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PD Dr. med. Anne Hilgendorff
und
der Stanford Medical School, Division of Medicine - Pulmonary and Critical Care Medicine, Stanford University
Prof. Dr. Vinicio de Jesus Perez

PDK-4 overexpression in pericytes extracted from idiopathic pulmonary arterial hypertension lung tissue could be the cause of altered cellular behavior, migration and proliferation

Dissertation
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I would like to dedicate this thesis to my brother Florian.
He was, is and always will be part of my life, although he is missed every day.
I can't share this moment with him, but I can make him part of it.
In loving memory of a kind, wonderful and exceptionally person,
my beloved brother who died too soon – Florian Richter.

Mama und Papa

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1. Abstract

Abstract (English):

Idiopathic arterial hypertension (IPAH) is a seldom but rigorous disorder, which relates to arteriopathy, transformation and preferentially thickening of the medial vessel layer. Moreover, altered behavior in migration and proliferation as well as resistance to apoptosis has been observed in IPAH pericytes. The molecular mechanisms leading to the named physiological changes are still sparingly researched. To explore the possible cause of seen pathology, we extracted pericytes from IPAH- and healthy donor lung tissue and did a gene analysis using a microarray. The test showed that the expression of PDK-4, an enzyme involved in the regulation of glucose metabolism, was highly upregulated. We hypothesized that the observed behavioral changes in IPAH-pericytes were caused by an inhibited glucose shift into the mitochondria. Our further tests showed an increased membrane potential and lowered reactive-oxygen-species-levels in IPAH pericytes. These results could be explained by the observed PDK4-upregulation. Following investigation could target the regulation of PDK-4 and further provide new therapeutic approaches in PAH-treatment.

Abstract (German):

Idiopathische arterielle Pulmonale Hypertonie (IPAH) ist eine seltene doch drastisch verlaufende Erkrankung, welche mit Arteriopathien und Veränderungen – vornehmlich Verdickung der medialen Gefäßwand – einhergeht. Weiters wurden verändertes Verhalten der Proliferation und Migration sowie eine Resistenz gegenüber Apoptose beobachtet und beschrieben. Die zugrundeliegenden molekularen Mechanismen sind derzeit noch spärlich erforscht. Um die möglichen Ursachen der beobachteten Pathologien zu untersuchen, extrahierten wir Perizyten aus IPAH- und gesundem Spenderlungengewebe. In Folge wurde eine Genanalyse mittels Microarray durchgeführt. Es zeigte sich eine – im Vergleich zum Gesunden – deutlich erhöhte Expression von PDK-4, ein Enzym welches in die Regulation des Glucosemetabolismus involviert ist. Wir vermuten, dass die beobachteten Verhaltensänderungen in IPAH-Perizyten einer Inhibition des Glukosetransports in die Mitochondrien zugrunde liegt. Unsere weiteren Tests zeigten ein erhöhtes Membranpotential und einen verminderten Wert von reaktiven Sauerstoffspezien in IPAH-Perizyten. Zukünftige Untersuchungsziele könnten sich auf die Regulation von PDK-4 beziehen und in Folge neue Therapieoptionen und -ziele liefern.

2. Background

2.1 Pulmonary Arterial Hypertension

2.1.1 Definition

"*Pulmonary arterial hypertension has been defined as an increase in mean pulmonary arterial pressure (PAP) > 25mmHg at rest assessed by right heart catheterization*."
(taken from Simonneau G. et al. 2016).

Definition	Characteristics[a]	Clinical group(s)[b]
PH	PAPm ≥25 mmHg	All
Pre-capillary PH	PAPm ≥25 mmHg PAWP ≤15 mmHg	1. Pulmonary arterial hypertension 3. PH due to lung diseases 4. Chronic thromboembolic PH 5. PH with unclear and/or multifactorial mechanisms
Post-capillary PH	PAPm ≥25 mmHg PAWP >15 mmHg	2. PH due to left heart disease 5. PH with unclear and/or multifactorial mechanisms
Isolated post-capillary PH (Ipc-PH)	DPG <7 mmHg and/or PVR ≤3 WU[c]	
Combined post-capillary and pre-capillary PH (Cpc-PH)	DPG ≥7 mmHg and/or PVR >3 WU[c]	

CO = cardiac output; DPG = diastolic pressure gradient (diastolic PAP – mean PAWP); mPAP = mean pulmonary arterial pressure; PAWP = pulmonary arterial wedge pressure; PH = pulmonary hypertension; PVR = pulmonary vascular resistance; WU = Wood units.
a) All values measured at rest; see also section 8.0.
c) Wood Units are preferred to dynes.s.cm25.

Figure 1 – Definition of PAH [taken and modified from Galiè N. et al. 2016, Table 3 used with permission from Dr. Galiè]

2.1.2 Pathophysiology and Pathomechanisms

The pathology of pulmonary arterial hypertension was first characterized in the late 19th century by E. von Romberg (1891). During the autopsy of patients with unknown lung disease he found lesions in the pulmonary vasculature, which he later called "Sclerosis of pulmonary arteries". After declaring sclerosis to be the cause of this particular lung disease, a variety of studies to explain the pathophysiology and pathomechanism were conducted. One of the first explanations was a raise in blood pressure in the lung

circulation. It was suspected that an increase of pressure led to a remodeling of arteries and small vessels due to the fact that lung vasculature was construed to low pressure (Hornowski J. 1914, Posselt A. 1925).

TABLE 1.—*Basis of Grades of Hypertensive Pulmonary Vascular Disease Found in Association with Large Ventricular Septal Defects and Functionally Related Diseases*

	Grade of hypertensive pulmonary vascular disease					
	1	2	3	4	5	6
Type of intimal reaction	←—None —→	←————	————	—Cellular—	————	————→
			←————	— Fibrous and fibroelastic—	————	————→
				←———— "Plexiform lesion" ————		————→
State of media of arteries and arterioles	←————	————	——— Hypertrophied ———		————	————→
			←———	— Some generalized dilatation ———		————→
				←——— Local "dilatation lesions" ———		————→
					←———— P H * ————	————→
						←—N A †—→

* Pulmonary hemosiderosis associated with distended, thin-walled, arterial vessels throughout the lung.
† Necrotizing arteritis.

Figure 2 - Classification of PAH in 6 grades [taken from Heath D. et al. 1958, Table 1]

Further investigations in animal models (rats) showed that a hypoxic environment led to a remodeling of small vessels and pulmonary hypertension in pulmonary circulation. The results were consistent with clinical and pathological changes observed in PAH patients and autopsy results of PAH patients (Bennet GA. Et al. 1934).

On the basis of previous studies and histological findings first approaches to classify the pathology of PAH were made. Heath D. et al. (1958) invented a classification in 6 stages, based on histological changes in arteries and arterioles. These histological alterations covered hypertrophied arterioles and arteries, intimal reactions such as fibrosis, plexiform lesions, pulmonary hemosiderosis and, in the end stage, necrotizing arteritis.

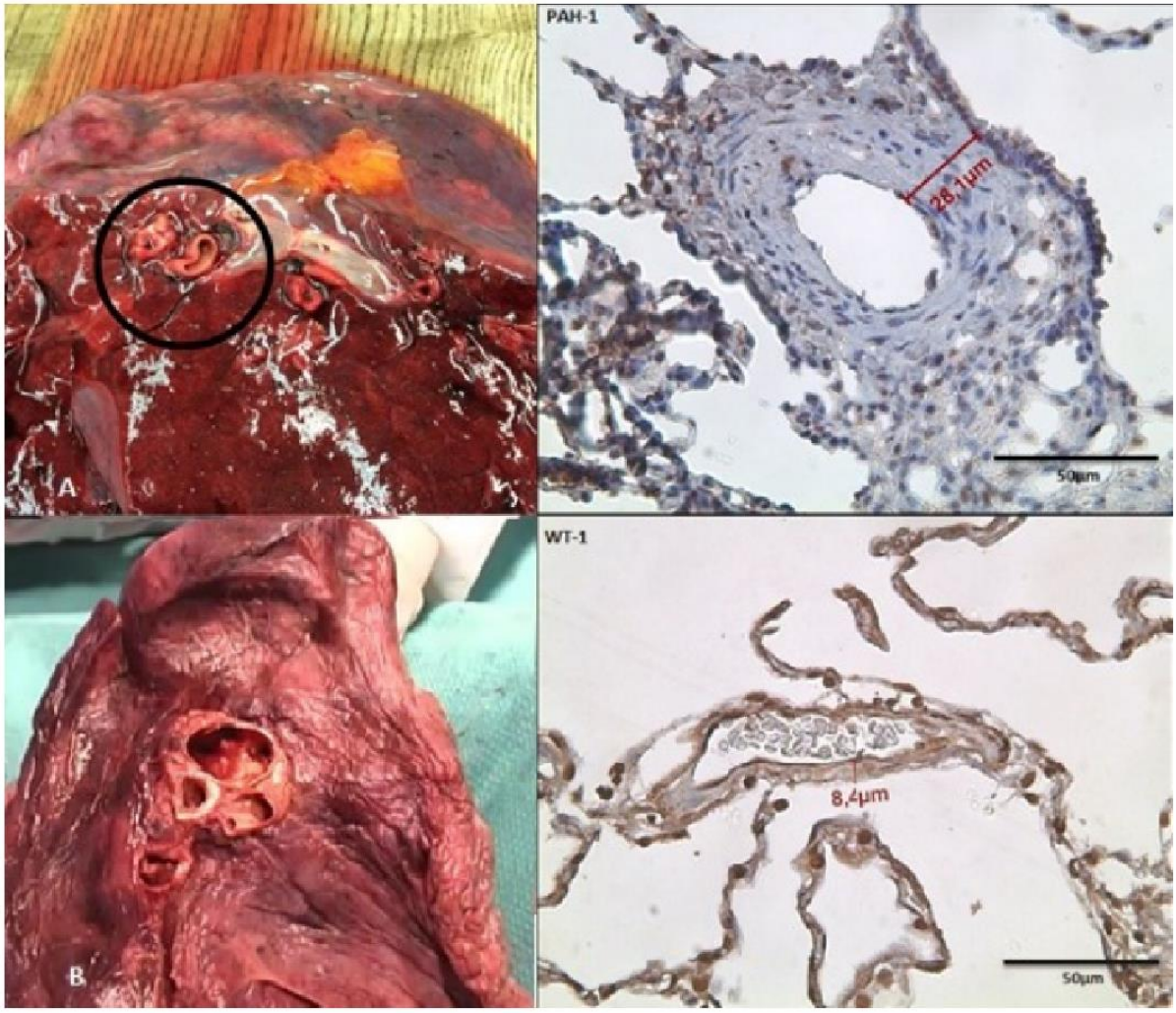

Figure 3 - Macroscopic and microscopic comparison of PAH- and healthy tissue. Images A and PAH-1 were taken from PAH lung tissue and visualize the vessel-wall thickening (diameter 28,1µm) and lumen narrowing known as pathological causation. In contrast to Images B and WT-1 (taken from healthy lung tissue), where a significant decreased vessel-wall diameter and wider lumen is seen. [A – thankfully provided by the department for Pathology, University Hospital St. Pölten; B- taken from Video Library – University of Wisconsin School of Medicine and Public Health; PAH-1/WT-1 thankfully provided by the de Jesus Perez Lab, Stanford University.

Morphological hallmarks for pulmonary arterial hypertension are plexiform lesions. Plexiform lesions are complex vascular alterations originating from remodeled pulmonary arteries and small vessels. They are characterized by endothelial cell proliferation and muscular hypertrophy - preferentially thickening of the medial vessel layer – resulting in increased lumen - constriction and elevating the pressure as a result (Pietra G.G. et al. 1989, Tuder R.M. 2009, Jonigk D. et al 2011).

Heightened stages of vascular endothelial growth factor (VEGF) are suspected as being one cause of vascular remodeling. Elevated levels of VEGF, hypoxia inducible factor 1α

(HIF α 1) (Tuder R.M. et al. 2001, Tuder R.M. et al. 2009) vasoconstrictive mediators such as endothelin and thromboxane have been shown (Christman B.W. et al. 1992; Montani D. et al. 2013). Furthermore, alterations in proliferation, migration and a higher resistance to physiological apoptosis have been demonstrated (Masri F.A. et al. 2007, Malefant S. et al. 2013).

To explore the cause of vascular remodeling, overexpression of angiogenesis inducing factors and resistance to apoptosis, studies to investigate a possible genetic background were conducted.

Table 1 – Pulmonary Arterial Hypertension: Risk Factors
[Taken and modified from Galiè et al. 2016, thankfully provided by Dr. Gerald Simonneau]

1. Pulmonary arterial hypertension
1.1 Idiopathic
1.2 Heritable
1.2.1 BMPR2
1.2.2 Other mutations
1.3 Drugs and toxin induced
1.4 Associated with:
1.4.1 Connective tissue disease
1.4.2 HIV infection
1.4.3 Portal hypertension
1.4.4 Congenital heart diseases
1.4.5 Schistosomiasis
1′ Pulmonary veno-occlusive disease and/or pulmonary capillary hemangiomatosis
1′ .1 Idiopathic
1′. 2. Heritable
1′ .2.1 EIF2AK4 mutation
1′ .2.2 Other mutations
1′ .3 Drugs, toxins and radiation induced
1′ .4 Associated with:
1′ .4.1 Connective tissue disease
1′ .4.2 HIV infection
1″. Persistent pulmonary hypertension of the newborn
2. Pulmonary hypertension due to left heart disease
2.1 Left ventricular systolic dysfunction
2.2 Left ventricular diastolic dysfunction
2.3 Valvular disease
2.4 Congenital/acquired left heart inflow/outflow tract obstruction and congenital cardiomyopathies
3. Pulmonary hypertension due to lung diseases and/or hypoxia
3.1 Chronic obstructive pulmonary disease
3.2 Interstitial lung disease
3.3 Other pulmonary diseases with mixed restrictive and obstructive pattern
3.4 Sleep-disordered breathing
3.5 Alveolar hypoventilation disorders
3.6 Chronic exposure to high altitude
3.7 Developmental lung diseases
4. Chronic thromboembolic pulmonary hypertension (CTEPH)
4.1 Chronic thromboembolic pulmonary hypertension
4.2 Other pulmonary artery obstructions
4.2.1 Angiosarcoma
4.2.2 Other intravascular tumors
4.2.3 Arteritis
4.2.4 Congenital pulmonary arteries stenoses
4.2.5 Parasites (Hydatidosis)
5. Pulmonary hypertension with unclear multifactorial mechanisms
5.1 Hematologic disorders: chronic hemolytic anemia, myeloproliferative disorders, splenectomy
5.2 Systemic disorders: sarcoidosis, pulmonary histiocytosis, lymphangioleiomyomatosis, neurofibromatosis
5.3 Metabolic disorders: glycogen storage disease, Gaucher disease, thyroid disorders
5.4 Others: pulmonary tumoral thrombotic microangiopathy,, fibrosing mediastinitis, chronic renal failure (with/without dialysis), segmental pulmonary hypertension]

2.1.3 Symptoms

Patients with pulmonary arterial hypertension often present with unspecific symptoms, therefore the diagnosis is often delayed, and patients are diagnosed in an advanced stage of the disease. First signs appear as breathlessness, fatigue, weakness, decrease in exercise capacity, dizziness or syncope. Symptoms can be presented mildly, so the diagnosing process may be slow making the final diagnosis often happens in progressed stages (Rich S. et al. 1987, Galiè N. et al. 2009, Montani D. et al. 2013).

2.1.4 Diagnosis

The physical signs of the disease vary and depend on the stage of the illness. The clinical inspection in early stages is mostly without pathological findings, in advanced stages it repeatedly shows jugular vein distension, peripheral edema, cool extremities, ascites and hepatomegaly. Auscultation in early stages usually is inconspicuous, nevertheless several pathologies such as a pan-systolic cardiac murmur of tricuspid regurgitation or a diastolic cardiac murmur of pulmonary insufficiency in later stages. Further diagnostic- and disease monitoring tools are electrocardiograms (ECG), chest-x-rays, echocardiographic imaging, right heart catheterization, 6-min-walk-distance and blood values (Rich S. et al. 1987, Montani D. et al. 2013).

