Role of Lactate Transporters in Pulmonary Hypertension

Inauguraldissertation
zur Erlangung des Grades eines Doktors der Medizin
des Fachbereichs Medizin
der Justus-Liebig-Universität Gießen

vorgelegt von
Daniel Gerd Bermes
aus Trier

Gießen 2022

Aus dem Fachbereich Medizin der Justus-Liebig-Universität Gießen

Zentrum für Innere Medizin

Medizinische Klinik und Poliklinik II

Gutachter: Prof. Dr. Dr. Gall

Gutachter: Prof. Dr. Borggrefe

Tag der Disputation: 31.07.2023

Table of contents

1 Introduction

1.1 Pulmonary Hypertension

1.1.1 Definition

Pulmonary hypertension (PH) compromises all circumstances leading to an elevated pressure in the pulmonary circulation [51]. The pressure threshold is based on the mean pulmonary arterial pressure (mPAP). The latest "world symposium on pulmonary hypertension" from 2018 defined the upper limit of normal at 20 mmHg, based on scientific work of normal mPAP values in healthy individuals [213]. Normal values of mPAP are about 14 ± 3 mmHg and independent of sex or ethnicity [124–126]. This is considered in the current guidelines, defining PH whenever the mPAP increases above 20 mmHg [106]. Elevated pressure in the pulmonary circulation results in an increased right ventricular (RV) afterload, ultimately leading to life-threatening RV failure [51,221,232]. Hence, life expectancy is drastically reduced for any patient suffering from PH [102,136,144]. Worldwide 50 – 70 million individuals are affected by PH, emphasizing the relevance of the disease [101,102].

According to the definition, mPAP must be determined at rest [106]. However, an abnormal elevated mPAP during exercise can be useful to detect mild forms of pulmonary vascular disease [93]. A definition of exercise induced PH has been re-introduced in the current guidelines (mPAP/cardiac output (CO) slope >3 mmHg/L/min between rest and exercise) [106].

Another, clinically relevant, hemodynamic definition of PH addresses the distinction between pre- and postcapillary PH (table 1). This differentiation enables the delimitation of the underlying cause of elevated mPAP. Precapillary PH arises from pulmonary vasculopathy, whereas postcapillary PH is a consequence of pulmonary venous congestion [148,162]. The two entities can be distinguished by measuring the pulmonary arterial wedge pressure (PAWP) and pulmonary vascular resistance (PVR) during right heart catheterization (RHC) [106]. In healthy individuals the PAWP is about 8 ± 3 mmHg [124,126]. In postcapillary forms, the PAWP is elevated due to venous congestion. The PVR is about 0,9 ± 0,4 WU in healthy subjects [124,126]. In the guidelines the thresholds are set to 15 mmHg for PAWP and 2 Wood Units (WU) for PVR [106].

Table 1 Hemodynamic definition of PH, adapted from [106]

Definition	Hemodynamics	Clinical groups
PH	mPAP > 20 mmHg	All
Pre-capillary PH	mPAP > 20 mmHg PAWP ≤ 15 mmHg PVR ≥ 2 WU	1, 3, 4, 5
Isolated post-capillary PH	mPAP > 20 mmHg PAWP > 15 mmHg PVR < 2 WU	2, 5
Combined pre- and post-capillary PH	mPAP > 20 mmHg PAWP > 15 mmHg PVR ≥ 2 WU	2, 5

1.1.2 Classification

The reasons leading to elevated mPAP are various, which is reflected in the classification of the disease into 5 different groups (table 2) [106,125,212]. Assessment of the PH group is important, as it has direct impact on the available therapeutic possibilities (see 1.4) [60,106]. The different forms are classified according to their etiology or a pathophysiological overlap. The current classification refers to the recommendation of the „world symposium on pulmonary hypertension 2018“ [213].

Group 1, also referred to as pulmonary arterial hypertension (PAH), is a rare disease with a prevalence of 15 to 50 per million adult inhabitants, depending on the registry [108,136,169]. Patients with PAH have a poor life expectancy. If untreated, the median survival is considered to be only 2,8 years [42,136]. According to the Giessen PH registry the overall 5 year survival of PAH patients is only 52,7% [62]. The hemodynamic definition of PAH implies, besides mPAP and PAWP, an elevated PVR of more than 2 WU [106]. The subgroup idiopathic pulmonary arterial hypertension (IPAH) is to be seen as an exclusion diagnosis by the lack of known reasons for other forms of PAH [100].

Left heart diseases are the most common causes for PH (PH-LHD) and they are referred to as group 2. In 50 to 80% of the cases, LHD account for PH [102,196–198]. Any type of LHD can result in increased mPAP [153,198]. If patients with an underlying LHD disease develop PH, their prognosis is drastically hampered [53,196]. Patients with PH associated to ventilation disorders or exposure to chronic hypoxia, like in high altitude, are summed up in Group 3. The most frequent lung diseases include chronic obstructive pulmonary disease (COPD), interstitial lung disease (ILD) and

combined pulmonary fibrosis and emphysema (CPFE) [160]. Interestingly, 47 to 90% of CPFE patients develop PH [159]. Lung disease patients who acquire PH have a higher mortality. According to the Swiss PH registry even the worst prognosis of all the PH groups [144,160,163].

Patients are classified as group 4, if the elevated mPAP is caused by an obstruction in pulmonary arterial vessels. Chronic thromboembolic pulmonary hypertension (CTEPH) is attached to this group. CTEPH is commonly initiated by single or recurrent pulmonary embolism, mostly derived from deep vein thrombosis [61,104,168,236]. About 3,8% of patients with an acute episode of pulmonary embolism develop CTEPH [170]. It is still unknown why some of the patients develop PH, although some risk factors have been identified [21]. Among all the PH subgroups, CTEPH is the only potentially curable form (see 1.4.2) [122].

Group 5 consists of all forms of PH with unclear mechanisms.

Table 2 Detailed clinical classification of PH, adapted from [213]

Detailed clinical classification of PH	

1 Pulmonary arterial hypertension
• Idiopathic
• Heritable
• Drugs/toxins induced
• Associated with:
  ○ Connective tissue disease
  ○ HIV infection
  ○ Portal hypertension
  ○ Congenital heart disease
  ○ Schistosomiasis
• long-term responders to CCB
• overt features of venous/capillaries involvement
• persistent PH of the newborn
	3 PH due to lung diseases and/or hypoxia
	• Obstructive lung disease
	• Restrictive lung disease
	• Other pulmonary diseases with mixed restrictive and obstructive pattern
	• Hypoxia without lung disease
	• Developmental lung disorders
	
	4 PH due to pulmonary artery obstructions
	• Chronic thromboembolic PH
	• Other pulmonary artery obstructions

2 PH due to left heart disease • Left ventricular systolic dysfunction (HFrEF) • Left ventricular diastolic dysfunction (HFpEF) • Valvular disease • Congenital/acquired cardiovascular conditions leading to post-capillary PH	**5 PH with unclear and/or multifactorial mechanisms** • Hematological disorders • Systemic and metabolic disorders • Others • Complex congenital heart disease

1.1.3 Pathophysiology

As PH is a heterogenous disease, it is difficult to address all specific pathological features of each group. Most of our knowledge about PH pathophysiology, pathobiology and pathology have originated from studying PAH and hypoxia induced PH [39,219]. Although there are some key differences, especially regarding the pathogenesis of the disease, there are important similarities at molecular and histological level [39,49,72,104,122,143,196]. Therefore, the following sections will give an overview of the general changes observed in PH, focusing on the knowledge that was generated from PAH and hypoxia induced PH.

The pulmonary circulation is a high-flow, low-pressure and low-resistance system [72,110]. Even with increasing cardiac output (CO), PVR and pressure remains almost constant [146]. This is achieved by a pronounced compliance of the thin walled precapillary arterioles and the recruitment of non-perfused vessels [110,146,147]. The RV is adapted to these circumstances and a thin walled flow generator [146].

Pathophysiological changes observed in PH consists of vasoconstriction, vascular remodeling and in situ thrombosis [51,104]. Initially, vasoconstriction was believed to be a major feature of PH [104,154]. Although vasoconstriction is one of the driving forces, it is only the predominant cause in a small subset of PAH patients. The ones responding to CCB treatment, which accounts for less than 10% of the PAH patients [109]. The current understanding considers pulmonary vasoconstriction as an early event of the PH process. This is followed by obstructive remodeling of the pulmonary vasculature, causing reduced cross-sectional area of the pulmonary circulation, alongside with pruning and increased stiffness of the vessels, associated with high shear stress, pulse intensity and disturbed flow [105,218]. High shear stress has direct impact on endothelial cell function by inducing further proliferative signaling, promoting inflammation and pro-thrombotic environment, ultimately leading to an increased PVR and pressure [90,107].

The RV needs to adapt to the altered conditions. Initially with concentric hypertrophy and eventually with maladaptive eccentric hypertrophy, accompanied by decreased systolic and diastolic function resulting in RV failure [99,232].

1.1.4 Histological changes

Pulmonary arteries (PA) accompany air-conducting structures in pulmonary parenchyma. Near the hilus, PA are of an elastic type and have approximately the same size as bronchi. Peripheral, starting at the transition from bronchus to bronchiole, elastic arteries become muscularized. In the alveolar region, PA lose their medial smooth muscle and internal elastic layer. Pulmonary veins are found in interlobular septs and have a characteristic thin-walled structure [26,116,224]. As a hallmark of PH, vascular remodeling of each caliber is described, involving all 3 layers of pulmonary vasculature. Endothelial cells (EC), pulmonary arterial smooth muscle cells (PASMC) and fibroblasts are affected [72,116]. The cells acquire an antiapoptotic and hyperproliferative phenotype [5,85,207]. Already in 1958 Heath and Edwards described the histological changes in pulmonary vascular disease and introduced a classification into 6 grades (table 3) [88]. Still, this morphologic graduation can be used to get an overview of the pathologic changes occurring in PH.

Table 3 Histological classification of PH by Heath and Edwards, adapted from [116]

Classification by Heath and Edwards	
Grade 1	Medial hypertrophy and neo-muscularization
Grade 2	Cellular intimal proliferation
Grade 3	Fibrous vascular occlusion
Grade 4	Arterial dilatation with focal formation of plexiform lesion
Grade 5	Chronic arterial dilatation with numerous plexiform lesions
Grade 6	Necrotizing arteritis

Medial hypertrophy is an early onset of the disease and may be reversible [72,107]. It is found in all types of PH [26,37]. Histologically it is defined if the medial layer exceeds 10% of the cross-sectional diameter [72]. Thickening of the medial layer is correlating with hemodynamic parameters in PAH patients [49]. Neo-muscularization of formally non-muscularized arterioles is another early seen characteristic [26,107]. With ongoing disease, intimal proliferation occurs as a result of chronic pressure overload [26,72]. Followed by recruitment of subendothelial cells like fibroblasts and an “onion-like” concentric fibrosis of the intima, leading to an occlusion of the vessels [72,116]. Arteriolar dilatation, neointima formation, plexiform lesions and necrotizing arteritis are

terminal and irreversible pathologies [37,115,191]. Grade 4 – 6 are only found in really severe forms of PH [116].

1.1.5 Pathobiology

The exact molecular mechanisms leading to the PH phenotype are not completely understood yet. Various altered pathways are known to promote the disease [72,158,222,228]. Imbalance of vasoactive mediators, mainly derived from EC, is a well described feature. Impaired production of vasodilating agents like nitric oxide (NO) [66,131,241] and prostacyclin [33,200] alongside with increased release of vasoconstricting agents like thromboxane A_2 [33], endothelin [67,210] and serotonin [94] sustain pulmonary vasoconstriction. This mediator imbalance generates a hyperproliferative and pro-thrombotic condition [73,114,141]. Besides vasoactive mediators, several growth factors are involved in pulmonary vasculopathy. Vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), tumor necrosis factor α (TNFα), transforming growth factor β (TGFβ) and fibroblast growth factor (FGF) are all known to be upregulated in PH [87,105,209,222]. Additionally, changed transcription factor activity, like reduced p53 [234] and upregulated hypoxia inducible factor 1α (HIF-1α) [54,211], nuclear factor of activated T-cells (NFAT) [22] and nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) [166,204], just to name a few of the known, promote cell proliferation, migration, apoptosis resistance and inflammation. K^+ and Ca^{2+} channels are affected, as well. Downregulation of voltage-gated potassium channels (K_v) and high Ca^{2+} currents drive to membrane depolarization, inducing increased vasotonus and again, activation of transcription factors. Hyperpolarization of the mitochondria, further inhibits apoptosis signaling [85,244]. Inflammatory processes are considered as a trigger for the progression of PH [49,185]. Signs of inflammation are commonly found in PH lung vessels. Monocytes, macrophages, T lymphocytes and dendritic cells are especially found in plexiform lesions [90,158,224]. Higher concentrations of cytokines synthesized by inflammatory cells, like interleukin 6 (IL-6) and IL-1ß, are found in IPAH patients and animal models of PAH [191,206]. Just by touching a few selected players of the disease, the complexity of PH pathobiology is obvious. Collectively, altered vasoactive mediators, growth factors, transcription factors, inflammation and mitochondrial function are synergistically promoting the PH vasculopathy.

