Angiogenesis in Pulmonary Arterial Hypertension
- Imaging of the Right Heart in a New Mouse Model

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# Table of contents

1 **ACKNOWLEDGEMENTS** 5  
2 **ABSTRACT (DEUTSCH)** 6  
3 **ABSTRACT (ENGLISH)** 6  
4 **BACKGROUND** 7  
   4.1 **Pulmonary Hypertension** 7  
      4.1.1 Definition 7  
      4.1.2 Classification 7  
      4.1.3 Diagnosis of PH 10  
   4.2 **Pulmonary arterial hypertension (PAH) – WHO Group I** 15  
      4.2.1 Definition 15  
      4.2.2 Diagnosis and evaluation of severity 15  
      4.2.3 Epidemiologie of PAH 17  
      4.2.4 Risk Factors in PAH 17  
      4.2.5 Histology 18  
      4.2.6 Pathogenesis 18  
      4.2.7 Therapy 21  
      4.2.8 Pulmonary veno-occlusive disease (PVOD) and pulmonary capillary hemangiomatosis (PCH) 24  
   4.5 **The VEGF signaling pathway in PAH** 25  
      4.5.1 Compounds of the VEGF signaling system 25  
      4.5.2 Control of the VEGF signaling system 26  
      4.5.3 VEGF in human and experimental PAH 28  
      4.5.4 The Paradox of PAH following VEGFR Blockade 28  
      4.5.5 Conclusion – the role of VEGF in PAH 30  
   4.6 **Animal models mimicking human PAH** 31  
      4.6.1 Chronic Hypoxia (CHP) 32  
      4.6.2 Sugen 5416 and CHP 33  
      4.6.3 Summary of current animal models of PAH 36  
5 **RATIONALE AND AIM** 37  
6 **MATERIALS AND METHODS** 38  
7 **RESULTS** 42  
   7.2.1 Right ventricular ejection fraction 43  
   7.2.2 Pulmonary acceleration time / ejection time (PAT/ET) ratio 44  
   7.2.3 Midsystolic notching 45  
   7.2.4 Left ventricular function – cardiac output (CO) 46  
8 **DISCUSSION** 47  
9 **CONCLUSION AND FUTURE PROSPECTS** 49
11 APPENDIX

11.1 Animal Models for human PH
   11.1.1 Classical single pathogenic insult (SPI) animal models
   11.1.2 New MPI animal models
   11.1.3 Knockout models in mice
   11.1.4 Overexpression models

11.2 Figures

11.3 Tables

11.4 Abbreviations and acronyms
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2 Abstract (Deutsch)

Hintergrund

Methoden und Ergebnisse:

Conclusio

3 Abstract (English)

Background
Pulmonary arterial hypertension is a subtype of pulmonary hypertension that is a sequel of defective vascular remodelling – with an endothelial cell specific monoclonal expansion, leading to continuous vessel occlusion and therefore an increase in pulmonary vascular resistance. Affected patients suffer from symptoms caused by progressive right heart failure which ultimately leads to death.

Methods and Results
The understanding of pathogenesis of pulmonary arterial hypertension is limited by the absence of precise animal models exhibiting aspects of pulmonary arterial hypertension including the typical pulmonary vasculopathy. In this study we developed a novel model combining a conditional knock-out of vascular endothelial growth factor receptor type 2 in endothelial cells (Kdr<sup>Δ<sub>end</sub></sup>) of C57/BL6J mice with exposure to either normoxia or chronic hypoxia in an environmental chamber with FiO<sub>2</sub> of 10% for 4 weeks.

Echocardiographic and magnetic resonance imaging revealed an equal reduction of cardiac output in Kdr<sup>Δ<sub>end</sub></sup> and control mice in response to hypoxia, a decrease in pulmonary acceleration time/ ejection time ratio in response to hypoxia that was most significant in Kdr<sup>Δ<sub>end</sub></sup> mice. Pulse-wave Doppler measurements of the right ventricular outflow tract of hypoxic Kdr<sup>Δ<sub>end</sub></sup> mice showed midsystolic notching, indicating severe pulmonary hypertension. Cardiac magnetic resonance imaging furthermore revealed a reduced right ventricular ejection fraction in hypoxic Kdr<sup>Δ<sub>end</sub></sup> mice signifying right ventricular dysfunction.

**Conclusion**

The combined pathogenic stimulus of direct ablative gene manipulation of Kdr and chronic hypoxia in C57/BL6J mice leads to pulmonary arterial hypertension.

## 4 Background

### 4.1 Pulmonary Hypertension

#### 4.1.1 Definition

Pulmonary Hypertension (PH) comprises a variety of different diseases. It is most recently defined as an increased mean pulmonary artery pressure (mPAP) >20 mmHg at rest assessed by right heart catheterization (RHC) interpreted in combination with pulmonary arterial wedge pressure (PAWP) and pulmonary vascular resistance (PVR) (1).

#### 4.1.2 Classification

**4.1.2.1 Hemodynamic Classification**

Clinical measurements set the base for a hemodynamic classification of PH as demonstrated in Table 1 (1).

<table>
<thead>
<tr>
<th>Definition</th>
<th>Characteristics</th>
<th>Clinical group(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-capillary PH</td>
<td>mPAP &gt;20 mmHg</td>
<td>1) PAH</td>
</tr>
<tr>
<td></td>
<td>PAWP ≤15 mmHg</td>
<td>3) PH due to lung disease</td>
</tr>
</tbody>
</table>
### Haemodynamic definition of PH

<table>
<thead>
<tr>
<th>Isolated post-capillary PH (IpcPH)</th>
<th>mPAP &gt;20 mmHg</th>
<th>2) PH due to left heart disease</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PAWP &gt;15 mmHg</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PVR &lt;3 WU</td>
<td>5) PH with unclear and/or multifactorial mechanisms</td>
</tr>
<tr>
<td>Combined pre- and post-capillary PH (CpcPH)</td>
<td>mPAP &gt;20 mmHg</td>
<td>2) PH due to left heart disease</td>
</tr>
<tr>
<td></td>
<td>PAWP &gt;15 mmHg</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PVR ≥3 WU</td>
<td>5) PH with unclear and/or multifactorial mechanisms</td>
</tr>
</tbody>
</table>

**Table 1** Haemodynamic definition of PH.

mPAP = mean pulmonary arterial pressure; PAH = pulmonary arterial hypertension; PAWP = pulmonary arterial wedge pressure; PH = pulmonary hypertension; PVR = pulmonary vascular resistance; WU = Wood units (1).

### 4.1.2.2 Clinical classification

A clinical classification has been established that categorizes numerous different clinical conditions into 5 groups according to similar clinical presentation, pathological findings, haemodynamic characteristics and treatment strategy (Table 2) (1).

<table>
<thead>
<tr>
<th>I. PAH</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Idiopathic PAH</td>
</tr>
<tr>
<td>II. Heritable PAH</td>
</tr>
<tr>
<td>III. Drug- and toxin-induced PAH</td>
</tr>
<tr>
<td>IV. PAH associated with:</td>
</tr>
<tr>
<td>i. Connective tissue disease</td>
</tr>
<tr>
<td>ii. Human immunodeficiency virus infection</td>
</tr>
<tr>
<td>iii. Portal hypertension</td>
</tr>
<tr>
<td>iv. Congenital heart disease</td>
</tr>
</tbody>
</table>
v. Schistosomiasis

V. PAH long-term responders to calcium channel blockers

VI. PAH with overt features of venous/capillary (PVOD/PCH) involvement

VII. Persistent PH of the newborn syndrome

II. PH due to left heart disease

I. PH due to heart failure with preserved LVEF

II. PH due to heart failure with reduced LVEF

III. Valvular heart disease

IV. Congenital/acquired cardiovascular conditions leading to postcapillary PH

III. Pulmonary hypertension due to lung disease and/or hypoxia

I. Obstructive lung disease

II. Restrictive lung disease

III. Other lung disease with mixed restrictive/obstructive pattern

IV. Hypoxia without lung disease

V. Developmental lung disorders

IV. PH due to pulmonary artery obstructions

I. Chronic thromboembolic PH (CTEPH)

II. Other pulmonary artery obstructions
   i. Sarcoma (high or intermediate grade) or angiosarcoma
   ii. Other malignant tumours
       Renal carcinoma
       Uterine carcinoma
       Germ cell tumours of the testis
       Other tumours
   iii. Non-malignant tumours
       Uterine leiomyoma
   iv. Arteritis without connective tissue disease
   v. Congenital pulmonary artery stenoses
   vi. Parasites
       Hydatidosis

V. PH with unclear and/or multifactorial mechanisms

I. Haematological disorders
   i. Chronic haemolytic anaemia
   ii. Myeloproliferative disorders

II. Systemic and metabolic disorders
   i. Pulmonary Langerhans cell histiocytosis
   ii. Gaucher disease
   iii. Glycogen storage disease
iv. Neurofibromatosis
v. Sarcoidosis

III. Others
i. Chronic renal failure with/without haemodialysis
ii. Fibrosing mediastinitis

IV. Complex congenital heart disease

Table 2  Clinical classification of PH.

| LVEF = left ventricular ejection fraction; PAH = pulmonary arterial hypertension; PCH = pulmonary capillary haemangiomatosis; PVOD = pulmonary veno-occlusive disease (1). |

4.1.3 Diagnosis of PH

When symptoms and signs raise suspicion for PH, a set of diagnostic investigations is needed to confirm the presence of PH, to describe aetiology and to assess the functional and haemodynamic severity of disease. A diagnostic algorithm is shown in Figure 1.

![Diagram of diagnostic algorithm](image)

**Figure 1**  Diagnostic algorithm of pulmonary hypertension.

mPAP = mean pulmonary artery pressure; PAH = pulmonary arterial hypertension; PAWP = pulmonary artery wedge pressure; PH = pulmonary hypertension; PVR = pulmonary vascular resistance; RHC = right heart catheterization; V/Q scan = ventilation/perfusion scan (1,2).
4.1.3.1 Diagnostic algorithm of pulmonary hypertension (Figure 1)

If clinical suspicion of PH arises, transthoracic echocardiography (TTE) should be the first diagnostic step. If an intermediate to high level of probability of PH (see 4.1.3.6) has been determined, further examinations should follow. Additional testing should aim at excluding the more common groups 2 and 3 (History, symptoms, signs, electrocardiogram (ECG), chest radiograph, pulmonary function test (including lung diffusion capacity for carbon monoxide (DLCO), arterial blood gases analysis, ± nocturnal oximetry, high-resolution computed tomography (HR-CT) of the chest). If no cause could be identified, PH due to pulmonary artery obstructions should be excluded next via a ventilation/perfusion (V/Q) scan. If still no etiology could be identified, RHC should be performed to verify a hemodynamic state consistent with pulmonary arterial hypertension (PAH) or group 5. Lastly a variety of specific diagnostic tests can help distinguish between different causes of disease.

4.1.3.2 Clinical presentation

Symptoms of PH are unspecific and mostly related to subsequent right ventricular (RV) dysfunction. Early signs become apparent on exertion and include shortness of breath, fatigue, weakness, angina and syncope, in rare cases also dry cough and nausea/vomiting. In advanced stages of disease symptoms at rest might present including abdominal distension and ankle oedema (2).

Some symptoms associated with PH will evolve due to mechanical complications: Haemoptysis in case of ruptured hypertrophied bronchial arteries, hoarseness due to compression of the laryngeal nerve by a dilated pulmonary artery (PA), dissection or even rupture of the afore-mentioned PA, wheezing caused by compressed airways and angina as a result of left coronary artery compression.

During physical examination various signs can be found: A left parasternal lift (due to RV hypertrophy (RVH)), an accentuated pulmonary component of the second heart sound, a pansystolic murmur (tricuspid regurgitation), a diastolic murmur (pulmonary regurgitation), elevated jugular venous pressure, hepatomegaly, ascites, peripheral oedema and cool extremities (2).

4.1.3.3 Electrocardiogram (ECG)

Even though a normal ECG does not exclude PH some abnormalities may occur, especially in severe PH with subsequent right heart failure: P pulmonale, right axis deviation, signs of RVH including RV strain and right bundle branch block (2,3). QTc and QRS prolongation (4,5) such as supraventricular arrhythmias (6) imply advanced disease.

4.1.3.4 Chest radiograph

If PH is considered a possible differential diagnosis, certain abnormalities in a chest radiograph like central pulmonary artery dilatation, ‘pruning’/ loss of peripheral blood vessels and right heart enlargement support the diagnosis. Additional signs of pulmonary venous congestion in left heart disease (Table 2, group 2) or interstitial lung disease (Table 2, group
3) provide further information on etiology. However PH cannot be excluded by a normal chest radiograph only (7).

4.1.3.5 *Pulmonary function tests and arterial blood gases*

Due to multiple possible pathomechanisms leading to symptoms compatible with PH, it is crucial to exclude underlying airway or parenchymal lung disease with pulmonary function tests and arterial blood gases. In PAH a reduction of lung volumes is common, as well as a decrease in DLCO (8). Alveolar hyperventilation at rest causes normal partial oxygen pressure ($\text{p}a\text{O}_2$), but decreased partial carbon dioxide pressure ($\text{p}a\text{CO}_2$) in arterial blood gas analysis (9). Furthermore, a high prevalence of nocturnal hypoxaemia and central sleep apnoeas in PAH has been reported (10).

4.1.3.6 *Transthoracic Echocardiography (TTE)*

TTE is recommended as a first-line non-invasive investigation if PH is suspected. Conclusions derived from TTE should procure a level of probability and thereby clarify the necessity for RHC. The main variable is the estimated systolic pulmonary artery pressure (sPAP) based on continuous wave (CW) Doppler measurements of peak tricuspid regurgitation velocity (TRV) (Table 3). Right atrial pressure (RAP) is another valuable parameter, estimated using the simplified Bernoulli equation taking into account right atrial (RA) diameter and respiratory variation in diameter of the inferior vena cava (IVC). A number of additional signs compatible with a diagnosis of PH are listed in Table 4. It is important to emphasize that treatment decisions cannot be made based on echocardiography alone but must follow precise examination and diagnosis via RHC (11).