Definition and diagnostic criterion for pulmonary arterial hypertension: "*Pulmonary hypertension (PH) is defined by a mean pulmonary artery pressure ≥ 25 mm Hg at rest, measured during right heart catheterization.*" (taken from Hoeper M.M. et al. 2013).

Table 2 - New York Heart Association Functional Classification
[taken and modified from AHA, 2017]

NYHA-I	Asymptomatic patient; no limitations of physical activity
NYHA-II	Slight limitation of physical activity; normal physical activity results in dyspnea, fatigue, palpitations; comfortable at rest;
NYHA-III	Distinct limitation of physical activity; Mild physical activity results in dyspnea, fatigue, palpitations; Comfortable at rest;
NYHA-IV	Unable to carry on any physical activity without discomfort; Symptoms persist at rest; Physical activity increases discomfort;

2.1.4.1 Electrocardiogram (ECG / EKG)

The ECG frequently shows a right axis deviation, a positive Sokolov Index for right ventricular hypertrophy (R in V1 + S in V5 > 1,05mV or R in V2 + S in V6 > 1,05mV) and due to the right atrial enlargement supraventricular arrhythmias in late stages. (Sokolov et al. 1949, Rich S. et al. 1987, Rich J.D. et al. 2013, Tonelli A. et al. 2014).

Furthermore, a prolonged QTc interval (>440ms) and widespread QRS (>100ms) complex have been shown in ECGs of PAH patients (Rich J.D. et al. 2013).

The comparison of ECGs taken at diagnosis point and close to death shows a progression of PR-interval-duration and QRS-duration (Tonelli A. et al. 2014).

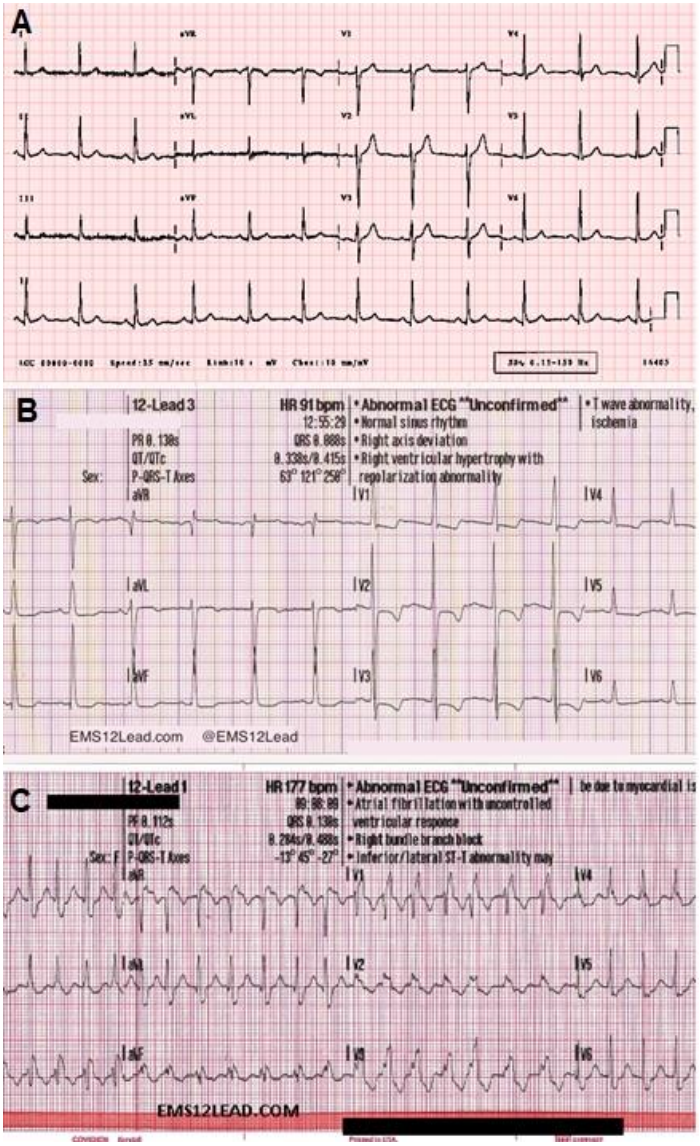

Figure 4 - Electrocardiogram
A - Sinusrhythm, normal axis, PQ- / QRS- / and QTc Values within the normal range, no ST- abnormalities, no T-wave abnormalities
B- Sinusrhythm, right axis deviation, prominent R wave in V1-3 and T wave inversions in V1-6
C - Atrial fibrillation, enlarged QRS complexed (>120ms = complete RBBB, > 100ms = incomplete RBBB), enlarged and slurred lateral S waves in I, aVL, V5-6 (ind]icating delayed RV depolarization)

[thankfully provided by Prof. Dr. Vinicio de Jesus Perez]

2.1.4.2 Chest x-ray

The initial chest x-ray at diagnosis point shows abnormalities in approximately 90% of patients. Radiological findings can appear as enlargement of central pulmonary arteries, loss of peripheral blood vessels and/or enlarged right heart cavities (atrium/ventricle) (Rich S. et al. 1987, Galiè N. et al. 2016, Asha M. et al. 2017).

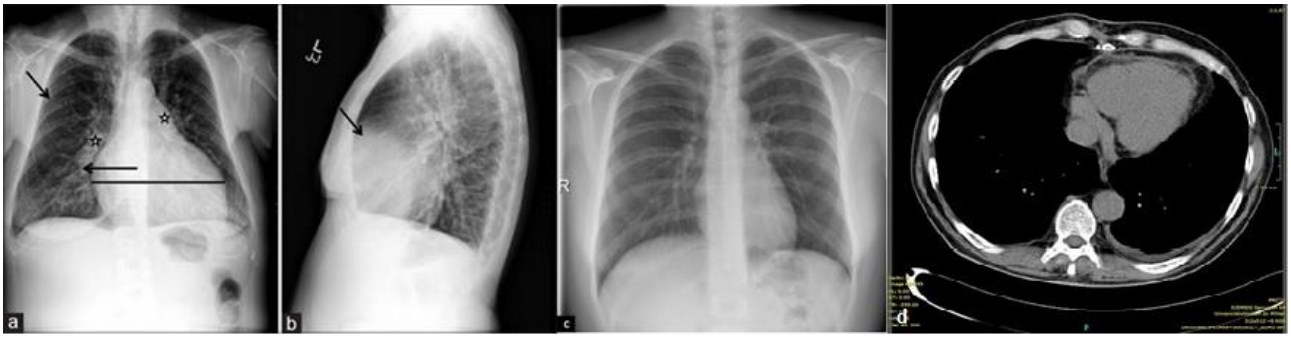

Figure 5 - Chest X-Ray in PAH
a) Postero-anterior projection showing dilated pulmonary arteries (stars), cardiomegaly (horizontal line), and rapid tapering (pruning) of right pulmonary artery (arrow). In addition, there is a decrease in the pulmonary vasculature in the periphery of the lung (arrow). (taken and modified from Asha M. et al. 2017, Fig. 2, figure used with permission from Dr. Alaa Gauda and Dr. Tonelli).
(b) Lateral projection depicting a decrease in the retrosternal air space (arrow); (taken and modified from Asha M. et al. 2017, Fig. 2, figure used with permission from Dr. Alaa Gauda and Dr. Tonelli).
(c) 27a, female, PA projection (standing); Bone structure is normal, no signs of fractures, mediastinum is slim and centered, no tracheal shift, no sign of pulmonary congestion, heart configuration normal, heart diameter is regular, pulmonary vessels extend to the periphery, no pneumothorax, recussus clear, no signs of infiltrates or effusions, diaphragmatic cupola definable; [thankfully provided by the department of radiology, university hospital St. Pölten, Austria]
(d) Radiological Imaging of pericardial effusion in a thoracic CT scan
[thankfully provided by the department of radiology, university hospital St. Pölten, Austria]

2.1.4.3 Echocardiography

Echocardiography is an excellent diagnostic method for visualizing the anatomy in cardiology. With the transthoracic echo (TTE) the size of the chambers of the heart can be measured, also the valve- and pump function and the ejection fraction. Further the aorta ascendens, the arcus aortae and pericardial effusion can be visualized. With the Doppler-sonography the blood flow can be evaluated and possible valve stenosis insufficiencies as well as the pulmonary arterial pressure can be identified.

Especially in obese patients, the transesophageal echo (TEE) facilitates a clearer image of the heart. Also, alterations in the atrial auricles for instance blood clots or vegetations in endocarditis are detected easier.

For evaluating the right ventricular function several parameters must be determined. The size, ejection fraction and pump function, as well as the myocardial performance index and the tricuspid annular systolic excursion must be assessed. The cardiac pump function and ejection fraction give information about contractility, the tricuspid annular systolic excursion correlates with right ventricular function and the myocardial performance index is a parameter regarding the cardiac function.

Tricuspid annular systolic excursion (TAPSE) is regularly evaluated during cardiac echocardiography and correlates with right ventricular systolic function. Low values (< 15mm) indicate a decreased right ventricular systolic function and show a poorer prognosis in PAH patients (Forfia P.R. et al. 2006, Rudski L.G. et al. 2010, Brunner N.W. et al. 2015, Haddad F. et al. 2016).

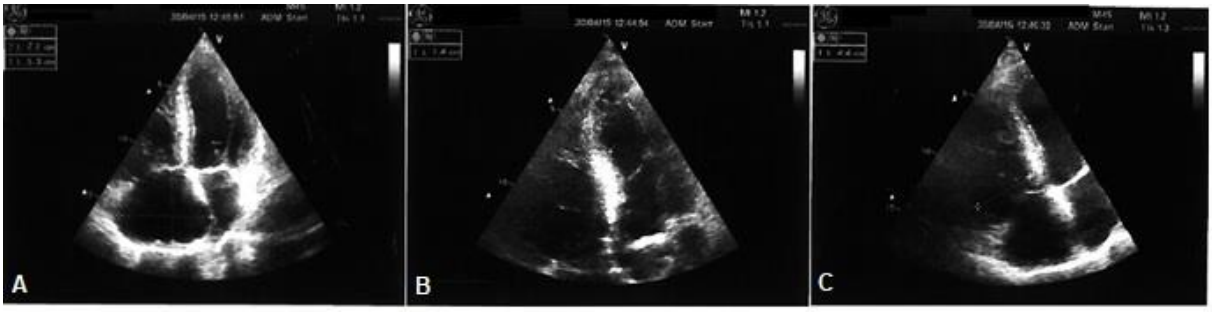

Figure 6 - Trans-thoracic-echocardiography in PAH patients
(A) septum hypertrophy: diameter 14mm
(B) tricuspid valve diameter: 44mm
(C) right atrium diameter: 48mm
[Images thankfully provided by the department of cardiology, university hospital St. Pölten, Austria]

The myocardial performance index is a parameter that combines systolic and diastolic values of the right ventricle and gives information about the function. Values above >0,40 (pulsed Doppler method) or > 0,55 (tissue Doppler method) are seen as pathological, limitations are irregular heart rates (Rudski LG et al. 2010).

The echocardiography of PAH patients shows a variety of pathological findings: right atrial and ventricular enlargement, increase of ventricular mass, tricuspid valve regurgitation and septum deviation and hypokinesis (Rich J.D. et al. 2013). Functional tricuspid-valve regurgitation leads to increased systolic volume in the right atrium, and (if not treated) results in right atrial enlargement. Reduced right ventricular systolic function and an extended right atrium can be seen as a manifestation of high pressure. Further a diastolic septum deviation towards the left ventricle is a sign of elevated right heart pressure and volume overload due to decreased cardiac output (Raymond R.J. et al. 2002, Rich J.D. et al. 2013, Haddad F. et al. 2016).

Table 3 - Echocardiography in PAH
[Taken and redrawn from Galiè N. et al. 2016, Tbl. 8A; used with permission from Dr. Galiè]

Echocardiographic probability of pulmonary hypertension in symptomatic patients with a suspicion of pulmonary hypertension		
Peak tricuspid regurgitation velocity (m/s)	**Presence of other echos 'PH signs'**	**Echocardiographic probability of PH**
≤2.8 or not measurable	No	Low
≤2.8 or not measurable	Yes	Intermediate
2.9–3.4	No	Intermediate
2.9–3.4	Yes	High
>3.4	Not required	High

Doppler sonography is a tool to measure the pulmonary artery systolic pressure (PASP). During the investigation, the maximum tricuspid regurgitant jet velocity (TRV) is measured

and following the PASP is calculated: PASP = (4 x [TRV]²) + RAP). Pulmonary hypertension is unlikely if the PASP is below 36mmHg, suspect above 40mmHg and likely if above 50mmHg (Chemla G. et al. 2004, Galiè N. et al. 2009, Amsallem M et al. 2016). However, the validity of calculated PASP is limited by the tricuspid regurgitant jet – a deficient jet value can lead to false PASP calculations or interpretations (Amsallem M et al. 2016).

2.1.4.4 Right Heart Catheterization

Table 4 – Pressure values
[taken from Klinke et al. 2005]

Anatomic location	Physiological pressure values
Central venous pressure (CVP)	8-10mmHg
Right atrial pressure (RAP)	2-28mmHg
Right ventricular pressure (RVP)	Systolic: 15-30mmHg Diastolic: 3-8mmHg
Pulmonary artery pressure (PAP)	Systolic: 15-30mmHg Diastolic:4-12mmHg
Pulmonary capillary wedge pressure (PCWP)	2-15mmHg
Left ventricular pressure (LVP)	Systolic: 80-140mmHg Diastolic:3-12mmHg

In 1970 the cardiologists William Ganz and Jeremy Swan invented the Swan-Ganz-Catheter, a catheter which is inserted percutaneously via the venous system into the right heart. The method is used to determine the pulmonary capillary wedge pressure (PCWP) - a balloon is inflated and wedged into a small arterial vessel (Swan H.J. et al. 1970). From the gained pressure values conclusions about the left-heart-function, blood volume and flow velocity or oxygen saturation can be drawn.

An elevated PCW and PA pressure was described in several studies, focusing on PAH-patients or -animal models (Rich S et al. 1987, Montani D. et al. 2013).

As mentioned before, PAH is defined as PAP > 22mmHg, PCWP ≤15mmHg and PVR >3 Wood Units (Badesch D.B. et al. 2009, Hoeper M.M. et al. 2009, Galiè N. et al. 2016).

Another very strong parameter in the diagnostic process of PAH is the cardiac output. The cardiac output per minute can be roughly calculated by multiplying the current heartrate with the stroke volume. However, the cardiac output per minute is influenced and determined by several variables such as anatomical conditions, pre- and afterload and contractility (Klinke et al. 2005). In addition to measure valid pressure values for the right heart, right heart catheterization gives clinicians also the possibility to estimate the patient's cardiac output by evaluating pulmonary artery and pulmonary wedge pressure (Hoeper M.M. et al. 2009, Galiè N. et al. 2016). By using thermodilution, a bolus of e.g. sterile ice-cold saline is injected via the catheter in the right atrium. Father distal a thermo-sensor integrated in the catheter measures the drop of temperature. The measured time delay is used to calculate the ejection fraction and cardiac output (Hoeper M.M. et al. 1999 and 2009, Galiè N. et al. 2016). Also, the cardiac output can be calculated by using the direct Fick-method: "..*as the quotient of oxygen uptake (VO2) and the difference of the arterial and mixed venous oxygen content.*" (taken from Hoeper M.M. et al. 1999).