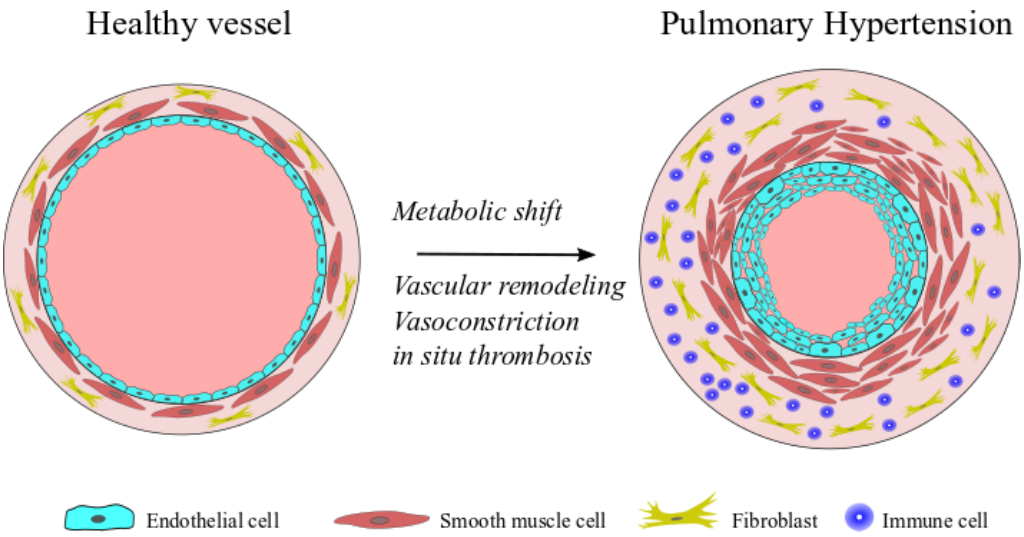

Figure 1 Scheme of vascular remodeling in PH, adapted from [206]

1.1.6 Metabolic background

In normal conditions cells cover up to 70% of their energy demand using oxidative phosphorylation [247]. In response to hypoxia, every mammalian cell changes from predominantly oxidative phosphorylation to glycolysis and lactate fermentation to cover their adenosine triphosphate (ATP) demand. The hypoxia induced metabolic shift is known as "Pasteur effect" [192]. Already in 1920s Otto Warburg described this metabolic shift to take place in tumor cells, even under normoxic conditions. Therefore it is called Warburg effect [184,235]. Initially it seems contradictory as glycolysis only generates a minority of the energy, with 2 ATP, compared to oxidative phosphorylation, with 36 ATP [247]. Though, this metabolic shift is reasonable for highly proliferative cells as the generated metabolites are used to fuel anabolic pathways, like synthesis of DNA or amino acids. Additionally, it is faster, in case there is enough glucose supply [6,13,84,103]. A similar metabolic phenotype, with enhanced cytosolic glycolysis, is well described for PAH and highly investigated recently [6,39,167,203,220]. An increased glucose consumption of pulmonary vasculature has been shown, using ^{18}F-fluordeoxyglucose (FDG) uptake, in PAH patients and monocrotaline induced PH [135,245]. Gene expression studies of PAH lungs showed significant upregulation of various glycolytic genes [246]. A closer look at the cellular level revealed, that the metabolic change takes place in all PH affected cells. Namely EC [28,54,242], PASMC [240], fibroblasts [41,180] and immune cells [41]. The RV usually generates 60 to 90% of energy from fatty acid oxidation and is more flexible regarding its energy source compared to

pulmonary vasculature. Affected by PH the RV adapts towards an increased glycolysis [89,175]. Dysregulated cellular metabolism is seen as a contributor to PH progress [6]. Consequently, targeting the metabolic pathways became a promising therapeutic approach. Dichloroacetate (DCA) is one of the used compounds. It restores oxidative phosphorylation by inhibiting pyruvate dehydrogenase kinase (PDK). DCA showed great potential in monocrotaline induced animal models of PH and pulmonary arterial banding induced RV failure [138,174].

Many different pathways and stimuli are identified to induce the metabolic change in PH. One of the key players is HIF-1α. As hypoxia dependent transcription factor, HIF-1α primes the cells to glycolysis by upregulating various glycolytic enzymes [117,120]. In PAH HIF-1α is even active under normoxic conditions [54,74]. Further less known factors are the downregulation of p53, activation of NFAT via the Ca/calcineurin axis or activation of the myc-c pathway. Direct disturbances of the mitochondria and its enzymes are described as well, like downregulation of the superoxide dismutase (SOD2), miRNA derived inhibition of glycolytic enzymes, disturbed iron homeostasis and disrupted endoplasmic reticulum – mitochondria interaction [4,6,85,167,203,215,220].

Down the road comes lactate, the end-product of glycolysis. But it is much more than merely an end-product [45]. Lactate induces the expression of various genes, as it was shown in L6 myocytes [86]. There is evidence that lactate induces VEGF expression by mimicking hypoxia signaling [10,47,216]. In glioblastoma cells lactate was found to induce TGFβ expression [9]. Studies of the central nervous system showed that lactate modifies prostaglandin derived vascular regulation [14]. Additionally, lactate has strong impact on the immune cells. Lactate inhibits the differentiation of immune cells and prevents cytokine release, which contributes to the immune escape in cancer cells [69,96]. Via induction of IL-8 and NF-κB in EC, lactate supports cell migration and angiogenesis [50,133]. Not surprisingly, high intratumoral lactate concentrations correlate with poor prognosis of the patients in various cancer entities [20,165,217,233,243,248]. On the other hand, lactate is a health hazard for the cell itself. Intracellular accumulation of lactate drops the cytosolic pH, which results in inhibition of further glycolysis, leading into an energy crisis and eventually cell death [45,50,80]. Obviously, the cells need to possess possibilities to maintain pH and lactate homeostasis. Little is known how PH affected cells escape the energy crisis. But these metabolic alterations were initially described in cancer cells and have been extensively studied in this field. Cancer cells

induce different members of their pH regulation system, like Na^+ - H^+ exchanger, carbonic anhydrases or Na^+ - HCO_3^- co-transporter [29,119]. Among these pH regulators, only one family of transporters regulate lactate homeostasis, namely monocarboxylate transporters.

A simplified scheme of the metabolic background of PAH is displayed in figure 2.

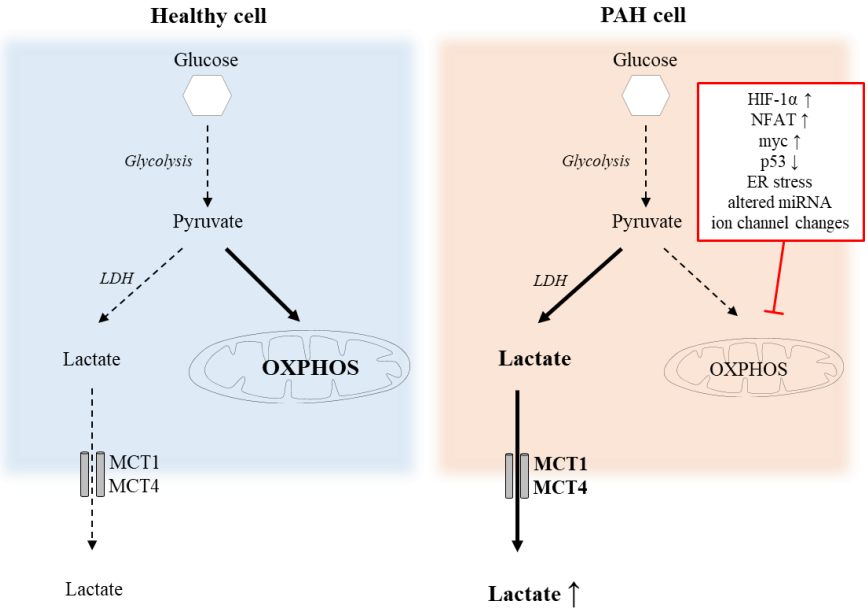

Figure 2 Simplified scheme of the metabolic background of PAH. LDH – Lactate dehydrogenase, OXPHOS – Oxidative phosphorylation, MCT – Monocarboxylate Transporter

1.2 Monocarboxylate Transporters

In cellular metabolism, monocarboxylic acids, such as pyruvate, ketone bodies and lactate, play a major role. The importance of lactate was previously described. Originally, lactate was thought to cross the plasma membrane by free diffusion. It was just when Halestrap and Denton chemically inhibited lactate and pyruvate transport in human erythrocytes in 1974, that a specific monocarboxylate transporter was discovered [78].

Monocarboxylate transporters (MCT), also known by their gene family "solute carrier 16" (SLC16A), is a heterogenous group of transporters. 14 proteins are currently attached to the MCT family. Only MCT1 (SLC16A1), MCT2 (SLC16A7), MCT3 (SLC16A8) and MCT4 (SLC16A3) facilitate the transport of monocarboxylates [81]. By far, the predominant role of MCT1-4 is the transport of lactate [24,80]. Other members whose substrates have been characterized are MCT8 (SLC16A2), which transports thyroid hormone [56,231] and MCT10 (SLC16A10) also known as t-type amino acid transporter

(TAT1) shuttling aromatic amino acids [134]. The substrates and the function of the remaining MCTs are still unknown [77].

The topology of all MCTs is comparable. All of them have a structure of 12 transmembrane (TM) domains, intracellular N and C terminal end and a large cytosolic loop between TM domain 6 and 7. The TM regions have a conserved structure, differences are mainly found at the N- and C-terminal end and the loop between TM domain 6 and 7 [77,80,186]. For their proper expression and attachment to the plasma membrane the MCTs require interaction with ancillary protein, mainly basigin (CD147). Basigin consists of a single TM domain, a short intracellular C-terminal end and a large glycosylated extracellular domain, whereas MCTs have no reported posttranslational modifications [77,79,123,239]. The transport is mediated as a proton linked symport of monocarboxylates in a 1:1 stoichiometry. Driving force is the concentration gradient of the substrates, making it ATP independent [14,75,182]. Thus, they are not only important for lactate export, but also for pH homeostasis. Studies from skeletal muscles showed that MCT activity is responsible for 40% of the pH regulation [29]. The transport mechanism has been extensively studied for MCT1. First, the proton binds to a lysine residue (K38) at the suspected substrate binding site to provide a binding site for lactate anion. Then, MCT1 performs a rearrangement to a "closed" state that exposes substrates to the outer surface, where first lactate and then the proton is released. Rate limiting step is the return to the "open" conformation [75,76,238].

Despite their similarities, MCT1 – 4 differ in their substrate affinity (table 4) and tissue distribution, explaining their individual physiological functions [55,81].