<table>
<thead>
<tr>
<th>Peak tricuspid regurgitation velocity (m/s)</th>
<th>Other echocardiographic signs of PH (Table 4)</th>
<th>Probability of PH</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤2.8 or not measurable</td>
<td>No</td>
<td>Low</td>
</tr>
<tr>
<td>≤2.8 or not measurable</td>
<td>Yes</td>
<td>Intermediate</td>
</tr>
<tr>
<td>2.9 - 3.4</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>2.9 - 3.4</td>
<td>Yes</td>
<td>High</td>
</tr>
<tr>
<td>&gt;3.4</td>
<td>Not required</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3** Echocardiographic probability of PH.
PH = pulmonary hypertension (2).
Table 4  Other echocardiographic signs compatible with PH.
IVC = inferior vena cava; IVS = interventricular septum; LV = left ventricle; PA = pulmonary artery; RA = right atrium; RV = right ventricle (2).

4.1.3.7 Ventilation/perfusion (V/Q) lung scan

The V/Q scan is the screening method of choice for PH due to pulmonary artery obstructions (group IV), superior to computed tomography pulmonary angiogram (CTPA). In PAH small peripheral unmatched or non-segmental perfusion defects are common (12).

4.1.3.8 Computed tomography  (CT)

CT imaging provides important additional information often needed for a diagnosis of PH. RV enlargement, increased pulmonary artery (PA) diameter ≥29 mm, pulmonary:ascending aorta diameter ratio ≥1.0, a segmental artery:bronchus ratio >1:1 in three or four lobes are signs of PH (13). HR-CT is able to detect signs of different pathomechanisms like interstitial lung disease and emphysema, pulmonary veno-occlusive disease (PVOD) and pulmonary capillary haemangiomatosis (PCH) (14,15). Contrast CT angiography of the PA helps determine interventional accessibility of lesions of PH due to pulmonary artery obstructions (yet does not replace pulmonary angiography) (16).

4.1.3.9 Cardiac magnetic resonance imaging (CMRI)

CMRI provides highly accurate information on RV size and morphology and furthermore constitutes a non-invasive assessment of RV function (blood flow, cardiac output (CO) and PA distensibility). Especially in collectives unsuitable for other diagnostic means e.g. pregnant women, children, contraindicated iodine-based contrast media or in case of follow-up examination CMRI has proven a valid diagnostic tool. Signs like late gadolinium
enhancement, reduced PA distensibility and retrograde flow are consistent with a diagnosis of PH and should imply further examination by RHC (17–19).

4.1.3.10 Blood tests and immunology

Blood tests give information on various aetiologies of PH and possible end organ damage. A routine set of biochemistry, haematology and thyroid function tests (PAH is associated with thyroid disease) is mandatory when proposing a diagnosis of PAH. Besides, liver function tests are often abnormal due to elevated hepatic venous pressures, hepatitis or endothelin receptor antagonist (ERA) therapy. Furthermore serology provides information on the presence of connective tissue diseases (CTD), hepatitis, human immunodeficiency virus (HIV), systemic lupus erythematosus and systemic sclerosis (high prevalence in PAH). Patients with a diagnosis of PH due to pulmonary artery obstructions should be referred to thrombophilia screening (antiphospholipid antibodies, anticaardiolipin antibodies, lupus anticoagulant). Lastly, elevated N-terminal pro-brain natriuretic peptide (NT-proBNP), representing cardiac involvement, constitutes an independent risk predictor (2).

4.1.3.11 Right heart catheterization (RHC) and vasoreactivity

To confirm a diagnosis of either PAH or PH due to pulmonary artery obstructions, to evaluate suitable treatment options, in case of congenital cardiac shunts and prior to organ transplantation (group 2 and 3), RHC should be the final diagnostic step after having completed any other necessary investigations. Ideally it should be performed in expert centers due to technical complexity. Pressure measurements in the PA, in PA wedge position using a balloon catheter (= surrogate for left atrial (LA) pressure), in the RV and RA are taken at the end of normal expiration. Blood samples for oxymetry are taken from the superior vena cava (SVC), IVC and PA in addition to systemic arterial oxygen saturation (SaO₂), especially in case of a suspected left-to-right-shunt (2). CO is estimated by either (preferably) Thermodilution or (in-) direct Fick method (20). In case of IPAH, HPAH or drug-induced PAH vasoreactivity testing of pulmonary circulation is indicated: A minority (10% of IPAH) of patients react to high-dose calcium channel blocker (CCB) treatment. If administration of inhaled nitric oxide (NO) at 10-20 parts per million (ppM) or alternatively i.v. epoprostenol results in a decrease of mPAP ≥10 mmHg to ≤40 mmHg with unchanged or increased CO, patients show an acute positive response (2). In case of left heart disease (LHD), diuretics frequently reduce PAWP <15 mmHg and therefore mask an initially postcapillary PH. A volume challenge with a 500 ml fluid bolus can help discriminate between PAH and LV diastolic dysfunction. It is important to note that PAWP may underestimate LV end-diastolic pressure (21). Pivotal derived variables are the transpulmonary pressure gradient (TPG) and PVR. In case of angina, risk factors for coronary artery disease, LV diastolic or systolic dysfunction and heart failure with preserved ejection fraction (HFpEF) left heart catheterization should be performed liberally (2).
4.1.3.12 Genetic testing

Genetic counselling and screening for disease-causing mutations is available to patients with sporadic, familial or PAH induced by anorexigen agents. Besides information on reproductive risk there is also a psychological impact of either positive or negative results that justifies genetic counselling. If no bone morphogenetic protein receptor type 2 (BMPR-2) mutation can be identified in either a patient <40 years of age or a patient with associated hereditary haemorrhagic teleangiectasia, screening for ACVRL1 (encoding for serine/threonine-protein kinase receptor R3) and ENG (encoding for endoglin) mutations is indicated. Screening for rare mutations like potassium channel subfamily K member 3 (KCNK3), caveolin 1 (CAV1) is considered only if no mutations were identified (22). Patients with a diagnosis or family history of PVOD or PCH should be tested for bi-allelic eukaryotic translation initiation factor 2 alpha kinase 4 (EIF2AK4) mutation (23).

4.2 Pulmonary arterial hypertension (PAH) – WHO Group I

4.2.1 Definition

PAH is a subset of pulmonary hypertension. Per definitionem PAH is a precapillary PH with a mean pulmonary artery pressure >20 mmHg, pulmonary vascular resistance (PVR) ≥3 Wood units (WU) and a PAWP ≤15 mmHg in absence of other possible causes such as PH due to lung disease and/or hypoxia, due to left heart disease, due to pulmonary artery obstructions or other rare diseases. The definition of PAH comprises a group of disease entities that differ in etiology but share characteristics such as precapillary PH, pulmonary hypertensive arteriopathy including plexiform lesions, slow clinical onset and chronic progression such as similar response to treatment (1,24).

4.2.2 Diagnosis and evaluation of severity

4.2.2.1 Clinical parameters, imaging and hemodynamics

Variations in symptoms (changes in exercise capacity, episodes of chest pain, arrhythmia, haemoptysis or syncope) and signs (cyanosis, enlarged jugular veins, oedema, ascites and pleural effusions, heart rate, rhythm and blood pressure) such as adherence to prescribed drugs help determine disease stability and a level of severeness. World Health Organization functional class (WHO-FC) is mainly determined by RV function and constitutes a reliable predictor of survival (25). In case of clinical deterioration a comprehensive assessment of RV function via echocardiography should ensue (Table 5). If needed cardiac magnetic resonance imaging (CMRI) can further distinguish RV morphology and function (26). RHC should be considered for diagnosis and follow-up in case of changed medication or any decisions regarding listing for transplantation (2).
Of note, echocardiographic measurements of sPAP or RHC measurements of mPAP do not predict disease progression well and therefore are considered negligible (27).

<table>
<thead>
<tr>
<th>TTE (28)</th>
<th>CMRI</th>
<th>RHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Chamber sizes</td>
<td>- RV volume</td>
<td>- RAP</td>
</tr>
<tr>
<td>- Tricuspid regurgitation</td>
<td>- LV volume</td>
<td>- CI</td>
</tr>
<tr>
<td>- LV eccentricity index</td>
<td>- RV EF</td>
<td>- SvO₂</td>
</tr>
<tr>
<td>- RV contractility</td>
<td>- SV</td>
<td></td>
</tr>
<tr>
<td>- RV longitudinal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>systolic strain/ strain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rate (29)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- RV fractional area change</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Tei index</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- TAPSE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Speckle tracking</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5  Specific indicators of RV function.

CI = cardiac index; CMRI = cardiac magnetic resonance imaging; LV = left ventricle; RAP = right atrial pressure; RHC = right heart catheterization; RV = right ventricle; RV EF = right ventricular ejection fraction; SV = stroke volume; SvO₂ = mixed venous oxygen saturation; TAPSE = tricuspid annular plane systolic excursion; TTE = transthoracic echocardiography.

4.2.2.2  **Exercise capacity**

Exercise capacity can be measured using different standardized tests. The often used 6-minute-walking test constitutes a submaximal exercise test. Combined with the Borg Rating of Perceived Exertion scale in order to determine a level of effort it provides a first impression of exercise capacity (30). Cardiopulmonary exercise testing (CPET) constitutes a maximal exercise test. It provides concrete information on gas exchange, ventilator efficacy and cardiac function during exercise. In PAH a low end-tidal paCO₂, high ventilatory equivalents for carbon dioxide (VE/VCO₂), a low oxygen pulse (VO₂/HR) and low peak oxygen uptake (peak VO₂) are typical (31).

4.2.2.3 **Biochemical markers**

While there is no specific marker for PAH, brain natriuretic peptide (BNP) and NT-proBNP (correlation with myocardial dysfunction) are most commonly used, as they provide valid prognostic information if interpreted in clinical context (32). Various other markers of different areas have been explored, yet no specificity towards PAH could be distinguished in either one (Table 6).
Vascular dysfunction - ADMA
- Endothelin-1
- Angiopoietin
- von Willebrand factor

Inflammation - C-reactive protein
- Interleukin 6
- Chemokines

Myocardial stress - Atrial natriuretic peptide
- BNP/ NTproBNP
- Troponins

Low CO and/or tissue hypoxia - PaCO₂
- Uric acid
- GDF15
- Osteopontin

Secondary organ damage - Creatinine
- Bilirubin

<table>
<thead>
<tr>
<th>Table 6</th>
<th>Markers explored in context of PAH (2).</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADMA = Asymmetric dimethylarginin; BNP = brain natriuretic peptide; GDF15 = growth differentiation factor 15; NT-proBNP = N-terminal pro-brain natriuretic peptide; paCO₂ = arterial carbon dioxide pressure (2).</td>
<td></td>
</tr>
</tbody>
</table>

4.2.3 Epidemiologie of PAH

Studies among european populations suggest a prevalence of PAH from 15 to 60 cases per million and an incidence of 5-10 cases per million per year (33). The mean age at diagnosis ranges between 50 and 65 years. The often reported female predominance cannot be asserted due to notable variations among different registries. Survival improved in course of time, whereas there are different mortalities within the subgroups – PAH associated with congenital heart disease (CHD) shows higher survival rates than IPAH, whereas association with CTD has a worse, PVOD and PCH the worst prognosis (34).

4.2.4 Risk Factors in PAH

Risk factors as such were defined as predisposing or facilitating factors in the development of PAH and were classified as definite, likely or possible (35). Various drugs and toxins with respective risk level ar shown in Table 7.
### Table 7  
Drugs and Toxins associated with PAH (1).

<table>
<thead>
<tr>
<th>Definite</th>
<th>Possible</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminorex</td>
<td>Cocaine</td>
</tr>
<tr>
<td>Fenfluramine</td>
<td>Phenylpropanolamine</td>
</tr>
<tr>
<td>Dexfenfluramine</td>
<td>L-tryptophan</td>
</tr>
<tr>
<td>Benfluorex</td>
<td>St John’s Wort</td>
</tr>
<tr>
<td>Methamphetamines</td>
<td>Amphetamines</td>
</tr>
<tr>
<td>Dasatinib</td>
<td>Interferon-α and -β</td>
</tr>
<tr>
<td>Toxic rapeseed oil</td>
<td>Alkylating agents</td>
</tr>
<tr>
<td></td>
<td>Bosutinib</td>
</tr>
<tr>
<td></td>
<td>Direct-acting antiviral agents against hepatitis C virus</td>
</tr>
<tr>
<td></td>
<td>Leflunomide</td>
</tr>
<tr>
<td></td>
<td>Indirubin (Chinese herb Qing-Dai)</td>
</tr>
</tbody>
</table>

#### 4.2.5 Histology

All forms of PH comprise arterial changes like intimal, medial and adventitial thickening, the appearance of muscle-like cells in the walls of smaller arteries and a loss of peripheral vascular diameter. In comparison, pulmonary hypertensive arteriopathy in PAH is more severe and specifically characterized by „plexogenic arteriopathy“. The characteristic 'plexiform' and other complex lesions emerging from pulmonary vascular remodeling in human PAH comprise intimal hyperplasia, medial hypertrophy, adventitial proliferation/fibrosis, occlusion of small arteries, in situ thrombosis and infiltration of inflammatory/progenitor cells. The plexus is formed by slit-like channels which are lined by ECs and myofibroblasts within a dilated segment of the affected artery. An increased expression of transcription and growth factors known from angiogenesis like vascular endothelial growth factor (VEGF) and hypoxia inducible factor (HIF)-1α is commonly found (36,37). Many heterogenous types of lesions exist that all together lead to a severe luminal reduction, stiffening of proximal and remodelling of distal vessels (36–39).

#### 4.2.6 Pathogenesis

The pathogenesis of PAH is not fully understood, yet the combination of sustained vasoconstriction, arterial wall remodeling and in situ thrombosis are considered to be the main contributing factors (40). The consequence is pulmonary arterial obstruction, causing an increased PVR which ultimately results in RV failure and death (2).

##### 4.2.6.1 PAH affects all vascular layers

The molecular pathology of PAH affects all vessel layers and is therefore often referred to as panvasculopathy (41).

The pulmonary vascular endothelium (intima) is affected by an imbalance of vasodilation and constriction favoring the latter (42–44) and increased levels of prothrombotic tissue factor
Furthermore apoptosis-resistant endothelial cells (EC) proliferate extensively, causing the formation of plexiform and other complex lesions that occlude the vascular lumen (46). In the media, pulmonary arterial smooth muscle cell (PASMC) proliferation is increased whereas apoptosis is suppressed (47,48). It is influenced by a number of pathologic mechanisms: Mutation or downregulation of BMPR-2 (49,50), mitochondrial metabolic abnormalities, increased expression of survivin (anti-apoptotic) (51), serotonin transporter (SERT) (52) and platelet-derived growth factor receptor (PDGFR) (53), increased tyrosine kinase activation (54), decreased expression or loss of Kv1.5, a voltage-gated, O₂-sensitive potassium channel that is inhibited by hypoxia to initiate hypoxic pulmonary vasoconstriction (55) and normoxic activation of HIF-1α (56).