To evaluate the oximetry, Galiè N. et al. 2016 recommended to take (blood) samples from vena cava superior and inferior as well as samples of the pulmonary artery. Further they suggested a stepwise evaluation of PAH patient's oxygen saturation.
In addition to evaluating pressure levels and taking blood samples to determine oxygen saturation values, testing vasoreactivity is recommended for patients belonging to group 1 PAH. The test is administered via giving intravenous epoprostenol or nitric oxide and considered as positive, if a drop of minimum 10mmHg and reaching a pulmonary artery pressure equal or lower than 40mmHg without a worsening of cardiac output is registered. Positive testing prognosticates better survival and effect of calcium channel blocker therapy (Tonelli A.R. et al. 2009, Goldberg A.B. et al. 2017).

2.1.4.5 6-Minute-Walk-Distance (6MWD)

By measuring the 6-min-walk-distance, the patient's' exercise capacity and symptomatology under physical stress in measured. In 2002 in an official statement the American Thoracic society showed, that the 6MWD of healthy adults was >500m in

average. PAH patients in NYHA-stages II-IV usually have lower testing values. Raymond R.J. et al. 2002 and Wensel R. et al. 2002 showed, that the results of the 6-min-walk-test directly correlate with the outcome. A high value (>380m) is a positive prognostic criterion, patients with a decreased 6-min-walk-distance are more likely having a worse outcome.

Nevertheless the 6-minute-walk-distance is a test that is influenced by a variety of extrinsic factors such as age, gender, general physical condition and previous diseases especially to the musculoskeletal-system. Further mental aspects such as compliance and motivation can also limit the 6MWD. The test value is given in absolute numbers, which limits the potential to individualize scores to the patient and show improvement or deterioration over time and therapy (Sitbon O. et al. 2002, Benza RL. et al. 2010, Grünig E. et al. 2012, Demir R. et al. 2014, Galiè N. et al. 2016).

Table 5 - 6-Min-Walk-Distance
[Taken from Badesch D.B. et al. 2010]

NYHA-I	475.5 ± 9.3m
NYHA-II	419.2 ± 3.7m
NYHA-III	323.1 ± 3.8m
NYHA-IV	214 ±13.7m

2.1.5 Therapy

Thanks to intensive research and development novelties in pharmaceuticals the therapy options for pulmonary arterial hypertension patients have increased. Nowadays PAH treatment is a multimodal concept which combines specific pharmacotherapy with supportive therapy – for example the use of diuretics and/or oxygen – and general measures such as physical therapy, rehabilitation, infection prevention, birth control and psychosocial support.

The available therapeutics target the main vasoconstrictive pathways: the prostacyclin pathway and the endothelin-pathway. In addition, PAH therapy addresses phosphodiesterase-V levels and influences the vessel-tone via calcium-channel-blockers (Venteluolo C.E. et al. 2012, Seferian A. et al. 2013).

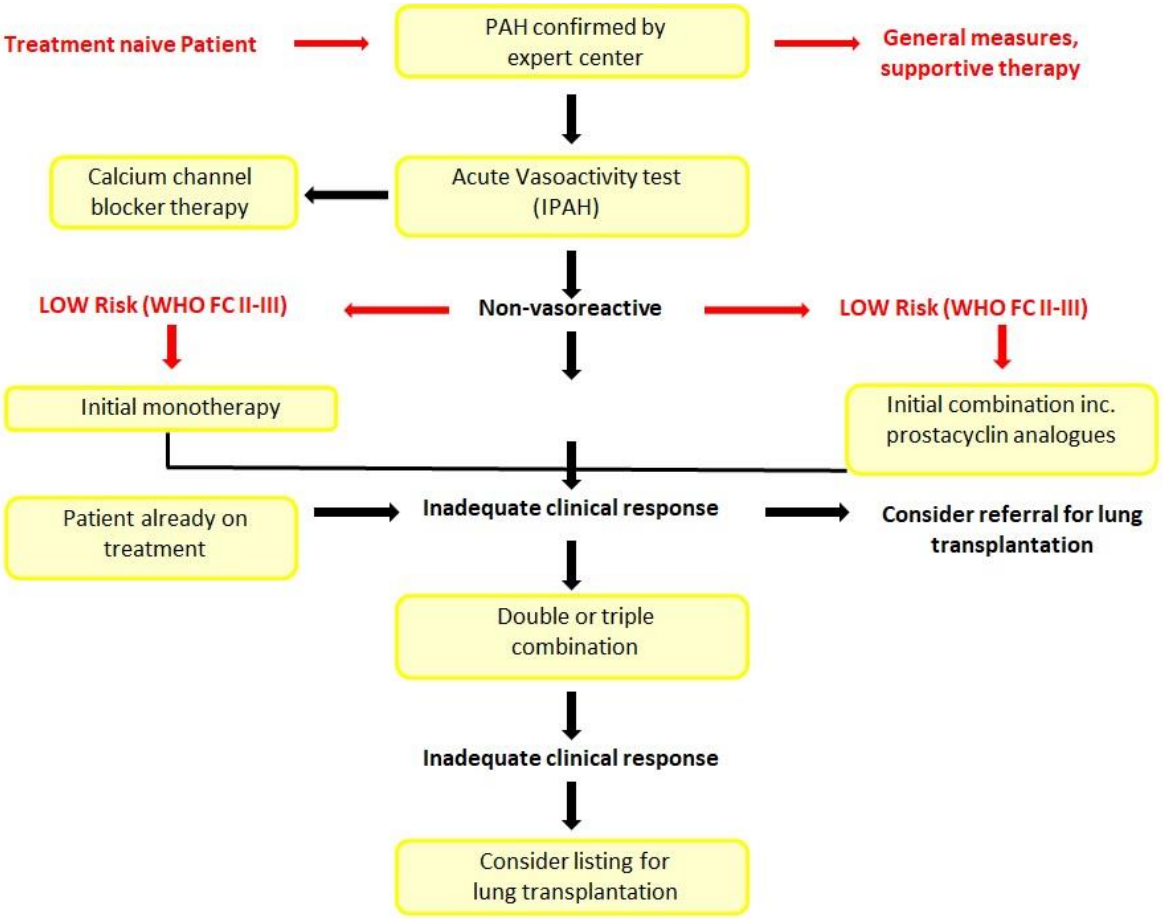

Figure 7 - Evidence based treatment algorithm for pulmonary arterial hypertension patients - for group 1 patients only.
[Taken and redrawn from Galie N. et al. 2015, Fig. 2, figure used with permission from Dr. Galiè]

2.1.5.1 Prostacyclin pathway

The enzyme cyclooxygenase transforms arachidonic acid to prostaglandin PGH2, which is metabolized via prostacyclin synthase to the transmitter prostacyclin. By binding to the prostacyclin receptor, the transmitter activates a G-protein coupled pathway and enhances the intracellular cAMP levels. As a consequence of the second messenger elevation, vasoconstriction decreases and so does the blood pressure. Furthermore, prostacyclin inhibits the platelet aggregation and has an antiproliferative effect.

The medication can be applied intravenously (Epoprostenol), subcutaneously (Teprostinil) and by inhalation (Teprostinil, Iloprost) and orally (Selexipag).

Epoprostenol treatment is administered via a permanently implanted venous access system (e.g. a central venous catheter) via using a portable pump. The initial dosage is 1

to 2 ng/kg per min and is increased continuously, based on the patient's tolerance. Barst R.J. et al. showed in 1996 in a prospective, randomized, multicenter open trial the effectiveness of continuous epoprostenol infusion compared to conventional therapy. Patients presented an improvement in physical resilience and a depletion in mean PAP and PVR.

Side effects are documented and include arthralgia, jaw pain and flush. In addition, clinicians should keep the side effects caused by the implanted catheter such as infection, thrombosis or pump malfunction in mind (Barst R.J. et al. 1996, Rich S: et al. 1999, Oudiz R.J. et al. 2004, Galiè N. et al. 2016).

Treprostinil - a prostacyclin analog - can be administered subcutaneously via a micro-infusion pump or a subcutaneous catheter (Galiè N. et al. 2016). The initial dosage is 1 to 2 ng/kg per min, however an increase in dosage is limited by severe pain in the area of injection or the placed subcutaneous catheter as well as other side effects such as flush and headaches (Simonneau G. et al. 2002, Galiè N. et al. 2016). The substances half-value period is longer compared to epoprostenol, following a catheter blockage or a dislocated catheter is not as life threatening and – according to the data - a transition from epoprostenol to treprostinil is possible and efficacious (Gomberg-Maitland M. et al. 2005). Simonneau G. and his group presented a randomized controlled trial in 2002, showing an advancement in physical fitness, a refinement in hemodynamics as well as a reduction of symptoms, such as dyspnea.

The synthetic analog iloprost can be applied orally, per inhalation or intravenously. Inhalation of Iloprost showed an advancement in 6MWD and in addition an enhancement in hemodynamic parameters and symptoms such as dyspnea and following in the NYHA class (Olschewski H. et al. 2002, Galiè N. et al. 2016). Yet due to the high frequency of inhalations – app. six to nine times per day – the practicability of this treatment method is disputable.

Selexipag belongs to the group of non -prostanoid prostacyclin receptor agonists and is orally administered. It reduces the pulmonary vascular resistance and the systemic vascular resistance and increases the cardiac index (Simonneau et al. 2012).

Sitbon O. and his team published a large randomized, placebo-controlled study in 2015 with 1156 PAH WHO-Group 1 participants. The selexipag dosages were individually aligned with a maximum of 1600µg two times per day. The study did not show a significant discrepancy in mortality comparing the two groups, however it showed a decrease in disease progression and hospitalizations for disease aggravation.

2.1.5.2 Endothelin pathway

The substance endothelin belongs to the class of peptide hormones and is produced in the endothelium of blood vessels. The hormone acts via a G-protein-coupled pathway and induces local vasoconstriction, therefore it increases the intravascular pressure. Moreover, it provokes cellular proliferation and remodeling and is conductive to cardiac hypertrophy.

Three subtypes are known so far, Endothelin-1 -2 and 3-, ET-1 is the most potent vasoconstrictor.

The synthetic endothelin-receptor-antagonists (ERA) bosentan, macitentan and ambrisentan unfold their effect via inhibiting the binding of endothelin to the receptor. Therefore, the reaction cascade can’t take place and the vascular tone decreases. Common side effects are hypotension, increase in transaminases, headache, edema and pruritus. (Hoeper MM et al. 2005, Herold G et al. 2011, Venteluolo CE 2012).

Bosentan belongs to the group of oral non-selective endothelin receptor antagonists. Rubin L.J. et al. issued a double-blind, placebo-controlled study in 2002 where patients were treated with either a placebo or 62.5mg bosentan twice a day for four weeks. After that, the treatment continued with an ongoing treatment with 125 or 250mg twice daily for an additional twelve weeks. The study demonstrated amelioration of physical capacity, dyspnea and following functional class. In 2008, Galiè N. et al. also found several differences between the bosentan group compared to the placebo reagent group. The research team demonstrated improvement of physical capacity (measured by the 6MWD), as well as a lowered pulmonary vascular resistance.

Also, the reagent macitentan belongs to the group of oral non-selective endothelin receptor antagonists. Pulido T. et al. released in 2013 the SERAFIN trial. The study included patients with moderate to severe PAH-group 1 patients. They received either 3mg or 10mg macitentan once a day over two years. The findings demonstrated a decelerated disease progression and further an enhancement of physical capacity. The effectiveness of macitentan treatment was proven regardless of additional PAH treatment.

Ambrisentan belongs to the oral selective endothelin-1-receptor type A antagonists. Galiè N. et al. published a double-blind study in 2005 about the effects of orally administered ambrisentan for twelve weeks (doses were 1, 2.5, 5 or 10mg given once a day). The patient cohort incorporated patients with idiopathic PAH, PAH associated with collagen vascular disease, anorexigen use or HIV infection. Results showed a substantial elevation of 6-min-walk-distance, as well as a decrease in mean pulmonary artery pressure and cardiac index.

2.1.5.3 Phosphodiesterase-V-Inhibitors

Phosphodiesterase-V-inhibitors prohibit the effect of the enzyme Phosphodiesterase-V on the cGMP pathway. The second messenger cGMP (cyclic Guanosin-Monophosphate) activates intracellular protein kinases and induces vasodilation in vascular smooth muscle cells in its wake. The PDE-V-inhibitors Sildenafil and Tadalafil are approved drugs for mono- and combination therapy of pulmonary arterial hypertension. Common side effects are headaches, hypotension, dizziness and priapism. (Herold G et al. 2011, Venteluolo CE 2012, Herold G et al. 2011, Galiè N. et al. 2016).

Sildenafil is a powerful suppressor of phosphodiesterase-5, orally administered. Galie N. et al. published a double-blind, placebo-controlled study about the effects of Sildenafil given to systematic pulmonary arterial hypertension patients in 2005. The substance-receiving-group was treated with sildenafil orally, three times a day for twelve weeks (either 20, 40 or 80mg). The sildenafil-group demonstrated an enhancement of exercise capacity

(improvement of 6-min-walk-test), a decline in mean PAP and a revision of functional class. Similar results were seen in a later published randomized controlled study assessing the efficiency of orally administered sildenafil (Singh T. et al. 2010).

Tadalafil is also an orally administered phosphodiesterase-5-inhibitor, but in contrast to sildenafil given only once a day. Galiè N. et al. published a double-blind, placebo-controlled study evaluating the impact of tadalafil in 2009. Given the maximum of 40mg tadalafil once a day, the patients showed an improvement in 6-min-walk-distance, an enhancement in time to clinical worsening and better quality of life. Side effects such as headache, myalgia and flush were reported.

Riociguat is a novel therapeutic agent, that belongs to the soluble guanylate cyclase (sGC) stimulators. By stimulating the sGC the synthesis of cyclic guanosine monophosphate is triggered and following as vascular vasodilation is achieved. In addition to its direct stimulation, riociguat also increases the body's own sensitivity for endogenous nitric oxide (Grimminger F. et al. 2009). Ghofrani H.A: et al. published a study in 2013 with 443 assigned patients. The maximum dose of riociguat was 2.5mg given three times a day orally. The participants showed substantial ameliorated exercise capacity, and enhancements in PVR.

2.1.5.4 Calcium-channel-blockers

Calcium-channel-blockers are therapeutic reagents which inhibit the signal-transduction in L-type calcium-channels by binding to the receptor. The decreased intracellular calcium-level causes a vasodilation in the vasculature. In the myocardium, the reduced calcium level results in decreased inotropy, chronotropy and blood pressure as well as the oxygen consumption sinks.

Calcium-channel-blockers have a large therapeutic margin and the application spectrum ranges from migraine prevention to coronary heart disease, arterial hypertension, Morbus Raynaud and Prinzmetall-Angina to pulmonary hypertension.

Side effects may be edema, hypotension, dizziness, headaches and flush- or tachycardia. (Herold G. et al. 2011, Herold G. et al. 2011, Venteluolo C.E. 2012).

Depending on the patient's' heart rate, different substances are available. Patients who tend to bradycardia favor amlodipine and nifedipine, tachycardia patients favor diltiazem. The effective dose rate is relatively high compared to other indications for calcium channel blockers.

Starting with lower dose rates, the increase of dosage goes up to a maximum of 20mg for amlodipine, 240mg for nifedipine and 720 mg for diltiazem (Galiè N. et al. 2016).

However, the indication for calcium-channel-blocker therapy in pulmonary arterial hypertension is limited and only for patients with a positive vasoreactivity (Galiè N. et al. 1995, Galiè N. et al. 2016). Further, patients who receive treatment with calcium channel blockers should be observed and monitored strictly and undergo a follow up examination approximately three to four months after starting the treatment, including another right heart catheter assessment (Galiè N. 2016). Properly managed and given to a patient with vasoreactivity, a therapy with this substance group can decrease the mean pulmonary arterial pressure and the pulmonary vascular resistance (Sitbon O. et al. 2005).

2.1.5.5 Combination therapy and additional measurements

The combination of two or more PAH-specific therapeutics is recommended if the patients response to a single drug treatment is poor or no improvement has been shown at all. If adjusted adequately, combination therapy is well tolerated. (Hoeper MM et al. 2005, Galiè N et al. 2013, Montani D et al. 2014, Venteluolo CE 2012, Müller-Mottet S et al. 2015).