Table 4 Substrate affinity of MCT isoforms

Substrate	**MCT1**	**MCT2**	**MCT3**	**MCT4**	**Plasma**	**Cytosolic**
	K_m [mM]					[mM]
Lactate	3,5	0,74	6	28	1,0	<1 (up to 30x fold increase)
Pyruvate	1,0	0,08	-	153	0,1	-

MCT1, 2 and 4 measured in Xenopus oocytes, MCT3 in Yeast. Values taken from [148]. Pyruvate and lactate concentrations taken from [150]

MCT1 has the broadest tissue distribution and is nearly ubiquitous expressed. The medium high affinity for L-lactate enables uptake and efflux, taking plasma and cytosolic lactate concentrations into account. It can be described as the universal lactate transporter [75]. MCT2 is a high affinity lactate transporter and has been mainly studied in the brain, where it enables the uptake of lactate at postsynaptic membranes. Furthermore, it mediates lactate uptake in specialized cells facilitating lactate as energy fuel. For example in liver cells in the context of Cori-cycle or astrocytes, where Brooks proposed a metabolic symbiosis with surrounding cells [24,25]. Making MCT2 a specialized transporter for lactate uptake with restricted tissue distribution [14,24]. MCT3 is only found in retinal pigment epithelium (RPE) and choroid plexus epithelium, where lactate efflux is mediated [79,152]. The physiological role of MCT4 is the lactate export in highly glycolytic tissues, such as white skeletal muscle, astrocytes or white blood cells. The low affinity for pyruvate prevents its loss from the cell and ensures that it can be used for the conversion into lactate. Comparing to the other lactate transporter the affinity for L-lactate is rather low [77,79].

The only other known lactate transporters are sodium-coupled monocarboxylate transporter (SMCT). SMCT1 (SLC5A8) and SMCT2 (SLC5A12) shuttle monocarboxylic acids together with sodium across the plasma membrane. They are mainly found in the intestine, kidney, or brain, where they ensure substrate uptake. In cancer they are seen as tumor suppressors and therefore downregulated in the disease state, as they are also responsible for the uptake of pro-apoptotic mediators like butyrate and propionate [64].

Among the lactate transporters, MCT1 and 4 are the only ones theoretically capable to maintain lactate homeostasis in pulmonary vasculature. In cancer research their importance is well examined (see 1.2.2). Therefore, this study and the next sections will focus on MCT1 and 4.

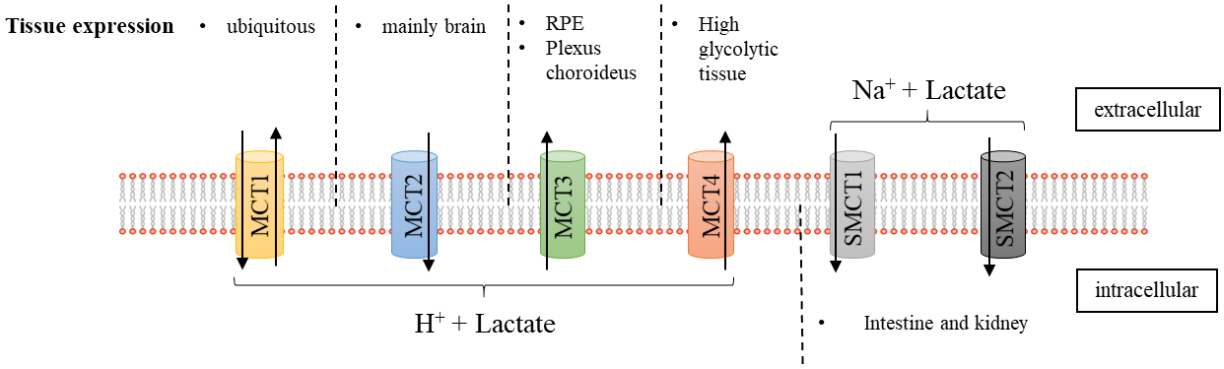

Figure 3 Overview of known lactate transporters [39,55,64,80,186]. RPE – Retinal pigment epithelium, MCT – Monocarboxylate Transporter, SMCT – Sodium-coupled Monocarboxylate Transporter

1.2.1 Regulation

Having an universal promoter, MCT1 is expressed in nearly every tissue. Some cells, like β cells of the pancreas, do not express MCT1 at all [80]. The 5' untranslated region of the promoter contains multiple CpG sequences. Methylation of these sequences leads to tissue specific silencing of MCT1 [81]. The first insight into transcriptional regulation originated from numerous studies in skeletal muscle. Showing that MCT1 expression is upregulated in response to stimulation, whereas denervation causes reduced MCT1 expression. This promoted the hypothesis that MCT1 expression is regulated in an AMP-activated protein kinase (AMPK) and calcium-dependent pathway [75,95]. Indeed, following studies proved that AMPK and NFAT (downstream of calcium/calcineurin) induces MCT1 expression [77,145,171]. Studies in several cancer entities with myc gain of function mutations revealed a myc dependent upregulation of MCT1 [35,48]. Hypoxia as a stimulus for cytosolic glycolysis is known to induce MCT4 expression. Ullah et al. found that MCT4 to be a direct target of HIF-1α, but not MCT1 [227]. Although MCT1 mRNA is reported to increase in hypoxic conditions, the protein expression is not changed or even reduced, pointing to post-transcriptional regulation. P53 was shown to alter MCT1 mRNA stabilization and thus suppress MCT1 protein expression under hypoxic conditions. The loss of p53 in hypoxia resulted in an increased MCT1 expression, which was induced by NF-κB [19]. Interestingly, lactate induced NF-κB activation also resulted in an increased MCT1 expression [193]. In contrast, an opposite effect was described by other authors, showing an increased MCT1 protein expression under hypoxic condition [31].

1.2.2 Monocarboxylate Transporter 1 and 4 in PH and cancer

The knowledge about the role of MCT1 and 4 in PH pathobiology is still very limited. Caruso et al. found that MCT1 mRNA is significantly upregulated in endothelial cells derived from BMPR2 mutant patients and in the lungs of sugen-hypoxia rats [28]. While focusing on miRNA in PH, they only investigated MCT1 mRNA as an indicator for a highly glycolytic phenotype and did therefore not further investigate the expression profile, like protein expression.

In contrast there are a lot of publications dealing with the role of MCT1 and 4 in cancer and as previously described the altered metabolism observed in PH is similar to that seen in many cancer entities. In general, PH is considered as a cancer like disease, sharing many of the so called “hallmarks of cancer”, like sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, angiogenesis, genome instability and mutation [41,84,189,202]. Therapeutic approaches derived from cancer therapy like tyrosine kinase inhibitor imatinib showed promising potential [65,206]. Therefore, a glance at the knowledge derived from cancer research is reasonable.

About 70% of all cancer entities have an overexpression of glycolytic enzymes [1]. Pinheiro et al. published a review about the role of MCTs in cancer [177]. He and other investigators found MCTs to be upregulated in many different cancer entities. MCT1 expression is upregulated and associated with a poor prognosis in breast [19,176], lung [52,181], ovarian [30], intestinal [178,179] and endometrial cancer [127]. The same accounts for MCT4, its expression is associated with poor prognosis in pancreatic [7], prostate [32,172], colorectal [151] and lung cancer [199]. Interestingly, some entities only express one of the isoforms, some express both [46,133,177].

The current theory propose a metabolic symbiosis between hypoxic or glycolytic tumor cells and oxidative tumor cells. In this concept MCT4 is expressed in the glycolytic cells, mainly found in the tumor stroma in distance to oxygen supplying vessels, mediating the export of lactate. Extracellular lactate is then taken up by oxidative cells via MCT1 and serving as respiratory fuel. Keeping the mediatory effects of lactate in mind, this microenvironment, maintained by MCT1 and 4, supports proliferation, migration and angiogenesis and further ensures glucose supply for glycolytic cells [45,47,50,55].

1.2.3 Inhibitors

First insight into the efficacy of MCT inhibition was generated using small molecules like α-cyano-4-hydroxycinnamate (CHC). This compound inhibits MCT1 and was successfully used in different *in vitro* and *in vivo* cancer models [20,133,216]. However, the disadvantage of these early described inhibitors for clinical use were their non-specificity for MCTs. The next generation of MCT1 inhibitors was developed by AstraZeneca. The first one, AR-C155858, initially conceived as an immunosuppressive agent, showed potential in preclinical cancer treatment [128,164]. This new generation is highly specific and potent in inhibiting MCT1 and 2 with K_i values in the nmol/l range. A second developed MCT1 inhibitor, AZD3965, is even more potent [40]. It showed great anticancer efficacy in a variety of cancer cell lines and *in vivo* models [20,40,157,181]. Furthermore, AZD3965 was tested in a phase I/II clinical trial for the treatment of patients with prostate cancer and diffuse large B-cell lymphoma (NCT01791595). MCT1 inhibitors are ineffective in cells and cancer entities expressing MCT4 [7,20,181]. Diseased cells escape the MCT1 inhibition and become treatment resistant by upregulating MCT4 to maintain lactate efflux. Unfortunately, no specific MCT4 inhibitor is available, yet. Although it is reported that AstraZeneca succeeded in developing one (AZ93), it is still not commercially available [133]. MCT4 is an attractive anti-cancer target. In a head and neck squamous cell carcinoma and pancreatic cancer mouse model MCT4 knockout showed less severe disease burden [7,17]. Just recently, Benjamin et. al found an already approved anti-hypertensive drug, syrosingopine, to inhibit MCT1 and 4. Syrosingopine has higher potency for MCT4 over MCT1 [11,12]. Besides MCT1 and 4, syrosingopine inhibits the vesicular monoamine transporter (VMAT), which is responsible for anti-hypertensive treatment. Benjamin et. al showed that the anti-cancerous effect is independent of VMAT inhibition and due to the MCT1 and 4 inhibition. Oxidative phosphorylation and glycolysis are tightly coupled, if glycolysis is inhibited by blocking lactate export the cells can survive by inducing oxidative phosphorylation [7,247]. The current hypothesis is that in order to drive the abnormal cells into metabolic crisis and death oxidative phosphorylation needs to be slowed down as well. This approach was already tried by different investigators, mainly using metformin to inhibit mitochondrial complex I [7,48,132]. Therefore, combination of metformin and MCT inhibitors can be a promising future therapeutic approach.

Table 5 Overview and characterization of MCT inhibitors, adapted from [171]

Molecule	Target	K_i	Off target	References
DIDS	MCT1	434 µM	Aquaporin, Cl^- /HCO_3^- exchanger	[27,238]
CHC	MCT1 MCT2 MCT4	155 µM 24 µM 991 µM	Mitochondrial pyruvate exchanger	[27,34,140,216]
AR-C155858	MCT1 MCT2	1,2 nM <10 nM*		[128,164]
AZD3965	MCT1 MCT2	1,6 nM 9,6 nM		[20,40,157]
Bindarit	MCT1 MCT4	>450 µM 30,2 µM		[58]
Syrosingopine	MCT1 MCT4	IC_{50} 2,5 µM IC_{50} 40 nM	VMAT	[11,12]

DIDS – diisothiocyanostilbene-2.2'-disulphonate, CHC – α-cyano-4-hydroxycinnamate, VMAT – Vesicular Monoamine Transporter

* No inhibitory effect when MCT2 is associated with embigin instead of basigin

1.3 Diagnosis of Pulmonary Hypertension

A diagnostic algorithm is provided in figure 4 [153]. Every patient with otherwise unexplained dyspnea on exertion, syncope or signs of right ventricular dysfunction should be considered suspicious for PH [100]. An early diagnosis improves the prognosis of the patients [57,101,125]. Tragically, due to the unspecific symptoms, it is still lately diagnosed [125].

The most important diagnostic tools, besides mandatory RHC, to detect PH are clinical appearance, echocardiography and ventilation/perfusion scanning. Although further clinical alterations in electrocardiogram [8,125,153,161] or radiograph of the chest [8,44,125,153] are frequently observed, they are non-specific for PH.