The adventitia is damaged by metalloprotease activation permitting cell migration and synthesis of mitogenic petides like tenascin (57).

4.2.6.2 Molecular mechanisms

Based on the current state of scientific knowledge a number of basic science concepts try to explain the development of typical lesions and progression of PAH (41):

1) Endothelial dysfunction with an imbalance between vasodilation and vasoconstriction (favoring the latter), increased mitogenesis and thrombosis were often described. Restoration of balance is the target of most current therapeutic options (42,43,58).

2) A number of genetic mutations was associated with a higher susceptibility to develop PAH. Numerous members of the transforming growth factor (TGF)-β family are affected in PAH. Amongst them heterozygous BMPR-2 mutations make up for 75% of familial PAH and 25% of sporadic cases of PAH (22). BMPR-2 is a constitutively active serine-threonine kinase receptor that forms heterodimers with one of four existing type 1 receptors (BMPR-1A, BMPR-1B, activin receptor-like kinase 1 (Alk1) or Alk2) in case of ligand (BMP) attachment. The ensuing phosphorylation of the intracellular part of the type 1 receptor leads to a Smad protein-signaling cascade, ending in the formation of a complex with Smad4 that translocates into the nucleus and controls gene transcription. BMPR-2 mutations lead to promoted cell proliferation and suppressed apoptosis and a predisposition for PAH (59). Penetrance is low at approximately 25%. Thus, there must be gene-gene or gene-environment interactions that either enhance or prevent the development of vascular disease in people carrying a mutation (60). In patients with PAH and associated hereditary haemorrhagic teleangiectasia a mutation of the gene encoding for Alk1 is most common, followed by mutations in ENG, BMPR1B and SMAD9. Further associated genetic mutations outside the TGFβ superfamily have been found in CAV1 and KCNK3 (22,61).

3) PAH displays a variant of similarities with cancer. Excessive cell proliferation and impaired apoptosis in PASMCs, fibroblasts and ECs (47,48,62) are common in both disease entities (62), as are mitochondrial changes. An impaired mitochondrial O₂-sensing mechanism causes normoxic activation of HIF-1α which suppresses the oxidative metabolism by increased pyruvate dehydrogenase kinase (PDK)
transcription and activates glycolytic genes. Increased activity of PDK, an enzyme that inhibits pyruvate dehydrogenase, leads to a shift from oxidative phosphorylation to glycolysis despite adequate oxygen supply (37,56,63). These metabolic abnormalities can be partially corrected by a mitochondrial-targeted strategy with Dichloroacetate, a PDK inhibitor (64). In contrast to cancer, PAH does not metastasize or disrupt tissue boundaries (41).

4) Another contributing mechanism is refractory vasoconstriction due to rho-Kinase activation. Calcium/ calmodulin binds an enzyme called myosin light chain (MLC) kinase which phosphorylates MLC and causes PASMC contraction. It’s antagonist, MLC phosphatase, dephosphorylates MLC and therefore causes relaxation. Rho-kinase inhibits MLC phosphatase, causing refractory vasoconstriction. Rho-kinase inhibitors (Y-27632, fasudil) help reduce PAH in various models and human PAH, but can cause systemic vasodilatation (65,66).

5) Pulmonary veno-occlusive disease (PVOD) and pulmonary capillary hemangiomatosis (PCH)

PVOD and PCH represent a rare subgroup of PAH (Table 2) showcasing obliterative remodeling of pulmonary venules and capillary infiltration of the pulmonary interstitium (1). PCH/PVOD are regarded varied expressions of one disease entity due to widely overlapping clinico-pathological characteristics, an elevated risk of acute pulmonary oedema in response to PAH treatment, worse prognosis and especially due to bi-allelic mutations in EIF2AK4 causing both heritable PVOD and PCH (1). PAH and PVOD/PCH share clinical, pathological and modelucal characteristics (i.e. over-activation of the PDGF/PDGFR axis (2,3), serotonin-induced SMC hyperplasia (4)). Both diseases are associated with increased EC proliferation contributing to the formation of vascular lesions (1), however unlike the characteristic plexiform lesions in PAH, lesions of PVOD/PCH present markers of cell growth suppression (i.e. peroxisome proliferation-activated receptor-γ, caveolin-1) and hence display a distinct EC phenotype (5).

Since the discovery of bi-allelic mutations of EIF2AK4 causing heritable PVOD/PCH in 2014, its implication in PCH/PVOD pathogenesis is thoroughly investigated. EIF2AK4 is a gene coding for general control nonderepressible 2 (GCN2) – a kinase phosphorylating the α-subunit of eukaryotic translation initiation factor 2 (eIF2α). The phosphorylation of eIF2α is considered to have a protective effect regarding cellular stress and therefore a decrease in activity might signify an increase in vulnerability to stressors like inflammation and oxidative stress (6). Intrestingly, alkylating substances like mitomycin-C (a known risk factor for PVOD) lead to decreased levels of GCN2 in animal models (7).

Morover the knockout of compartments of the ERG–apelin receptor (APLNR) axis – modulating EC differentiation - could be associated with pulmonary venous specific EC proliferation. Interestingly no connection between GCN2 and ERG/APLNR could be made, indicating two distinct pathogenetic pathways (8).
Due to the association of PVOD/PCH and inflammatory conditions, immune mediated vascular injury has been proposed as a potential phogenetic influence, too. Indeed inflammatory cell proportions were different in PVOD and the serum level of Granulysin – a potent effector protein of inflammatory cells - was increased (9), indicating that deregulation of Granulysin and differences in inflammatory response might play a role in pathogenesis of PCH/PVOD, too.

4.2.7 Therapy

4.2.7.1 Treatment goals and follow-up strategy

Therapy of PAH aims at keeping patients at low risk status with good exercise capacity, RV function, a low mortality risk and in general good quality of life, albeit these goals might not be attainable by some collectives (co-morbidities, old age, advanced disease) (2). The treatment of PAH should include 1) general measures, 2) specific medical treatment and in case of inadequate response to the latter 3) escalation of therapy (combination therapy, lung transplantation).

4.2.7.2 Treatment algorithm in PAH

As soon as a patient has been diagnosed with PAH, referral to an expert center is advisable. Besides general measures and required supportive therapy, patients with IPAH, HPAH or drug/toxin induced PAH will first undergo acute vasoreactivity testing. Initial treatment of responders will consist of high-dose CCB and should be evaluated for adequacy after a period of 3-4 months. Non-responders (and patientens with inasquate response to CCB) will receive specific PAH medical treatment. Patients at low or intermediate risk are suited for initial monotherapy, whereas no first-line monotherapy can be advised due to lacking comparison of different compounds. Another possibility for this collective might be upfront combination therapy with ambrisentan and tadalafil (67). Non-responders at high risk should start on initial combination therapy including an parenteral prostacyclin analogon like epoprostenol (68). In case of inadequate clinical response to initial treatment, sequential double or triple combination therapy should follow. If maximal combination therapy still fails to produce an adequate clinical response, patients should be referred for lung transplantation if not contraindicated.
Figure 2  Treatment algorithm in PAH.
CCB = calcium channel blockers; HPAH = hereditary PAH; IPAH = idiopathic PAH; PAH = pulmonary arterial hypertension (2).

4.2.7.3 Specific drug therapy

Calcium channel blockers
Few patients with IPAH, HPAP or drug-induced PAH are responders to acute vasoreactivity testing during RHC. Responders are treated with high doses of CCBs (in case of relative bradycardia nifedipine and amlodipine, in case of relative tachycardia diltiazem) (69). The efficacy of CCB treatment – measured by WHO-FC I or II, pronounced haemodynamic improvement - is assessed after 3-4 months of therapy. In case of insufficient response treatment must be completed by specific PAH therapy. Regular monitoring is advisable due to severe side effects (e.g. hypotension, syncope, RV failure) (2).

Endothelin receptor antagonists (ERA)
Over-activation of the endothelin system plays an important role in the pathogenesis of PAH (44). Endothelin 1 binds to typ A and B receptor isoforms located on pulmonary vascular smooth muscle cells, leading to vasoconstriction and elevated mitosis rates. ERAs such as
Ambrisentan, Bosentan and Macitentan counteract these effects (70–72). Treatment with ERAs (except Macitentan) should enclose regular liver function testing.

Phosphodiesterase type 5 (PDE-5) inhibitors and soluble guanylate cyclase (sGC) stimulators

PDE-5 degrades cyclic guanosine monophosphate (cGMP) which leads to vasodilation as a second messenger to NO. Hence PDE-5 inhibitors such as sildenafil, tadalafil and vardenafil augment the NO/cGMP pathway by slowing down cGMP degradation (73). Besides PDE-5 inhibitors have antiproliferative properties on PASMCs (74). Riociguat, a sGC stimulator, increases cGMP production and has additional antiproliferative and antiremodelling effects. It is worth mentioning that a combination of riociguat and PDE-5 inhibitors leads to hypotension and therefore is contraindicated (75,76).

Prostacyclin analogues and prostacyclin receptor agonists

Prostacyclin is synthesized mainly by endothelial cells and acts as a potent endogenous vasodilator and platelet aggregation inhibitor with additional cytoprotective and antiproliferative properties (77). Dysregulation of prostacyclin metabolic pathways plays a significant role in the pathogenesis of PAH (78). Hence prostacyclin analogues (Epoprostenol, Illoprost, Treprostenil, Beraprost) and selective prostacyclin IP receptor agonists (Selexipag) constitute a vital part of therapy (79–83).

Combination therapy

The combination of drugs each targeting one of three associated pathways - the prostacyclin pathway (prostanoids), the endothelin pathway (ERAs) and the NO pathway (PDE-5 inhibitors and sGC stimulators) - has become a valid option if monotherapy cannot effectuate the desired improvement. There is evidence on the superiority of initial therapy with tadalafil and ambrisentan compared to monotherapy with each of the substances (67). In case of sequential drug combination evidence for benefit was found in numerous cases with WHO-FC II and III (Macitentan added to Sildenafil, Riociguat added to bosentan, selexipag added to ERA and/or PDE-5 inhibitors) and WHO-FC III respectively (Sildenafil added to epoprostenol) (72,75,83,84).

Drug interactions

Drug interactions may occur with Bosentan, an inducer of cytochrome P450 (CYP) isoenzymes CYP3A4 and CYP2C9. While Bosentan will reduce plasma levels of drugs metabolized by the isoenzymes, CYP3A4/2C9 inhibitors (e.g. ketoconazole, ritonavir, amiodarone, fluconazole, fresh grapefruit juice) will lead to increased plasma bosentan levels. Sildenafil is equally metabolized by CYP3A4 and CYP2C9 and its levels may decrease in coadministration with CYP3A4 substrates or inhibitors, whereas increased levels can be expected with CYP3A4 inducers (e.g. carbamazepine, phenytoin, phenobarbital, rifampicin, St John’s wort). In case of additional antihypertensive therapy systemic hypotension must be anticipated (2).
4.2.7.4 Transplantation

In advanced stages of PH and RV failure the implantation of a veno-arterial extracorporeal membrane oxygenation (ECMO), improving oxygenation and decompressing the RV, can bridge to transplantation (85). Transplantation should be considered in case of persisting WHO-FC III and IV despite maximal combination therapy or in case of diagnosed PVOD or PCH. As disease progresses during often long waiting times due to lacking donor organs, listing for either double-lung or heart-lung transplantation should be considered timely (86). Post-transplant survival was promising with 39–75% at 5 years and to 27–66% at 10 years (87).

4.2.8 Pulmonary veno-occlusive disease (PVOD) and pulmonary capillary hemangiomatosis (PCH)

PVOD and PCH represent a rare subgroup of PAH (Table 2) caused by obliterative fibrosis of pulmonary venules and capillary infiltration of the pulmonary interstitium (1). Knowledge on etiology is lacking, however association with bi-allelic EIF2AK4 mutation (autosomal recessive inheritance (2)), certain diseases (i.e. SSc, HIV) and drugs/toxins (i.e. cyclophosphamide, mitomycin) has been reported (3). Differentiation between PVOD and PCH is vague - both diseases share a series of pathologic characteristics and a similarly high risk of drug-induced pulmonary oedema in response to PAH therapy (4,5). Moreover PVOD and PCH overlap in the majority of cases (6), hence unsurprisingly a link between both diseases has been proposed in that angioproliferative processes of PCH could occur secondary to obstruction due to PVOD (6). In general PVOD/PCH are underdiagnosed - in part due to clinical signs and symptoms shared with PAH (5). However some findings like basal crackles in pulmonary auscultation, digital clubbing and more severe hypoxaemia and lower DLCO set PVOD/PCH apart (5). Besides clinical findings the diagnosis of PVOD/PCH is based on imaging modalities like chest radiography and the more specific HR-CT (subpleural thickened septal lines, centrilobular ground-glass-opacification and mediastinal lymphadenopathy), bronchoscopy and bronchoalveolar lavage (diagnostic tools for the often associated diagnosis of occult alveolar hemorrhage (5,7). Nowadays, these non-invasive diagnostic means in combination with genetig testing for bi-allelic EIF2AK4 mutation often render lung biopsy unnecessary (5). Of note, RHC will reveal a hemodynamic situation similar to PAH except for less elevated PAWP (due to changes residing in small pulmonary venules and capillaries) but comes with greater risk for acute pulmonary oedema in response to vasoreactivity testing (5). Therapy is difficult in PCH/PVOD and in general, the prognosis is worse when compared to PAH (3). A combination of diuretic treatment, O2 administration and gradually increasing doses of epoprostenol is recommended however unsatisfying due to high risk of pulmonary oedema (4). Therefore patients with a diagnosis of PVOD/PCH should be evaluated for eligibility for lung transplantation – the only curative approach (8).
4.5 The VEGF signaling pathway in PAH

It is hypothesized that PAH is caused by dysfunctional angiogenesis resulting in pulmonary obliterator vasculopathy. The pathomechanisms underlying PAH include pressure, flow and shear stress, but also cell growth/death and cell phenotype plasticity (92). The main players involved in the development of PAH are considered 1) the extensively researched BMPR-2, and 2) the less attended VEGF signaling system (92,93). The role of the complex VEGF signaling system in the pathogenesis of PAH has not fully been elucidated. Especially the paradox results of the Sugen 5416 (SU5416) and chronic hypoxia (CHP) rodent model where VEGF receptor (VEGFR) inhibition induced PAH demand further explanation (94). Ultimately, antiangiogenic drugs might be a new treatment option.