In 2016, Sitbon O. and his team published a retrospective analysis, demonstrating a therapeutic approach using initial dual oral combination. The majority (in total 61) received a treatment combination of bosentan plus sildenafil, 17 patients were treated with bosentan plus tadalafil,11 patients were treated with a combination of ambrisentan plus tadalafil and 8 patients received a combination of ambrisentan plus sildenafil. The study participants were initially assessed 4 months after start of dual oral treatment. According to the authors, the patients showed significant improvements in physical shape and exercise capacity, symptoms such as dyspnea, WHO functional class and hemodynamic parameters (reduction of pulmonary vascular resistance). The median follow-period was 30 months

and the results supported the initial findings. In summary the research presented, that first line oral dual therapy (endothelin receptor antagonist plus Phosphodiesterase-V-Inhibitor) increases exercise capacity, cardiac index and on the other hand decreases right atrial pressure and pulmonary vascular resistance (e.g. a reduction of pulmonary vascular resistance of 40-50% in dual therapy compared to a reduction of 30% in monotherapy).

Furthermore, infection prevention, birth control and the choice of anesthesia in elective surgery can influence the progress of the disease. Although efforts were made to find a proper treatment algorithm, the options are still limited, and the treatment plan is oriented at individual response, medical preconditions and drug interactions (Galiè N. et al. 2016).

Summarizing, only the symptoms but not the origin of the disease can be treated. It is still a progressive and fatal disease and the only known cure so far is lung or combined heart-lung-transplant.

2.1.6 Prognosis

During the past years approaches to find new treatment options and algorithms were made. The response to new therapeutic reagents and the reduction of side effects increased the livability and therapeutic management. Prognosis and survival of PAH patients are still limited and correlates with the NYHA-stage at diagnosis point, the response to pharmacotherapy, lifestyle changes and supportive therapy such as infection prevention, birth control and psychosocial support.

In 1991 D'Alonzo A. et al. described the median survival time for PAH in correlation to the NYHA stage as following: "*NYHA I + II: 58,6 months, NYHA III: 31,5 months, NYHA IV: 6 months*". The mortality is correlated with right ventricular hemodynamic function, pulmonary artery pressure and right arterial pressure as well as with the cardiac index.

In 2002 McLaughlin V.V. described the survival of PAH patients with epoprostenol treatment subsequently: "*1-year survival: 88%, 2-year survival: 76%, 3-year survival: 63%*".

In 2002 Sitbon O. et al. also investigated the survival of PAH patients with epoprostenol treatment: "*1-year survival: 85%, 2-year survival: 70%, 3-year survival: 60%*".

Benza R.L. et al. published a study in 2010 evaluating the causal variables of one-year-survival in PAH patients. The study included 2716 patient who previously registered in the US Registry to evaluate Early and Long-Term PAH Disease management (REVEAL). The investigators determined five risk groups. Patients with low risk belonged to the IPAH subgroup, were demographically 35 y/o females. Renal insufficiency was an optional comorbidity, the vital parameters showed a regular heart rate and normotensive blood pressure values. In cardiac echo no pericardial effusion was seen, the mRAP was app. 8mmHg and exercise capacity was around 360 (6-MWD). In contrast to these values, the group with very high risk belonged to the Scleroderma-subgroup, the patients were generally 55y/o females with renal insufficiency, pericardial effusion seen in echocardiogram, increased mRAP (app. 22mmHg) and decreased exercised capacity (app. 200m in 6-MWD). Further the authors stated that "..*only an elevated mean right atrial pressure within the year preceding study enrollment and a markedly increased pulmonary vascular resistance were independent risk predictors."* (taken from Benza R.L. et al. 2010).

The survival numbers of PAH patients have increased, and their prognosis has improved over the years compared to earlier periods (Müller-Mottet S. et al. 2015), nevertheless treatment options are still limited.

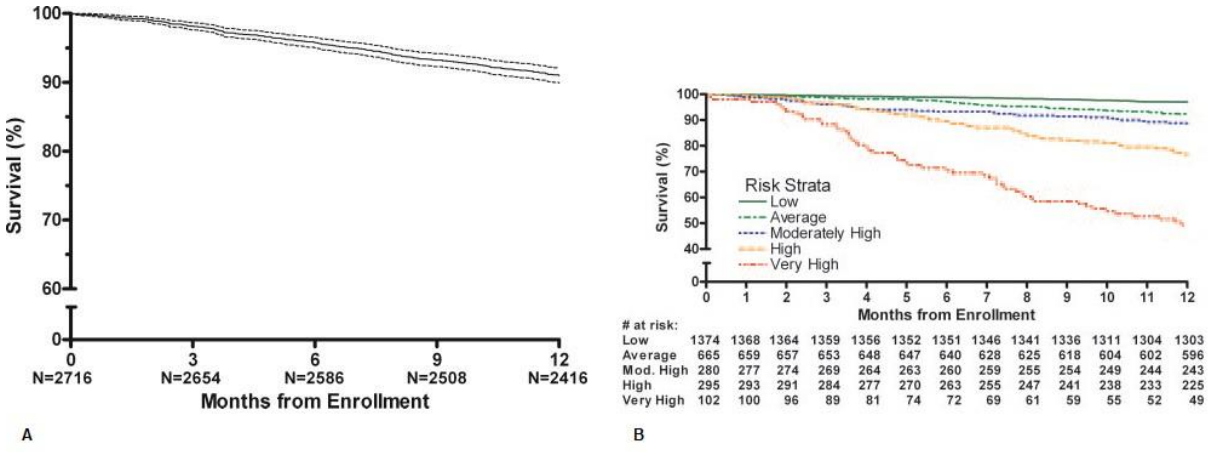

Figure 8 - Prognosis and Survival in PAH
(A) Kaplan-Meier estimates of 1-year survival from time of enrollment. Dashed lines represent the 95% CI for the Kaplan-Meier estimates.
(B) Observed 1-year survival from time of enrollment according to predicted risk strata.
[Taken from Benza R.L. et al. 2010 A=Fig. 1 and B=Fig.2, figures used with permission from Dr. Benza]

2.2 The Connection between PAH and Pericytes

Besides finding new treatment options for IPAH patients, the focus of research has turned to the initial cause and origin of cellular changes in small- and micro vessels.

Alteration in migration, proliferation and apoptotic behavior has been observed (Malefant S. et al. 2013), and with these new findings, not only vascular muscle cells and endothelial cells, but also pericytes have been brought into focus. Pericytes originate from mesenchymal stem cells (Shakib et al. 1966; Díaz-Flores L. et al. 2009) and are mostly found in the layer of non-muscular microvessels and capillaries (Weibel E.R. et al. 1974). Pericytes envelope lumen-building endothelial cells (Weibel E.R. et al. 1974), evolve cell junctions (gap junctions) and are suspected to influence angiogenesis, differentiation and multiplication of endothelial cells by expressing cytokines such as TGFbeta-1 or PDGFR-beta-1 (Song S. et al. 2005, v. Tell D. et al. 2006, Díaz-Flores L. et al. 2009) and other vasoactive substances (Peppiat C.M. et al. 2006).

Pericytes dysfunctions or mutations lead to vessel wall instability (Weibel E.R. et al. 1974; v. Tell D. et al. 2006) and increased endothelial membrane permeability (Bell R.D. et al. 2010). Pericytes maintain their plasticity for differentiation into other cell types like endothelial or smooth muscle cells. Prospectively causing higher amounts of proliferating cells in IPAH vessels, which constrict the lumen and finally raise the resistance and pressure (Lee S.D. et al. 1998).

2.3 Vascular Alterations and Enhancement of Angiogenesis

As previously written on, the first description of pulmonary arterial hypertension was done by E. von Romberg in 1891, who characterized the alterations in lung vasculature found in the autopsy of his patients as sclerosis. Ever since, a variety of studies was published whose authors tried to understand the histological changes observed in patients with so called “arteriosclerosis pulmonalis” (Hornowski J. 1914, Posselt A. 1925). Thanks to the new awareness in physiology and pathology as well as development of new technologies,

the focus of research turned from histologic findings to investigations in biochemical background.

The disease is also associated with so called plexiform lesions. These lesions are characterized by an overexpression of the vascular endothelial growth factor (VEGF) by endothelial cells as well as additional angiogenesis influencing factors (Geraci M.W. et al. 2001; Tuder R.M. 2009). Furthermore, heightened expression of VEGF and hypoxia inducible factor 1α (HIF α 1) (Tuder R.M. et al. 2001) as well as vasoconstrictive mediators such as endothelin and thromboxane and muscularization of arterioles and capillaries have been shown (Christman B.W. et al. 1992; Montani D. et al. 2013). The development of IPAH due to mutations in the bone morphogenetic protein pathway (BMP-pathway) and the coding gene for bone morphogenetic protein receptor type 2 (BMPR2) has been proven (Deng Z. et al. 2000; Morrell N.W. 2006).

Intensified angiogenesis and overexpression of angiogenesis stimulating and influencing factors are believed to be the cause of vascular remodeling and following causing the microscopically and macroscopically observed alterations.

As mentioned above, pericytes originate from mesenchymal stem cells (Shakib et al. 1966; Díaz-Flores L. et al. 2009), keep their ability to differentiate into other cell types (Lee S.D. et al. 1998) and envelope lumen-building endothelial cells (Weibel E.R. et al. 1974). Due to these realizations, the focus of research turned towards pericytes and their potential involvement in lumen-narrowing and pressure elevating processes in idiopathic pulmonary arterial hypertension (Lee S.D. et al. 1998).

2.4 Pericytes

The history of pericytes date back to the late 19th century and was first described in 1871 by the scientists C.J. Eberth and in 1873 by C. Rouget as "..*population of contractile cells surrounding the endothelial cells of small blood vessels (Rouget, 1873)*." (taken from Armulik A. et al. 2011). In the 1920s K.W. Zimmermann described so called "Rouget-cells" (later pericytes) and their anatomic location in his scientific paper "Der feine Bau der Blutcapillaren".

Pericytes originate from mesenchymal stem cells (Shakib et al. 1966; Díaz-Flores L. et al. 2009) and are primarily found in the layer of non-muscular microvessels and capillaries (Weibel E.R. et al. 1974). In addition, they enclose lumen-building endothelial cells (Weibel

E.R. et a. 1974) and develop cell-connections such as gap-junctions. By expressing mediators and messengers such as TGF-beta-1 or PDGFR-beta-1 or other partial vaso-active substances, they are suspected to affect and/or manipulate differentiation and multiplication of endothelial cells and angiogenesis (Díaz-Flores L. et al. 2009, Peppiat C.M. et al. 2006, v. Tell D. et al. 2006). Moreover, pericytes retain their ductility to differentiate into other cell lines such as endothelial- or smooth muscle cells.

Suspicion is entertained that pericytes dysfunction or mutation induce vessel-wall instability (Weibel E.R. et al. 1974; v. Tell D. et al. 2006) and increased endothelial membrane permeability (Bell R.D. et al. 2010). Also altered behavior in proliferation, migration and apoptosis has been found (Malefant S. et al. 2013). An increased level of proliferation for example in IPAH lung vasculature leads to vessel-wall-thickening and therefore lumen-constriction which following leads to an increased vessel-wall-resistance and elevated intravascular pressure (Lee S.D. et al. 1998).

The new knowledge of pericytes-function and altered behavior in diseases puts this cell type in the focus of research.

2.5 Mitochondria

2.5.1 Overview

The mitochondrion is very often characterized as the power station of the cell – it contains a large variety of metabolic pathways, processes metabolites and in the end, generates ATP. The number of mitochondria in a cell is dependent on the metabolic activity of the particular tissue, the amount can be increased by training for example in skeletal- and heart-musculature.

2.5.2 Endosymbiotic Theory

The endosymbiotic theory was founded by Konstantin Mereschkowsky at the beginning of the 20th century and is based on the assumption that mitochondria used to be individual (aerobe) organisms that migrated into (anaerobe) host cells to live in a symbiosis

(Mereschkowsky K. 1910, Margulis L. 2004). The term “endosymbiosis” origins from the Greek words “endo” – inside and “symbiosis” – living together.

Closer inspection of mitochondria shows significant differences to other cell organelles and similarities to bacteria. Mitochondria are organized into 2 membranes, which differ in their structural composition – the inner membrane is rich in cardiolipin. This lipid is almost only found in the inner membrane of mitochondria (Pangborn M.C. 1942, Horn F. 2002). They proliferate independently by division and contain their own circular mitochondrial DNA (mt-DNA). Furthermore, the mitochondrial ribosomes enclose 70S and 30S subunits in contrast to eukaryotic ribosomes (Horn F. et al. 2002, Rassow J. et al. 2006).

Differencesx in structure, mt-DNA and mt-ribosomes and proliferation, support the theory of prokaryotes taken inside other cells and developing a symbiotic lifestyle (Horn F. et al. 2002, Martin W. et al 2012, Zimorski et al. 2014).

2.5.3 Histology and structure

Mitochondria are small, oval, vermicular cell organelles and occur in most eukaryotic organisms (Henze K. et al. 2003). They can be discriminated by their appearance into two groups: the cristae-type and the tubulus-type (Luft R. et al. 1962, Frey T.G. et al. 2000, Lüllmann-Rauch R. et al. 2006). The inner membrane is compartmentalized into a large number of lamellae or tubes to expand and enlarge the surface, which in due course increases the ATP production. Cristae-type mitochondria are found for example in renal cells, tubulus-type mitochondria are only found in steroid-hormone-producing cells (Lüllmann-Rauch et al. 2006, Rassow et al. 2006).

The organelles are composed of two membranes that separate them into two different compartments. The outer membrane – a phospholipid bilayer – contains several so called porins that facilitate the diffusion for proteins <5000kDa. Furthermore, the outer membrane contains translocase of the outer membrane (TOM), a protein-complex that allows and helps transporting proteins into the intermembrane space (Fleischer et al. 1967, Horn et al. 2002, Rassow et al. 2006, Herrmann et al. 2000).

The inner membrane is rich in Cardiolipin a lot less permeable for molecules, it contains a large variety of transport mechanisms - for example the translocase of the inner membrane (TIM), the Pyruvate-translocator, the ADP/ATP-translocator, Ca^{2+}-Na or H-antiporter - to

enable protein-exchange and metabolic pathways. Moreover, the inner membrane contains the respiratory chain and the ATP-Synthase (Bernardi P. 1999, Rassow J. et al. 2006, Horn F. et al. 2002).

The Pyruvate-Dehydrogenase-Complex is located in the mitochondrial matrix, as well as the beta-oxidation and the Krebs-Cycle. In addition, parts of the Heme-Biosynthesis and the Urea-Cycle take place in the matrix, which is an important Ca^{2+}-store (Bernardi P. 1999, Rassow J. et al. 2006, Horn et al. 2002). Further, it contains mitochondrial DNA (mDNA), a circular plasmid which stores genes for mitochondrial proteins (Rassow J. et al. 2006, Horn F. et al. 2002).

2.5.4 Reproduction and inheritance

Mitochondria are capable of reproduction via growing and developing septa that lead to separating the organelle into 2. The average lifespan of a mitochondrion is approximately 10-20 days, afterwards they are degraded in the lysosomes. Due to the maternal inheritance of mitochondria, defects and diseases are only passed along in the female line (Giles R.E. et al. 1980, Yaffe M.P. 1999, Sutovsky P. et al. 2000, Horn F. et al. 2002, Rassow J. et al. 2006).