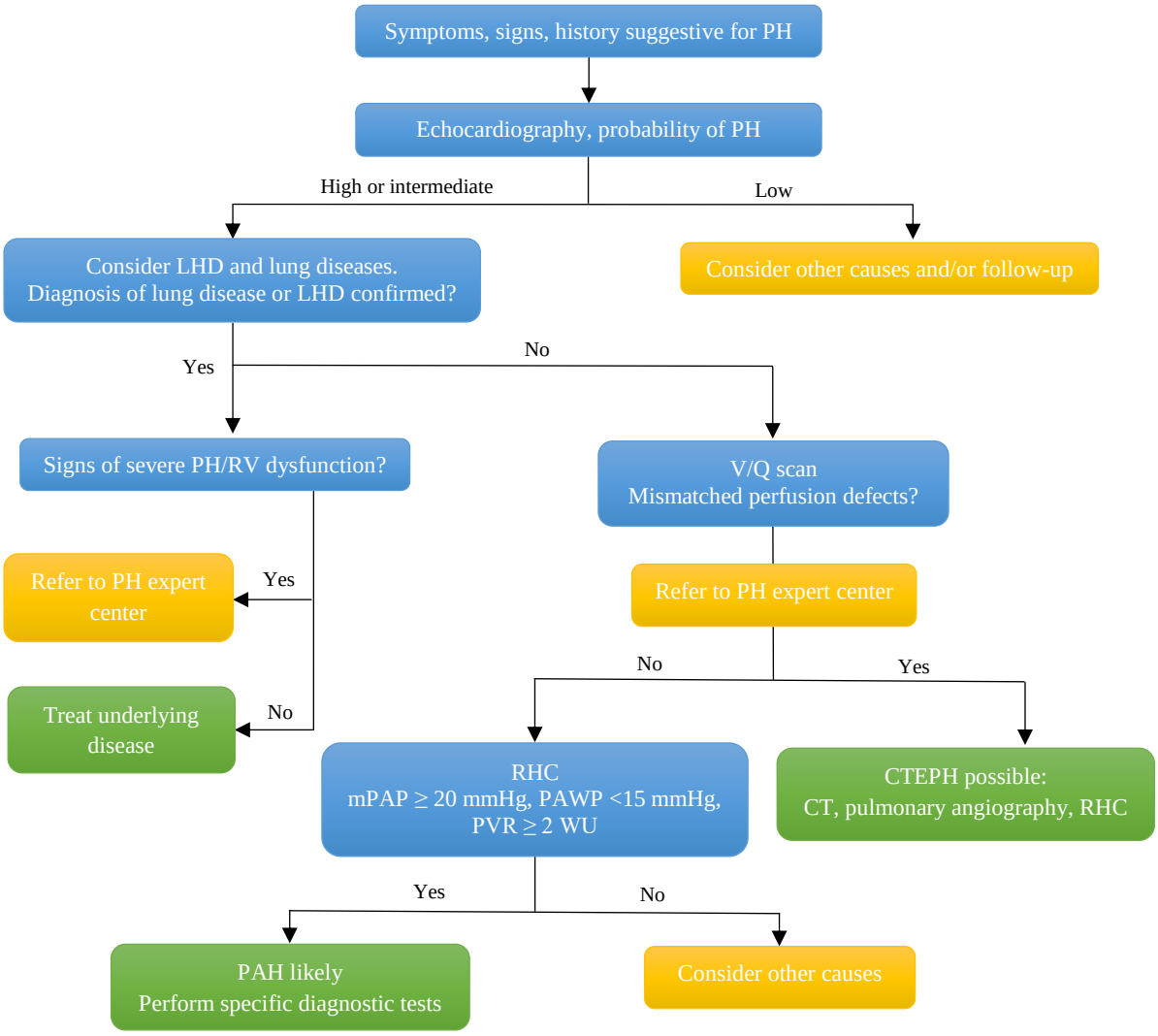

Figure 4 Simplified PH diagnostic algorithm, adapted from [153]

1.3.1 Clinical appearance

Clinical appearance of PH patients is non-specific, mostly conform to progressive right heart failure [106,153]. Initially dyspnea, fatigue, dry cough, nausea, syncope or angina pectoris complaints are described [8,57,125]. With deterioration of right heart function peripheral and central oedema, ascites and engorgement of the neck veins can arise. Exertional intolerance is quantitated with the World Health Organization (WHO) functional class (FC) [8].

1.3.2 Ventilation/Perfusion scanning

Ventilation/Perfusion Scanning (V/Q) is the commonly recommended tool to detect CTEPH [121,122,237]. V/Q mismatch of at least one segment or two subsegments is typical for embolic obstruction. With high sensitivity (96-97%) and specificity (90-95%) CTEPH can be barred with an inconspicuous V/Q scan [225]. Following the diagnostic

algorithm, V/Q scan should be performed in each PH patient before performing RHC [125].

1.3.3 Echocardiography

Transthoracic echocardiography (TTE) is an important, non-invasive method to judge the probability of PH. TTE is not used to confirm diagnosis of PH, but to decide the need for further investigations, most importantly RHC [57,106]. In TTE systolic pulmonary arterial pressure (sPAP) is estimated based on the peak tricuspid regurgitation velocity (TRV) and right atrial pressure (RAP) [43]. TRV can be measured using Doppler, RAP needs to be estimated by inferior vena cava respiratory index [201]. Estimated sPAP is highly associated with invasive measured values when performed by experienced investigators [44,70,155]. Further values, such as tricuspid annular plane systolic excursion (TAPSE), RV and right atrial (RA) size, should be measured to evaluate right heart function.

European guidelines recommend a graduation by probability of PH primarily based on TRV and other "PH signs" in TTE. Based on TTE derived PH probability and further clinical findings, the decision to perform RHC is made [125,153].

1.3.4 Right heart catheterization

To confirm PH diagnosis, RHC is mandatory and determined in the definition of PH [8,57,125]. Collected data helps to identify PH group, delivers prognostic information and is essential for therapy planning [153]. The pressure measurements are performed in PA, PA wedge position, RV and RA, at the end of normal expiration [100]. CO is measured in triplets using thermodilution or the direct Fick method. Blood samples for oximetry from defined spots (inferior and superior vena cava and PA) are performed to detect shunts. Derived from these data, PVR, transpulmonary pressure gradient (TPG) and diastolic pressure gradient (DPG) are calculated [100,125]. PVR, TPG and DPG can be used to identify presence of pulmonary vasculopathy in PH-LHD patients [106,149]. To prevent false high values, patients underlying diseases should be mastered before RHC [106].

Additionally, pulmonary vasoreactivity testing is recommended for IPAH, heritable PAH and drug induced PH. It is performed to detect patients eligible for high-dose calcium channel blocker (CCB) treatment [60,125]. Inhaled NO is usually used for vasoreactivity

testing [98]. The testing is successful if mPAP decreases by more than 10 mmHg and absolute mPAP is under or equal 40 mmHg with stable or increased CO [8]. These patients are referred to as “responder” and have a much better prognosis than “non-responder” [194,214].

1.4 Therapy

The therapy aims to improve patient’s quality of life, prognosis and time to clinical worsening [153]. Risk stratification is performed to steer treatment and verify treatment success. Depending on patient’s baseline following goals should be achieved by therapy: WHO FC I or II, near-normal RV size and function, normalization of hemodynamic parameters (RAP <8mmHg and CI >2,5 to 3,0 l/min/m²), 6MWD of >380 to 440 m, normal BNP levels and near-normal exercise testing results [60,137].

The current drug treatment is only approved for PAH patients in Germany, with two exceptions being approved for CTEPH [59]. CTEPH is the only form of PH that can potentially be cured using pulmonary endarterectomy (PEA) [237]. Although the prognosis of the PH improved with the developed drugs, there is still no curative medical treatment [60,136]. In all secondary forms of PH, therapy of the underlying disease should be in focus, as they are not eligible for PH specific therapy [101,198].

1.4.1 Supportive therapy

Oral anticoagulants: Abnormalities in coagulation are described in some PH groups [61]. Post-mortem examination of IPAH patients showed high prevalence of vascular thrombotic lesions [26]. Evidence for oral anticoagulation is only confined to IPAH, heritable PAH, PAH due to anorexigens and CTEPH. Anticoagulation can be reasonable depending on patient’s comorbidities or therapy associated (i.e. catheter associated thrombosis) [71].

Diuretics: PH eventually leads to decompensated right heart failure with fluid retention [57]. Diuretics are recommended to control fluid balance, if necessary [71].

Oxygen: Substitution of O_2 is not generally indicated, as oxygen saturation is typically at normal level in PH patients. In case arterial oxygen pressure is consistently below 60 mmHg (arterial O_2 saturation < 91%), the patients should achieve O_2 therapy, in analogue to the recommendations from COPD [60,71].

1.4.2 Specific therapy

The specific therapy includes different drug treatments, PEA for CTEPH patients and as ultima ratio lung transplantation [51,59,98]. The currently approved drugs for PAH treatment are comprehensively reviewed by Galiè et al. and are just briefly described [60].

CCB: As mentioned before, “responder” benefit from long term therapy with CCBs and have a better outcome than “non-responder”. By inhibiting calcium channels, PA vasotonus is decreasing, leading to reduced PVR. But less than 10% of PAH patients are suitable for CCB therapy [214].
Endothelin pathway: Endothelin levels are elevated in PAH patients [67]. Endothelin exerts via binding to endothelin receptors (type A, B), forcing vasoconstriction and proliferation [210]. Endothelin receptor antagonists (ERA) promote vasodilatation and have antiproliferative effects. (ERA: Ambrisentan, Bosentan, Macitentan)
NO pathway: NO is released by endothelial cells. The endothelial NO synthase (eNOS) is induced by shear stress. NO activates soluble guanylate cyclase (sGC), which in turn produces cyclic guanosine monophosphate (cGMP) and mediates vasodilatation. Decomposition of cGMP is proceeded by phosphodiesterase-5 (PDE-5) [142]. NO/cGMP pathway is disturbed in PH [66,131,241]. Some available drugs either induce the pathway by stimulating sGC or inhibition of PDE-5. Additionally, PDE-5 inhibitors have antiproliferative effects. (PDE-5 inhibitors: Sildenafil, Tadalafil. sGC stimulator: Riociguat).
Prostacyclin pathway: Prostacyclin mediates vasodilatation, inhibits platelet aggregation and has antiproliferative effects [114,141]. In PH prostacyclin synthesis is reduced [33]. As PAH approved drugs, prostacyclin receptor agonists and prostacyclin analogues (PRA) are in use (Analogues: Epoprostenol, Iloprost, Treprostinil, PRA: Selexipag).

A combination therapy of the mentioned drugs can be performed depending on severity of the disease or missing therapy success [60].

Pulmonary endarterectomy: PEA is the treatment of choice for CTEPH, if patients are eligible for operation [101,121,237]. In case PEA is not successful or possible, CTEPH patients can benefit from PAH medication. Riociguat and Treprostinil are the only approved drugs for CTEPH in Germany [122].

1.5 Biomarker

The „Biomarker Definitions Working Group“ from the National Institute of Health defined a biomarker in 2001 as “*a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention*“ [16].

This definition implies different features of a biomarker. It might be used as a tool for diagnosis, classification, prognostic information, risk stratification or progression of a disease. On the other hand, the nature of a biomarker is not limited by this definition. Meaning a biomarker can originate from laboratory values derived from body liquids (urine, blood, ascites, liquor), functional data (6MWD, NYHA class) or hemodynamic measures (mPAP, PVR, CI) [68,91].

For PH patients, a diagnostic biomarker would be a big relief, as diagnosis of PH requires invasive diagnostics, which is emphasizing the benefit of a disease indicating biomarker [15,91]. On the other hand, survival or mortality are ideal clinical endpoints for treatment success but are challenging to assess in rare diseases like PH and ethically questionable [91]. Therefore, a surrogate biomarker is desirable. A surrogate biomarker is defined as “*a biomarker that is intended to substitute for a clinical endpoint*” [16]. Accordingly, PH biomarkers should be eligible as diagnostic biomarker and surrogate parameter for treatment success, prognosis, progression of the disease and identify pulmonary vascular remodeling [16,97,129]. Different pathways have been covered during the search for a PH biomarker, including endothelial dysfunction, in situ thrombosis, oxidative stress, end-organ failure, cardiac function or metabolism [3,208].

The already investigated pathways and biomarkers are extensively discussed by Anwar, Ruffenbach et al. [3] and Pezzuto et al. [173]. Some of the investigated biomarkers showed significant correlation with hemodynamic data or prognosis. Still, there is no existing biomarker or surrogate biomarker for PH with the previously defined requirements [91].

Currently, the only frequently used biomarker in PH is brain-natriuretic peptide (BNP) or its inactive signal peptide N-terminal pro-brain-natriuretic peptide (NT-proBNP) [125]. Because of the relevance in PH, the peptides will be described in more detail.

