Ilo2=measurement at 30 minutes after iloprost inhalation. Figures A-F depict individual patients. Triangles represent values measured by thermodilution, squares represent values measured by inert gas rebreathing as assessed by Innocor.
According to the Fick principle, pulmonary blood flow is linearly correlated with the arterio-venous oxygen difference (AVDO₂) as assessed during right heart catheterization. Therefore we also compared the changes in CO as assessed by thermodilution and inert gas rebreathing with the changes in AVDO₂ during the pharmacologic testing (Figure 11).
**Figure 11.** Acute changes of CO (L/min) in seven patients during pharmacologic testing measured by inert gas rebreathing and thermodilution and assessed by the change of AVDO\textsubscript{2} (vol\%) at four different time points (A: at 30 minutes after sildenafil intake, B: at 60 minutes after sildenafil intake, C: at 15 minutes after iloprost inhalation, D: at 30 minutes after iloprost inhalation). Triangles represent the difference between cardiac output values, squares represent the difference between AVDO\textsubscript{2} values. Abbreviations: AVDO\textsubscript{2} = arterio-venous oxygen difference, Therm = thermodilution, Sil = sildenafil, Ilo = iloprost.

This analysis revealed that changes in CO assessed by the inert gas rebreathing method were not consistent with changes in CO assessed by thermodilution or with changes in AVDO\textsubscript{2} at all time points. 30 and 60 minutes after Sildenafil intake, modest changes in CO as assessed by both by thermodilution and...
Innocor were observed and a similar modest change in AVDO$_2$. After iloprost inhalation, however, the modest increase in CO was not followed by the increase in AVDO$_2$. Due to the low number of subjects an exact statistical analysis is not possible, but a reliable matching of the parameters appears to be unlikely.

In order to test for possible factors that may influence the CO measurements with the rebreathing method and explain inconsistencies described above, measurements were carried out in healthy volunteers using different settings. During these measurements, the size of the bag which was used for the inhalation and body position were changed and the effect of these changes documented.

**Figure 12.** X-axis represents the volume of the rebreathing bag (L). Values of cardiac output are shown on the Y-axis (CO (L/min)) Black and blue lines represent the measured CO values of the same subject in sitting position at different time points. Red line represents the measured CO values of the same
healthy subject in lying position. Individual values are depicted as dots on the black, red and blue lines, respectively.

We found that the change in body position and in breathing bag volume was associated with a change in pulmonary blood flow. A representative figure of the measurements in a healthy volunteer is shown in Figure 12. In this subject, increasing balloon size led to an increase in cardiac output. It can be also observed that the use of a bag volume size of 2.2 L (suggested by the Innocor system for this subject based on the lung function and BMI) lead to the most constant cardiac output measurements.

### 3.2 Pilot study for blood-derived biomarkers

Serum levels of apelin isoforms (apelin-12, apelin-13, apelin-17, apelin-36), TNF-like weak inducer of apoptosis (TWEAK) and growth differentiation factor-15 (GDF-15) were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits. Blood samples from n=10 patients with IPAH, n=10 patients with CTEPH and n=10 age- and sex-matched (to the IPAH group) controls were used for the pilot studies. For the prospective validation study, sample size calculation was performed based on the results of the pilot study. Patient characteristics are given in Material and Methods.

Apelin-12 was detectable in only one patient and one healthy control, and apelin-36 was detectable in about half of the study participants. Apelin-13 and TWEAK showed no difference between the groups in the pilot study while Apelin-17 and GDF-15 were significantly elevated in IPAH (apelin-17: p<0.01, GDF: p<0.007) and CTEPH (apelin-17: p<0.002, GDF-15: p<0.001) as compared to controls (Figure 13). Sample size calculation revealed that n=31 patients per group are needed to be included for a power of 80% for Apelin-17, Apelin-36 and GDF-15.
Figure 13 Serum levels of A. Apelin-13 B. Apelin-36 C. Apelin-17 D. growth differentiation factor-15 (GDF-15) and E. Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) in healthy controls, idiopathic pulmonary arterial hypertension (IPAH) and chronic thromboembolic pulmonary hypertension (CTEPH) patients. p<0.05 was considered statistically significant. N=10 patients per group.
3.3 Prospective study for apelin isoforms and GDF-15

According to the power calculation based on the results of the pilot study, n=31 IPAH patients, n=31 age- and sex-matched healthy subjects and 24 CTEPH patients were included in the validation study. Apelin-13 did not show any difference between the investigated groups (Figure 14A). Apelin-36 showed significantly higher levels in CTEPH as compared to IPAH ($p<0.05$ CTEPH vs. IPAH Figure 14B), but not versus the control population. Apelin-36 was not detectable in a relevant number of examined subjects (n=7 controls, n=2 IPAH and n=16 CTEPH patients). Apelin-17 was elevated in IPAH and in CTEPH as compared to control ($p<0.0001$ CTRL vs. IPAH, $p<0.001$ CTRL vs. CTEPH, Figure 14C) and there was no significant difference between CTEPH and IPAH. Similarly, GDF-15 was elevated in IPAH and in CTEPH as compared to control ($p<0.0001$ CTRL vs. IPAH, $p<0.001$ CTRL vs. CTEPH, Figure 14D) and there was no significant difference between IPAH and CTEPH. Out of the investigated apelin isoforms, apelin-17 showed the largest elevation versus controls in the serum of IPAH as well as in CTEPH patients. The observed pattern was very similar to that seen with GDF-15.