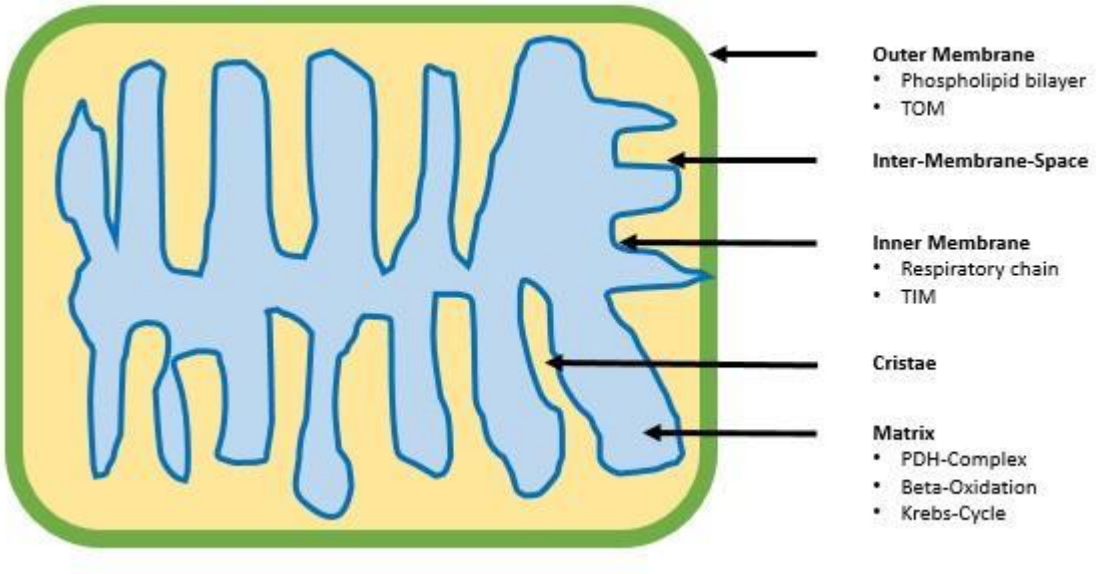

Figure 9 - Schematic image of a mitochondrion

2.5.5 Mitochondrial Functions and Pathways

The mitochondrion plays a very important role in the cellular metabolism and energy gain. Several metabolic pathways take – partly or fully – place in the mitochondrion and supply the electron chain with substrates to generate the needed amount of ATP.

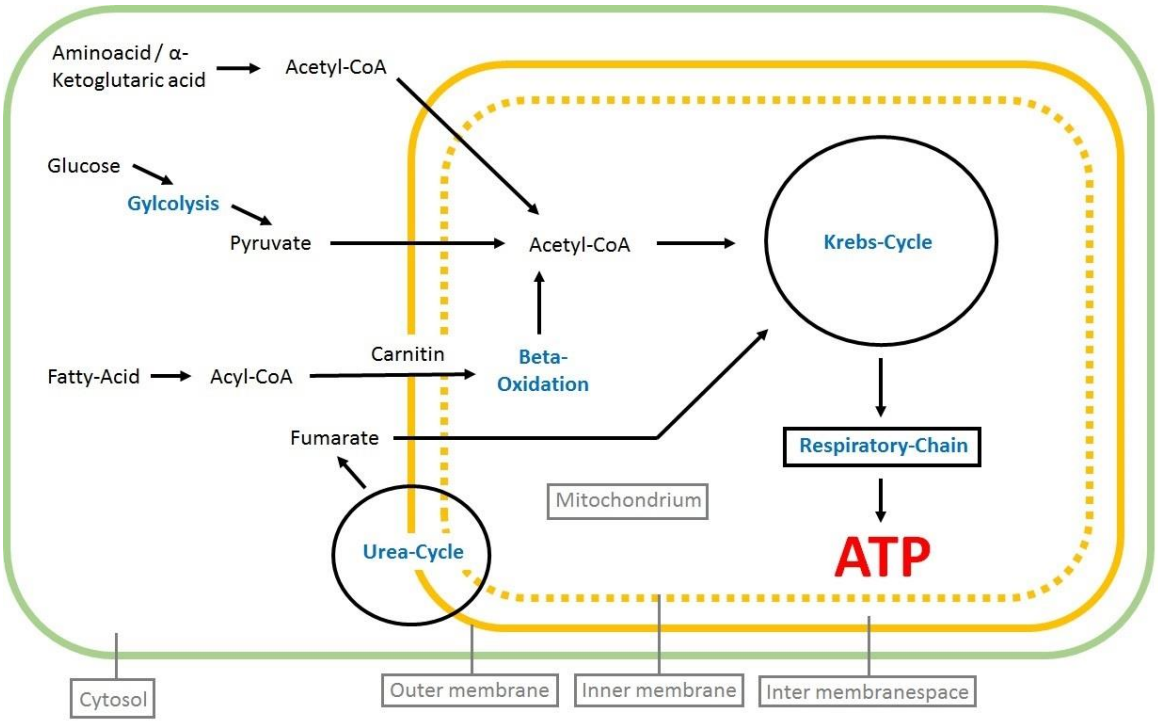

Figure 10 - Schematic image of mitochondrial pathways and interactions

Ingested carbohydrates are split by amylases in the small intestine, glucose-blood-levels are increasing, and glucose is transported into the pancreatic beta-cells. As a result, glucose is metabolized by glycolysis to ATP, the increased intracellular ATP levels lead to an inhibition of potassium efflux and following the cell depolarizes. This conducts an activation of voltage-dependent Calcium-channels which induce an exocytosis of insulin-filled vesicles (Horn F. et al. 2002, Rassow J. et al. 2006).

The binding of Insulin to the Insulin-receptor effectuates an increased glucose-uptake and –storage, and in its course, decreases the blood-glucose-values. Once inside the cell, glucose is – as mentioned previously – metabolized via glycolysis, the gained pyruvate is transported into the mitochondria, channeled into the Krebs-cycle, and further on to the electron chain. Glucose oxidation delivers approximately 36 ATPs per molecule glucose instead of 2 ATPs via glycolysis only (Horn F. et al. 2002, Rassow J. et al. 2006).

However, the mitochondrion not only is the "power station of the cell". Parts of the steroid-hormone-biosynthesis, reactive oxygen species production, regulation of apoptosis and calcium-homeostasis also take place in the mitochondria. The large variety of functions and metabolic interactions show the importance of mitochondrial integrity within the cells.

Alterations, mutations or damage can lead to major dysfunctions not only at a cellular level but can also can be a threat for a whole organism (Horn F. et al. 2002, Rassow J. et al. 2006).

2.5.5.1 Krebs Cycle

The Krebs-Cycle is a pathway consisting of several reaction steps to utilize Acetyl-CoA and generate FADH2, which is channeled into the respiratory chain and further disposed to gain ATP. The Krebs-Cycle is an ongoing reaction cascade which is fueled by pyruvate, Acetyl-CoA and amino-acids. In addition to these substrates, this pathway also reuses reaction products such as NAD+, FAD, and H2O provided by the respiratory chain, the protein catabolism or other metabolism channels (Horn F. et al. 2002, Rassow J. et al. 2006).

Also, the Krebs-Cycle and its products are involved in a lot of further metabolic pathways: The transformation of Oxaloacetate to Aspartate or α–Ketoglutarate to Glutamate provides substrates for the amino-acid-biosynthesis. The condensation of Oxaloacetate with Acetyl-CoA forms Citrate, this product is transported to the cytosol to be divided back to Acetyl-CoA which is channeled to the Fatty-Acid-Biosynthesis and Oxaloacetate which is transported back into the mitochondrion. Succinyl-CoA is used as substrate for the Porphyrin-Biosynthesis, Malate in the gluconeogenesis (Horn F. et al. 2002, Rassow J. et al. 2006).

Table 6 – energy efficiency of Krebs-Cycle

FADH2	1	App. 1.5 ATP
NADH	3	App. 7.5 ATP
GTP	1	App. 1 ATP
Total ATP = 10		

2.3.5.2 Beta-Oxidation

The beta-oxidation serves as a catabolic pathway to reduce fatty acids and generate Acetyl-CoA. The gained molecule is channeled into the Krebs-Cycle and metabolized to

NADH, FADH2 which are further used for the production of ATP through the respiratory chain.

The beta-oxidation itself takes place in the mitochondrial matrix, to start a reaction the substrate Acyl-CoA has to be transported into the Mitochondria. In the cytosol, free fatty acids bind to coenzyme A and forms Acyl-CoA, which then binds to the transporter Carnitine and is transported into the mitochondrial matrix (Horn F. et al. 2002, Rassow J. et al. 2006).

The energy efficiency of one beta-oxidation cycle is approximately 14 ATP.

Table 7 – energy efficiency of Beta-Oxidation

FADH2	1	App. 1.5 ATP
NADH	1	App. 2.5 ATP
AcetylCoA	1	App. 10 ATP
Total ATP = 14		

2.3.5.3 Pyruvate-Dehydrogenase-Complex

The Pyruvate-Dehydrogenase-Complex, a multi-enzyme-complex situated in the mitochondrial matrix. By catalyzing Pyruvate to Acetyl-CoA, PDH-Complex it links the depletion of glucose (Glycolysis) to and provides substrates for the Krebs-Cycle and further supplies the electron chain to gain ATP.

The regulation of the Pyruvate-Dehydrogenase-Complex functions via product regression – sufficient amounts of Acetyl-CoA in the mitochondrion effectuate a reversible phosphorylation via a Kinase and in its course the PDH is inhibited. As soon as the Acetyl-CoA amounts decrease, a phosphatase is activated and separates the phosphate-rest from the enzyme-complex. By this separation the inhibition is reversed, and the enzyme-complex is active again (Horn F. et al. 2002, Rassow J. et al. 2006).

2.5.5.4 Respiratory Chain

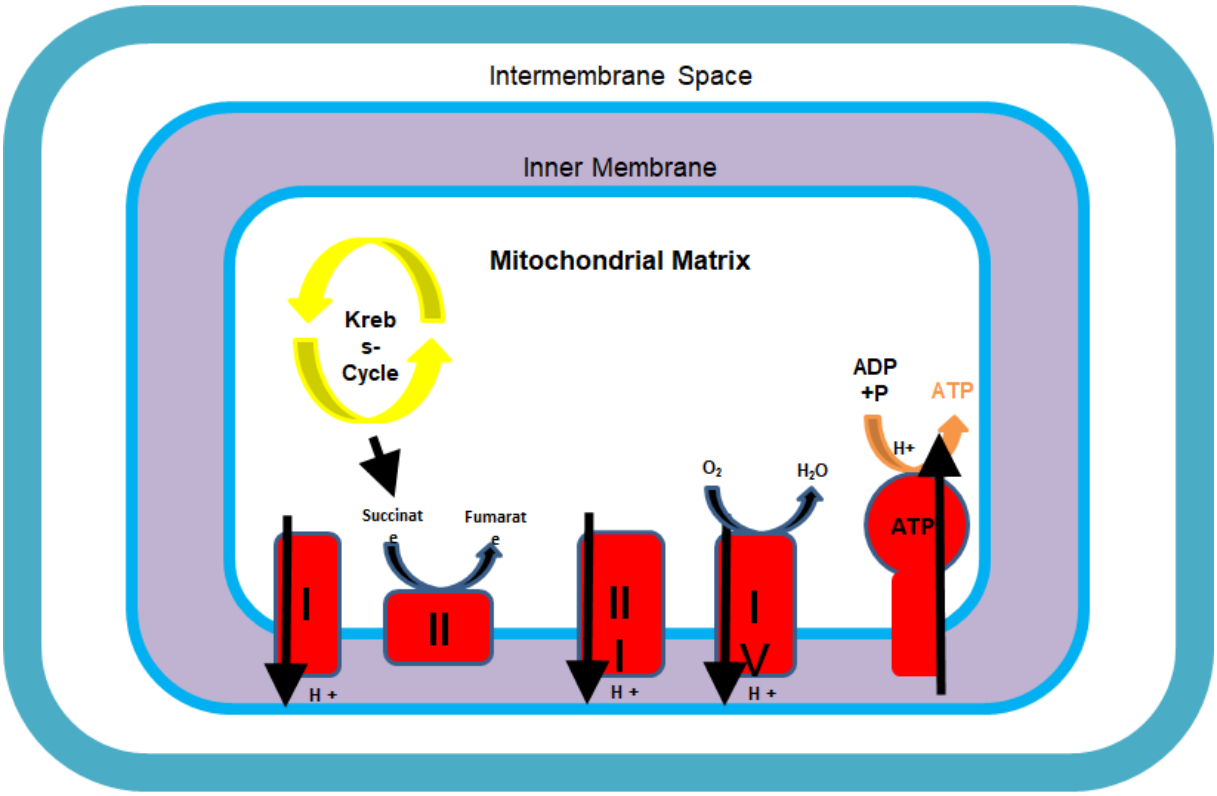

Figure 11 - graphical illustration of the respiratory chain

Electron chain (also called respiratory chain) is situated in the inner mitochondrial membrane and composed of four interacting complexes. Different metabolic pathways channel their reaction products and generated electrons into the electron chain, then they are then used for a cascade of redox-reactions to create a proton-gradient. This proton-gradient is needed to power the ATP-Synthase-Complex, an enzyme-complex which synthesizes ATP from ADP and Pi.

Complex-I (NADH-Dehydrogenase) accepts electrons donated from NADH-molecules from various catabolic pathways. Using four electrons Ubichinon is further reduced to Ubihydrochinon and 4 additional electrons can be transported to the inter-membrane-space (Horn F. et al. 2002, Rassow J. et al. 2006).

The connection between respiratory chain and Krebs-cycle is the enzyme Succinate-Dehydrogenase. It is a citric-acid-cycle enzyme and at the same time Complex-II of the electron chain. Here Succinate is transformed to Fumarate, while reducing FAD to FADH2 and Ubichinon is reduced to Ubihydrochinon (Horn F. et al. 2002, Rassow J. et al. 2006).

Complex-III, the Cytochrome-C-Reductase is responsible for regenerating the reduced Ubichinon-molecules via the q-cycle, a reaction cascade that finally transfers two electrons onto Cytochrome-C and two electrons are transported back into the inter-membrane-space (Horn F. et al. 2002, Rassow J. et al. 2006).

The last step, Complex-IV, the Cytochrome-C-Oxidase, is responsible for oxidizing the electron-loaded Cytochrome-C-molecules. Transmitting 2 electrons onto oxygen is a highly exergonic reaction step, H2O emerges (Horn F. et al. 2002, Rassow J. et al. 2006).

The reaction cascade and its individual reaction steps generate – as mentioned previously – a proton-gradient that is necessary to synthesize ATP. This glucose oxidation is a highly efficient way to utilize pyruvate and Acetyl-CoA and generate a significantly higher amount of energy (Horn F. et al. 2002, Rassow J. et al. 2006). In addition, a sufficient capacity of glucose oxidation or mitochondrial respiration facilitates the manufacturing of reactive oxygen species, necessary for the apoptotic behavior and pathway within the cell (Feng J. et al. 2001).

2.5.6 Mitochondrial diseases

These mainly pediatric or neurologic disorders are clinical pictures caused by a dysfunction or alteration in mitochondria. Due to the key role of mitochondria in energy gain, many patients show symptoms such as weakness, muscle weakness, fading muscle-control and neurological deficiencies, limited exercise capacity, heart- lung- liver- and kidney-disease, hearing- or vision-loss, cognitive deficits, or short stature.

Examples for mitochondrial diseases (taken from Dimauro S. et al. 2005):

***"LHON** – **L**eber's **H**ereditary **O**ptic **N**europathy"*

"Leigh-Syndrome"

***"MERRF** - **M**yoclonic **E**pilepsy with **R**agged **R**ed **F**ibers"*

***"MELAS** - **M**itochondrial myopathy, **E**ncephalomyopathy, **L**actic **A**cidosis, **S**troke-like symptoms"*

2.5.7 Mitochondria in cancer

The mitochondrion holds and important key function in the cellular metabolism. It handles pyruvate by channeling it into the Krebs-Cycle and therefore fueling the respiratory chain to generate ATP. The aerobic glucose-utilization is a very sufficient and highly developed but complex pathway and not only provides energy but also produces reactive oxygen species (ROS). Every developed and complex system is also prone to error. In case of mitochondria, alterations in biochemical and metabolic pathways or enzyme organization can result in lowered capacity for respiratory control (Luft R. et al. 1962), changed behavior and resistance to apoptosis (Bonnet S. et al. 2007).