1.5.1 BNP and NT-proBNP

In clinical daily routine NT-proBNP and BNP are commonly used at PH patients. The evaluation of BNP in PH is even addressed in the European guidelines [106]. Human BNP is a polypeptide acting as a hormone, regulating cardiovascular homeostasis. BNP is dominantly secreted from heart ventricles. It gets synthesized as a preprohormone of 134 amino acids. ProBNP (108 amino acids) is generated by cleaving the C-terminal signal sequence. When secreted, the initially synthesized precursor proBNP gets cleaved in the biological active 32 amino acid long BNP and the N-terminal rest called NT-proBNP, which has 76 residue amino acids. Secretion stimulus is a volume overload of the myocytes resulting in stretch strain [15,23,118]. After secretion into the circulation, BNP binds to membrane bound guanylate cyclase inducing an increase of intracellular cGMP concentration, mediating vasodilatation and diuretic effects [83]. BNP and NT-proBNP are approved prognostic markers for heart failure, correlating with severity of the disease [156,188]. In PAH circulating BNP levels correlate with mPAP and PVR values, if the pressure is high enough to cause right heart strain, which occurs in most of the patients with progression of the disease [15,18,150]. High NT-proBNP values identifies the presence of PH with a sensitivity and specificity of about 90% [15]. High BNP or NT-proBNP levels are associated with higher mortality and predicts a poor prognosis [91,150]. Therefore, BNP is part of the recommended risk stratification. Normal natriuretic peptide concentrations are one of the goals to achieve in PH treatment [98,129]. However, BNP only indicates myocardial stress, no matter if left or right. It does not reflect pulmonary vascular disease itself and is therefore not seen as specific PH biomarker [91].

1.6 Research questions

Up to date nothing is published specifically about the role of MCT1 or 4 in PH, although altered metabolism with increased lactate concentrations in pulmonary vascular cells is well known. On the other hand, there is good scientific evidence about the importance of MCT1 and 4 in different cancer entities. As PH is a cancer like disease, we hypothesize that lactate transporters play an important role in PH. Objective of this study is to investigate the role of MCT1 and 4 in pulmonary hypertension with the following approaches:

- Examine the expression of MCT1 and 4 in the lungs of IPAH patients compared to healthy donor lungs.
- Investigate the expression profile of MCT1 and 4 in human pulmonary arterial smooth muscle cells after hypoxic and non-hypoxic stimulation, such as PDGF, TNFα or TGFβ.
- Evaluate the effect of MCT1 and 4 inhibition or silencing on functional characteristics of human pulmonary arterial smooth muscle cells.
- Examine the potential of MCT1 and 4 as a biomarker for pulmonary hypertension.

2 Materials and Methods

2.1 Cell culture

The *in vitro* experiments were performed using human pulmonary arterial smooth muscle cells (hPASMC) purchased from Lonza (PASMC-Pulmon.Art.SM Cells, SmGM-2, cryo amp, cat. no. CC-2581). Until usage the cells were stored in liquid nitrogen. For the experiments, the cells have been unfrozen, dissolved in 12 ml of growth medium and seeded in 60 mm plates in 3 ml per plate. The growth medium (Lonza, SmGM-2 Smooth Muscle Growth Medium-2, cat. no. CC-3182) consists of an addition of growth factors. In specific, recombinant human fibroblast growth factor-B (rhFGF-B), recombinant human epidermal growth factor (rhEGF), as well as insulin and antibiotic mixture of gentamicin and amphotericin. The cells were cultured at 37°C and 5% CO_2. The medium was changed the following day and from then on, every second day. Cell growth was tracked using microscope until cells covered approximately 80% of the plate in a monolayer. Usually, this was achieved after 6 to 7 days. All the *in vitro* experiments were performed in passage 7.

2.1.1 Cell splitting

For cell splitting the medium was removed and the cells were washed one time with 3 ml of prewarmed phosphate buffered saline solution (PBS, Pan Biotech, DPBS, cat. no. P04-36500). Then 1,5 ml of warm trypsin (dissolved to 1x in PBS) was added to the cells and incubated for 1 min at 37°C. Following the trypsinization, 1,5 ml of growth medium was added and the detached cells were transferred into a 50 ml falcon. To ensure complete harvest of the cells, additional 3 ml of PBS was rinsed over the plate and transferred to the falcon as well. The falcons were centrifuged at 1200 rpm for 5 min at 20°C to collect the cells in a pellet. The supernatant was removed and the cell pellet was dissolved in 1 ml of growth medium. Using counting chambers, total number of cells was determined. For the calculation, the medium of 2 counts have been used. The appropriate volume of cells for the specific experimental design was transferred to the appropriate plates.

2.1.2 Hypoxic and non-hypoxic stimulation

To generate hypoxic stimulation, the cells were grown for 3 days in hypoxic conditions at 1% O_2 at 37°C. An appropriate control in normoxic conditions was used. As

non-hypoxic stimulus PDGF, TNFα and TGFβ treatment was used. Depending on the experimental setup, cells were either exposed to PDGF (R&D Systems, Recombinant Human PDGF-BB Protein, cat. no. 220-BB-010) at a concentration of 40 ng/ml, TNFα (R&D Systems, Recombinant Human TNF-alpha Protein, cat. no. 210-TA-005/CF) at a concentration of 20 ng/ml or TGFβ (R&D Systems, Recombinant Human TGF-beta 1 Protein, cat. no. 7754-BH-005/CF) at a concentration of 10 ng/ml for 2 days. Each of the stimulators was dissolved in basal medium (Lonza, SmBM Smooth Muscle Cell Growth Basal Medium, cat. no. CC-3181). Control hPASMCs were incubated without any of the mentioned stimulators in basal medium for corresponding period of time. Cells were grown in normoxic conditions at 37°C.

2.1.3 Cell lysis and preparation of western blot samples

To create western blot samples, 40.000 cells were seeded in 2 ml per well in 6 well plates. Following the experimental conditions, the plates were placed on ice immediately. The medium was removed and the cells were washed one time with 2 ml of ice-cold PBS. Then 150 µl of lysis buffer was added to each well. The lysis buffer consists of Radioimmunoprecipitation assay (RIPA) buffer, 1% v/v Vanadat, 1% v/v Phenylmethylsulfonylfluorid (PMSF) and 2% v/v protease inhibitor. After mechanical scratching of the cells, the mixture was added to a new 1,5 ml tube and placed on ice. Homogenization was performed by aspirating the cells using 1 ml syringe and 20G needle. The homogenized mixture was centrifuged at full speed for 15 min at 4°C. The supernatant was transferred into a new tube and the protein amount was determined using Biorad DC protein assay (cat. no. 5000111). Depending on to the protein amount, the cell samples were diluted in lysis buffer, laemmli sample buffer (1:5, Biorad, cat. no. 1610747) and ß-mercaptoethanol (1:20, Roth, cat. no. 4227.3). The final amount of protein used for western blot samples was between 12 and 20 µg per well. Eventually, the samples were boiled at 99°C for 10 min and a spin-down centrifugation was performed.

2.1.4 siRNA transfection

HPASMCs were transfected with siRNA to knockdown MCT1 and 4 expression. After 1 day of recovery from splitting, the cells were starved for 16 h in basal medium, containing 1% penicillin and streptomycin. After 16 h starvation, the basal medium was removed and replaced by basal medium without antibiotics. Transfection was performed using lipofectamine 2000 (invitrogen, cat. no. 11668-019), diluted in Opti-MEM (gibco,

cat. no. 31985-047) to 0,5% v/v. siRNA against MCT1 and 4 and scramble siRNA (scRNA) were diluted in Opti-MEM as well and a final concentration of 100 nM was created. scRNA (cat. no. D-001810-01-20) and siRNA (MCT1 cat. no. L-007402-00-0005, MCT4 cat. no. L-005126-02-0020) were purchased from dharmacon. The mixture of lipofectamine, siRNA and Opti-MEM was added to the basal medium in each well and incubated for 8 h at 37°C. Upon the incubation the mixture was replaced with growth medium and the experimental conditions were started. scRNA was used as a control at the same concentrations as the siRNA. Successful knockdown of MCT1 and 4 was confirmed using western blot analysis.

2.1.5 Pharmaceutical treatments

AZD3965 (selleckchem, cat. no. 1448671-31-5) and syrosingopine (sigma aldrich, cat. no. SML1908-25MG) were used to inhibit MCT1 and 4 pharmacologically. The inhibitors were dissolved in Dimethylsulfoxid (DMSO, sigma aldrich, cat. no. D4540-100-1) to create a stock solution of 50 mM for both AZD3965 and syrosingopine. Aliquots of the stock solutions were stored at -80°C. The same amount of DMSO as the highest used concentration of AZD3965 or syrosingopine was added to the cell culture medium and represented the treatment control.

2.1.6 BrdU assay

Bromdesoxyuridin (BrdU) incorporation assay was used to determine the proliferative capacity of the cells. BrdU is thymidine analogue and gets integrated into the DNA during cell replication. The amount of integrated BrdU reflects the proliferative capacity of the cells. BrdU assay was purchased from roche (Cell Proliferation ELISA, cat. no. 11647229001). For the BrdU assay, 5000 cells were seeded in a 24 well plate and following 2 days of recovery, the cells went through starvation period of 24 hours prior the addition of appropriate compound or DMSO control, dissolved in cell culture medium. Chronic hypoxic incubation for 3 days or PDGF stimulation for 2 days were used to induce a hyperproliferative phenotype of the cells (see 2.1.2). 18 h prior to the assay read-out, BrdU labelling reagent was added to cell culture medium. 50 µl of 20x BrdU labelling reagent was added to each well for a total volume of 1 ml. After the incubation time the cell medium was removed by inverting and tapping on paper towels and 450 µl of fixation solution was added into each well and incubated for 30 min at room temperature. Next, fixation solution was replaced with 450 µl of anti-BrdU diluted in

antibody dilution solution and incubated for 1,5 h at room temperature. Finally, the plates were washed 3 times with 450 µl washing buffer and 450 µl of substrate solution was added to each well. Optical density at 370 nm with a reference wavelength of 492 nm was determined after 5, 10, 15 and 20 min using tecan infinite M2000 plate reader. The background control was subtracted from the values to generate the final relative BrdU incorporation.

2.1.7 Cell viability assay

The amount of viable cells was determined using alamarBlue Cell Viability reagent purchased from invitrogen (Cat no. DAL1100). The assay is based on a non-fluorescent resazurin solution. Resazurin is a non-toxic and cell permeable substrate. When entering the cell plasma, it is reduced via reducing enzymes in living cells to resorufin. Resorufin is highly fluorescent. The fluorescent intensity in turn is used to quantitively measure cell viability. In the absence of reducing enzymes from living cells, no fluorescent resorufin is generated. Otherwise, the generated fluorescent signal is proportional to the number of viable cells.

For the assay 10.000 hPASMCs were seeded in growth medium into a black-walled 96 well plate. After 1 day of recovery the experimental conditions were started for 24 h. Untreated control, background control and positive control were included. As positive control staurosporine (SSP, cayman, cat. no. 84590) was added at a final concentration of 1 µM 3 h prior to the assay quantification. $1/10^{th}$ volume of alamarBlue cell viability reagent was added to each well, followed by an incubation of 4 h at 37°C in darkness. The fluorescent signal was detected using tecan infinite M2000 plate reader. Excitation wavelength was set to 550 nm and emission wavelength to 600 nm. Multiple reads per well were performed. Results were subtracted with fluorescence values of the background control.

2.1.8 Cell migration assay

Cell migration was quantified using Culture-inserts 2 well for self-insertion purchased from ibidi (cat. no. 80209). In this assay a physical gap is generated in between cell monolayers and the process of cell migration into this gap is monitored by live cell imaging. Live cell imaging was performed by IncuCyte ZOOM Live-Cell microscopy imaging system.

For the assay, 7500 hPASMCs were seeded in each of the 2 defined wells in a 96 well plate. After cell recovery and attachment for 24 hours, the culture-insert was removed and the physical gap was thereby created. After 1 time of washing with warm PBS, the respective experimental conditions were started by adding syrosingopine or DMSO to growth medium. As mentioned above, the cell migration was then monitored using IncuCyte ZOOM Live-Cell microscopy imaging system. An image was taken every 20 minutes for a period of 12 hours. The images were analysed by IncuCyte ZOOM software and the results shown as the percentage of gap coverage by migrated cells. A scheme of the assay principle is provided by ibidi company, see figure 5 [111].