In the following table, these results are shown for each marker as well as for each group.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CTEPH</th>
<th>IPAH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Apelin-13</strong></td>
<td>median 245.6</td>
<td>326.4</td>
<td>212.3</td>
</tr>
<tr>
<td><strong>Apelin-17</strong></td>
<td>median 921</td>
<td>1561 $#$</td>
<td>1591 $#$</td>
</tr>
<tr>
<td><strong>Apelin-36</strong></td>
<td>median 252.8</td>
<td>523</td>
<td>179.3 $$</td>
</tr>
<tr>
<td><strong>GDF-15</strong></td>
<td>median 497.1</td>
<td>1355 $$</td>
<td>1798 $#$</td>
</tr>
</tbody>
</table>

Table legend:

# Significant difference between Control and IPAH

$\$ Significant difference between CTEPH and IPAH

$\$ Significant difference between Control and CTEPH
Figure 14. Serum levels of apelin isoforms (A, B, C) and GDF-15 (D) in n=31 IPAH patients, n=31 age- and sex-matched controls as well as n=24 CTEPH patients represented as Whisker-Blot plots. Points above the plots represent outliers. p<0.05 was considered statistically significant.

In order to assess potential clinical relevance of the individual markers, their correlation with invasive hemodynamic data was analyzed (heart rate (HF), mean pulmonary arterial pressure (mPAP), right atrial pressure (RAP), pulmonary arterial wedge pressure (PAWP), pulmonary vascular resistance (PVR), cardiac output (CO), cardiac index (CI)). In addition, their correlation with blood gas parameters (oxygen partial pressure (pO₂), carbon dioxide partial pressure (pCO₂)), blood derived markers (N-Terminal pro-brain natriuretic peptide (NT-proBNP), uric acid, C-reactive protein (CRP), bilirubin) as well as six-minute walking distance (6MWD) and World Health Organization functional class (WHO class) was analyzed.
Results for IPAH and CTEPH are summarized in Table 10 and Table 11 respectively.

<table>
<thead>
<tr>
<th>Spearman ρ</th>
<th>Apelin-13</th>
<th>Apelin-17</th>
<th>Apelin-36</th>
<th>GDF-15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>-0.39</td>
<td>0.21</td>
<td>0.31</td>
<td>0.21</td>
</tr>
<tr>
<td>HF</td>
<td>0.46</td>
<td>-0.02</td>
<td>0.27</td>
<td>-0.27</td>
</tr>
<tr>
<td>mPAP</td>
<td>0.27</td>
<td>-0.05</td>
<td>-0.38</td>
<td>0.20</td>
</tr>
<tr>
<td>RAP</td>
<td>-0.22</td>
<td>-0.30</td>
<td>-0.09</td>
<td>0.35</td>
</tr>
<tr>
<td>PAWP</td>
<td>-0.19</td>
<td>-0.15</td>
<td>0.20</td>
<td>0.46</td>
</tr>
<tr>
<td>PVR</td>
<td>0.13</td>
<td>0.02</td>
<td>-0.50</td>
<td>0.29</td>
</tr>
<tr>
<td>CO</td>
<td>0.29</td>
<td>-0.16</td>
<td>0.18</td>
<td>-0.39</td>
</tr>
<tr>
<td>CI</td>
<td>0.25</td>
<td>0.07</td>
<td>0.32</td>
<td>-0.44</td>
</tr>
<tr>
<td>pO₂</td>
<td>0.43</td>
<td>-0.34</td>
<td>0.74</td>
<td>0.05</td>
</tr>
<tr>
<td>pCO₂</td>
<td>0.18</td>
<td>0.13</td>
<td>-0.05</td>
<td>0.22</td>
</tr>
<tr>
<td>NT-proBNP</td>
<td>0.14</td>
<td>-0.23</td>
<td>0.25</td>
<td>0.56</td>
</tr>
<tr>
<td>uric acid</td>
<td>-0.04</td>
<td>-0.05</td>
<td>-0.03</td>
<td>0.63</td>
</tr>
<tr>
<td>CRP</td>
<td>0.26</td>
<td>-0.30</td>
<td>0.44</td>
<td>0.37</td>
</tr>
<tr>
<td>bilirubin</td>
<td>-0.10</td>
<td>0.00</td>
<td>0.05</td>
<td>0.13</td>
</tr>
<tr>
<td>6MWD</td>
<td>-0.10</td>
<td>0.08</td>
<td>0.10</td>
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<tr>
<td>WHOclass</td>
<td>0.17</td>
<td>-0.10</td>
<td>0.10</td>
<td>0.57</td>
</tr>
</tbody>
</table>

Table 10. Spearman correlation analysis results for IPAH patients. Cells filled with green indicate a significant (p<0.05) correlation.

Although levels of apelin-13 did not differ between the groups, there was a negative correlation between apelin-13 levels and the age of IPAH patients (ρ = -0.39). Furthermore apelin-13 levels were positively correlated with heart rate (ρ = 0.46) and pO₂ (ρ = 0.43). The main apelin isoform, Apelin-17 did not show any correlation with demographic characteristics and invasive hemodynamics. In IPAH, Apelin-36 was positively correlated to pO₂. Serum levels of GDF-15 were negatively correlated to cardiac index (ρ = -0.44) and six minute walk distance (ρ = -0.4). Positive correlations were found between GDF-15 levels and PAWP (ρ = 0.46), NT-proBNP (ρ = 0.56), uric acid (ρ = 0.63) and WHO class (ρ = 0.57).
Table 11. Spearman correlation analysis results for CTEPH patients. Cells filled with green indicate a significant (p<0.05) correlation.

In CTEPH apelin-13 did not correlate with any patient characteristic whereas apelin-17 was positively correlated with markers of right heart failure (RAP ρ = 0.45, NT-proBNP ρ = 0.44) and bilirubin (ρ = 0.45). Apelin-36 did not correlate with the investigated patients’ characteristics and hemodynamics. GDF-15 showed a negative correlation with pCO₂ (ρ = -0.58) and six minute walking distance (ρ = -0.54). Moreover, serum levels of GDF-15 were correlated with invasive hemodynamics like mPAP (ρ =0.44), RAP (ρ = 0.44), PVR (ρ = 0.4) and NT-proBNP (ρ =0.58).

As elevated right atrial pressure (RAP) and NT-proBNP values are indicative of right heart failure and are established prognostic parameters in PH, individual plots were selected for both IPAH and CTEPH for the two most promising markers, apelin-17 and GDF-15. The analyses showed that apelin-17, similarly to GDF-15, was significantly correlated with RAP and NT-proBNP in CTEPH, but not in IPAH.