4.5.1 Compounds of the VEGF signaling system

VEGF signaling plays a pivotal role in vasculogenesis and angiogenesis. The compounds of the VEGF signaling pathway include VEGFs (ligands), VEGFRs (receptors) and coreceptors (i.e. neuropilin) (93).

Among VEGF isoforms, VEGF-A is the most common ligand. It binds VEGFR-1 (also known as fms-related tyrosine kinase 1 (Flt-1)) and VEGFR-2 (also known as kinase insert domain receptor = kdr) (95). In general, VEGF-A is known to enhance vascular permeability, angiogenesis and vascular cell survival (96). However during the process of alternative splicing, proangiogenic and antiangiogenic variants are created (97). Other ligands include VEGF-B (antiangiogenic effect), VEGF-C and VEGF-D (lymphangiogenesis) and placental growth factor (PGF, vasculogenesis and angiogenesis during embryogenesis) (98,99). Further related molecules include VEGF-E, a viral homologue, and VEGF-F, a component of snake venom (100,101). Like VEGF-A, all VEGF ligand isoforms bind one or two of three existing VEGF-receptors with different affinity (Table 8).

The VEGFRs are tyrosine kinases with a characteristic structure of 7 extracellular Immunoglobulin-like domains, a cytoplasmatic tyrosine kinase domain and a long kinase insert region. Upon ligand-mediated dimerization VEGFRs activate, translocate intercellularly and transmit any given signal (93). VEGFR-1 undergoes weak tyrosine autophosphorylation in case of ligand binding. It is considered a decoy receptor as it prevents VEGFR-2 activation, furthermore the soluble VEGFR-1 is known to sequester VEGF (102,103). All together, VEGFR-1 activation has a negative effect on VEGF activity. VEGFR-2 is the main receptor responsible effectuating the mitogenic, prosurvival, proangiogenic, and permeability-enhancing functions of VEGF-A. In case of ligand bindig, dimerization and tyrosine phosphorylation ensue (93,96). VEGFR-3 (also known as fms-related tyrosine kinase 4 = Flt4) binds VEGF-C and VEGF-D and serves the genesis and maintenance of lymphatic endothelium (99).

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Receptor</th>
<th>Function</th>
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</table>

<p>| VEGF-A | VEGFR-1, VEGFR-2 | Enhances vascular permeability, angiogenesis and vascular cell survival |
| VEGF-B | VEGFR-1, VEGFR-2 | Antiangiogenic effect |
| VEGF-C | VEGFR-3 | Lymphangiogenesis |
| VEGF-D | VEGFR-3 | Placental growth factor |
| PGF | VEGFR-3 | Vasculogenesis and angiogenesis during embryogenesis |
| VEGF-E | VEGFR-1, VEGFR-2 | Viral homologue |
| VEGF-F | VEGFR-1, VEGFR-2 | Component of snake venom |</p>
<table>
<thead>
<tr>
<th>VEGF-A</th>
<th>VEGFR-1</th>
<th>VEGFR-2</th>
<th>Vascular permeability, angiogenic, vascular cell survival (17)</th>
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<tbody>
<tr>
<td>VEGF-B</td>
<td>VEGFR-1</td>
<td></td>
<td>Antiangiogenic (14)</td>
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<tr>
<td>VEGF-C</td>
<td>VEGFR-2</td>
<td>VEGFR-3</td>
<td>Lymphangiogenesis, tip cell sprouting during angiogenesis (40)</td>
</tr>
<tr>
<td>VEGF-D</td>
<td>VEGFR-2</td>
<td>VEGFR-3</td>
<td>Lymphangiogenesis</td>
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<td>PGF</td>
<td>VEGFR-1</td>
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<td>Angiogenesis and vasculogenesis during embryogenesis</td>
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<td>VEGF-E</td>
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<td>VEGF-F</td>
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**Table 8**  Receptor affinity and function of VEGF ligands.

PGF = placental growth factor; VEGF = Vascular endothelial growth factor; VEGFR = Vascular endothelial growth factor receptor.

### 4.5.2 Control of the VEGF signaling system

Upon ligand-bound activation VEGFR-2 promotes mitosis, angiogenesis and cell-survival by phosphorylating downstream proteins in ECs. Thereafter VEGFR-2 is endocytosed and undergoes either proteasome degradation of recycling back to the plasma membrane. A variety of influencing factors regarding VEGF-A expression, VEGFR-2 activation and signaling are listed in Table 9.

<table>
<thead>
<tr>
<th>Influencing Factor</th>
<th>Effect</th>
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<tbody>
<tr>
<td><strong>Factors influencing VEGF-A expression</strong></td>
<td></td>
</tr>
<tr>
<td>HIF-1α and -2α</td>
<td>Induced angiogenesis and NO production (104).</td>
</tr>
<tr>
<td>p53</td>
<td>Up-regulated VEGF expression in response to hypoxia, then down-regulation (105).</td>
</tr>
</tbody>
</table>
### Factors influencing VEGFR-2 activation and signaling

<table>
<thead>
<tr>
<th>Factor</th>
<th>Effect</th>
</tr>
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<tbody>
<tr>
<td>Flt activation</td>
<td>Prevention of Kdr activation, Sequestration of VEGF ligands (102,103).</td>
</tr>
<tr>
<td>s-Flt activation</td>
<td>Prevention of Kdr activation, Sequestration of VEGF ligands (102,103).</td>
</tr>
<tr>
<td>Notch-receptor activation</td>
<td>Decreased Kdr and VEGFR-3 expression, increases Flt expression (108).</td>
</tr>
<tr>
<td>Neuropilin-1 (upon binding of VEGF)</td>
<td>Enhances binding of VEGF₁₆₅ to Kdr and increases signal transduction (109).</td>
</tr>
<tr>
<td>Sphingosine 1</td>
<td>Forms complexes with and phosphorylated Kdr (110).</td>
</tr>
<tr>
<td>Ephrin-B₂&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Endosomal degradation of Kdr; absent ephrin-B₂ disturbs VEGF signaling (111).</td>
</tr>
<tr>
<td>Integrins&lt;sup&gt;b&lt;/sup&gt;</td>
<td>VEGF signaling independent of VEGFR tyrosine kinases: Cell spreading, migration and survival (112).</td>
</tr>
<tr>
<td>FoxO1</td>
<td>Kdr activation deactivates FoxO1 - EC growth, Kdr inhibition activates FoxO1 – apoptosis (113).</td>
</tr>
<tr>
<td>COX-2, Cytosolic PLA₂</td>
<td>Kdr-dependent activation (link between inflammation, hypoxia and angiogenesis) (96)</td>
</tr>
</tbody>
</table>

**Table 9**  
Influencing Factors of VEGF signaling.

COX-2 = cyclooxygenase type 2; EC = endothelial cell; Flt = fms-related tyrosine kinase 1 (= VEGF receptor type 1); FoxO1 = forkhead box protein O1 (transcription factor of phosphatidylinositol-3-kinase/ mammalian target of rapamycin/ complex 2 axis); HIF1/2α = hypoxia inducible factor 1α/2α; Kdr = kinase insert domain receptor (= VEGF receptor type 2); NFκB = nuclear factor 'kappa-light-chain-enhancer' of activated B-cells transcription factor associated with inflammation; NO = nitric oxide; p53 = tumor protein 53; PLA₂ = phospholipases A₂; PGC-1α = peroxisome proliferator-activated receptor coactivator 1 alpha; s-Flt = soluble Flt; VEGF = vascular endothelial growth factor; VEGFR-3 = VEGFR receptor type 3.
a RTKs like Kdr are able to signal not only at plasma membrane level but also after endocytosis. Hence undisturbed endocytosis is an important factor for regular VEGFR-2 signaling.

b VEGF signaling can function independently of VEGFR tyrosine kinases via integrins (glycoproteins that transpass the plasma membrane and act as adhesion molecules between cells and the extracellular matrix) (112).

4.5.3 VEGF in human and experimental PAH

VEGF activity is modulated by many factors, including its own expression level, the presence of certain proteins that inactivate the ligand and the ratio of the different ligands competing for VEGFR binding sites (98). As a multitude of different VEGF ligands and receptors, coreceptors and structurally similar receptor tyrosine kinases (RTK) contribute, the VEGF signaling pathway remains highly complex and versatile.

While elevated VEGF levels are expected in several conditions like tumor angiogenesis (114) and intraocular neovascular disorders (115), a similar link between VEGF and PAH is highly probable. VEGF plasma levels of PAH patients were equally found to be elevated (116). On top of that, the characteristic complex vascular lesions displayed increased expression of VEGF and VEGFR-2 (37). The question is whether VEGF is the cause or the consequence of PAH pathogenesis. Assuming that an increased activity of the VEGF signaling system stands at the beginning of PAH development, a number of experimental models tried to provoke PAH development by amplifying VEGF signaling. However the unexpected paradoxical results revealed lacking knowledge: Experimental overexpression of VEGF-A did not increase, but attenuate the development of CHP-induced PAH in a CHP rat model (117); correspondingly, the combined stimulus of VEGFR-2 blockade via SU5416 and CHP in an experimental rat model resulted in the development of severe angio-obliterrative PAH (94).

4.5.4 The Paradox of PAH following VEGFR Blockade

The established rat model of Tarasiviciene et al. (118) used a combined stimulus of VEGFR-2 inhibition via SU5416 and CHP and observed initially increased EC apoptosis rates followed by increased EC proliferation rates. Over time rats developed severe angio-obliterrative PAH and right heart failure (RHF). As these findings do not correlate with our understanding of the VEGF signaling system, multiple theories circulate, trying to explain the paradoxical outcome.

Rebound effect

One possible explanation for the excessive EC proliferation might be a “rebound” effect after administration of an anti-VEGF therapy, leading to increased stimulation of the VEGF system and triggering of angiogenesis. This theory is based on a study of cancer patients treated with the RTK inhibitor Dasatinib who displayed increased expression of VEGFRs and developed PAH (119). This theory gives rise to speculations concerning the existence of endogenous „SU5416-like“ inhibitors of VEGF signaling (93). Several potential molecules
might entail a function similar to the VEGFR-2 inhibitor.

The splice variant VEGF\textsubscript{165b} binds and blocks VEGFR-2. Increased plasma concentration or an imbalance between VEGF\textsubscript{165a} and VEGF\textsubscript{165b} were found in systemic sclerosis (Ssc) and could be responsible for initial EC apoptosis (120).

VEGFR-1/Flt-1 (membrane-bound) and its soluble isoform s-Flt are known for their antiangiogenic properties and several findings suggest a link with disturbed VEGFR-1 signaling and the occurrence of excessive EC proliferation in later stages of PAH: s-Flt levels were found elevated in human PAH (121); VEGFR-1 is known to induce the antiapoptotic protein survivin which has been linked to the formation of plexiform-like lesions in PAH (122); pluripotent endothelial progenitor cells were found to express both VEGFR-1 and s-Flt and to secrete a cytokine (tumor necrosis factor superfamily member 15 = TNFSF15 = vascular endothelial growth inhibitor) that simultaneously induces Flt-1 degradation and increases s-Flt expression (123).

Ligands other than VEGF binding VEGFR-2 could have an impact: Decorin is a leucin-rich proteoglycan that regulates the activity of various growth factors and acts as a pan-RTK-inhibitor. If soluble Decorin binds to VEGFR-2, it induces autophagy in ECs and suppresses angiogenesis (124,125).

Ligand-dependent VEGFR-2 endocytosis might impact the development of PAH. Internalized VEGFR-2 is capable of activating the phosphatidylinositol-3-kinase/ protein kinase B (PI3K/Akt) prosurvival pathway (67). A disruption of the endosomal-dependent internalization might cause increased intracellular activation of VEGFR-2 and result in proliferation of ECs.

Another suspicious molecule is the antiangiogenic chemokine CXCL4 (platelet factor 4) which had predictive value in the development of PAH associated with Ssc (126).

Two consecutive pathogenic ‘hits’

An alternative explanation for the vast proliferation of ECs after VEGFR blockade (first ‘hit’) might be a second pathogenic ‘hit’. Physiological stressors associated with PAH (i.e. shear stress, drugs, infections, inflammation) could increase circulating VEGF levels. In the SU5416 + CHP setting, SU5416 (an unselective inhibitor of VEGFR1/2) is considered the first ‘hit’ that inhibits VEGFR-2, causes EC apoptosis and as consequence thereof selects apoptosis-resistant ECs. Hypoxia constitutes the second ‘hit’ that promotes the proliferation of apoptosis-resistant cells (46).

In both scenarios, increased VEGF would signal through an uninhibited VEGFR-3 (127), and/or alternatively via integrins (128).

Receptor Tyrosine Kinase (RTK) Remodeling

Recent reports from large registries suspect anti-cancer treatments like tyrosine kinase inhibitors (TKI) or antiangiogenic therapies to induce pulmonary hypertension that is
independent of isolated vasoconstriction suggesting pulmonary vascular toxicity (129). The paradoxical upregulation of several RTKs in response to VEGFR inhibition might be another possible mechanism contributing to increased EC proliferation (113). Besides VEGF and VEGFRs other RTKs and ligands like PDGF, c-kit (protoonkogen encoding for KIT tyrosine kinase) and endothelial growth factor (EGF) are involved in the pathogenesis of PAH (130–132). The question regarding different RTK inhibitors is in what way each substance individually affects PAH development. Imatinib positively affected the hemodynamic state, however clinical state remained unchanged (133); on the contrary Dasatinib (a pan-RTK-Inhibitor) and Bevacizumab (a humanized monoclonal antibody against VEGF) appeared to promote pathogenesis (119,134); Sunitinib and Sorafenib have been shown to increase VEGF levels (135).