In 2002 Carew J.S. et al. suspected that reduced apoptosis rates in cancer cells and tumors could be explained by impaired function of the respiratory chain – down-regulated activation of mitochondria lead to decreased oxidative phosphorylation, this further results in less production of pro-apoptotic factors and decreased levels of apoptosis. In addition, in 2002 Carew J.S. et al. hypothesized that mitochondrial DNA mutations and changes in respiratory activity appear to be characteristics of cancer cells.

The adequate function of the respiratory chain is essential to produce reactive oxygen species. A reduction in respiratory chain activation leads to a reduced ROS level, which can result in altered apoptotic behavior, aging processes and mutagenesis of mtDNA (Carew J.S. et al. 2002, Sandeep R. et al. 2006).

Mitochondrial DNA alterations are found in several cancer types such as gynecological tumors (breast- and ovarian-cancer), gastrointestinal-tumors (esophageal-, gastric-, colorectal-cancer), malignant growth in liver and pancreas as well as renal-cell-cancer, prostate-cancer and brain-tumors (Carew J.S. et al. 2002, Thierbach R. et al. 2005).

2.6 Warburg-Effect

In 1924 Otto Warburg, a German physician and biochemist, and his team investigated the differences between healthy muscle tissue and carcinoma muscle tissue extracted from frogs. The investigation showed, that carcinoma cells had increased levels of lactate in relaxed and active state compared to healthy ones. The team also proved a correlation between elevated glycolysis rates at higher pH levels (Warburg O. et al 1924). Further

investigations in animal models and tests with human cancer cells showed a shift from respiration to fermentation energy. It was suspected that either an irreversible injury of respiration had occurred, or the coupling of respiration and ATP formation was damaged (Lynen F. 1942, Warburg O. 1956).

Oxidative use of glucose is a more effectively way to generate ATP in the presence of high oxygen levels than generating ATP via anaerobic glycolysis; however, Warburg's' investigations showed a transition of metabolism and energy gain in cancer cells.

Increased glycolysis in cancer cells leads to the assumption that the mitochondrial function could be impaired and explain the metabolic shift. Further investigations showed altered mitochondrial function, affected glucose oxidation as well as elevated glycolysis and lactate rates. Also decreased oxidative capacity (and less ROS production) could lead to apoptosis resistance. The conclusion was that reduced Krebs-cycle, decreased mitochondrial function and hampered apoptosis could promote cancer (Cuezva J.M. et al. 2002, Thierbach R. et al. 2005, Schulz T.J. et al. 2006).

The Warburg-Effect has never been distinctly confirmed, although recent studies report a connection between metabolic and genetic changes observed during malignant growth.

Considering the Warburg-Theory, a line to the changed behavior seen in IPAH such as resistance to apoptosis, changes behavior in proliferation and transition from glucose oxidation towards anaerobic glycolysis (Sutendra G. et al. 2014).

3. Hypothesis, aims and goals

Suppression of mitochondrial bioenergetics are associated with abnormal pericyte behavior and impaired angiogenesis in PAH.

3.1 Exploring the Hypothesis

To explore significant changes in IPAH pericytes compared to healthy ones, we extracted this particular cell type from healthy and diseased lung tissue and cultured them in pericyte full media, until 100% confluency was reached. The cells were lysated, collected and prepared for the microarray. The microarray was conducted with the Ambion Illumina RNA amplification kit and the slides were analyzed by using BeadStudio (Illumina, Inc). The gained raw data was analyzed using GeneSpring.

Results: The analyzed data showed an up-regulation of PDK-4 by 6.92-fold.

The high up-regulation of PDK-4 led us to further investigations and provided an incentive for us to choose this enzyme as our goal of research.

3.1.1 Pyruvate-Dehydrogenase-Kinase-4

The enzyme pyruvate-dehydrogenase-kinase-4 (PDK4) is located in the mitochondrial matrix and in mammalians it is mainly found in skeletal- and heart-muscle (Pilegaard H. et al. 2004). It consists of 411 amino acids and has a molecular weight of approximately 46kDa.

The PDK-4 inhibits the Pyruvate-Dehydrogenase-Complex via phosphorylation, therefore the pyruvate shift and thus the substrate flow into the mitochondria is blocked (Rowles J. et al. 1996, Sugden M.C. et al. 2006). By decreasing or blocking the fueling of Krebs-cycle and respiratory chain, the manufacturing of ATP and reactive oxygen species (ROS) cannot take place. A lack of ROS can lead to a higher resistance to apoptosis or altered apoptotic behavior. Furthermore, the lack of ATP must be counterbalanced by activating anaerobe glycolysis and causes increased intracellular lactate levels. This elevated lactate

generation - as a result of diminished glucose oxidation in the respiratory chain - is often seen in malignant cells and tumors (McFate T. et al. 2008). Also, an elevation of the PDK-4 gene is associated with other diseases, for example pulmonary arterial hypertension, rectal prolapse and non-insulin dependent Diabetes Mellitus (Jeoung N.H. et al. 2008; Piao L. et al. 2013, Deol P. et al. 2015).

The PDK-4 gene expression depends on a large variety of surrounding conditions. Increased levels of PDK-4 expression are found during fasting or exercise - short term high and prolonged low-intensity exercise – as well as catabolic conditions. In addition, the expression is also regulated via glucocorticoids, retinoic acid and insulin. Glucocorticoids and retinoic acid enhance PDK-4 whereas increased levels of insulin inhibit the expression of PDK-4 (Wu P. et al. 1998, Huang B. et al. 2002, Pilegaard H. et al. 2004, Kwon H.S. et al. 2006, Connaughton S. et al. 2010).

3.2 Aim 1: Investigating PDK-4 regulation

Is PDK-4 up-regulated in IPAH-pericytes compared to healthy pericytes?

To measure the PDK-4 expression in healthy and sick IPAH pericytes, we cultured cells of both tissues in full media and seeded them on Collagen I plates (10µg/ml). We conducted an RNA-extraction with an RNeasy-Mini-Kit, measured the gained RNA with NanoDrop2000 and converted it into c-DNA. A q-PCR was conducted with TaqMan.

Results: The q-PCR showed a significantly up-regulated PDK-4 activation by 3.46-Fold-change.

To verify our q-PCR results we did 2 Western-Blots, neither of them showed a significant band for the PDK-4 antibody, however we tested the Anti-PDK-4-Antibody Human-Heart-Lysate and got a strong band in blot 2.

Although none of the western blots showed any significant result, we decided to further investigate PDK-4 as possible cause of different behavior in IPAH pericytes. To verify changes in apoptosis, proliferation, migration and metabolism we started experiments targeting these actions.

3.3 Aim 2: Investigating Metabolic Alterations

Does PDK-4 up-regulation cause further changes in cell metabolism? Can we prove our assumption, that PDK-4 up-regulation inhibits the PDH-complex and in its course, decreases the pyruvate shift into mitochondria? The lack of substrate will shut down the Krebs-Cycle and electron chain – can we show change in membrane potential and a decrease in ROS production?

Measuring Mitochondrial Transmembrane Potential ($\Delta\Psi m$) with TMRE in WT- and IPAH pericytes:

Cell cultures of Healthy- and IPAH-pericytes had been cultivated and treated with TMRE (tetramethylrhodamine ethyl ester) to label the membrane potential in mitochondria (Invitrogen/Molecular Probes) and PDK-4 to visualize the amount in healthy and sick cells. Furthermore, the stained pericytes were analyzed with an immunofluorescence microscope and the analyzing program Image J.

Results: The mean CFTF for TMRE was elevated ca. 28% in IPAH pericytes compared to WT pericytes. The mean CFTF for PDK-4 was elevated approx. 50% in IPAH pericytes compared to WT pericytes.

Measuring ROS-activity in WT-pericytes and IPAH-pericytes

WT- and IPAH-pericytes had been cultivated in full-pericytes-media and then treated with CellROX-Reagent (Molecular Probes by life technology) to explore the possible alterations in reactive oxygen species production in sick cells. After staining the cells with the dye, the pericytes were analyzed with an immunofluorescence microscope and the analyzing program Image J.

Results: The mean CFTF for CellROX-Reagent was decreased ca. 42.9% in IPAH-pericytes compared to WT-pericytes.

3.4 Aim 3: Further Investigations

With our previous experiments we have already shown the changes in metabolism at highly elevated PDK-4 levels. DCA as PDK-inhibitor should reverse the observed alterations in cell metabolism. Which experiments would give use proof of this assumption?

Dichloroacetate is a small molecule inhibiting PDK-isoenzymes and therefore releasing the inhibition of PDH-complex previously done by PDK. Several studies and clinical trials using DCA as an inverter of the effects on the metabolism of PDH-complex inhibition have been done.

3.3.4 Future Attempts

DCA-treatment of IPAH-pericytes compared to non-treated IPAH-pericytes and WT-cells:

We suspect that altered behavior of IPAH-pericytes in migration, apoptosis and proliferation is a result of a PDK-4 up-regulation and therefore an inhibition of the PDH-Complex. With an inhibited substrate flow into the mitochondria, the activation decreases and with it the ATP- and ROS-production. DCA as PDK-inhibitor should reverse those effects.

Can we visualize the supposed impact of DCA on IPAH-pericytes by treating cultivated IPAH-cells with DCA and comparing them to an IPAH-reference plate and a WT-pericytes plate? If this DCA-hypothesis proved true, we would see cell-networks on the IPAH-plate similar to healthy cells.

Measuring $\Delta\Psi m$ after treating IPAH-Pericytes with DCA:

We have already shown the difference between $\Delta\Psi m$ in WT- and IPAH-pericytes. Would it be possible to demonstrate the mitochondria-activating effects of DCA in IPAH-pericytes, if we – hypothetically speaking – treated one IPAH-pericytes group with a certain concentration of DCA, then stained this group with TMRE, measured the CFTF and compared the gained value to the baseline-value of an untreated IPAH-pericytes group?

Theoretically speaking, the ΔΨm of DCA-treated sick cells should be revised to (depolarized) normal values.

Measuring reactive oxygen species production after treating IPAH-Pericytes with DCA:

In one of our experiments we showed the transformation of reactive oxygen species production in IPAH pericytes. We could prove a significant decrease in CellROX-CFTF IPAH-pericytes compared to WT-pericytes.

If we compiled a similar hypothesis to the TMRE-experiment – treating IPAH pericytes with DCA, later measuring the ROS-production and comparing the gained data to a baseline value of non-treated IPAH-pericytes – could we also prove a difference?

If DCA activated the mitochondria via inhibiting PDK-4 and indirectly activating the PDH-complex, sufficient substrate flow should be regained. With an abundant flow of fuel into the Krebs-Cycle and the electron chain, the ROS-production should increase significantly.

4. Material and Methods

4.1 Used cell types

Lonza: vascular smooth muscle cells
Sciencell: vascular smooth muscle cells
ST0: iPAH pericytes
CC0: iPAH pericytes

4.2 Media for Pericytes

The used media for pericytes contained fetal bovine serum, pericytes growths supplements and penicillin/streptomycin solution.

4.3 Cultivation and Growth of Pericytes

Liquid full media plates were incubated at 37°C, overnight. The pericytes-full-media was changed every two to three days, after the plates had reached 100% confluency; the cells were washed with PBS, treated with 2ml Trypsin and incubated at 37C for one minute. Afterwards the cells were washed off the plate with full media and collected in a 10ml tube. The fluid was centrifuged for 5min with a speed of 400rpm at 25°C, the protrusion was abolished. The remaining pellet was resuspended with 5ml full media and the liquid was separated onto 5 plates, 1ml per plate, filled up with 9ml of full fresh media each.

4.4 RNA-Extraction with Rneasy Mini Kit (EN-Rneasy Mini Handbook, QUAIGEN, 2010)

The pericytes were grown on fluid 10ml-full-media plates, incubated at 37°C. When they reached 100% confluency, the plate was washed with PBS twice and afterwards the cells were lysated with 600 µl of lysate buffer. The lysatebuffer contained 10µl beta-

Mercaptoethanol in 1ml RTL-Buffer. The cells were scratched off the plate and pipetted into a 2ml tube, homogenized with a 5ml syringe and a 0.9mm needle 10 times, then the same volume of 70% ethanol was added to the solution and mixed well.
700µl were transferred to a RNeasy-Mini-spin-column (placed in a 2ml collection tube), and spinned for 15 seconds at ≥ 8000 x g. After spinning, the fluid was abolished, another 700µl were added to the tube and centrifuged again for 15s at ≥ 8000 x g. The fluid was abolished, 500µl of RPE-Buffer were added to the tube and spinned for 15s at ≥ 8000 x g again. Another 500µl RPE-Buffer were added and spinned for 2min at≥ 8000 x g. The column was placed in a new collection tube (1.5ml), 50µl of RNase-free water were added and spinned for 1min at ≥ 8000 x g. The collected RNA was measured and stored at -80°C.

4.5 RNA-Measurement with NanoDrop 2000

For measuring the RNA concentration, the NanoDrop2000 with a wavelength of 190-840nm and a concentration range of 0.4-15,000ng/µl was used. The tool was blanked with RNAse-free water first, afterwards each sample was mixed gently and 0.5µl were pipetted on the measuring surface. Between each sample the surface was cleaned carefully.
The NanoDrop2000 software measured the RNA concentration; it was marked on the samples itself and filed on the computer. The samples were stored at -80°C.

4.6 Gene-Expression Measurement with Microarray

RNA labeling and microarray hybridization – using an Illumina HumanHT-12_v4_Beadchip
Staring material: 250ng of total RNA.

Each RNA sample was amplified using the Ambion Illumina RNA amplification kit with biotin UTP labeling. The Ambion Illumina RNA amplification kit uses T7 oligo(dT) primer to generate single stranded cDNA followed by a second strand synthesis to generate double-stranded cDNA, which is then column purified. In vitro transcription was done to synthesize biotin-labeled cRNA using T7 RNA polymerase. The cRNA was then column purified. The cRNA was then measured and total of 750 ng of cRNA was hybridized for

each array using standard Illumina protocols with streptavidin-Cy3 being used for detection. Slides were scanned on an Illumina Beadstation and analyzed using BeadStudio (Illumina, Inc).

4.7 Analyzing the Data with GeneSpring

The GeneSpring software (Agilent, Santa Clara, CA) was used for statistical analysis and normalization of microarray data. Raw array data (.txt) files were uploaded to GeneSpring and it was analyzed using the GeneSpring Advanced Workflow.
The raw data of WT-pericytes was compared to the raw data of IPAH-pericytes and analyzed with the Advanced-Workflow. Further a list of differentially regulated genes was created, a fold change of >2 was used as a cut off. The list was narrowed by choosing the 10 highest up- and down-regulated genes and following sorted by fold change. To name a gene of interest, the most elevated fold change was used as marker.

4.8 TaqMan q-PCR

PDK-4 primer: TaqMan primer with unknown sequence.
The collected RNA was turned into cDNA (Fermentas cDNA reaction) and a sample, 20µl with 50ng/µl was used for the following q-PCR.
The q-PCR reaction was prepared with using the following formula:

2X TaqMan PCR Master Mix	25µl
20X FAM Primer	2.5µl
Sample DNA	2µl
H^2O	20.5µl
Total	**50µl**

15µl from the total volume of 50µl were loaded onto the plate as triplicates, after the loading the plate was centrifuged for 1-2min with 1300rpm. The computer settings were done and the program was started for 1.5h. Afterwards the data was analyzed and exported.