Figure 15. represents the correlation plots for Apelin-17 and GDF-15 with right heart failure specific markers in IPAH.
Figure 15. Correlation analysis of apelin isoforms and GDF-15 levels with hemodynamic characteristics of IPAH patients’ obtained by right heart catheterization (ρ-Spearman correlation coefficient, p<0.05 was considered statistically significant)

Figure 16 represents the correlation plots for Apelin-17 and GDF-15 with right heart failure specific markers in CTEPH.
Figure 16. Correlation analysis of apelin isoforms and GDF-15 levels with CTEPH patients’ hemodynamic characteristics obtained by right heart catheterization ($\rho$-Spearman correlation coefficient, $p<0.05$ was considered statistically significant).

In order to assess the diagnostic value of these markers for PAH, area under the curve (AUC) for the receiver operating curve (ROC) was calculated. This revealed that apelin-13 and apelin-36 could not reliably differentiate between healthy and IPAH (Figure 17 A, B) whereas apelin-17 and GDF-15 were similarly able to identify IPAH and controls with an AUC of 0.86 for Apelin-17 and 0.83 for GDF-15 (Figure 17 C,D). A cut-off value of 983.5 for Apelin-17 could detect IPAH with a sensitivity of 100% and a specificity of 55.2%.
Figure 17. A (IPAH) Receiver operating curves and area under the curve analysis of the investigated markers. Values of sensitivity (%) are represented on the X axes. Y axes represent values of 100% - specificity (%). A. ROC curve for apelin-13 B. ROC curve for apelin-36 C. ROC curve for apelin-17 D. ROC curve for GDF-15. AUC=area under the curve

Table 12 summarizes the cutoff values for Apelin-17 in IPAH.

Table 13 summarizes the cutoff values for GDF-15 in IPAH.
### Table 12. Apelin-17 in IPAH

<table>
<thead>
<tr>
<th>Apelin-17 Cut-off (pg/mL)</th>
<th>Sensitivity%</th>
<th>95% CI</th>
<th>Specificity%</th>
<th>95% CI</th>
<th>Likelihood ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 983.5</td>
<td>100</td>
<td>88.78% to 100.0%</td>
<td>55.17</td>
<td>35.69% to 73.55%</td>
<td>2.23</td>
</tr>
<tr>
<td>&gt; 1145</td>
<td>77.42</td>
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<td>72.41</td>
<td>52.76% to 87.27%</td>
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<td>&gt; 1487</td>
<td>67.74</td>
<td>48.63% to 83.32%</td>
<td>93.1</td>
<td>77.23% to 99.15%</td>
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<tr>
<td>&gt; 1767</td>
<td>38.71</td>
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<td>96.55</td>
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<td>&gt; 3637</td>
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<td>&gt; 3969</td>
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<td>0.08164% to 16.70%</td>
<td>96.55</td>
<td>82.24% to 99.91%</td>
<td>0.94</td>
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</table>

### Table 13. GDF-15 in CTEPH

<table>
<thead>
<tr>
<th>GDF-15 Cutoff (pg/mL)</th>
<th>Sensitivity%</th>
<th>95% CI</th>
<th>Specificity%</th>
<th>95% CI</th>
<th>Likelihood ratio</th>
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<td>&gt; 226.3</td>
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<td>6.667</td>
<td>0.8178% to 22.07%</td>
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<td>&gt; 467.6</td>
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<td>46.67</td>
<td>28.34% to 65.67%</td>
<td>1.81</td>
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<td>&gt; 836.0</td>
<td>80.65</td>
<td>62.53% to 92.55%</td>
<td>73.33</td>
<td>54.11% to 87.72%</td>
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<td>&gt; 991.5</td>
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<td>&gt; 1743</td>
<td>51.61</td>
<td>33.06% to 69.85%</td>
<td>93.33</td>
<td>77.93% to 99.18%</td>
<td>7.74</td>
</tr>
<tr>
<td>&gt; 2444</td>
<td>38.71</td>
<td>21.85% to 57.81%</td>
<td>96.67</td>
<td>82.78% to 99.92%</td>
<td>11.61</td>
</tr>
</tbody>
</table>

### Graphs

#### A
AUC = 0.57 - not significant

#### B
AUC = 0.56 - not significant

#### C
AUC = 0.75 - p<0.002

#### D
AUC = 0.76 - p<0.002
Figure 18. B (CTEPH) Receiver operating curves and area under the curve analysis of the investigated markers for CTEPH. Values of sensitivity (%) are represented on the X axis. Y axis represent values of 100% - specificity (%).

A. ROC curve for apelin-13  B. ROC curve for apelin-36  C. ROC curve for apelin-17  D. ROC curve for GDF-15. AUC=area under the curve

As seen on Figure 18B receiver operating curve analysis was performed for CTEPH patients. Apelin-13 and Apelin-36 could not differentiate between CTEPH and healthy controls whereas Apelin-17 and GDF-15 could, although a relatively high specificity was coupled with a moderate sensitivity (Table.14 and Table 15.).

Table 14 Apelin-17 in CTEPH

<table>
<thead>
<tr>
<th>Apelin-17 Cutoff (pg/mL)</th>
<th>Sensitivity%</th>
<th>95% CI</th>
<th>Specificity%</th>
<th>95% CI</th>
<th>Likelihood ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 568.3</td>
<td>100</td>
<td>85,75% to 100,0%</td>
<td>10,34</td>
<td>2,186% to 27,35%</td>
<td>1,12</td>
</tr>
<tr>
<td>&gt; 664.2</td>
<td>91,67</td>
<td>73,00% to 98,97%</td>
<td>17,24</td>
<td>5,846% to 35,77%</td>
<td>1,11</td>
</tr>
<tr>
<td>&gt; 1145</td>
<td>70,83</td>
<td>48,91% to 87,38%</td>
<td>72,41</td>
<td>52,76% to 87,27%</td>
<td>2,57</td>
</tr>
<tr>
<td>&gt; 1500</td>
<td>62,5</td>
<td>40,59% to 81,20%</td>
<td>93,1</td>
<td>77,23% to 99,15%</td>
<td>9,06</td>
</tr>
<tr>
<td>&gt; 1782</td>
<td>29,17</td>
<td>12,62% to 51,09%</td>
<td>96,55</td>
<td>82,24% to 99,91%</td>
<td>8,46</td>
</tr>
<tr>
<td>&gt; 1861</td>
<td>25</td>
<td>9,773% to 46,71%</td>
<td>96,55</td>
<td>82,24% to 99,91%</td>
<td>7,25</td>
</tr>
</tbody>
</table>