A closer look into the molecular mechanisms initiated by antiangiogenic treatments was taken by Ferrara et al. (113) who incubated ECs with VEGF and investigated the phosphoproteome. As a result they found several differences in phosphorylation patterns. The authors suggest an association with the PI3K/mammalian target of rapamycin (mTOR)/complex 2 axis, especially with a transcription factor called forkhead box protein O1 (FoxO1). FoxO1 is deactivated in case of VEGF binding to VEGFR-2, resulting in EC growth. Inversely VEGFR-2 inhibition leads to increased FoxO1 activation that causes apoptosis. Apart from regulating cell survival, activated FoxO1 was found to induce a process of RTK reprogramming, causing a VEGFR blockade–induced resistance of ECs that might partially be responsible for the formation of apoptosis-resistant cells in later stages of disease.

4.5.5 Conclusion – the role of VEGF in PAH

Clearly the VEGF signaling system is complex - equally modulating pro- and antiangiogenic pathways. While the paradoxical outcome of the SU5416+CHP and similar models demands further exploration, it seems evident that unimpaired VEGF signaling is of great importance for maintained homeostasis in the pulmonary vasculature. It can be concluded, that besides the pivotal role of BMPR-2 in PAH development, the VEGF signaling system constitutes an important influencing factor.
4.6 Animal models mimicking human PAH

The development of an animal model for PAH with high fidelity to human disease is an absolute premise when investigating the pathogenetic processes underlying PAH. So far most animal models displayed certain hemodynamic and structural changes compatible with human PAH but failed to replicate complex vascular remodeling as found in human PAH. Current treatment options reflect our level of understanding - targeting endothelial dysfunction and vasoconstriction but failing to affect vascular remodeling. Hence further studies on the pathogenesis of PAH are needed to increase knowledge and to identify potential therapeutic targets that do affect pulmonary vasculopathy.

Animal models are categorized into single-pathological-insult (SPI) models (i.e. hypoxia or monocrotaline), multiple-pathological-insult (MPI) models (i.e. SU5416 and Hypoxia), knock-out models and overexpression models (136). These models aim to develop histological, hemodynamic and structural features seen in human PAH. The classical SPI animal models expose rodents to hypoxia or monocrotaline, but fail to establish pulmonary hypertensive arteriopathy as seen in human PAH. Newer MPI models exhibit more severe PH including arteriopathy and therefore correlate better with human disease. The manipulation of specific genes further provides insight into the pathogenetic impact of certain molecules. Relevant animal models for a closer understanding of the pathomechanisms underlying human PAH are listed in Table 10. A closer explanation of all models is given below and in appendix 11.1.

<table>
<thead>
<tr>
<th>Models</th>
<th>Species</th>
<th>Usage</th>
<th>PH Group</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SPI models</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCT</td>
<td>Rats</td>
<td>Very common</td>
<td>Group 1</td>
</tr>
<tr>
<td>CHP</td>
<td>Rats</td>
<td>Very common</td>
<td>Group 3</td>
</tr>
<tr>
<td>Schistosomiasis</td>
<td>Mice</td>
<td>Common</td>
<td>Group 1</td>
</tr>
<tr>
<td>Fawn-hooded rats</td>
<td>Rats</td>
<td>Uncommon</td>
<td>Group 3</td>
</tr>
<tr>
<td>Pulmonary arterial banding</td>
<td>Rats</td>
<td>Common</td>
<td>Group 2</td>
</tr>
<tr>
<td><strong>MPI models</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCT + pneumonectomy</td>
<td>Rats</td>
<td>Uncommon</td>
<td>Group 1</td>
</tr>
<tr>
<td>MCT + CHP</td>
<td>Rats</td>
<td>Uncommon</td>
<td>Group 3</td>
</tr>
<tr>
<td>CHP + SU5416</td>
<td>Rats</td>
<td>Common</td>
<td>Group 1/3</td>
</tr>
</tbody>
</table>
**Knockout models**

<table>
<thead>
<tr>
<th>Model</th>
<th>Species</th>
<th>Frequency</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMPR-2</td>
<td>Mice</td>
<td>Uncommon</td>
<td>Group 1</td>
</tr>
<tr>
<td>VIP</td>
<td>Mice</td>
<td>Uncommon</td>
<td>No defined group</td>
</tr>
<tr>
<td>Neprilysin</td>
<td>Mice</td>
<td>Uncommon</td>
<td>No defined group</td>
</tr>
<tr>
<td>Endothelin receptor-B</td>
<td>Mice</td>
<td>Common</td>
<td>No defined group</td>
</tr>
<tr>
<td>Apolipoprotein-E</td>
<td>Mice</td>
<td>Uncommon</td>
<td>No defined group</td>
</tr>
</tbody>
</table>

**Overexpression models**

<table>
<thead>
<tr>
<th>Model</th>
<th>Species</th>
<th>Frequency</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interleukin-6</td>
<td>Mice</td>
<td>Uncommon</td>
<td>No defined group</td>
</tr>
<tr>
<td>Angiopoeitin-E</td>
<td>Rats</td>
<td>Common</td>
<td>No defined group</td>
</tr>
<tr>
<td>Serotonin/5-HTT</td>
<td>Mice</td>
<td>Common</td>
<td>No defined group</td>
</tr>
<tr>
<td>S100A4/Mts-1</td>
<td>Mice</td>
<td>Uncommon</td>
<td>No defined group</td>
</tr>
</tbody>
</table>

**Table 10** Animal models of pulmonary hypertension.

BMPR-2 = bone morphogenetic protein receptor-2; CHP = chronic hypoxia; MCT = monocrotaline; MPI = multiple-pathological-insult; SPI = single-pathological-insult; SU5416 = Sugen 5416 (inhibitor of VEGFR-2, which acts as a prosurvival signal on pulmonary ECs); S100A4/Mts-1 = a metastasis-promoting protein implicated in PH vascular remodeling; VIP = vasoactive intestinal peptide; 5-HTT = 5-hydroxytryptamine transporter.

a PH groups according to Nice classification (see Table 2).

**4.6.1 Chronic Hypoxia (CHP)**

Chronic normobaric or hypobaric hypoxia constitutes a classical SPI animal model for group 3 (PH due to lung disease and/or hypoxia) (137). CHP impaires the signaling pathway responsibiel for vasodilation from different angels. The genes and mechanisms involved are HIF-1, oxidant stress, enothelin-1 upregulation, stimulation of RhoA and Rho kinase and the NO-sGC-cGMP signaling pathway (138–141). Vascular remodeling induced by CHP is also influenced by the expression the genes of HIF-1α, endothelial nitric oxide synthase (eNOS), complement-3 and BMPR-2 (142–144). Especially mouse models of CHP proved invaluable when researching the moelcular processes involved in vascular remodeling (i.e. growth factors, reactive oxygen species, and the nitric oxide pathway (145–147). However other factors such as haemodynamics (i.e. PA banding (148)) and inflammatory and progenitor cell
recruitment (149) play a role in the occurring pulmonary structural modifications as well.

Hypoxia induces similar structural changes in most mammals: Muscuarisation of small, normally unmuscular arteries (150), thickening of previously muscularized precapillary PAs, a PA-specific vascular inflammatory response (151), thickening and fibrosis of the large proximal PAs leading to significant stiffening (152), mPAP elevation and RVH but not RV failure. Yet it fails to recapitulate pulmonary vascular disease including obstructive intimal lesions as found in human disease (153). Moreover there are differences depending on age, species and strains. Younger age is associated with higher susceptibility to hypoxia-triggered PH. Mice only show minimal vascular remodelling (154). Fawn hooded rats (FHR) develop the most severe spectrum of CHP-induced PH in rodents - probably due to increased endothelin production in PASMCs (155), but in contrast to human PAH also develop systemic hypertension. Experimental treatments successfully mended in CHP models include digoxin, A-17 (an inhibitor of microRNA-17), hypercapnia, bosentan, dichloroacetate, and targeted gene delivery of BMPR-2 (138,139,157–160).

On a final note, the most important differences of CHP models, when compared to human PAH, include reversibility of PH when returning to normoxic conditions, absent formation of irreversible intimal fibrosis or plexogenic lesions (161) and a generally less severe level of disease. An attempt to improve the CHP model is by adding another trigger (e.g. Monocrotaline (MCT) or SU5416) which shows increased severity and histological features more similar to human disease.

4.6.2 Sugan 5416 and CHP

The hallmarks of PAH - increased vasoconstriction and remodeling of arterioles - are based on abnormal function of pulmonary ECs as well as SMCs, fibroblast, platelets, and inflammatory cells (162). VEGF and VEGFR-2 have an important prosurvival and antiapoptotic role towards ECs and an increased level of VEGF were found on ECs forming the angioproliferative lesions that are typical for human PAH (163).

The idea of a rodent model manipulating the VEGF signaling pathway was captured in 2001 by Taraseviciene-Stewart et al.: A rat model using the combined pathogenic stimulus of VEGFR-2 inhibition via SU5416 and CHP (118) was established. Normoxic rats developed mild PH and slight pulmonary vascular remodelling whereas hypoxic animals recapitulated human PAH better than prior models, especially regarding pulmonary vascular remodeling. Once induced PAH persisted and progressed even after cessation of hypoxia, ultimately leading to RV failure and death in some animals. Besides, a particular time course of pulmonary vasculopathy could be observed (only in the combined stimulus group of CHP and SU5416): EC apoptosis was increased in early stages, followed by extensive proliferation of apoptosis-resistant ECs which then formed complex vascular lesions (118). The only difference between human PAH and disease in CHP-SU5416-treated rats lies in an accelerated time course of disease progression of the latter.

A modified model by Ciuclan et al. (164) exposed mice to the same combined stimulus of SU5416 and CHP. However a modified protocol of gradual SU5416 administration allowed
for closer investigation of angioproliferative and hemodynamic changes. The echocardiographic assessment displayed severe PAH with RVH and characteristic hemodynamic changes (i.e. increased RVP, changed flow profile through the PA valve including an elevated PAP, decreased pulmonary acceleration/ejection time (PAT/ET) ratio and signs of midsystolic notching). In contrast to human disease however the CO of study mice consistently decreased, indicating incipient heart failure. Pulmonary vascular remodeling with striking similarities to human disease was found again (i.e. neomuscularization of pulmonary arterioles, medial hypertrophy and concentric neointimal thickening/ occlusion, accumulation of collagen in the media, adventitia and pulmonary parenchyma, inflammatory cell infiltrates). Also, the time course of increased endothelial proliferation after increased apoptosis rates could be observed again. Systemic arterial pressure remained unchanged. When compared to the rat model, the mouse model displayed less severe PAH and showed better reversibility under normoxia (gradual decrease of RVP, RVH and vascular remodeling) (118,164).

Regarding the molecular pathways underlying PAH pathogenesis, Ciuclan et al. found dysregulated expression of genes associated with the TGF-β/BMP/Smad axis, vasoconstriction, inflammation and proliferation (Table 11). Some changes appear based on CHP alone (i.e. decreased BMPR-2) whereas others (i.e. plasminogen activator inhibitor-1 (PAI-1)) are further amplified under SU5416 exposure. Hence a parallel functioning of both stimuli is probable.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>CHP</th>
<th>CHP+SU5416</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMPR-2</td>
<td>TGF-β signaling</td>
<td>Down-regulated</td>
<td>-</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Fibrinolysis, fibrosis, TGF-β signaling</td>
<td>Up-regulated</td>
<td>Enhanced</td>
</tr>
<tr>
<td>Phosphorylated Smad2</td>
<td>TGFβ signaling</td>
<td>Up-regulated</td>
<td>Enhanced</td>
</tr>
<tr>
<td>Phosphorylated Smad1</td>
<td>TGFβ signaling</td>
<td>Up-regulated</td>
<td>-</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Growth factor family, mitogenic</td>
<td>Up-regulated</td>
<td>-</td>
</tr>
<tr>
<td>Tph 1</td>
<td>Rate-limiting enzyme of peripheral 5-HT synthesis</td>
<td>Up-regulated</td>
<td>-</td>
</tr>
</tbody>
</table>
Inflammation

Up-regulated

HIF-1α

Transcription factor mediating effects of hypoxia

Up-regulated

Enhanced

Phosphorylated AKT

VEGF-dependent EC survival

- 

Down-regulated

MAPK system (54)

Proliferation, stress-mediated signaling

Up-regulated

Enhanced

Table 1

| Gene expression rates of mice under chronic hypoxia ± Sugen 5416 (164). Akt = protein kinase B; BMPR-2 = bone morphogenetic protein receptor type 2; HIF-1α = hypoxia inducible factor 1α; IL-6 = interleukin 6; MAPK = mitogen-activated protein kinase; PAI-1 = Plasminogen activator inhibitor-1; PDGFR = platelet derived growth factor receptor; Smad 1/2 = "small mothers against decapentaplegic" - signal transducers for receptors of the TGF-β superfamily; TGF-β = transforming growth factor β; Tph 1 = Tryptophan hydroxylase 1.

Diverging gene expression evokes functional changes demonstrated by differential biomarker expression. These biomarkers are candidates to serve as prognostic tools in the future (Table 12).

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Description</th>
<th>CHP</th>
<th>CHP+SU5416</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRP 5-HT</td>
<td>Vascoconstrictor, mitogen on PASMCs and fibroblasts</td>
<td>increased</td>
<td>enhanced</td>
</tr>
<tr>
<td>BNP</td>
<td>Decreases central venous pressure, natriuresis</td>
<td>increased</td>
<td>extremely enhanced</td>
</tr>
<tr>
<td>ET-1</td>
<td>Vascoconstrictor, stress response</td>
<td>increased</td>
<td>enhanced</td>
</tr>
<tr>
<td>PAI-1 protein</td>
<td>Fibrinolysis, fibrosis, TGF-β</td>
<td>increased</td>
<td>enhanced</td>
</tr>
<tr>
<td>signaling</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDGF-AA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growth factor family, potent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mitogen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>increases</td>
<td>enhanced&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC mitogenesis and migration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>increased</td>
<td>-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 12** Biomarker levels in mice under chronic hypoxia and/or Sugen 5416 (164).

BNP = brain natriuretic peptide; ET-1 = endothelin 1; PAI-1 = Plasminogen activator inhibitor-1; PDGF-AA = platelet derived growth factor AA; PRP 5-HT = Serotonin levels in platelet-rich plasma; VEGF = vascular endothelial growth factor.

<sup>a</sup> Unlike PAH patient serum.