4.9 Western Immunoblot

The cells were washed three times with ice-cold 1xPBS, and lysates were prepared by adding boiling lysis buffer (1xRIPA and 1mM PMSF). The cells were scraped off the plate, pipetted into a 1.5-ml microcentrifuge tube, and boiled for 10 min before spinning.
The resulted overlaps were pipetted to fresh microcentrifuge tubes and stored at -80° C. The protein concentration was measured by the Lowry assay (Bio-Rad Laboratories).
A Bis-Tris-Gel (4-12%; Life Technologies, USA) was prepared and even amounts of protein were loaded onto each lane. Then the gel was exposed to SDS-Page electrophoresis under reducing conditions.
Following PVDF membranes were blocked for 1h in blocking buffer (5% milk powder in 0.1% PBS/Tween20) and afterwards incubated with primary antibodies for PDK-4 (rabbit antibody) and Human Heart Lysate (HHL) overnight at 4 °C. HRP conjugated secondary antibodies were visualized by ECL (Amersham, USA). Signal normalization was performed with a mouse monoclonal antibody against α-tubulin (Sigma-Aldrich, USA).

4.10 Measuring mitochondrial membrane potential with TMRE (Invitrogen/Molecular Probes)

Healthy and IPAH-pericytes were cultivated in full media. The preparations were done as described in the protocol and a staining solution with a concentration of 600nM TMRE in full media was prepared.
The culture media was aspirated, the cells were immersed in 600nM TMRE at 37°C for 10mins. Then the dyeing solution was removed and the cells were washed once with PBS. For fixation, the cells were treated with formaldehyde (4%) for 10mins at room-temperature. Following the formaldehyde was removed, the plates were washed one time with PBS, and then the cells were covered with fresh PBS and stored at 4°C.
The cells were imaged by an immunofluorescence microscope (λ-excitation 548 nm; λ-emission 573 nm), pictures were taken and later analyzed by Image J.
[Used Protocol, changes were made (see above): Labelling Mitochondria with TMRM or TMRE by Brad Chazotte; Cold Spring Harb. Protoc; 2011; doi:10.1101/pdb.prot5641].

4.11 Measuring ROS-Production with CellROX (Molecular Probes by life technology)

Healthy and IPAH-pericytes were cultivated in full media. The preparations were done as described in the protocol and a staining solution with a concentration of 5µM ROX reagent (Green reagent) in warmed full media was prepared.

The cells were washed once with PBS, 250µl staining solution was pipetted on each well and incubated for 10mins at 37°C. After staining dyeing solution was removed and the cells were washed once with PBS. To fixate the cells, they were immersed in formaldehyde (4%) for 10mins at room temperature. Then the formaldehyde was removed, the cells washed with PBS and following covered with fresh PBS and stored at 4°C. The cells were imaged by an immunofluorescence microscope (λ-excitation ≈ 500 nm; λ-emission ≈ 530 nm), pictures were taken and later analyzed by Image J.

[Used Protocol, changes were made (see above): CellROX Oxidative Stress Reagents (Catalog nos. C10422, C10443, C10444, C10448); CellROX Green Reagent C10444 molecular probes by life technologies].

5. Results

5.1 Analyzing Microarray with GeneSpring

A

Regulation	Fold Change	Symbol	Gene Name
UP	6,921982	PDK-4	Pyruvate dehydrogenase kinase, isozyme 4
	5,4344397	G0S2	Homo sapiens G0/G1switch
	5,208488	HLA-A29.1	Major histocompatibility complex class I HLA-A29
	5,070739	IL6	Interleukin 6 (interferon, beta 2)
	4,835987	GPR64	G protein-coupled receptor 64 (GPR64)
	4,7084017	ADH1A	Alcohol dehydrogenase 1A (class I)
	4,148623	CXCL6	chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)
	4,056757	CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
	3,9300213	CSF3	Colony stimulating factor 3 (granulocyte)
	3,81221	CXCL6	chemokine (C-X-C motif) ligand 6

Regulation	Fold Change	Symbol	Gene Name
DOWN	6,206931	FBN2	Fibrillin 2 (FBN2)
	5,548494	ACTG2	Actin, gamma 2
	5,4344397	IGFBP2	Insulin-like growth factor binding protein 2
	5,208488	CLDN11	Claudin 11 (oligodendrocyte transmembrane protein)
	5,070739	CYGB	Cytoglobin (CYGB)
	4,835987	IGFBP5	Insulin-like growth factor binding protein 5
	4,71632	IGFBP5	Insulin-like growth factor binding protein 5
	4,7084017	PLAT	Plasminogen activator, tissue
	4,148623	KRT19	Keratin 19
	4,119265	TMEM35	Transmembrane protein 35

B

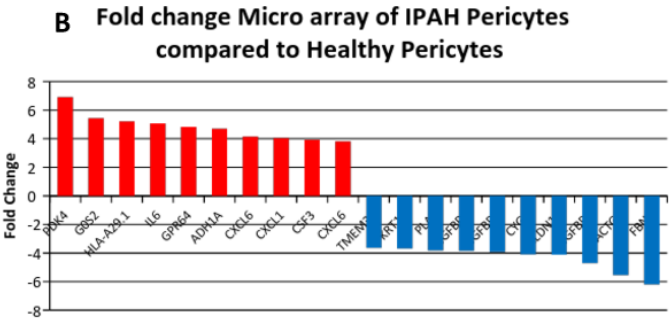

Figure 12 - Microarray data
(A) Microarray Data analyzed with GeneSpring, list of top 10 up- and down-regulated Genes. The Genes were filtered by fold change, a t-test was not done.
(B) Diagram of microarray data analyzed with GeneSpring, filtered by fold change. The gene expression of IPAH pericytes and healthy cells were compared by a microarray and the raw data was analyzed by GeneSpring. PDK-4 showed the highest regulation in IPAH pericytes, compared to healthy ones it was up-regulated with a fold change of 6.92.

To analyze the gained Microarray data, we used the GeneSpring software (Agilent, Santa Clara, CA). The Microarray provided raw data (.txt) files, which was uploaded in and analyzed by GeneSpring (Advanced Workflow, fold change of >2 was used as cut off). By comparing the gene expression in WT-pericytes with IPAH-pericytes, the analysis procured a list of up- and down-regulated genes, which was narrowed down by choosing the 10 most elevated up- and down-regulations using the particular fold change.
PDK-4 showed the highest peak in fold change overall with an up-regulation by 6.92-fold. We chose PDK-4 as our gene of interest and started further investigations.

5.2 TaqMan q-PCR

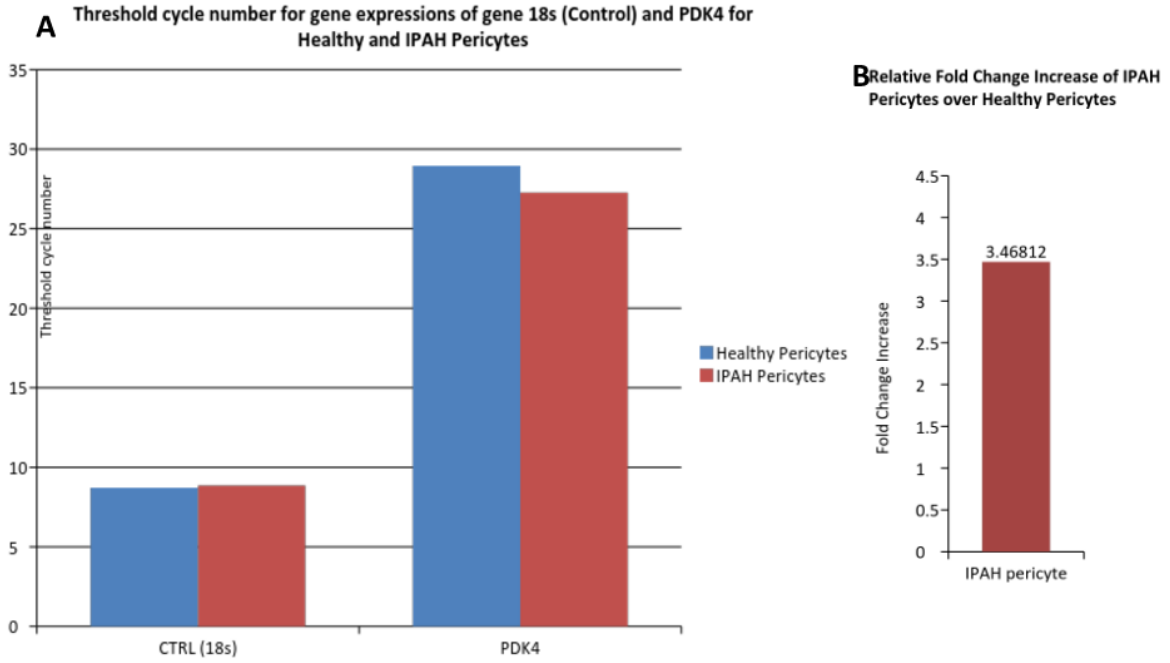

Figure 13 – TaqMan q-PCR
(A) - Experimental results for TaqMan qPCR comparing healthy and IPAH pericytes. The control gene 18s was chosen for its consistent gene expression over time. The data shows that the gene PDK4 was expressed significantly more in IPAH pericytes, since it took a lower number of cycles to reach the threshold.
(B) -The fold change Increase was calculated by subtracting the CTRL gene expression cycle number from the PDK4 value and for both Healthy and IPAH pericytes. These values were subtracted from each other, and the result inserted in following formula: Relative fold change increase=2^x
The data showed a significantly up-regulation of PDK-4 in IPAH pericytes and underlined our previously gained data

(A - Experimental results for TaqMan qPCR comparing healthy and IPAH pericytes. The control gene 18s was chosen for its consistent gene expression over time. The data shows that the gene PDK4 was expressed significantly more in IPAH pericytes, since it took a lower number of cycles to reach the threshold.
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The data showed a significantly up-regulation of PDK-4 in IPAH pericytes and underlined our previously gained data.)

To verify our gained Microarrays data – a highly up-regulated PDK-4 in IPAH pericytes in comparison to healthy cells - we performed a TaqMan q-PCR with a PDK-4 (TaqMan) primer of unknown sequence. The q-PCR was done and showed an up-regulation of PDK-4 by 3.46-fold. These results strongly supported our GeneSpring data and led to further investigation.

5.3 Western-Immunoblot

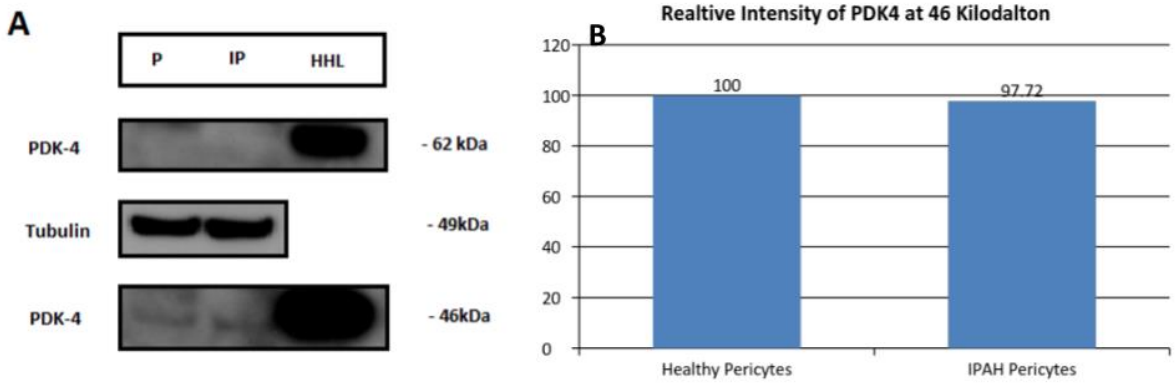

Figure 14 – Western Immunoblot
(A) – experimental results for Western-Immunoblot with PDK-4 Antibody and Human Heart Lysate (HHL). The specific weight for PDK-4 Antibody as well as for HHL is 46kDa. As expected the Human Heart Lysate (HHL) is clearly visible 46kDa. However, we expected there to be a significant band for PDK-4 Antibody at 46kDa in healthy pericytes (P) and IPAH pericytes (IP). WesternBlots are known to be more effective in detecting proteins rather than enzymes, this could be an explanation for the lack of significant bands for PDK-4.
(B) – The graphical analysis of the Western-Immunoblot using the program Image-J resulted in a minimal higher relative intensity of PDK-4 in IP (IPAH pericytes) compared t healthy pericytes. The result is not significant, therefore a statement cannot be made

As verification for the TaqMan q-PCR data, 2 Western-Immunoblots were done, using PDK-4 Antibody (rabbit antibody), Human Heart Lysate (HHL) and Tubulin as control. Neither of the blots showed a distinct band for PDK-4, but HHL showed a significant and strong band at 46 and 62kDa as well as Tubulin at 49kDa.

5.4 Measuring Mitochondrial Transmembrane Potential with TMRE in WT- and IPAH pericytes

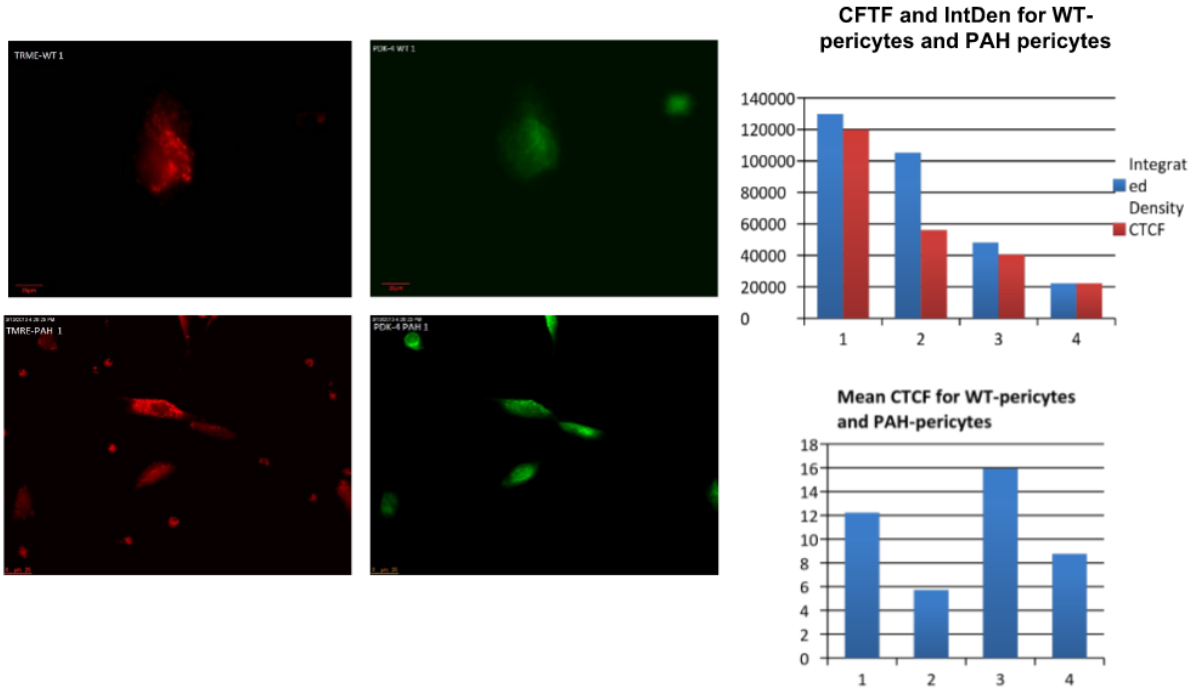

Figure 15 –Staining WT- and IPAH-pericytes with TMRE and PDK-4.
Staining IPAH- and WT-pericytes with TMRE, the IPAH-cells show a 28% increased value. Staining with PDK-4 reveals a 50% elevation in IPAH cells compared to WT-cells

To visualize the changes in mitochondrial membrane potential we stained WT- and IPAH-pericytes with TMRE at a concentration of 600nM for 10mins. To image the cells an immunofluorescence microscope was used (λ-excitation 548 nm; λ-emission 573 nm). The pictures were analyzed with Image J. using a Histogram. The fluorescence was measured and the mean CFTF (Corrected total cell fluorescence = Integrated Density – (Area of selected cell X Mean fluorescence of background readings)) was calculated.