Table 15 GDF-15 in CTEPH

<table>
<thead>
<tr>
<th>Cutoff GDF-15 for CTEPH</th>
<th>Sensitivity%</th>
<th>95% CI</th>
<th>Specificity%</th>
<th>95% CI</th>
<th>Likelihood ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 319.4</td>
<td>100</td>
<td>85,75% to 100,0%</td>
<td>13,33</td>
<td>3,755% to 30,72%</td>
<td>1,15</td>
</tr>
<tr>
<td>&gt; 437.5</td>
<td>91,67</td>
<td>73,00% to 98,97%</td>
<td>43,33</td>
<td>25,46% to 62,57%</td>
<td>1,62</td>
</tr>
<tr>
<td>&gt; 886.5</td>
<td>62,5</td>
<td>40,59% to 81,20%</td>
<td>76,67</td>
<td>57,72% to 90,07%</td>
<td>2,68</td>
</tr>
<tr>
<td>&gt; 1299</td>
<td>54,17</td>
<td>32,82% to 74,45%</td>
<td>86,67</td>
<td>69,28% to 96,24%</td>
<td>4,06</td>
</tr>
<tr>
<td>&gt; 1364</td>
<td>50</td>
<td>29,12% to 70,88%</td>
<td>90</td>
<td>73,47% to 97,89%</td>
<td>5</td>
</tr>
<tr>
<td>&gt; 2097</td>
<td>33,33</td>
<td>15,63% to 55,32%</td>
<td>96,67</td>
<td>82,78% to 99,92%</td>
<td>10</td>
</tr>
</tbody>
</table>
3.4 Assessment of circulating endothelial microparticles in PH

The level of endothelial microparticles as well as the microparticle activity was assessed in an explorative study including 15 controls, 16 CTEPH and 36 PAH patients. For patients characteristics see Materials and Methods. As shown in Figure 15, microparticle activity showed no statistically significant difference among the investigated groups (Control: mean=5.44±2.18 nM, CTEPH mean=4.77±2.43 nM, PAH mean=4.6±1.9 nM).

![Boxplot of MPactivity](image)

**Figure 19.** Plasma endothelial microparticle activity (as expressed in nM) in patients without PH (control), as well as in CTEPH and PAH patients.

Next, we analyzed whether microparticle activity correlated with the obtained invasive hemodynamics. There were no statistically significant correlations between microparticle activity and patients’ demographic or hemodynamic data. Of note, no invasive hemodynamics were available from the controls.

When we analyzed the number (not the activity) of endothelial microparticles in the PAH cohort, there was a significant elevation as compared to controls (median=43.45 pg/mL, IQR:38.05-51.33 vs. median= 34.5 pg/mL, IQR:32.6-42.6)
(Figure 20). However, the level of plasma endothelial microparticles did not differ between controls and CTEPH patients (median=35 pg/mL, IQR:31.95 - 38.15).

![MP TF](image)

**Figure 20.** Level of plasma endothelial microparticles (as expressed in pg/mL) in patients without PH (control), CTEPH and PAH patients.

Searching for a potential link between the concentration of the endothelial microparticles and pulmonary hemodynamics, we performed correlation analysis for both PAH and CTEPH. This revealed that in PAH the concentration of endothelial microparticles was negatively correlated with right atrial pressure (Fig. 21 p<0.02, r=-0.49), but there was no significant correlation with mean PAP, PAWP, PVR or any other relevant hemodynamic parameter.
Figure 21 Spearman correlation analysis between right atrial pressure (RAP) and concentration of endothelial microparticles (MP TF).

As shown in Figure 22, in CTEPH there was a positive correlation between MP TF and PAWP (p<0.0001, r=0.84) and between MP TF and pCO₂ (p<0.04, r=0.55), but there was no significant correlation with mean PAP, RAP, PVR or any other relevant hemodynamic parameter.
Figure 22. Correlation analysis between plasma microparticle concentration, PAWP and pCO$_2$ in CTEPH patients.

3.5 Characterization of circulating progenitor cells

In order to assess the number and phenotype of circulating progenitor cells, peripheral blood from 20 patients with pulmonary arterial hypertension and from their age- and sex-matched controls was used. Patients’ characteristics are given in the Materials and Methods section.

3.5.1 Flow cytometry

Using a novel gating strategy, we identified putative progenitors as CD34+CD133+ double positive cells. These cells did not express the chemokine receptor CXCR2, the macrophage/granulocyte marker CD16, vascular endothelial growth factor receptor-2, CD309 (also known as KDR) or CD117 (c-kit) i.e. there were no triple positive events (CD34+CD133+CXCR2+, CD34+CD133+CD16+, CD34+CD133+CD309+, CD34+CD133+CD117+) neither in PAH nor in controls. This suggests that cells expressing both CD34+ and CD133+ are undifferentiated in the peripheral blood.