### 4.6.3 Summary of current animal models of PAH

PAH itself is a heterogenous group of disease entities and its pathogenesis has not fully been elucidated until today. Hence the development of a precise animal model replicating all facets of disease is difficult to accomplish. SPI models were developed first, displaying a lesser extent of disease probably present in early stages of human disease. Later on MPI models evolved and accomplished a better correlation with severe human disease. So far however, no ideal animal model for human PAH has been established. Furthermore one has to keep in mind that the significance of all animal models is limited by differences in response to stimuli depending on species, sex, age and environment. A one-to-one comparison between human disease and PAH in other animal species is therefore difficult. Nevertheless, animal models provide an unvaluable platform to investigate the pathophysiological processes influencing the development and persistence of PAH: Besides they are irreplaceable in the testing of new experimental treatments with the goal to prevent progression and even reverse established disease.
5 Rationale and Aim

The SU5416+CHP model was the first to imitate human PAH precisely, including specific pulmonary vascular remodeling and therefore led the way regarding the affiliation of disrupted VEGF signaling in PAH (118,164). Because administration of SU5416 is inhibits VEGFR-2 in all cell types, the aim of our study was to further dissect and clarify the pathogenetic role of VEGFR-2 inhibition in EC alone via a conditional knockout in pulmonary ECs of mice.

Further research into the role of VEGF signaling in the pathogenesis of PAH seems imperative for better understanding of underlying molecular mechanisms, paving the way for new therapeutic targets and more successful treatment.
6 Materials and methods

6.1 Mouse model

All aspects of this experimental mouse model were carefully chaperoned by the Department of Biomedical Research of the Medical University Vienna. Every treatment process was assented by the Institutional Animal Care Committee and the Austrian Ministry of Science (BMBWF 66.009/0141-II/10b/2010).

6.2 Kdr knockout and hypoxic breeding

C57/BL6J mice with a tamoxifen (TX) inducible CRE (CreER), controlled by a Tie2-promotor, were used. A targeted deletion of the first Exon of Kdr in ECs was initiated by administration of TX and resulted in EC-specific CRE activity. Mice were backcrossed at least 6 generations into the C57/BL6J background. Breeding resulted in a litter of both Kdr^{flox/flox}/Tie-2CreER (study group) mice and Cre negative littermates (Kdr^{flox/flox}/Tie-2, control group).

Deletion of Kdr in pulmonary ECs was performed by injecting 100 µL TX (20 mg/mL in 10 % ethanol and 90 % sunflower seed oil, all Sigma Aldrich, Austria) intraperitoneally (i.p.). TX was administered 3 Weeks prior to the experimental stage, first once a day for 5 days, then once a week for 2 weeks. Subsequently mice were labeled accordingly Kdr^{end} (Kdr^{flox/flox}/Tie-2CreER) or Cre-negative controls (Kdr^{flox/flox}/Tie-2) (165).
Figure 3 The CRE-LoxP system.

The CRE-LoxP system represents an important tool for genetic manipulation originating from bacteriophage P1. Cre recombinase is an enzyme that recognizes loxP sites (locus of X-over of P1) flanking a specific gene. The 34 bp long loxP sequence marks the intervening gene and depending on the orientation of both loxP sites (same orientation for excision, opposite orientation for inversion) Cre effectuates the excision of a targeted gene. In order to trigger a conditional (controlled time and cell/tissue type) excision the Cre recombinase is bound to a cell type-specific promoter (i.e. endothelial cell-specific Tie2) and a modified estrogen receptor (ER) which keeps the unactivated enzyme in the cytoplasm. The administration of TX, an antiestrogen that will "unlock" Tie2CreER by binding to the modified ER, enables the enzyme to transfer to the nucleus and see through the excision. In order to receive mice expressing both genetic features, one transgenic mouse strand expressing Tie2CreER and another expressing the floxed (flanked by LoxP sequences) gene are...
intercrossed. Initially CRE and the floxed gene are introduced into the genome via homologous recombination or using an adenovirus as vector (166). HSP90 = heat shock protein 90; CRE = Cre recombinase; TX = Tamoxifen; loxP = loxP site; Kdr = vascular endothelial growth factor receptor type 2.

6.3 Chronic hypoxia

As soon as the TX administration period was terminated mice were exposed to either chronic normobaric hypoxia (10% FiO₂) or normoxia (21% FiO₂) in a ventilated chamber (Biospherix A chamber®, Lacona, NY, USA) for 28 days. Exact O₂ concentration was monitored using an oxygen analyzer (Proox 110, Biospherix®, Lacona, NY, USA) and sustained by controlling nitrogen inflow rates. All results from hypoxic time-points indicate measurements taken after 6 weeks of hypoxic exposure.

6.4 Echocardiographic assessment

As soon as the treatment period was completed, mice underwent assessment via TTE. First, mice were anaesthetized with inhaled isoflurane 1,5% (Baxter Healthcare, Vienna, Austria). After removing hair from the chest area using depilatory cream mice were fixated on a flat surface for steady measuring conditions. A Vevo 2100 imaging system (VisualSonics Inc, Toronto, Canada) with a MS 400 ultrasound probe was used.

For depiction of the PA outflow tract, the ultrasound transducer was placed in a parasternal long axis position. Pulse wave Doppler was employed to visualize and analyse haemodynamic flow through the PA valve and measure the ratio of pulmonary acceleration time (PAT) to total pulmonary ejection time (ET). PAT/ET ratio was determined by the time taken from start of flow to maximal velocity or to the end of flow. A set of three cardiac cycle measurements ensured an average ratio of PAT/ET (which varies inversely with PAP). The degree of midsystolic notch (degree of indent through deceleration flow and measure of tricuspid regurgitation) was assessed by applying a score between 0 and 1 to each wave profile observed for each animal (167). Left ventricular function was measured via cardiac output. A 2-D image of the LV was obtained in a short axis position at the level of the papillary muscles. Then 2-D guided M-mode images were acquired at a sweep speed of 100 mm/s and stored digitally. The LV inner dimensions at diastole and systole and the LV external dimension at diastole were measured digitally on the M-mode trace (167).

6.5 Cardiac magnetic resonance tomodgraphy (CMRT)

All CMRT measurements were taken on a 9.4 Tesla Biospec 94/30 USR system (Bruker Biospin, Ettlingen, Germany), using a gradient insert with inner diameter of 116 mm. A maximum gradient strength of 667 mT/m could be accomplished. A transmitter volume resonator with an inner diameter of 86 mm was used for radiofrequency excitation. Images were obtained with a mouse heart coil array with 4 elements. After initial anesthesia in a chamber with isofluorane-influx mice were positioned on a heated mouse bed and continually anesthesized with 1,5-2% isofluorane per face mask. Cardiac function was visualized with a prospective ECG-gated cine gradient echo-based flow to compensate MR sequence (in-built
software ParaVision 6.0, Bruker Biospin, Ettlingen, Germany). The average of ten consecutive axial slices (long axis from cardiac apex to base) was used for all measurements. The imaging acquisition parameters used were: time of echo = 2.4 ms, time of repetition = 8 ms, averages = 6, field of view = 25 mm x 25 mm, slice thickness = 0.8 mm, flip angle = 15°, partial Fourier Transformation = 1.45, measured matrix = 132 x 192, visualized matrix = 192 x 192, 18 movie frames.

Post processing. For the assessment of LV function the specific Segment-Software for Quantitative Medical Image Analysis (Segment Software, v1.8 R1172; Medviso AB, Lund, Sweden) was used. End-systole and end-diastole were determined in the cine sequence. End-systolic and end-diastolic volumes (ml) of the LV were demarcated manually on each axial slice (along the long axis, excluding papillary muscles) and LV EF (%) was calculated automatically.

6.6 Statistical analysis

All data were normally distributed. Hence the significance of differences between groups (n = 8 animal per group) was determined by an unpaired 2-tailed Student t test and analysis of variance. P values <0.05 (95% confidence interval) were deemed significant. Statistical analysis was performed using SPSS 23.0 (IBM corp., Chicago, IL, USA). All results are specified as means ± SEM.
7 Results

7.1 Characterization of Kdr\textsuperscript{\lambda end} mice

TX was injected into both Kdr\textsuperscript{\lambda end}/Tie-2CreER and control mice at an approximate age of 8-10 weeks. Subsequently the mice were exposed to either hypoxia or normoxia in respective housing modalities. All mice survived the described treatment (up until the scheduled time of sacrifice).

Heart Rate

TTE in anesthetized mice revealed an average heart rate of 396 ± 28 bpm with no statistically significant differences between Kdr\textsuperscript{\lambda end} and control mice.
7.2 Assessment of pulmonary hypertension by echocardiography and magnetic resonance imaging

7.2.1 Right ventricular ejection fraction

Decreased measurements of RV ejection fraction after hypoxic exposure observed in cardiac MRI (2F) indicate incipient RV dysfunction in $Kdr^{\Delta eno}$ mice.

![Graph showing RVF (%)](image)

**Figure 4** Right ventricular ejection fraction measured by cardiac magnetic resonance imaging.
7.2.2 Pulmonary acceleration time / ejection time (PAT/ET) ratio

The PAT/ET ratio was significantly decreased in all mice exposed to CHP. Kdr knockout caused further depletion.

**Figure 5** Echocardiographic measurements of pulmonary acceleration time / ejection time ratio.
7.2.3 Midsystolic notching

Midsystolic notching represents another typical sign of severe PH. In Doppler measurements of the RV outflow tract (RVOT) during systolic ejection a distinct notch within the first or second third of the deceleration flow profile can be observed. The visualized deceleration of systolic RV flow velocity can be understood as a pathological wave reflection in the presence of elevated pulmonary artery pressures (168).

Midsystolic notching was observed only in $Kdr^{\Delta\text{end}}$ mice exposed to hypoxia, but did not occur in control mice.

![Figure 6 Pulsed Doppler measurements of the right ventricular outflow tract (parasternal long axis) during systolic ejection.](image)

*Left:* Representative measurements of hypoxic control mice.

*Right:* Representative measurements of hypoxic $Kdr^{\Delta\text{end}}$ mice; white arrows direct at midsystolic notch.
7.2.4 Left ventricular function – cardiac output (CO)

In our model hypoxia induced a decrease in CO in both $Kdr^{\Delta_{end}}$ mice and controls, however $Kdr^{\Delta_{end}}$ mice did not display any signs of decreased CO before hypoxic exposure. Corresponding results were obtained by CMRI.

**Figure 7** Cardiac output measured by echocardiography.

**Figure 8** Cardiac output measured by cardiac magnetic resonance imaging.
8 Discussion

PAH is a severe and progressive disease that develops based on characteristic pulmonary hypertensive arteriopathy. It is caused by a combination of sustained vasoconstriction, in situ thrombosis and a negative pulmonary vascular remodeling process with monoclonal expansion of collateral ECs and total vessel occlusion. As a result the vascular lumen diminishes, PVR rises, increasing RV afterload and ultimately causing RV failure and death (2,40). The pathomechanisms underlying PAH include pressure, flow and shear stress, but also cell growth/ death and cell phenotype plasticity (92). So far, the extensively researched BMPR-2 and the less attended VEGF signaling system have been identified as key components in pathogenesis (92,93).

Results like elevated plasma levels of VEGF in PAH patients (116) and an increased expression of VEGF and VEGFR-2 in the characteristic complex vascular lesions (37) initiated first conclusions regarding a link between VEGF signaling and PAH. With the question of whether elevated expression of VEGF and VEGFR-2 were cause or consequence of PAH, Taraseviciene-Stewart et al. developed a rat model using Sugen 5416 and CHP (118). The surprising results of trigged severe angioobliterative PAH via VEGFR inhibition and the observation of initially increased apoptosis, followed by vast proliferation of apoptosis-resistant ECs gave rise to multiple theories.

Rodent models using the combined pathogenic stimulus of CHP and SU5416, an unselective inhibitor of VEGFR-1 and VEGFR-2, achieved to procure an astounding level of congruence with human PAH (118,164). However their outcome cannot be attributed to inhibition of VEGFR-1 or VEGFR-2 alone. Considering that these receptors have opposite effects on angiogenesis (VEGFR-2 is the main receptor mediating the proangiogenic signal of VEGF-A, VEGFR-1 acts as a decoy receptor that prevents the activation of VEGFR-2 and therefore is considered antiangiogenic) a model inhibiting only one receptor promises clarity.

In this study we evaluated a new transgenic mouse model combining CHP and a conditional knock out of VEGFR-2 in pulmonary ECs. We consider the SU5416 and CHP model an important reference and deem a comparison of outcomes conclusive. After all both models are based on VEGFR inhibition.

This diploma thesis elaborates results obtained via echocardiography and CMRI. Both imaging modalities represent non-invasive measures designated to procure a level of probability for PH. This means that although a variety of signs compatible with PH exist a valid diagnosis cannot be made without precise assessment of hemodynamic parameters via right heart catheterization. Of note, all measurements were taken at baseline and after hypoxic exposure respectively.

With regard to the typical hemodynamic changes in human PAH, our model displayed a decrease in PAT/ET ratio that further exacerbated in KdrΔend mice. Consistent with our results the echocardiographic assessment of mice exposed to CHP ± SU5416 revealed a decreased PAT/ET ratio in hypoxic animals that further exacerbated in SU5416-exposed animals. Considering the inverse proportionality of PAT/ET ratio and PAP these results indicate an elevation of PAP that is of course congruent with a diagnosis of PH.
Defined as indent in the deceleration flow profile of the RVOT during systolic ejection in Doppler measurements, midsystolic notch represents a sign of severe PH. Midsystolic notch became apparent in Doppler measurements of hypoxic Kdr<sup>Δ</sup>end mice. Equally, hypoxic SU5416-treated mice displayed incipient midsystolic notch. This sign was not reproducible in control mice.

In addition to assessment of the RV using TTE, we evaluated RV EF – a parameter for RV function - via CMRI. A significant decrease of RV EF at baseline and after hypoxic exposure of Kdr<sup>Δ</sup>end mice insinuated beginning RV dysfunction. Interestingly, Bogaard et al. (169) claim that the sole burden of elevated RV pressure causes RVH but is insufficient to provoke RV dysfunction. Additional angio proliferative changes as seen in PAH however would provoke RV dysfunction on top of RVH. In reference to Bogaard et al. we conclude that the observed decrease in RV EF could be considered a hint at pulmonary vasculopathy and PAH.

An important aberration regarding human PAH was observed in mice exposed to hypoxia ± Sugen 5416: CO consistently decreased under hypoxic exposure, even more so in response to SU5416. These results indicate that CHP alone and combined with SU5416 provokes heart failure, a pathologic development that is not present in human PAH (164). In contrast to these results our model did show lower CO in response to hypoxic exposure, however Kdr<sup>Δ</sup>end mice were not affected more severely. We obtained corresponding results via cardiac MRI.