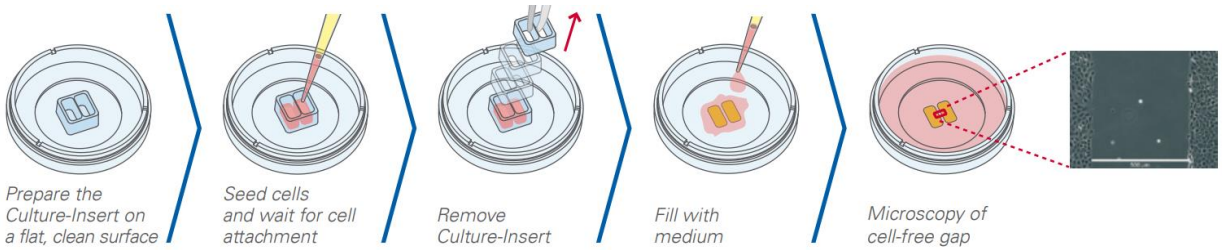

Figure 5 Scheme of cell migration assay, with courtesy of ibidi GmbH [111]

2.1.9 Apoptosis assay

Apoptosis of hPASMCs was assessed using Kinetic Apoptosis Kit purchased from abcam (cat. no. ab129817). The assay uses Annexin XII based probes, known as pSIVA (Polarity Sensitive Indicator of Viability and Apoptosis). The probe binds to phosphatidylserine when it gets exposed to the outer cell membrane, a phenomenon occurring in apoptotic cells. To visualize the binding, the probe is conjugated to a fluorescent dye (IANBD) that fluoresces only when bound to the cell membrane. For the assay, 7500 hPASMCs were seeded in a black-walled 96 well plate. After cell recovery for 24 hours in 37°C, the cells were exposed to syrosingopine or DMSO control together with pSIVA-IANBD probe. The cells were exposed for 12 hours in hypoxic conditions (1% O_2, 5% CO_2, 37°C). The generated fluorescent signal is proportional to cell apoptosis. Fluorescence was detected using IncuCyte ZOOM Live-Cell imaging system. The images were analyzed by IncuCyte ZOOM software and the results are presented as fluorescent-green positive cells normalized to cell surface confluence.

2.1.10 Extracellular lactate measurements

Extracellular lactate measurements were performed using Lactate-Glo Assay purchased from Promega (cat. no. J5021). This assay uses enzymatical coupling of lactate dehydrogenase (LDH) and a reductase to produce luciferin. A simplified scheme of the underlying reactions is shown in figure 6 [38]. The luciferin signal is detected by measuring luminescence.

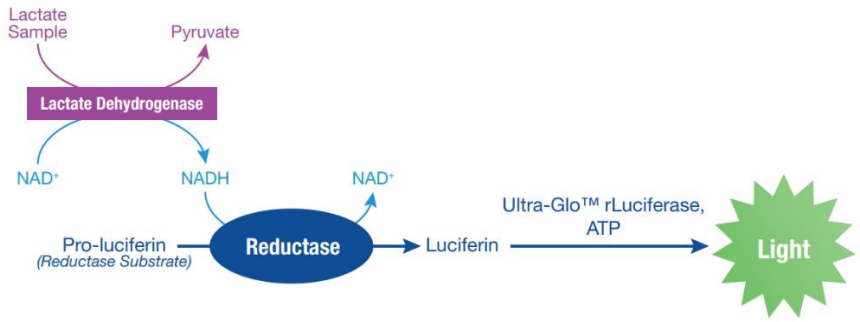

Figure 6 Schematic diagram of the Lactate-Glo assay principle, with courtesy of Promega Corp. [38]

7500 hPASMCs were seeded in 96 well plate in growth medium. The cells were exposed to the experimental conditions for 2 days in normoxic or hypoxic conditions (1% O_2) at 37°C. To measure extracellular lactate concentration, the cell culture medium was diluted 100 times in PBS. 30 µl of the diluted medium was added into a new 96 well plate together with 30 µl of lactate detection reagent and mixed by shaking for 60 sec. The plate was incubated for 60 min and luminescence was recorded using tecan infinite M2000 plate reader. Lactate standards at given concentrations, diluted in PBS, were added. Every well was measured in doublets. Lactate concentration of the samples were calculated using the inverse formula of plotted luminescence signal of the known lactate standards in a linear function.

2.2 Lung tissue samples

Lung tissue samples have been provided by the Giessen Biobank. The Ethics Committee of the Justus-Liebig-University School of Medicine approved all the studies involving human lung samples (AZ 58/15). In total, 9 samples of IPAH patient lungs and 8 of donors lungs were available to investigate the expression profile of MCT1 and MCT4. No further clinical data about the patients were available.

2.2.1 Tissue homogenization

Lung tissue samples were stored in liquid nitrogen. For the homogenization, a maximum of 30 mg of sample was used. If necessary, the lung tissue was chopped using mortar and pistil. The tissue was placed in 2ml tubes with 10 – 15 homogenization beads and 200 µl lysis buffer. The lysis buffer consists of 20% RIPA buffer, 80% distilled aqua and 0,1% PMSF. The tubes were placed in precellys tissue homogenizer. After homogenization, the samples were placed on ice for 10 min and then centrifuged at 4°C for 10 min at full speed. The supernatant was transferred into a new tube. Polymerize chain reaction (PCR) or western blot samples of the lung tissue homogenates were made as described in the according sections.

2.3 Reverse-transcription quantitative PCR

For preparation of cell culture samples, the cell culture plates were placed on ice and washed one time with 2 ml of ice-cold PBS. The cells were then detached by adding 300 µl lysis buffer and mechanical scratching. The lysis buffer derived from purchased RNA purification kit (Qiagen, RNeasy Mini Kit, cat. no. 74104). Cell lysates were homogenized using syringe and needle. Tissue samples were disrupted and homogenized with the supplied lysis buffer and rotor-stator homogenizer (precellys). The RNA was isolated according to the manufacturers protocol. The amount of RNA per sample was measured using nanodrop ND-1000 and all samples were diluted using RNAse free H_2O. In the next step the isolated RNA was reverse transcribed into cDNA using iScript cDNA synthesis kit (Biorad, cat. no. 170-8890). For the final PCR reaction 1 µl of cDNA sample was added to 9 µl master mix. The master mix consists of iTaq universal SYBR Green Supermix (Biorad Laboratories Inc., cat. no. 172-5124), RNAse free water and forward and reverse primer. Real-time PCR quantification was performed by using Biorad CFX connect. Cycling conditions were as follows: 95°C for 10 min, 40 cycles of 95°C for 10 sec, 59°C for 20 sec, 72°C for 10 sec. To confirm specific amplification of the target sequence melting curve analysis was performed, in addition to gel electrophoresis. Each sample was measured in doublets and 2 negative controls for each primer were included. Quantification cycle (Cq) values were determined and cycle of threshold (Ct) levels were set at the same level for gene of interest and reference gene at the exponential phase of the qPCR. Relative quantitative evaluation was performed using ΔCt and $2^{-\Delta\Delta Ct}$ to compare the expression levels.

Primers were designed using Primer-BLAST (NCBI) and ordered from metabion international AG. Following primer sequences were used:

Table 6 Primer sequences used for qPCR

Target		Sequence
MCT1	Forward	3’ TCT TTG CGG CTT CCG TTG TT 5’
	Reverse	3’ AAT ACG GAG CTG AGC CAC CC 5’
MCT4	Forward	3’ ACG AAG CGG AGG TCT GAA GG 5’
	Reverse	3’ ATG ACG AAA CAG CCG AAG AG 5’
PBGD	Forward	3’ CCC ACG CGA ATC CTC AT 5’
	Reverse	3’ TGT CTG GTA ACG GCA ATG CG 5’
β-actin	Forward	3’ CTG GGA CGA CAT GGA GAA AA 5’
	Reverse	3’ AGG GAT AGC ACA GCC TGG AT 5’

Porphobilinogen deaminase (PBGD) was used as reference gene for IPAH / donor lung tissue samples and β-actin for cell culture samples.

2.4 PCR amplicon gel electrophoresis

PCR amplicons and negative controls were run on gel electrophoresis to confirm the specificity of the amplification. The amplicons were separated using a 1,5% agarose gel, which consists of 1,5% m/v of agarose diluted in TRIS-Acetate-EDTA (TAE) buffer and 1:25.000 SYBR safe DNA gel stain (Invitrogen, Cat.no. S33102). 5 µl of sample or negative control were mixed with 1 µl of 6x DNA loading dye (thermofisher, cat. no. R0611) and 5 µl of the mixture was added to the gel, as well as 3 µl of a 100 bp gene ruler (thermofisher, cat. no. SM0241). Electrophoresis was performed with 120V, 150mA and 100W for 30min. Finally, the nucleotide signal was visualized using Biorad chemidoc XRS+.

2.5 Western Blot

Protein expression was investigated using Western Blot from lung tissue samples and hPASMCs. The preparation of western blot samples is described in section 2.1.3. The protein mixture was separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). 12% gels were self-made using 3,4 ml distilled H_2O, 4 ml 30% Acrylamide (Roth, cat. no. 3029.1), 2,5 ml of 1,5 mol Tris Buffer and 0,1 ml 10%

SDS (invitrogen, cat. no. 24730-020). 25 µl of sample and 3 µl of protein size marker (Biorad, precision plus protein dual color standard, cat. no. 161-0374) were loaded per well. Electrophoresis settings were as follows: 120 V, 400 mA, 150 W for approximately 1 h and 50 min. After electrophoresis the proteins were blotted on a polyvinylidene difluoride (PVDF) membrane, which was activated for a few seconds in methanol, using following settings: 100 V, 115 mA, 150 W for 1 h and 15 min. Unspecific bindings were blocked by incubating the membrane at room temperature for 1 h in 6% m/v milk buffer. Primary antibodies were incubated overnight at 4°C on a rolling shaker. After 4 times washing for 10min each, the membranes were incubated with secondary antibody for 1h at room temperature on a rolling shaker. As secondary antibodies horseradish peroxidase conjugated anti-rabbit or anti-mouse antibodies were used. After another 4 times of washing the bands were visualized using Amersham ECL Prime Western Blotting Detection Reagent (gehealthcare cat. no. RPN2232) and developed on Biorad chemidoc touch. Following antibodies have been used: anti-MCT1 (dilution 1:500 in milk buffer, rabbit, Merck Millipore, cat. no. AB3538P), anti-MCT4 (dilution 1:1000 in milk buffer, rabbit, proteintech cat. no. 22787-1-AP), anti-HIF-1α (dilution 1:1000 in milk buffer, rabbit, cayman, cat. no. 10006421), anti-β-actin (dilution 1:50000 in milk buffer, mouse, abcam, cat. no. ab8226), anti-mouse IgG (goat, Promega, cat. no. W4011) and anti-rabbit IgG (goat, Promega, cat. no. W4021).

β-actin was used as loading control. Incubation of β-actin was performed for 30 min at room temperature on a rolling shaker. In this case, secondary antibody incubation was performed for 30 min at room temperature.

The band intensity was quantified using Image Lab software (Biorad).

2.6 Biomarker studies

2.6.1 Collection of blood samples

Positive ethics committee vote was granted from Justus-Liebig-University Giessen for blood sample collection and further processing (see 12.1). The patients gave their written informed consent to use their clinical samples and data for scientific research (see 12.3). Blood samples were exclusively collected during RHC examination at pulmological ambulance of university hospital Giessen. With the written permission of the patients, central venous blood was drawn from inserted Swan-Ganz catheter, 2 EDTA tubes per

patient. Right after blood draw, the samples were centrifuged at 3000 rpm for 10 min and supernatant plasma was transferred in 1,5 ml Eppendorf-tubes. Until usage, the tubes were stored at -80°C. Collected data of RHC examination and further clinical data like VQ scan was used to assign samples to non-PH, IPAH and CTEPH group. Classification of the patients was performed by physicians from pulmological ambulance. The plasma samples were used to determine circulating MCT1 and 4 levels, using Enzyme-linked Immunosorbent Assay (ELISA). The investigated blood samples were collected from 23.04.2008 to 26.10.2017. Blood samples of patients were included, if PH was not diagnosed before to make sure patients did not receive any PH specific drugs, to avoid any interference with MCT1 or 4 levels. Besides hemodynamic data derived from RHC, additional data like WHO-FC, 6MWD and BNP was collected, if possible, from the same hospital visit, as well as survival data. The clinical data derived from Giessen PH database.