The mean CFTF for TMRE was ca. 28% elevated in IPAH pericytes compared to WT pericytes. The mean CFTF for PDK-4 was approx. 50% elevated in IPAH pericytes compared to WT pericytes.

5.5 Measuring ROS production

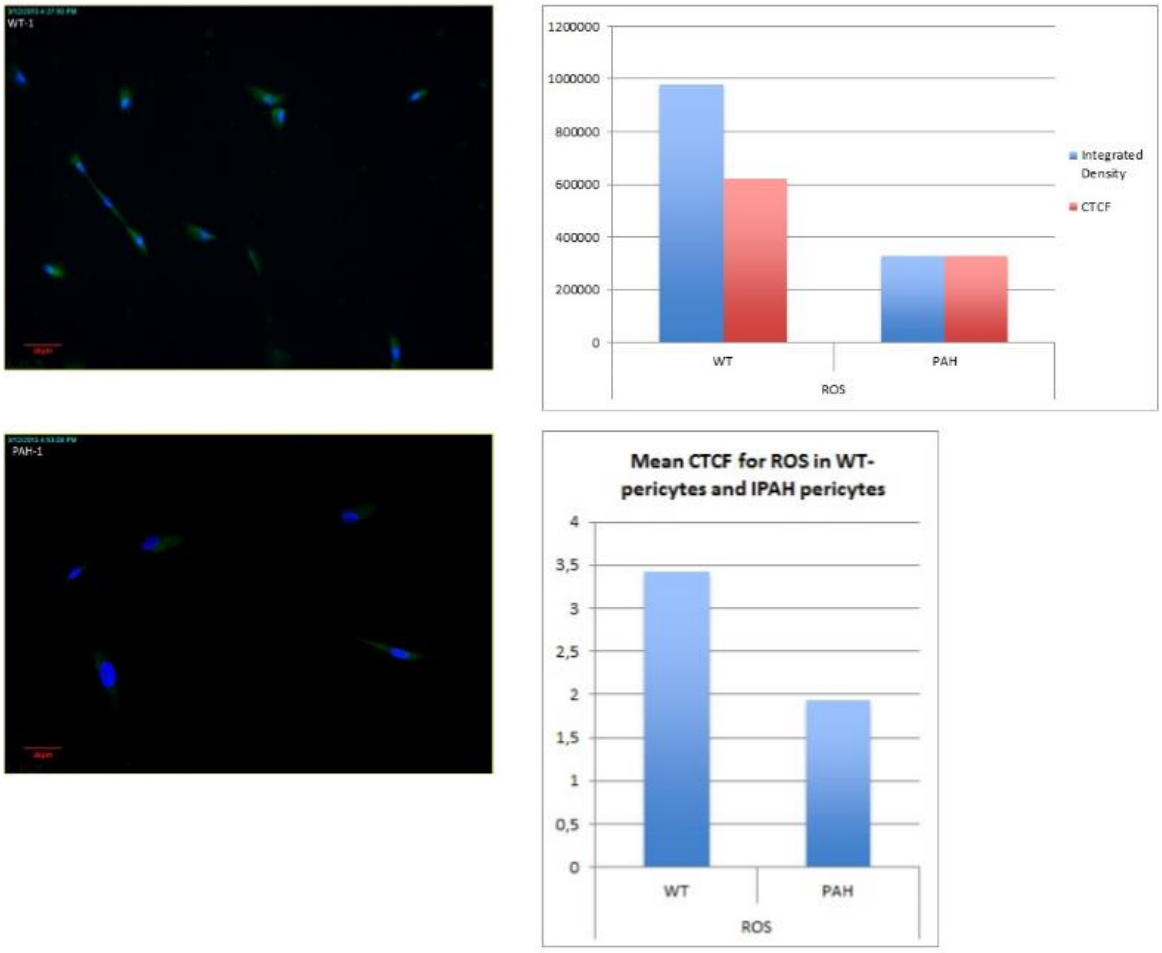

Figure 16 - Measuring ROS-production in IPAH- and WT-pericytes
Both, PAH- and WT-pericytes were stained with CellRox to visualize the ROS production and possible changes. After staining and imaging, the CFTF was calculated and showed a 42.9% decrease of ROS in IPAH-pericytes compared to WT-pericytes. (Corrected total cell fluorescence = Integrated Density – (Area of selected cell X Mean fluorescence of background readings)).

To visualize the changes in ROS production we stained WT- and IPAH-pericytes with CellRox at a concentration of 5µM for 10mins. To make a statement about ROS production in WT-pericytes and IPAH pericytes, we stained the cells with CellRox (green reagent) at a concentration of 5µM for 10mins. To evaluate the changes in ROS production in IPAH-pericytes the gained data an immunofluorescence microscope (λ-excitation ≈ 500 nm; λ-emission ≈ 530 nm) was used and pictures were taken for documentation. The images were uploaded into Image J, a histogram was made (measuring the fluorescence) and the gained data was analyzed. Again, the CFTF (Corrected total cell fluorescence = Integrated

Density – (Area of selected cell X Mean fluorescence of background readings)) was calculated.

The mean CFTF showed an approx. 42.9% decrease of ROS in IPAH-pericytes compared to WT-pericytes.

6. Discussion

Our initial focus of research was to investigate the origins and underlining mechanisms that cause the observed pathologies idiopathic pulmonary arterial hypertension.
We hypothesized that known macro- and microscopic alterations such as vessel-wall thickening and lumen narrowing could be caused by modified pericytes. As previously mentioned, increased proliferation of pericytes in IPAH lung tissue results in an elevated diameter of the vessel walls and subsequently increased intravascular pressure due to a higher resistance of the vessel wall (Lee S.D. et al. 1998). Therefore, our first goal was to explore the general differences of IPAH-pericytes compared to wild type pericytes. We started our investigation by extracting pericytes from idiopathic arterial hypertension lung tissue and healthy lung tissue following a MicroArray. The gained raw data was analyzed by using the GeneSpring software, the results showed an upregulation of PDK-4 by 6.92-fold in IPAH-pericytes compares to WT-pericytes. This finding supported our hypothesis of significant changes in IPAH pericytes. The enzyme PDK-4 is a suppressor of the pyruvate-dehydrogenase-complex and effectuates the downregulation of substrate flow (pyruvate) into the mitochondrion. By doing so, the Krebs-cycle and the electron chain lack of fuel and following decrease their production of ATP via oxidative phosphorylation and reactive oxygen species (Rowles J. et al. 1996, Sugden M.C. et al. 2006). To confirm our results of the MicroArray analysis, we decided to conduct another investigation, proving a PDK-4 upregulation in IPAH pericytes. We performed a TaqMan q-PCR, the investigation showed a PDK-4 activation by 3.46-Fold. In addition to the qPCR experiments, we conducted two Western-Immunoblots using a PDK-4 antibody. Both experiments did not show any significant result. Considering the strong results of the MicroArray data, we decided to proceed our investigations of possible PDK-4 overexpression related alterations in IPAH, despite the disappointing Western-Blot experiments. Since these first results

supported our assumptions - that an overexpression of PDK-4 could explain the observed pathological changes in IPAH pericytes compared to the cellular behavior in migration, proliferation and apoptosis in wild type pericytes – we decided to research the metabolic behavior. Our focus turned to the investigation of the cellular metabolism. To specifically target the metabolic pathways, influenced by a PDK-4 overexpression, the mode of action of this enzyme must be understood.

As previously mentioned, PDK-4 is responsible for the substrate flow into the mitochondrion. It inhibits the pyruvate-dehydrogenase-complex by phosphorylating a subunit. In a phosphorylated state, the PD-complex is no longer able to transform pyruvate to Acetyl-CoA and following Acetyl-CoA cannot be channeled into the Krebs-Cycle (Horn F. et al. 2002, Rassow J. et al. 2006). In a regular metabolic state, the Krebs-cycle is responsible for providing substrate for the respiratory chain and following facilitate oxidative phosphorylation. The ATP net balance undergoing oxidative phosphorylation is around 30 – 32 ATP per molecule glucose, compared to 2 ATP per molecule in anaerobe glycolysis (Rassow J. et al. 2006). In addition, the production of reactive oxygen species reduces under suppressed pyruvate-dehydrogenase-complex. Certain intracellular levels of ROS are important to maintain a normal apoptotic behavior in cells (Feng J. et al. 2001). Bonnet S. et al. (2007) showed that a depressed expression of potassium channels contributed to a higher resistance towards apoptosis and an inhibition of PDK indicated an increase in expression of potassium channels and reversed apoptotic behavior. Considering these information, a lack of ROS can lead to a higher resistance towards apoptosis and explain the findings in IPAH pericytes.

To investigate this assumption, we executed two additional experiments, targeting the metabolism in IPAH pericytes compared to WT pericytes. By measuring the mitochondrial transmembrane potential, our investigation yielded a 28% elevation (of membrane potential) in IPAH pericytes compared to WT pericytes. These findings support the assumption, that increased membrane potential is a prohibitory factor for apoptosis. Vice versa a lowered membrane potential increases apoptosis rates (Bonnet S. et al. 2007). Further we stained PDK-4 in both investigated cell populations - the enzyme was approximately 50% elevated in IPAH pericytes. These results underline the findings of our first two experiments – an increased rate of PDK-4 in IPAH pericytes in comparison with WT pericytes.

Our final trial targeted the fabrication of reactive oxygen species - the attempt was to visualize the alterations in IPAH pericytes. Both cell samples were stained with a green

reagent and later evaluated with an immunofluorescence microscope. The investigation showed a decrease of ROS by approximately 42.9% in IPAH pericytes. Taking this reduction of the amount of reactive oxygen species in the analyzed disease cell sample in comparison with the healthy cell sample, it strongly supports our hypothesis of alterations in IPAH pericytes due to the observed PDK-4 overexpression.

Summarizing our test series to investigate the possible origin of pathologies found in idiopathic pulmonary arterial hypertension, an overexpression of the enzyme pyruvate dehydrogenase kinase 4 explains the observed metabolic changes in IPAH pericytes.

We hypothesized that an inhibition of the pyruvate dehydrogenase complex by PDK-4 resulted in decreased activity of the respiratory chain and following diminished numbers of reactive oxygen species. In our investigations we proved a significant difference – a significant decrease – of reactive oxygen species in IPAH pericytes and therefore a lowered activity of the respiratory chain. Further we showed a significant distinction in membrane potential of wild type pericytes compared to IPAH pericytes. A higher membrane potential is associated with decreased values of apoptosis.

Both investigations and their results gave a valid explanation of the observed raised resistance to apoptosis and following altered behavior in proliferation and migration in IPAH pericytes.

Future investigations should concentrate on the effectiveness of PDK-4 and the reverse of its inhibition of the pyruvate dehydrogenase complex. By reversing this particular inhibition, a switch in metabolism from anaerobe glycolysis towards glucose oxidation, increase in ROS production, augmented expression of potassium channels and following decreased membrane potential could be achieved. Dichloroacetate - an inhibitor of PDK-isoenzymes - is already known and investigations concerning the potential have been researched in laboratory trials and clinical settings.

Dichloroacetate (CHCl2COOH) is a small, mitochondria targeting molecule, and indirectly activates the PDH by inhibiting PDK-isoenzymes. The reagent only targets cells with a PKD-4 overexpression and inhibited PDH, by reversing the effects of PDK a big variety of metabolic pathways are enhanced (Kato M. et al. 2007, Michelakis E.D. 2008, Piao L. et al. 2013). Indirect activation of PDH leads to an increased transport of pyruvate into the mitochondria and substitutes the Krebs-cycle with fuel, which further invokes the electron chain and raises the amount of gained ATP (McAllister A. et al. 1973). An activated electron chain and sufficient supply with substrates such as pyruvate, not only increases the ATP gain but also the production of reactive oxygen species, which leads to higher

apoptosis rates (Bonnet S. et al. 2007, Tong J. et al. 2011). DCA inhibits several anabolic pathways such as gluconeogenesis, lipogenesis and cholesterol genesis; hence the peripheral glucose and lipid utilizations are increased. (Stacpoole P.W. 1989).

DCA has already been used in clinical trials - Michelakis E.D. et al. published a study in 2010, where the team showed – both in vitro and in vivo – significant changes in glioblastoma cells after treatment with DCA. The mitochondrial hypopolarization was reversed, the level of ROS as well as the levels of apoptosis were increased. The familiar side effects of DCA, such as a peripheral neuropathy were dose dependent and reversible. Other secondary effects did not occur.

Further, positive effects of DCA given to patients with inherited mitochondrial diseases were proven. The patients not only showed significantly decreased levels of lactate and pyruvate, physical symptoms such as neurological disorders improved during therapy (DeStefano N. et al. 1995, Chinnery P. et al. 2006). Overall DCA is well tolerated in patients, possible side effects such as peripheral neuropathy have been shown as reversible (Stacpoole P.W. et al. 1998, Michelakis E.D. et al. 2010).

Considering our results and the achievements gained by clinical trials using DCA as an inhibitor of PDK-isoenzymes, this substance has great potential for future investigations and experiments reversing the effects of metabolically changes due to PDK overexpression.

7. Appendix

7.1 References

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7.2 List of Abbreviations

6MWD - 6-Minute-Walk-Distance

ATP= Adenosine Triphosphate

BMP-pathway - Bone Morphogenetic Protein Pathway

BMPR2 - Bone morphogenetic protein receptor type II

cAMP - Cyclic Adenosine Monophosphate

CFTF – Corrected Total Cell Fluorescence

cGMP - cyclic Guanosine Monophosphate

CTEPH - Chronic thromboembolic pulmonary hypertension

DNA - Deoxyribonucleic Acid

ECG – Electrocardiogram

FAD - Flavin Adenine Dinucleotide

HHL - Human Heart Lysate

IPAH - Idiopathic Pulmonary Arterial Hypertension

kDa – kilo Dalton

LHON – Leber's Hereditary Optic Neuropathy

MELAS - Mitochondrial myopathy, Encephalomyopathy, Lactic Acidosis, Stroke-like symptoms

MERRF - Myoclonic Epilepsy with Ragged Red Fibers

mPAP – median Pulmonary Arterial Pressure

mRAP – median Right Atrial Pressure

mtDNA – mitochondrial Deoxyribonucleic Acid

NAD - Nicotinamide Adenine Dinucleotide

NYHA – New York Heart Association

PAH - Pulmonary Arterial Hypertension

PASP - Pulmonary Artery Systolic Pressure

PDGFR-beta-1 - Beta-type Platelet-Derived Growth Factor Receptor

PDK-4 - Pyruvate-Dehydrogenase-Kinase-4

PGH2 - Prostaglandin H2

RAP – Right Atrial Pressure

RNA - Ribonucleic Acid

ROX - Reactive Oxygen Species

sGC - soluble Guanylate Cyclase

TAPSE- Tricuspid Annular Systolic Excursion

TEE - Transesophageal Echo

TGFbeta-1 - Transforming Growth Factor beta 1

TIM - Translocase of the Inner Membrane

TMRE - Tetramethylrhodamine Ethyl Ester

TOM - Translocase of the Outer Membrane

TRV - Tricuspid Regurgitant jet Velocity

TTE – Transthoracic Echo

VEGF - Vascular Endothelial Growth Factor

VO2 – Qotient of Oxygen Uptake

WHO – World Health Organization

WT – Wild Type

7.3 List of Figures

[Figures 9, 10 and 11 were designed and drawn by Alice Richter]

7.4 List of Tables

Eidesstattliche Versicherung

RICHTER ALICE

Ich erkläre hiermit an Eides statt,

dass ich die vorliegende Dissertation mit dem Thema

PDK-4 overexpression in pericytes extracted from idiopathic pulmonary arterial hypertension lung tissue could be the cause of altered cellular behavior, migration and proliferation

selbstständig verfasst, mich außer der angegeben keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Ich erkläre des Weiteres, dass die hier vorliegende Dissertation nicht in gleicher oder ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

Wien 15. April 2019 Alice Richter