On a final note, systemic arterial pressure remained unchanged in all study groups.

In conclusion, our results from TTE and CMRI of this new model of direct gene ablation of VEGFR-2 in pulmonary ECs in combination with chronic hypoxia are consistent with changes seen in human PH and PAH.
9 Conclusion and future prospects

While it is safe to say that unimpaired VEGF signaling is of great importance for maintained homeostasis in the pulmonary vasculature, further knowledge has been gained regarding the role of VEGFR-2 specifically in the pathogenesis of experimental PAH. VEGFR-2 is of particular interest as it is the main receptor effectuating the mitogenic, prosurvival, proangiogenic, and permeability-enhancing functions of VEGF-A (93,96).

The evaluation of this new mouse model using TTE and CMRI renders a variety of signs typical for PH (and even PAH) attributable to VEGFR-2 inhibition as first pathogenic ‘hit’ (CHP being the second ‘hit’). Besides, in contrast to the pre-existing SU5416+CHP mouse model (164), no signs of incipient heart failure (decreasing CO) in response to Kdr knock-out could be observed. Therefore, we conclude that all data collected using TTE and CMRI are in line with a diagnosis of human PH/PAH and imitate human PAH even better than the SU5416+CHP model.

Our findings can be applied to future experimental settings and will contribute to an overall better understanding of disease and the creation and applicability of future treatment strategies of human PAH. Considering persisting functional limitation and poor survival rates despite optimal treatment continuous research is invaluable to identify new pathways of pathogenesis as possible targets for therapy.
10 References


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11 Appendix

11.1 Animal Models for human PH

11.1.1 Classical single pathogenic insult (SPI) animal models

Monocrotaline (MCT) injury

MCT is an alkaloid from the plant Crotalaria spectabilis that causes hepatotoxicity and progressive PH in multiple animal species (170). Ingested MCT is activated by hepatic metabolization into monocrotaline pyrrole (MCTP) which causes pulmonary endothelial injury (171). In research settings a single subcutaneous or intraperitoneal injection of 60-80 mg/kg MCT was used (172). This endothelial injury then triggers pulmonary vasculitis and obstructive pulmonary vascular remodelling. Histologic assessment revealed intimal hyperplasia, medial hypertrophy and adventitial thickening. Moreover increased apoptosis of ECs, increased proliferation and restistance to apoptosis of PASMCs were discovered (137).

However, this model shows several flaws in recapitulating human PAH, exhibiting pathologies uncharacteristic of severe human PH. Gomez-Arroyo et al. summarized their findings of pulmonary interstitial edema, myocarditis, and hepatic veno-occlusive disease (all untypical for human disease) with the term "MCT syndrome". They also found that the impact of vasoconstriction was far more prominent in the model compared to human disease (172). Furthermore, MCT-induced PH is responsive to most treatments (i.e. administration of VIP genes (173), mesenchymal stem cell therapy (174) (stem cells are considered to replace or restore damaged cells in the pulmonary vasculature)). This might be explained by the highly accelerated disease progression towards death in the model, leaving too little time for the formation of compensatory mechanisms (137). In comparison, the newer multiple pathogenic insult (MPI) models (i.e. MCT and contralateral pneumonectomy (175)) do develop severe PH including RVH and increased hemodynamic parameters (i.e. mPAP, RV diastolic and systolic pressure). Nevertheless, the MCT-model permitted important insights into the processes of pulmonary vascular remodeling. Genetic mutations in genes responsible for PASMC and EC apoptosis and proliferation could be associated with PAH (i.e. impaired signaling of the TGF-smad pathway and the BMPR-2 pathway (176–178). Besides the important role of inflammatory cells and cytokines in early remodeling was discovered (179).

Chronic Hypoxia (CHP)

This model is described in section 4.6.1.

The fawn hooded rat (FHR)

The FHR is a strain of rat sensitive to hypoxia that is known to spontaneously develop PH. Already in early age FHRs show increased mPAP, PVR, and RV enlargement such as marked pulmonary vascular remodelling consistent with pathologic findings in PAH. The
underlying pathomechanisms include 1) immature lungs with a decreased number of alveoli, 2) defect serotonin uptake into platelets and 3) a chromosome-1 abnormality that disturbs the mitochondrial reactive oxygen species (ROS)–hypoxia inducible factor alpha (HIF-1α)–potassium channel pathway (56,180). Moreover superoxide dismutase-2 (SOD-2) is downregulated, whereas HIF-1α and pyruvate dehydrogenase kinase (PDK) are activated. The FHR model enables further research into aspects of PH like PASMC proliferation and apoptosis-resistance such as the tendency towards a glycolytic metabolism (Warburg effect) (181).

Résumé of SPI models

Patients with PAH are characterized clinically by progressive deterioration with severe PH and pathologically by a neointimal and plexogenic arteriopathy. As neither the hypoxic nor the monocrotaline model replicate these typical findings, newer models tried to modify these classic models.

11.1.2 New MPI animal models

Monocrotaline and pneumectomy

It is hypothesized that hemodynamic conditions play a role in the development of PAH. A subclavian to pulmonary artery shunt, increasing PAP towards systemic pressure levels, augement the effect of endothelial injury on pulmonary vessels by MCTP and lead to neointimal changes in large PAs. However increased pulmonary pressure alone did not suffice as a stimulus (182). The follow-up study consisted of a combined stimulus of left pneumonectomy and MCTP. Pneumonectomy only caused minimal elevation of PAP, but increased shear stress together with endothelial injury caused by MCTP lead to neointimal formation consisting of endothelial and SM-like cells, signs of inflammation and more severe PH. Furthermore BMPR signaling was found to be inactivated (175,183). Again, the consequences of pneumonectomy alone did not suffice to produce intimal changes, supporting the theory that multiple hits (i.e. endothelial injury and high flow) induce the formation of neointimal lesions in distal PAs. This model served investigations about etiology and response to treatment (e.g. the 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitor simvastatin (183)). Differences between this rat model and human PAH concern different disease progression rates (weeks versus years), the formation of plexogenic lesions (not present in rats) and the absence of genetic mutations. Furthermore the influence of severe organ involvement when using monocrotaline remains unclear (137). Of note, young individuals formed lesions in perivascular space, developed excess medial and adventitial thickening, probably due to fibroblasts more sensible to hypoxia and other stimuli (184,185).

Sugen 5416 and CHP

This model is described in section 4.6.2.

11.1.3 Knockout models in mice
Mouse models are suitable for specific gene manipulation via overexpression or knockout strategies, hence allowing further insight into genetic pathomechanisms of PAH.

**BMPR-2**

BMPR signaling is involved in the pathogenesis of heritable PAH (HPAH) and idiopathic PAH (IPAH) as 80% and 40% of patients respectively are carriers of BMPR-2 germline mutations. Although BMPR-2 mutations show autosomal dominant inheritance, only 20% of people carrying heterogenous BMPR-2 mutations develop clinical signs of PH (186,187). Hence additional pathogenic „hits“ (i.e. environment, genetic modifiers, inflammation) increase the risk of actual disease development. In 2004 Beppu et al. (188) introduced a BMPR-2 mutant allele (lacking exons 4 and 5 which encode for the transmembrane domain and part of the kinase of BMPR-2) to mice. These heterozygous mice (BMPR-2+/−) developed only mild signs of PH without RVH. In 2011 Yasuda et al. (189) investigated transgenic mice with a dominant negative allele for the BMPR-2 gene in PASCMs. These mice did develop plexiform-like lesions, marked PH including RVH. An experimental treatment with Fasudil, a Rho-kinase inhibitor, successfully decreased RVSP, RVH and muscularization of small PAs, whereas Smad-signaling remained unchanged. Hence Rho/Rho-kinase inhibition might be a promising target for the treatment of PAH associated with a BMPR-2 mutation.

**Vasoactive intestinal peptide (VIP) knockout mice**

VIP is responsible for SMC relaxation and appears to be downregulated in the serum and lungs of PH patients (190,191). Knockout mice with a deletion of VIP (VIP−/−) developed signs of PH and pulmonary vascular remodeling. Interestingly VIP deletion resulted in modified gene expression favoring vasoconstriction, proliferation, vascular remodeling and inflammation (192). VIP replacement therapy in VIP−/− mice has shown reversal of PH and even correction of gene expression (193). A first study on human PH patients with Aviptadil (VIP analog) inhaled prior to RHC showed mild oxygenation and hemodynamic improvements (191). It is important to note that marked differences exist regarding hemodynamic severity and histologic changes when compared to human disease. Nevertheless this model provides important insight into the role of VIP in molecular mechanisms underlying the development of PAH and other forms of PH.

**Neprilysin knockout mice**

Neprilysin or neural endopetidase (NEP) is a transmembrane mezzoendopeptidase found in lung and heart tissue such as peripheral blood vessels. In the lung NEP is expressed in PASMCs, fibroblasts and ECs and leads to growth and contraction. In the lungs of patients with PH, reduced activity and expression of NEP was found (194). To further investigate the effect of NEP on the pulmonary vasculature, a model of knockout mice with a deletion of NEP (NEP−/−) and either normoxia or hypoxia was created. Mice developed severe PH with muscularization of distal PAs, medial and adventitial thickening and RVH. Remarkably, isolated NEP−/− PASMCs stopped to hyperproliferate when exposed to NEP (195). In conclusion an important role of NEP in the pathogenesis of PH seems plausible.

**Endothelin receptor-B knockout model**
Endothelin is a vasoactive peptide that regulates pulmonary vascular tone. It operates via endothelin receptors A and B (ET\textsubscript{A} and ET\textsubscript{B}). ET\textsubscript{A} is located in PASMC and causes proliferation and vasoconstriction. ET\textsubscript{B} is located 1) in PASMC, where it causes pulmonary vasoconstriction and 2) in ECs, where it leads to vasodilation via NO and prostaglandin (196). Transgenic rats with an ET\textsubscript{B} deficiency exposed to hypobaric hypoxia developed severe PH (196). In the BMPR-2 knockout model reduced ET\textsubscript{A} and ET\textsubscript{B} expression could be shown besides elevated pulmonary ET levels (197). Similarly human PH is associated with elevated ET levels, however ET\textsubscript{A} and ET\textsubscript{B} is upregulated. Clearly endothelin signaling involved in PH development. This model was indespensable in the develop current treatment with ET receptor antagonists like bosentan (198).

**Apolipoprotein-E (ApoE) knockout model**

ApoE is known to reduce oxidized circulating low-density lipoprotein (LDL) and atherogenesis and decreased levels of ApoE are linked to insulin resistance and obesity (both risk factors for PH). Hansmann et al. (199) observed spontaneous development of PH and PA muscularization in Apo\textsuperscript{-/-} mice. Interestingly, PH patients display increased expression of ApoE (200,201). Adiponectin is synthesized by adipose tissue and has an advantageous effect on insulin resistance and atherosclerosis. Levels of adiponectin decrease with increasing body mass, and low levels are directly linked to the development of PAH in mice (201). Weng et al. (202) crossbred Apo\textsuperscript{-/-} mice with delta-Glycine-adiponectin mice in order to observe the effect of significantly increased adiponectin levels on PH. Study mice showed reduced PH and pulmonary vascular remodelling when PH was induced with ovalbumin. Hence adiponectin might be a valid therapeutic target.

**11.1.4 Overexpression models**

**Interleukin-6 (IL-6) overexpression**

IL-6 is a proinflammatory cytokine synthesized by T cells, macrophages and, to a lesser extent, by PASMCs. It influences immune processes, hematopoiesis and oncogenesis. Increased levels of IL-6 have been found in human and in animal PH and correlate well with disease severity (203,204). Hence a transgenic mouse model overexpressing lung-specific IL-6 was developed (205). On a molecular level, an activation of the proangiogenic VEGF, upregulation of the proliferation-promoting transcription factors c-Myc and Max, upregulation of antiapoptotic molecules like survivin and Bcl-2 and downregulation of TGF-beta were found (205). Mice displayed increased RVSP, RVH and pulmonary vascular remodeling (absent plexogenic lesions) that further exacerbated under hypoxic conditions. This development could be explained by IL-6 increasing EC proliferation and expression of antiapoptotic proteins. The efficacy of IL-6 antagonizing treatments in PH patients and their isolated PASMCs respectively has been shown in multiple studies (203,206,207). Considering all results the influence of IL-6 in hypoxia-induced lung inflammation and pulmonary vascular remodeling is highly probable.

**Angiopoietin-1 (Ang-1) overexpression model**
The angiopoietin signaling pathway consists of the ligands Ang-1 and Ang-2 and the endothelium-specific tyrosine kinase. Ang-1 is synthesized by SMCs and influences prenatal angiogenesis by signaling EC to stimulate SMC proliferation at sites of newly formed endothelial tubes. Furthermore Ang-1 and VEGF synergistically act upon the maturation of vascular networks after birth (208). In 2004 a rat model overexpressing Ang-1 was developed to further investigate the influence of Ang-1 on angiogenesis and vascular remodeling in PH (208). Rats developed severe PH and increased PASMC proliferation with obliteratorive vasculopathy. Multiple studies suggest an association between diverse forms of PH and changes in the expression of Ang-1 (116,209).

Serotonin transporter (5-HTT) overexpression model

Serotonin (= 5-hydroxytryptamine (5-HT)) signaling is composed of 5-HT, 5-HT receptors on the cell surface and 5-HTT which regulates intracellular levels of 5-HT. 5-HT is synthesized in PA ECs and acts as a vasoconstrictor and co-mitogen on PASMCs and PA fibroblasts. In PH the expression of all components of 5-HT signaling is upregulated (210–212). Furthermore 5-HTT is overexpressed in PASMCs of IPAH patients. Several rodent models investigated the effect of a disturbed 5-HT signaling pathway on the development of PH. Maclean et al. (213) created a mouse model using a combined stimulus of 5-HTT overexpression and CHP. Mice displayed RVH and pulmonary vascular remodeling (absent plexiform lesions). These findings inspired further studies that tested the 5-HTT inhibitor fluoxetine and other 5-HT receptor antagonists successfully as potential treatment for PH in MCT rats (214,215). It is important to acknowledge 5-HT signaling is coregulated by 5-HTT and 5-HT receptors (216), hence targeting both the receptor and the transporter might prove beneficial. Corresponding to these findings Morecroft et al. (217) found that vasoconstriction initiated by 5-HTT can be counteracted by administration of a 5-HT receptor antagonist.