Circulating CD133+ cells were significantly elevated in PAH as compared to controls (Fig 17A, median 0.9% IQR: 0.6 – 1.7 vs. 0.5% IQR: 0.2 – 0.6, p<0.001). A
CD133+ circulating EPC population is depicted on Figure 17 expressed as percentage of the gated lymphocytic cells. Circulating CD133+ cells positive for CD45 (CD133+CD45+), indicating their bone marrow origin, were significantly increased in PAH as compared to controls (Fig 17 B, median: 0.6 % IQR: 0.4 – 1.5 vs. 0.3% IQR: 0.2 – 0.3, p<0.001). The CD133+CD14+ population was also increased in PAH as compared to controls (Fig. 17 C, median 0.2 IQR: 0.1 – 0.5 vs. 0.0 IQR: 0.0 – 0.0 p<0.001). As depicted in Figure 17G, the total number of monocytes (CD14+) was higher in PAH as compared to control (p=0.008; median 90.1 IQR 88.2 – 92.8 vs. 82.7 IQR: 70.0 – 89.4). Similarly, the monocytes of bone marrow-origin (CD45+CD14+) were elevated (p<0.001; median 27.3 IQR 17.4 – 36.1 vs. 11.0 IQR: 4.7 – 16.8) (Fig 17E). In addition, circulating fibrocytes of bone marrow origin (CD34+CD45+CD14+) were elevated in PAH as compared to control (p=0.007; median 0.5 IQR 0.3 – 0.9 vs. 0.3 IQR: 0.2 – 0.3) (Fig. 17 F).

Figure 17. Percentage of circulating cells in PAH as compared to controls.
In an independent set of experiments peripheral and central blood was taken from patients with PAH and fluorescence activated cell sorting was performed as described above. There was no difference observed between the number of circulating progenitors between central and peripheral blood (data not shown).

3.5.2 Correlation analysis

As shown in Figure 18, in PAH, the level of CD133+ cells and the CD45+ subpopulation were positively correlated with mPAP (CD133+: $\rho = 0.654$, $p = 0.002$, CD133+CD45+: $\rho = 0.637$, $p = 0.003$) and pulmonary vascular resistance (PVR) (CD133+: $\rho = 0.521$, $p = 0.019$, CD133+CD45+: $\rho = 0.487$, $p = 0.030$). The subpopulation CD133+CD14+ showed only a significant correlation with the mPAP (mPAP: $\rho = 0.478$, $p = 0.033$; PVR: $\rho = 0.374$, $p = 0.104$). No significant correlations were observed between these biomarkers and NT-proBNP. There was a significant positive correlation between the number of fibrocytes and PVR ($\rho = 0.553$, $p = 0.011$) as well as NT-proBNP level ($\rho = 0.554$, $p = 0.011$). There was also a tendency towards a positive correlation with mPAP ($\rho = 0.442$, $p = 0.051$). Fibrocyte number did not show correlations with other invasive hemodynamics or demographic data.
Figure 18. Correlation of CD133 cell number and hemodynamics of PAH patients obtained by right heart catheterization (p-Spearman correlation coefficient, p<0.05 was considered statistically significant)

3.5.3 Characterization of CD133+ cells

Multiple cytospin preparations of mononuclear cells isolated from healthy controls as well as IPAH patients were examined. Positive staining for CD133 or CD34 was observed on the cell membrane on a small percentage of isolated cells. Phenotypically the nuclei of these cells were small and condensed. A representative image from an IPAH patient is shown in Figure 19.

Figure 19. Immunofluorescent staining of mononuclear cytospin preparations against CD133 (A) and CD34 (B). N=5 PAH patients were investigated. Representative images of an IPAH specimen are shown.

In order to characterize stemness of the circulating CD133 positive cells, cDNA was obtained from isolated CD133 cells. CD133 positive cells did not express SOX2, Nanog, CXCR4 and Ki67 as assessed by RT-PCR. The transcription factor Oct3/4 was expressed on the mRNA level in CD133 positive cells derived from PAH and controls with no significant differences (Fig. 20).
Figure 20. Real time PCR analysis of Oct3/4 from isolated CD133 cells in N=10 donor vs. n=10 PAH patients

3.5.4 Enzyme-linked Immuno Sorbent Assay (ELISA)

Plasma samples were analysed for the soluble form of CD133 (prominin-1). As shown in Figure 21 there was no significant elevation of prominin-1 in PAH (median: 0.027 pg/ml IQR 0.017 – 0.191 pg/ml vs. 0.037 pg/ml IQR 0.024 – 0.228, p=0.16).
Figure 21. Plasma levels of CD133 (prominin-1) in 20 PAH patients and their age- and sex-matched controls as determined by ELISA. Mann Whitney U test was used for the statistical analysis. P<0.05 was considered as statistically significant.

3.5.5 CD133 positive cells in the lung tissue include type 2 pneumocytes, monocytes and undifferentiated cells (stainings)

In the IPAH tissue the cells positively stained for CD133 were predominantly pneumocyte type 2 cells. The slides were analyzed by an expert in lung cytopathology (Prim. Dr. Martin Tötsch). Other cell types were also occasionally positively stained and may be described as inflammatory cells and undifferentiated cells (Figure 22). There were no differences regarding localization of CD133 positive cells between IPAH and donor lung tissues Figure 23.
Figure 22. Representative CD133 immunohistochemical staining of a donor (A) and an IPAH lung tissue (B). The insets are the zoom in regions for (a) pneumocyte type II (arrow), (b) undifferentiated cell (arrow) and (c) inflammatory cell. (C) Representative CD133 immunofluorescent staining of an IPAH lung tissue.
Figure 23 Serial staining of lung tissue from IPAH patients and donors.
4. Discussion

We presented non-invasive approaches including the non-invasive assessment of pulmonary blood flow and potential new blood-derived biomarkers which may contribute to the improved management of PAH patients and substantially add to our current understanding of the disease.

4.1 Inert gas rebreathing for non-invasive pulmonary blood flow measurement using the Innocor® System

We performed measurements with the use of inert gas rebreathing to non-invasively assess pulmonary blood flow. Generally, we confirmed a strong correlation between cardiac output values assessed by inert gas rebreathing and the thermodilution method. However, acute changes during pharmacologic testing induced by sildenafil and iloprost were not reliably detected.

4.1.1 Comparison with other methods in patients with PH

Currently the direct Fick and the thermodilution method are considered as gold standard for the measurement of cardiac output, whereas inert gas rebreathing may be one of the promising novel non-invasive techniques. Few studies exist which compared inert gas rebreathing with established methods in patients with PH.