2.6.2 Enzyme-linked Immunosorbent Assay

ELISA was used to determine plasma concentration of MCT1 and 4. For this purpose ELISA kits were purchased from mybiosource (MCT1 cat. no. MBS9392446, MCT4 cat. no. MBS9335997). Undiluted samples were transferred to provided 96 wells and each sample was measured in doublets. Negative controls were included. Assigned kits are one step kits, which means samples or standards (50 µl) and HRP conjugated antibody (100 µl) are transferred to the wells in the same step. After 1 h of incubation at 37°C chromogen solution (50 µl) was added and another 15 min incubated at 37°C, before the chromogen reaction was stopped by adding stop solution (50 µl). Optical density was measured at 450 nm with tecan infinite M2000 plate reader. The detection range of MCT1 was 0,625 ng/ml – 20 ng/ml (Standards: 0,625, 1,25, 2,5, 5, 10, 20 all ng/ml) and of MCT4 1,56 ng/ml – 50 ng/ml (Standards: 1,56, 3,12, 6,25, 12,5, 25, 50 all ng/ml). Sensitivity of both kits is given with 0,1 ng/ml. To determine the concentrations of the samples, concentration and optical density of the standards were plotted on a 4-parameter logistic curve and the corresponding regression graph was created. Using inverse formula final concentrations were calculated. Values that were out of detection range were excluded.

$$\text{Inverse Formula: } x = c\left(\frac{a-d}{y-d} - 1\right)^{\frac{1}{b}}$$

2.7 Statistical analysis

Statistical analysis of the biomarker experiments was performed using SPSS version 27 (IBM, NY, USA). Descriptive statistics of nominally and ordinally scaled values are stated as absolute and relative frequencies. Metric variables were tested for normal distribution using Shapiro-Wilk test and descriptive statistics of normally distributed variables are presented as mean ± standard deviation (SD). Non-normally distributed variables are described with median and interquartile range [IQR]. The measured biomarkers showed a right skewed distribution. For further statistical analysis the natural logarithmic values were calculated to generate normally distributed variables ("logarithmic transformation"). Homogeneity of variance was controlled using Levene-test. Normally distributed variables with variance homogeneity were compared using independent t-test or ANOVA test followed by Turkey HSD post-hoc test, depending on the number of groups that have been compared. Welch's t-test was used to compare two groups with normally distributed variables but unequal variances. In all other cases the mean comparisons were performed using non-parametric tests. Mann-Whitney U test was used to compare 2 groups and Kruskal-Wallis test, followed by Dunn-Bonferroni post-hoc test, for larger group comparisons. Association of the normally distributed variables were investigated by calculating Pearson correlation, all other associations were investigated using Spearman correlation. Correlation coefficient r is calculated in each case, with r value from 0,1 to 0,3 indicating weak, from 0,3 to 0,5 medium and above 0,5 strong correlations. For survival analyses, the survival time is defined as time from date of RHC until date of event. All other patients were censored at the day of last contact. Patients that were lost to follow-up were censored at the day of last contact, as well. Survival time was visualized using Kaplan-Meier curves and the groups were compared by Log-Rank test. Whenever differences of the survival time were observed, multivariate Cox-regression was performed. For multivariate analysis the variables age, PVR and WHO-FC were investigated.

All other statistics, in particular the molecular biology part, was analyzed using GraphPad Prism version 7 (GraphPad Software, CA, USA). Mean comparisons were performed using independent t test or ANOVA, followed by Turkey HSD post-hoc test. In general, p values <0,05 were considered as significant results. In the generated charts significant results are indicated by stars (*). * = $p<0,05$, ** = $p<0,01$, *** = $p<0,001$, **** = $p<0,0001$.

3 Results

3.1 Expression profile of MCT1 and 4

3.1.1 In the lungs of IPAH patients

The expression profile of MCT1 and 4 was investigated in IPAH and donor lung homogenates.

The mRNA expression of MCT1 and MCT4 was significantly increased in IPAH lung homogenates compared to donor lung samples by independent t-test. MCT1 mRNA expression was increased by 2,69 (±0,26) fold and MCT4 by 3,05 (±0,44) fold. The data is shown in figure 7A using $2^{-\Delta\Delta Cq}$ values, together with gel electrophoresis control in figure 7B.

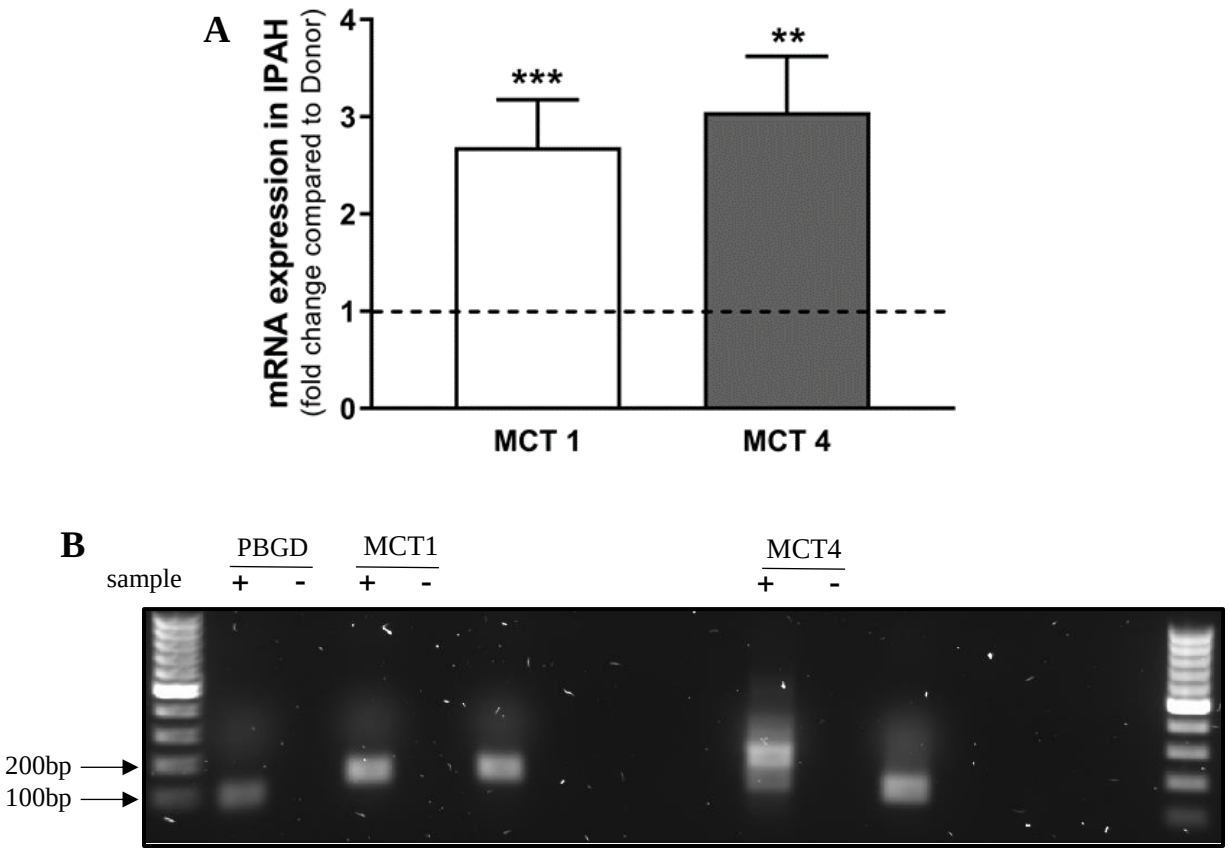

Figure 7 A mRNA expression of MCT1 and 4 in IPAH and donor lung homogenates; dotted line corresponds to donor expression, Donor n=8, IPAH n=9, independent t-test ** p<0,01, *** p<0,001
B Gel electrophoresis control of PCR amplicons; MCT1 127bp, MCT4 145bp

The protein expression of MCT1 and MCT4 was significantly increased in IPAH lung homogenates compared to donor lung homogenates by independent t-test. The respective blots and the quantification charts are shown in figure 8.

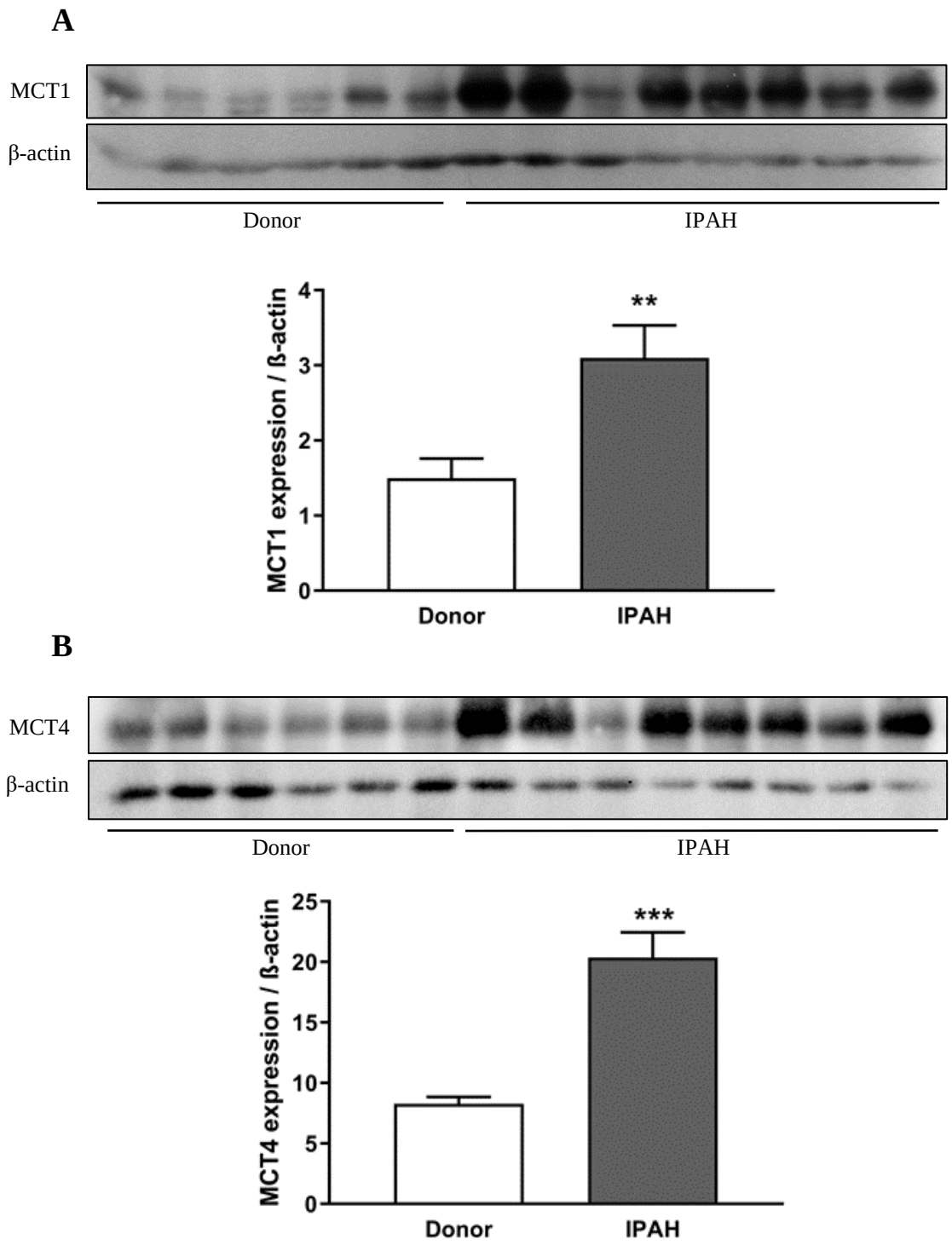

Figure 8 A Protein expression of MCT1 in IPAH and donor lung homogenates **B** Protein expression of MCT4 in IPAH and donor lung homogenates; Donor n=6, IPAH n=8, independent t-test, ** p<0,01, *** p<0,001

3.1.2 In hPASMCs after hypoxic and non-hypoxic stimulation

Besides lung tissue samples, the expression profile was investigated *in vitro*, focusing on hPASMCs. Therefore, hPASMCs isolated from healthy donors were exposed to hypoxic and non-hypoxic stimuli. After stimulation the mRNA and protein expression of MCT1 and 4 was determined.