S100A4/Mtx1 overexpression model

S100A4/Mts-1 is a calcium-binding protein that is known to promote metastasis. Initially, transgenic mice overexpressing S100A4/Mts-1 were used to study the role of this gene in metastatic mammary cancer. However 5% (notably only female mice) responded with elevated RVSP and pulmonary vascular remodelling including neointimal thickening and plexiform lesions (218,219). These findings were in line with the increased expression of S100A4/Mts-1 found in PASMCs of patients suffering from associated PAH due to CHD (219). As male individuals were not affected in the same way, these sex-dependent differences were further investigated by treating PASMCs of PH patients with 17β estradiol. As presumed higher S100A4/Mts-1 expression was the result, proving the influence of sex on this molecular pathway. These findings might partially explain gender differences in human IPAH (220). As this model displayed vascular remodeling with high precision, it was used in a number of other studies: For example, investigating the link between viral infections and PH. Based on the discovery that latent human herpes virus-8 infection (Kaposi's sarcoma associated) is often present in IPAH patients, S100A4/Mts-1-overexpressing mice were infected with murine gamma- herpesvirus-68. These mice developed perivascular inflammation, occlusive neointimal formation and degradation of elastin (221). Another study
exposed S100A4/Mts-1-overexpressing mice to CHP and showed differences in gene expression, amongst them Fibulin 5, a molecule associated with elastin fiber assembly, leading to thickening of the elastic laminae (222).
11.2 Figures

**Figure 1**    Diagnostic algorithm of pulmonary hypertension.

**Figure 2**    Treatment algorithm in PAH.

**Figure 3**    The Cre-Loxp system.

**Figure 4**    Right ventricular ejection fraction measured by cardiac magnetic resonance imaging.

**Figure 5**    Echocardiographic measurements of pulmonary acceleration time / ejection time ratio.

**Figure 6**    Pulsed Doppler measurements of the right ventricular outflow tract (parasternal long axis) during systolic ejection.

**Figure 7**    Cardiac output measured by echocardiography.

**Figure 8**    Cardiac output measured by cardiac magnetic resonance imaging.
11.3 Tables

**Table 1**  Haemodynamic definition of PH

**Table 2**  Clinical classification of PH

**Table 3**  Echocardiographic probability of PH.

**Table 4**  Other echocardiographic signs compatible with PH.

**Table 5**  Specific indicators of RV function.

**Table 6**  Markers explored in context of PAH.

**Table 7**  Drugs and Toxins associated with PAH.

**Table 8**  Receptor affinity and function of VEGF ligands.

**Table 9**  Influencing Factors of VEGF signaling.

**Table 10**  Animal models of pulmonary hypertension.

**Table 11**  Gene expression rates of mice under chronic hypoxia ± Sugen 5416.

**Table 12**  Biomarker levels in mice under chronic hypoxia and/or Sugen 5416.
### 11.4 Abbreviations and acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>2-D</td>
<td>Two dimensional</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine or serotonin</td>
</tr>
<tr>
<td>5-HTT</td>
<td>5-hydroxytryptamine transporter or SERT</td>
</tr>
<tr>
<td>6MWT</td>
<td>6 minute walk test</td>
</tr>
<tr>
<td>A-17</td>
<td>Inhibitor of microRNA-17</td>
</tr>
<tr>
<td>ACVRL1</td>
<td>Gene encoding for Serine/threonine-protein kinase receptor R3 (TGF-β superfamily)</td>
</tr>
<tr>
<td>ADMA</td>
<td>Asymmetric dimethylarginin</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>Alk1/2</td>
<td>Activin receptor-like kinase 1/2</td>
</tr>
<tr>
<td>APLNR</td>
<td>Apelin receptor</td>
</tr>
<tr>
<td>BAS</td>
<td>Ballon atrial septostomy</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BMPR-1A/1B/2</td>
<td>Bone morphogenetic protein receptor-1A/1B/2</td>
</tr>
<tr>
<td>BNP</td>
<td>Brain natriuretic peptide</td>
</tr>
<tr>
<td>ppm</td>
<td>Beats per minute</td>
</tr>
<tr>
<td>C57/BL6J</td>
<td>“C57 black 6 mice” - inbred strain of laboratory mouse.</td>
</tr>
<tr>
<td>CAV1</td>
<td>Caveolin 1</td>
</tr>
<tr>
<td>c-kit</td>
<td>Protoonkogen encoding for KIT tyrosine kinase</td>
</tr>
<tr>
<td>CCB</td>
<td>Calcium channel blocker</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphae</td>
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<tr>
<td>CHD</td>
<td>Congenital heart disease</td>
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<td>CHP</td>
<td>Chronic hypoxia</td>
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<tr>
<td>CI</td>
<td>Cardiac index</td>
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<tr>
<td>cm</td>
<td>Centimeter</td>
</tr>
<tr>
<td>CMRI</td>
<td>Cardiac magnetic resonance imaging</td>
</tr>
<tr>
<td>CO</td>
<td>Cardiac output</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase type 2</td>
</tr>
<tr>
<td>CpcPH</td>
<td>Combined pre- and post-capillary pulmonary hypertension</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
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<tr>
<td>CPET</td>
<td>Cardiopulmonary exercise testing</td>
</tr>
<tr>
<td>Cre</td>
<td>Cre recombinase</td>
</tr>
<tr>
<td>CreER</td>
<td>Tamoxifen-inducible Cre recombinase fused to the estrogen receptor (ER).</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>CTD</td>
<td>Connective tissue disease</td>
</tr>
<tr>
<td>CTPA</td>
<td>Computed tomography pulmonary angiogram</td>
</tr>
<tr>
<td>CW Doppler</td>
<td>Continuous wave Doppler</td>
</tr>
<tr>
<td>CXCL4</td>
<td>Platelet factor 4 (chemokine)</td>
</tr>
<tr>
<td>CYP 3A4/2C9</td>
<td>Cytochrome P&lt;sub&gt;450&lt;/sub&gt; 3A4/2C9</td>
</tr>
<tr>
<td>DLCO</td>
<td>Lung diffusion capacity for carbon monoxide</td>
</tr>
<tr>
<td>DPAH</td>
<td>Drug-induced pulmonary arterial hypertension</td>
</tr>
<tr>
<td>DPG</td>
<td>Diastolic pressure gradient</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
</tr>
<tr>
<td>ECMO</td>
<td>Extracorporeal membrane oxygenation</td>
</tr>
<tr>
<td>EDV</td>
<td>End-diastolic volume</td>
</tr>
<tr>
<td>EF</td>
<td>Ejection fraction</td>
</tr>
<tr>
<td>e.g.</td>
<td>exempli gratia (lat., for example)</td>
</tr>
<tr>
<td>EGF</td>
<td>Endothelial growth factor</td>
</tr>
<tr>
<td>eIF2α</td>
<td>eukaryotic translation initiation factor 2 (EIF2AK4)</td>
</tr>
<tr>
<td>ENG</td>
<td>Gene encoding for endoglin</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ERG</td>
<td>Ets transcription factor</td>
</tr>
<tr>
<td>ERA</td>
<td>Endothelin receptor antagonist</td>
</tr>
<tr>
<td>ESV</td>
<td>End-systolic volume</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin 1</td>
</tr>
<tr>
<td>Et al.</td>
<td>Et alii (lat., and others)</td>
</tr>
<tr>
<td>FHR</td>
<td>Fawn-hooded rat</td>
</tr>
<tr>
<td>FiO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Fraction of inspired oxygen</td>
</tr>
</tbody>
</table>
Flt-1  Fms-related tyrosine kinase 1 or vascular endothelial growth factor receptor type 1
Flox  Flanked by loxP sites
FoxO1  Forkhead box protein O1
GCN2  General control nonderepressible 2
GDF15  Growth differentiation factor 15
HFpEF  Heart failure with preserved ejection fraction
HIF-1α  Hypoxia inducible factor 1α
HIV  Human immunodeficiency virus
HPAH  Hereditary pulmonary arterial hypertension
HR-CT  High resolution computed tomography
ICU  Intensive care unit
i.e.  Id est (lat., that is)
IL-6  Interleukin-6
i.p.  Intraperitoneal
IPAH  Idiopathic pulmonary arterial hypertension
i.v.  Intravenous
IVC  Inferior vena cava
IVS  Interventricular septum
KCNK3  Potassium channel subfamily K member 3
Kdr  Kinase insert domain receptor or vascular endothelial growth factor receptor type 2
Kdr<sup>∆end</sup>  Kdr knockout
kPa  Kilopascal
Kv1.5  Oxygen-sensitive, voltage-gated K+ channel
LA  Left atrium
LHD  Left heart disease
LoxP site  Lox (locus of X-over P1) sequences
LV  Left ventricle
LVEF  Left ventricular ejection fraction
MCT  Monocrotaline
MI  Milliliter
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>MLC</td>
<td>Myosin light chain</td>
</tr>
<tr>
<td>mm</td>
<td>Millimeter</td>
</tr>
<tr>
<td>mmHg</td>
<td>Millimeter of mercury</td>
</tr>
<tr>
<td>mPAP</td>
<td>Mean pulmonary artery pressure</td>
</tr>
<tr>
<td>MPI</td>
<td>Multiple pathogenic</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>ms</td>
<td>Millisecond</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells</td>
</tr>
<tr>
<td>ng/l</td>
<td>Nanogram per liter</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>n.s.</td>
<td>Not significant</td>
</tr>
<tr>
<td>NT-proBNP</td>
<td>N-terminal pro-brain natriuretic peptide</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
</tr>
<tr>
<td>P53</td>
<td>Tumor protein 53</td>
</tr>
<tr>
<td>PA</td>
<td>Pulmonary artery</td>
</tr>
<tr>
<td>PaCO₂</td>
<td>partial carbon dioxide pressure</td>
</tr>
<tr>
<td>PaO₂</td>
<td>partial oxygen pressure</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor-1</td>
</tr>
<tr>
<td>PAP</td>
<td>Pulmonary artery pressure</td>
</tr>
<tr>
<td>PAT/ET ratio</td>
<td>Pulmonary acceleration time / ejection time ratio</td>
</tr>
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<td>PAH</td>
<td>Pulmonary arterial hypertension</td>
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<tr>
<td>PASMC</td>
<td>Pulmonary arterial smooth muscle cell</td>
</tr>
<tr>
<td>PAWP</td>
<td>Pulmonary arterial wedge pressure</td>
</tr>
<tr>
<td>PCH</td>
<td>Pulmonary capillary hemangiomathosis</td>
</tr>
<tr>
<td>pCO₂</td>
<td>Partial pressure of carbon dioxide</td>
</tr>
<tr>
<td>PDE-5</td>
<td>Phosphodiesterase type 5</td>
</tr>
<tr>
<td>PDE-5i</td>
<td>Phosphodiesterase type 5 inhibitor</td>
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<td>PDGF</td>
<td>Platelet derived growth factor</td>
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<tr>
<td>PDGFR</td>
<td>Platelet derived growth factor receptor</td>
</tr>
<tr>
<td>PDK</td>
<td>Pyruvate dehydrogenase kinase</td>
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</table>
PGC-1α  Peroxisome proliferator-activated receptor
Coactivator 1 alpha
PGF  Placental growth factor
PI3K  Phosphatidylinositol-3-kinase
PLA₂  Phospholipases A₂
PRP  Platelet-rich plasma
PH  Pulmonary hypertension
Ppm  Parts per million
P value
PVOD  Pulmonary veno-occlusive disease
PVR  Pulmonary vascular resistance
RA  Right atrium
RAP  Right atrial pressure
RCT  Randomized controlled trial
RHC  Right heart catheterization
RTK  Receptor tyrosine kinase
RV  Right ventricle
RVEF  Right ventricular ejection fraction
RVH  Right ventricular hypertrophy
RVOT  Right ventricular outflow tract
S100A4/Mts-1  S100 calcium-binding protein A4
SaO₂  Oxygen saturation
SEM  Standard error of the mean
SERT  Serotonin transporter
s-Flt  Soluble fms-related tyrosine kinase 1
sGC  Soluble guanylate cyclase
Smad  "Small mothers against decapentaplegic" -
Signal transducers for receptors of the TGF-β superfamily
SMC  Smooth muscle cell
sPAP  Systolic pulmonary artery pressure
SPI  Single pathogenic insult
Ssc  Systemic sclerosis
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
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<tbody>
<tr>
<td>SU5416</td>
<td>Sugen 5416</td>
</tr>
<tr>
<td>SV</td>
<td>Stroke volume</td>
</tr>
<tr>
<td>SVC</td>
<td>Superior vena cava</td>
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<tr>
<td>SvO₂</td>
<td>Mixed venous oxygen saturation</td>
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<tr>
<td>TAPSE</td>
<td>Tricuspid annular plane systolic excursion</td>
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<tr>
<td>TEE</td>
<td>Transthoracic echocardiography</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
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<tr>
<td>Tie2</td>
<td>Endothelial-specific promoter (Cre-loxp-system)</td>
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<tr>
<td>TKI</td>
<td>Tyrosine kinase inhibitors</td>
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<tr>
<td>TNFSF15</td>
<td>Tumor necrosis factor superfamily member 15</td>
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<tr>
<td>TPG</td>
<td>Tras pulmonary pressure gradient</td>
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<td>Tph 1</td>
<td>Tryptophan hydroxylase 1</td>
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<td>TRV</td>
<td>Tricuspid regurgitation velocity</td>
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<td>TX</td>
<td>Tamoxifen</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>VEGFR-2</td>
<td>Vascular endothelial growth factor receptor</td>
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<tr>
<td>VE/VCO₂</td>
<td>High ventilatory equivalents for carbon dioxide</td>
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<tr>
<td>VIP</td>
<td>Vasoactive intestinal peptide</td>
</tr>
<tr>
<td>VO₂</td>
<td>Oxygen consumption</td>
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<tr>
<td>VO₂/HR</td>
<td>Low oxygen pulse</td>
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<tr>
<td>V/Q scan</td>
<td>Ventilation/perfusion scan</td>
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<tr>
<td>WHO</td>
<td>World health organisation</td>
</tr>
<tr>
<td>WHO-FC</td>
<td>World health organisation functional class</td>
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<tr>
<td>WU</td>
<td>Wood unit</td>
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