A recent study compared the three methods: the Fick-method, the thermodilution and the inert gas rebreathing technique in a series of patients with suspected pulmonary hypertension (87). A good agreement among all methods was found in patients with arterial SO$_2$>90%. In addition, the authors proposed a new formula for the inert gas rebreathing to calculate the intrapulmonary shunt fraction. Interestingly, the inert gas rebreathing method overestimated the cardiac output in patients with pulmonary hypertension who presented with elevated wedge pressure which indicates that the method may be less reliable in certain subject groups. In our study we did not find systematic differences between thermodilution and inert gas rebreathing as assessed by Bland Altmann analysis, however, patients with elevated wedge pressure were excluded from the study.

In the study of Raissuni et al. three non-invasive methods, the inert gas rebreathing, 2D-Doppler echocardiography and the VISMO® method were compared
to assess cardiac output (88). Briefly, the VISMO method uses the ECG, a pulse oximetry wave, and sphygmomanometry to continuously estimate cardiac output based on pulse wave transit time assessed by the pulse oximetry and ECG. The authors found that cardiac output measurements with the inert gas rebreathing were comparable with 2D-Doppler echocardiography, with a mean difference of 0.31 L/min. Between the inert gas rebreathing method and the VISMO method, a considerably larger difference was found.

An interesting approach was the comparison of non-invasive stroke volume measurements by cardiac magnetic resonance imaging and inert gas rebreathing in thirty three patients with suspected pulmonary hypertension (84). In this study, acetylene was used as a tracer for inert gas rebreathing. Altogether four different methods were used in this study for cardiac output and stroke volume determination: thermodilution, inert gas rebreathing, as well as MRI derived pulmonary arterial and aortic flow. Bland-Altman analysis showed a good agreement among all methods.

An alternative approach to use inert gas rebreathing in the clinical routine might be its use as a potential prognostically relevant investigation. In the Scottish reference center for pulmonary hypertension, the Innocor device was used to monitor treatment response in patients with precapillary pulmonary hypertension at rest and during submaximal exercise and to examine the association between the pulmonary blood flow measurements and established prognostic parameters (89). The measurements were performed at baseline and after three months of the initiation of targeted therapy. While WHO functional class, N-terminal pro-brain natriuretic peptide and Cambridge Pulmonary Hypertension Outcome Review score were unchanged at follow-up, cardiac output, as assessed by the inert gas rebreathing system, increased. Six-minute walk distance tended to improve, however, probably due to a ceiling effect the improvement was not statistically significant. Although in this study there was no second right heart catheterization performed in order to compare thermodilution with inert gas rebreathing, the authors conclude that Innocor might be used for treatment response monitoring. Based on our study, however, cardiac output changes after drug administration may not always be detectable raising some doubt on the optimistic view by Lee WT et al.
4.1.2 Technical pitfalls

The Innocor® system provided by the company “Innovision” is a complex device that requires very good compliance from the patients and large experience from the operator. If these conditions are fulfilled, reliable repetitive measurements may be performed – even in the pediatric setting (90). According to a recent study, the Innocor device may be even used in pre-school aged children as a non-invasive tool for cardiac output determination (91). Although the accuracy of the method is quite good, the precision is not convincing. Therefore, individual values may be false high or false low. Averaging over repeated measurements may overcome this problem. In the absence of optimal patient compliance or experience of the user, however, the system may lead to major errors and false interpretations.

Among the important potential pitfalls is the choice of a wrong bag volume. This has been addressed in a study including 45 subjects with various pulmonary diseases. In these patients, due to the presence of obstruction or restriction, the predefined bag volume may not be always adequate. Interestingly, the authors did not find a significant difference in the assessed cardiac output using different bag volumes in any of the examined patient groups (92). Our measurements did not support this observation, as in our study, by varying the size of the rebreathing bag the reproducibility of the results became quite poor.

In conclusion, the inert gas rebreathing method provides a promising, yet not fully validated tool for cardiac output assessment. While the accuracy is good, the precision is too low to utilize it as a tool for pharmacologic testing.

4.2. Apelin and GDF-15 as blood-derived biomarkers in pulmonary arterial hypertension and CTEPH

We investigated a set of predefined markers in order to recognize novel circulating serum and plasma biomarkers in PH. In our study, apelin-17 and GDF-15 turned out to be elevated in both idiopathic pulmonary arterial hypertension and chronic thromboembolic pulmonary hypertension. These agents might serve as promising novel diagnostic markers in these diseases.
4.2.1 Apelin and GDF-15 as biomarkers of pulmonary hypertension

We investigated GDF-15 and the apelin isoforms as potential novel biomarkers in IPAH and CTEPH. We found that GDF-15 is elevated in IPAH patients and that its serum levels were correlated with some hemodynamic parameters such as cardiac index. This finding was in accordance with previous studies (93). As a novelty, we found that in addition to IPAH, GDF-15 may also be elevated in CTEPH. Moreover, in CTEPH, GDF-15 levels were correlated with mPAP, RAP and PVR.

The finding that apelin-13, the main isoform of the apelin family did not differentiate between healthy controls and patients with IPAH and CTEPH, was in line with previous investigations. The main finding was that apelin-17 was significantly elevated inpatients with IPAH and CTEPH as compared to controls. Remarkably, this marker was not significantly correlated with hemodynamic parameters, raising the question if apelin-17 is a specific marker for pulmonary hypertension. Based on the experience from earlier biomarker studies, however, the lack of correlation with invasive markers may not necessarily be a sign of missing specificity since the production and metabolism of apelin-17 may not directly be linked to hemodynamics and may reflect other, yet unrevealed aspects of the disease. Targeted PAH therapy may also influence the activity of the apelin-APJ axis, hence this may influence the production of the signal molecule and the cleavage of the main apelin form. This question has not been addressed in the literature, except that the pyruvilation, a common process in the apelin pathway, may be different between treatment naïve patients and patients on targeted therapy.

In CTEPH both apelin-17 and GDF-15 showed a strong correlation with heart failure markers like the right atrial pressure and the cardiac failure marker NT-proBNP. While the prognostic relevance of NT-proBNP has been established, the prognostic relevance of apelin-17 needs to be investigated in future studies.

In our study, the third apelin isoform, Apelin-36, was not detectable in many samples. This isoform was proposed as a general plasma marker in cardiopulmonary diseases and according to previous studies, in chronic heart failure and in IPAH, apelin-36 was decreased (94). Our data indicated that in those samples where it was
detectable, apelin-36 was rather increased in PH which might deserve further exploration. It cannot be excluded that differences in the analytic tests account for these discrepancies.

In summary, apelin and its isoforms have been shown to be differentially regulated in pulmonary hypertension. Apelin-17 is the isoform which may be the most promising marker for IPAH and CTEPH.

4.3 Endothelial microparticles in PAH and CTEPH

We found that the number of endothelial microparticles was elevated in a cohort of PAH patients undergoing right heart catheterization as compared to healthy controls and that it was correlated with some hemodynamic parameters.

It is generally accepted that the quantification and characterization of microparticles is not well standardized. Several methodological approaches have been used to quantify microparticles from the circulation. While we used ELISA as described by Raggam et al., others proposed the use of multicolor flow cytometry (95).

Smadja et al. investigated children with congenital heart disease and showed that the level of circulating endothelial cells was the only predictor of PH reversibility after heart surgery. Microparticles in this setting were not predictive (96). On the contrary, Amabile et al. showed that increased levels of CD62E+ endothelial microparticles were predictive of a poor outcome in treatment naive patients with pulmonary arterial hypertension. The patients were followed for 12 months and a cut-off for CD62e(+) EMPs levels above 353 events/microliter was associated with worse prognosis. Unfortunately, correlations with invasive hemodynamics were not available from this (97). Although we do not have long-term follow up data in our study, we found a weak but significant correlation with right atrial pressure in PAH, supporting the results of Amabile.

The same group showed that levels of circulating endothelial PECAM(+), VE-cadherin(+), E-selectin(+), and leukocyte-derived MPs, but not platelet and annexin V(+) MPs, were increased in subjects with PH compared with controls. There was a positive correlation between the level of these markers and hemodynamic parameters such as mPAP, PVR and RAP (97). These observations are partly in line
with our observations because we found correlations only with RA in PAH and with PAWP in CTEPH.

Endothelial and platelet-derived microparticles were assessed in patients with different forms of pulmonary arterial hypertension. The platelet-derived MPs, defined as positive for CD31 and CD41 and endothelial microparticles represented events which were single positive for CD31. Additionally, two populations of platelet-derived microparticles were identified according to their size: small and large platelet microparticles. Small PMPs and endothelial microparticles were increased in IPAH, HPAH, and SSc-PAH as compared to healthy controls, however, there was no difference between the subgroups. Levels of large PMP did not differ between the investigated populations. Levels of microparticles did not correlate with anthropomorphic and hemodynamic parameters. In thalassemia major patients, a subgroup of subjects with PH had higher levels of microparticles as compared to those without PH, suggesting that microparticles may be relevant in the cardiovascular complications of thalassemia (98).

4.4. Assessment of circulating progenitor cells in PAH

We showed that the relative number of CD133 positive progenitor cells among the mono-lymphocytic cells was elevated in pulmonary arterial hypertension and that this number was correlated with mean pulmonary arterial pressure and pulmonary vascular resistance. On the tissue level, CD133+ cells were identified as type 2 pneumocytes, inflammatory cells and undifferentiated cells.

4.4.1. Challenges of progenitor biology in PAH

The characterization of circulating EPCs is a matter of debate since many years. The main problem is that there is currently no specific marker or combination of markers that would reliably define them both phenotypically and functionally. In our study, phenotypically different subpopulations were detectable which were all elevated in PAH patients. This might also indicate that the pulmonary vasculopathy, especially the elevated pulmonary pressure, is the driving force for the mobilization of these cells from the bone marrow. On the other hand, it could indicate that the elevation of the total CD133+ population is pathogenic in PAH. Indeed, it was shown that CD133 cells from PAH patients caused pulmonary hypertension in the nude
mouse (99). Of note, in that study mostly patients with hereditary PAH were enrolled and the responses in the mouse seemed to be patient-specific suggesting that, apart from CD133 biology, there might be other relevant factors that determine the risk for PAH in the nude mouse.

### 4.4.2. Unbiased technical approach and study population

In fact, the CD133 positive population consists of phenotypically diverse subpopulations characterized by the presence of different leukocyte antigens (100). Therefore, in contrast to previous studies, we used a more comprehensive gating strategy. The leukocyte/monocyte antigens CD45 and CD14 were detectable on our cell populations leading to the conclusion that CD133+ progenitors are mobilized from the bone marrow. During the process of mobilization and maturation, CD133 positive cells may change their phenotype by releasing CD133-containing membrane vesicles (101). We showed that some subpopulations of CD133 positive cells are elevated in PAH whereas some are decreased as compared to controls. This supports the idea of continuous maturation and plasticity of these cells within the circulation. Thus, these EPCs are essentially hematopoietic cells with variable expression of CD34, CD133 and CD45. The value of multiple markers for different EPC populations reflects the heterogeneous nature of these cells (102).

### 4.4.3. Circulating progenitor cells and inflammatory cells as biomarkers

Circulating cells are easily accessible (i.e. from peripheral blood) and therefore suitable as biomarkers of disease severity and for PAH screening. A recent investigation has revealed that the relative number of progenitor cells in COPD patients with pulmonary hypertension was higher than in those without PH (103). Diller et al. found that the relative number of circulating CD34+, CD34+CD133+, CD34+KDR+, and CD34+CD133+KDR+ cells was lower in Eisenmenger patients as compared with healthy controls (80). IPAH patients showed elevated levels of circulating endothelial cells, but not of EPCs (CD34+, CD133+). Our results are in line with Toshner et al. who reported that the relative number of CD133+CD34+VEGFR2+ cells was elevated in 7 IPAH patients as compared to 7
controls (75). The discrepant results in these investigations may be due to the different patient populations studied. Whether targeted PAH therapy has an effect on the number and phenotype of the progenitor cells, remains elusive. One study showed that endothelial colony forming cells from patients treated with Treprostinil (eight children) had a hyperproliferative phenotype and showed enhanced angiogenic potential in a nude mouse model of limb ischemia (104). We found that the contribution of lymphocytes was significantly reduced in PAH patients whereas monocytes were elevated as compared to controls. We can only speculate on the nature of the lymphocytes but it could mean that such cells are abundantly recruited in the tissue or that their production is reduced in PAH.

4.4.4. Molecular characterization of progenitor cells

We did not find a significant increase in plasma levels of soluble CD133 in our patient cohort, however, we observed a trend towards increased levels in PAH. CD133 on the mRNA level in tissue homogenate did not show any difference between patients and controls which indicates that the contribution of these cells to the pathogenesis of pulmonary hypertension may not be structural but rather paracrine. This is supported by the finding that the transcription factor Oct3/4, a marker of stem cells is expressed in the CD133 positive cells from both PAH patients and healthy controls. Oct3/4 has often been used as a marker of self-renewal capacity (105).

4.4.5. Progenitor cells in the lung tissue

The main cell population expressing CD133 in the lung tissues was located in the alveolar-septal junction, consisting of type 2 pneumocytes. To our knowledge this has not yet been reported. Type 2 pneumocytes secrete surfactant and differentiate into type 1 pneumocytes, e.g. following lung injury. This may indicate that CD133 positive cells play a role in maintaining the cellularity of the alveolar surface. Our finding is supported by a previous study showing that in the bleomycin-induced fibrosis model, CD133 positive epithelial cells were protective against lung injury (106). Homing of the circulating CD133 positive progenitor cells remains unclear. It has been recently shown that in end-stage lung diseases associated with pulmonary hypertension there is an increase in the endothelial progenitors in the lung tissue as compared to donor samples (107). One non-exclusive hypothesis is that their homing
is independent of the *vasa vasorum* density and they might arise from the remodelled vessels instead of the bone marrow. Conversely, if they arise from the circulation of non-remodelled vessels, they act in a paracrine manner as hypothesized by Asosingh et al. It has been recently suggested that the expression of a serotonin receptor may be a pre-requisite for their ability to cause PAH (108). Whether they act in a paracrine manner or contribute directly by differentiating into mature cells, is still unclear.
5. Limitations

5.1 Inert gas rebreathing for non-invasive pulmonary blood flow measurement using the Innocor® System

We prospectively included patients undergoing right heart catheterization. This consisted of a mixed population of patients with and without PH. An obvious limitation is the small number of included patients. However, the data analysis did not focus on the general evaluation of the method, but rather on the assessment of acute changes induced by sildenafil or iloprost. To our best knowledge this is the first study evaluating the inert gas rebreathing method in such a setting.

5.2 Apelin and GDF-15 as blood derived biomarkers in pulmonary arterial hypertension and CTEPH

The IPAH cohort consisted partly of patients who underwent lung transplantation which means that a terminal disease was present. This is the reason why a follow-up analysis is not possible for the same subjects. Another limitation is that most of the included patients were under targeted therapy. Due to these reasons, we can not answer the question if apelin or GDF-15 levels change in the course of the disease and if therapy changes apelin levels.

5.3 Endothelial microparticles in PAH and CTEPH

The endogenous thrombin potential is an important parameter where microparticles could play a role. Some of our investigated patients received heparin before taking blood, therefore these results must be interpreted with caution. Due to the explorative nature of the study, the study groups were not matched, however, a study including 1:1 matching is already ongoing in our center.

5.4 Assessment of circulating progenitor cells in PAH

The low number of the included PAH patients is an obvious limitation, however, PAH is a rare disease and our patients were hemodynamically well characterized by right heart catheterization. In addition, our healthy controls were tightly age- and sex-matched to the investigated PAH patients. The lung histology was only performed on end-stage IPAH patients at the time of lung transplantation,
however, lung biopsies from patients at an earlier stage are not available for obvious ethical reasons.
6 Conclusions

1. Innocor

Inert gas rebreathing may represent an accurate non-invasive method for assessment of cardiac output in pulmonary hypertension, although the precision is too low to allow for assessment of acute changes of cardiac output. This questions the usefulness of the technique for follow-up investigations in patients with PH. Multicenter evaluation and the avoidance of pitfalls are essential.

2. Apelin

Apelin-17 and GDF-15 are promising circulating serum-derived biomarkers for idiopathic pulmonary hypertension and chronic thromboembolic pulmonary hypertension. Future studies are needed to assess the prognostic value of these novel markers.

3. Microparticles

Endothelial microparticles are elevated in the circulation of pulmonary arterial hypertension. Their level was negatively correlated with right atrial pressure. Whether they play a role in the pathogenesis of PH or their elevation is a result of the disease remains to be explored.

4. Circulating progenitor cells

The relative number of bone marrow-derived circulating CD133 progenitor cells is elevated in pulmonary arterial hypertension patients. In the lung tissue, CD133+ cells consist of phenotypically different populations including pneumocytes type II. Circulating CD133+ cells might represent a promising biomarker for PAH.
References


7. Delcroix M, Lang I, Pepke-Zaba J, Janssa P, D’Armini AM, Snijder R, et al. Long-Term Outcome of Patients With Chronic Thromboembolic Pulmonary Hypertension (CTEPH): Results From an International Prospective Registry. Circulation. 2016 Jan 29;


90. Gentile MA. Inhaled medical gases: more to breathe than oxygen. Respir Care. 2011 Sep;56(9):1341–57; discussion 1357–9.


98. Tantawy AAG, Adly AAM, Ismail EAR, Habeeb NM. Flow cytometric assessment of circulating platelet and erythrocytes microparticles in young thalassemia major patients.


100. Calloni R, Cordero EAA, Henriques JAP, Bonatto D. Reviewing and updating the major molecular markers for stem cells. Stem Cells Dev. 2013 May 1;22(9):1455–76.