3.1.2.1 Chronic hypoxic stimulation

After 3 days of chronic hypoxic incubation (Hox) MCT1 mRNA did not significantly change. Whereas a significant upregulation of MCT4 mRNA by 1,57 (±0,22) fold was observed when compared to appropriate normoxic (Nox) control by independent t-test. The mRNA expression is shown in figure 9.

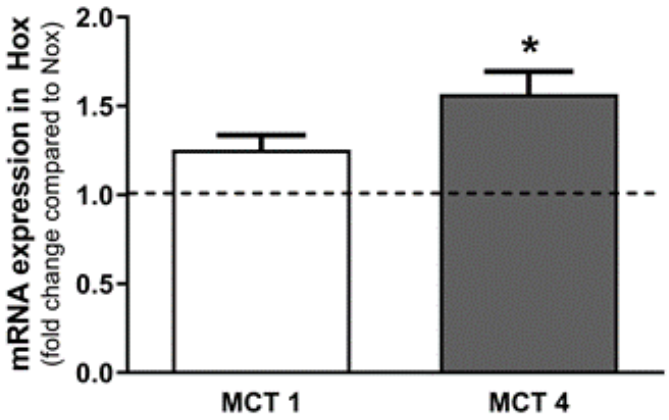

Figure 9 mRNA expression of MCT1 and 4 in hPASMCs after 3 days of chronic hypoxic stimulation; dotted line corresponds to Nox, each condition n=4, independent t-test, * $p<0,05$

On protein level, successful hypoxic stimulation was confirmed by significant HIF-1α upregulation in hypoxic conditions (figure 10A, D). MCT1 protein expression did not significantly change in hypoxic conditions compared to normoxic conditions (figure 10 A, B). MCT4 protein expression was significantly upregulated upon hypoxic stimulation for 3 days compared to normoxic control by independent t-test (figure 10 A, C).

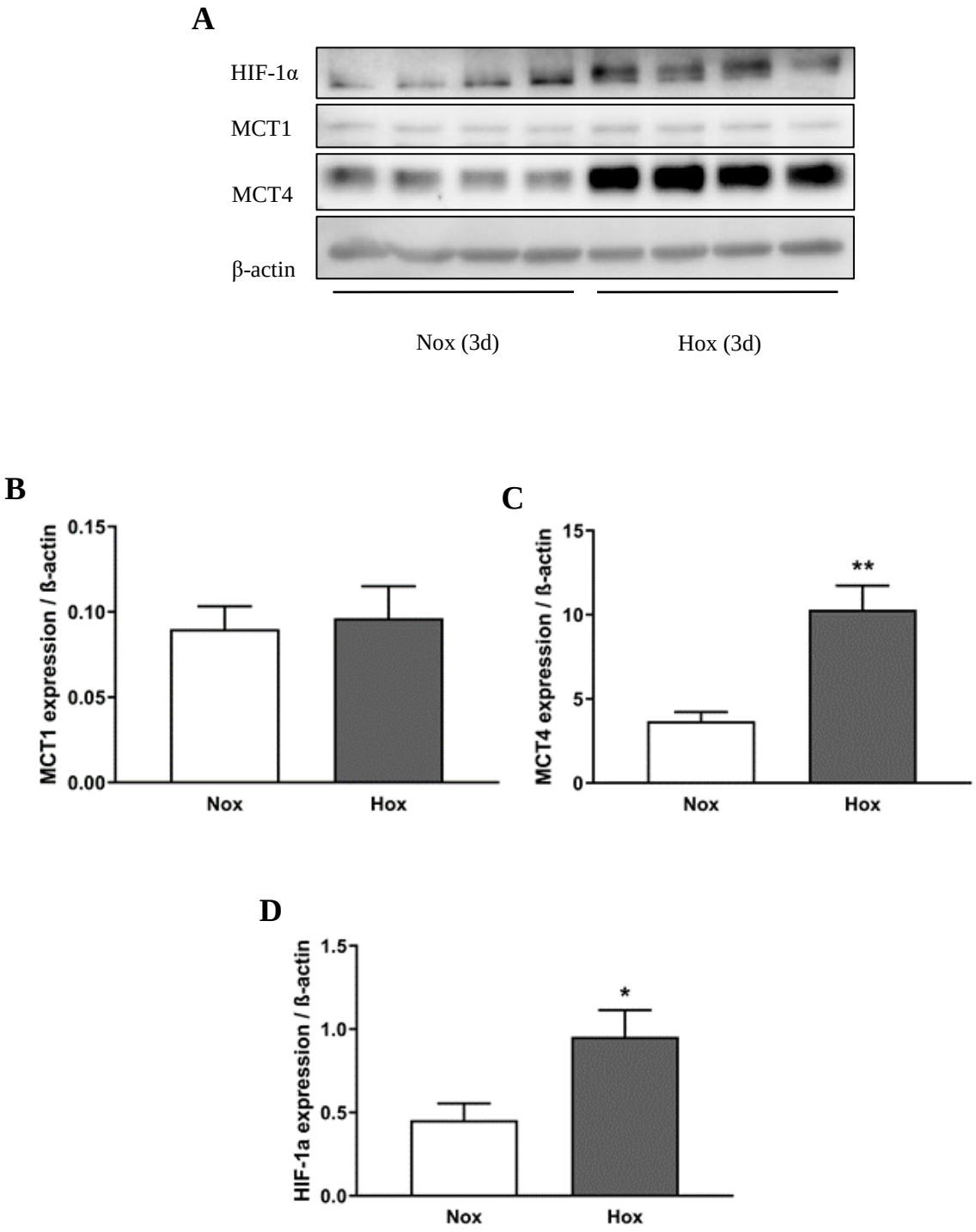

Figure 10 A Protein expression of MCT1, 4 and HIF-1α in hPASMCs after 3 days of chronic hypoxic stimulation **B** Quantification chart of MCT1 expression **C** Quantification chart of MCT4 expression **D** Quantification chart of HIF-1α expression; each condition n=4, independent t-test, * p<0,05, ** p<0,01

3.1.2.2 PDGF stimulation

HPASMCs were stimulated with PDGF for 2 days. Upon PDGF stimulation, MCT1 mRNA and protein expression did not significantly change in hPASMCs (figure 11, 12A). MCT4 mRNA was significantly downregulated by 0,66 (±0,08) fold when compared to basal medium treated cells by independent t-test (figure 11). In contrast, MCT4 protein expression was significantly upregulated compared to unstimulated control by independent t-test (figure 12B).

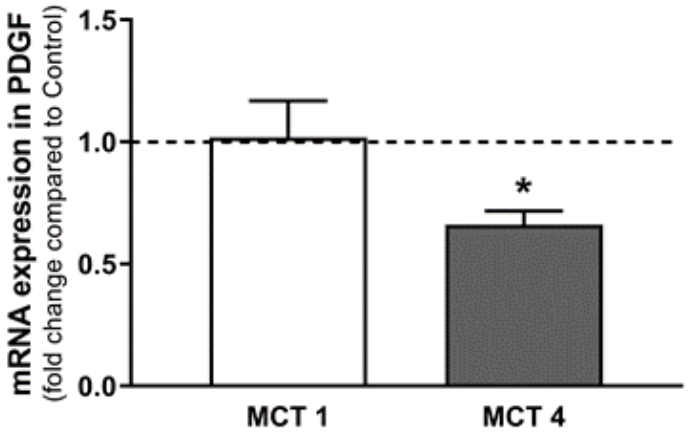

Figure 11 mRNA expression of MCT1 and 4 in hPASMCs upon PDGF treatment for 2 days; dotted line corresponds to basal medium control, each condition n=3, independent t-test, * $p<0,05$

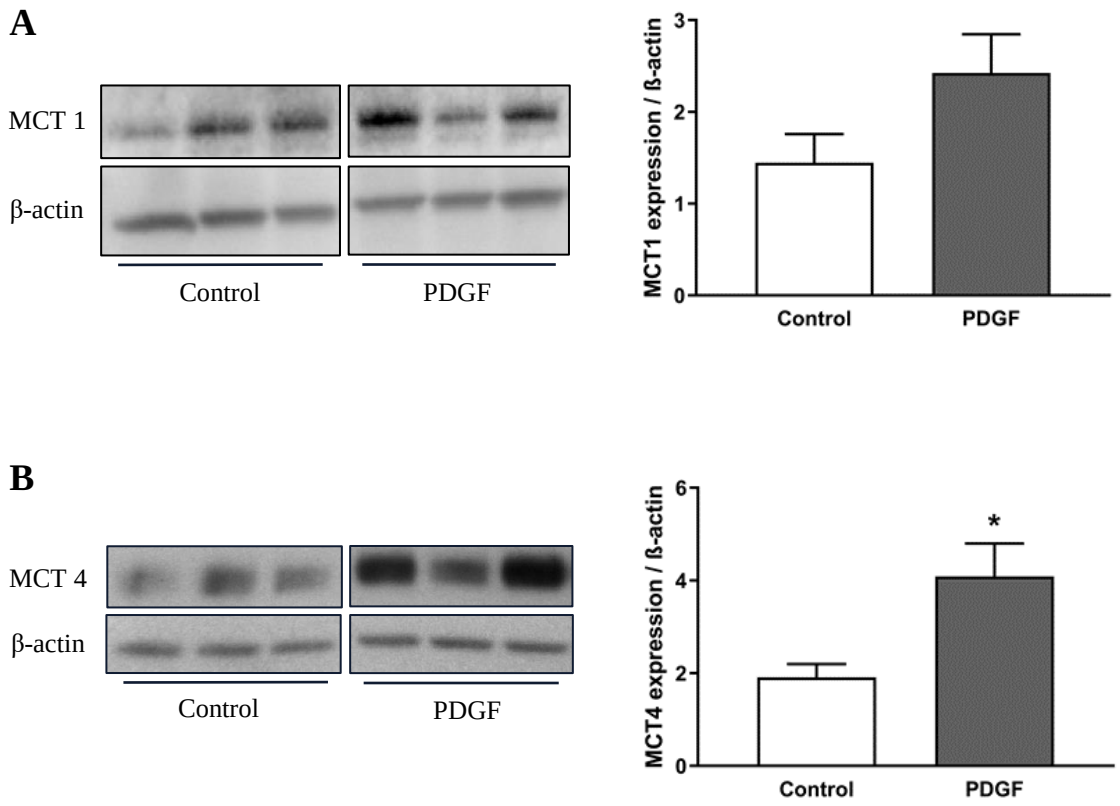

Figure 12 Protein expression of MCT1 and 4 in hPASMCs after 2 days of PDGF stimulation **A** Western Blot and quantification chart of MCT1 **B** Western Blot and quantification chart of MCT4; each condition n=3, independent t-test, * $p<0,05$

3.1.2.3 TNFα stimulation

MCT1 and 4 expression profile was not significantly influenced on protein and mRNA level by TNFα stimulation in hPASMCs for 2 days. The mRNA expression is shown in figure 13 and the protein expression in figure 14.

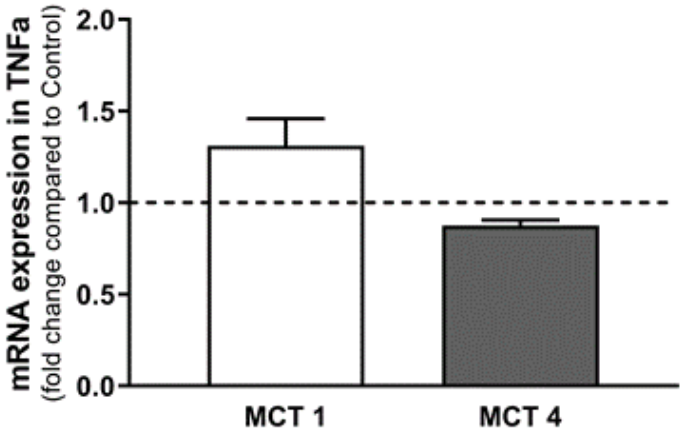

Figure 13 mRNA expression of MCT1 and 4 in hPASMCs upon TNFα treatment for 2 days; dotted line corresponds to basal medium control, each condition n=3

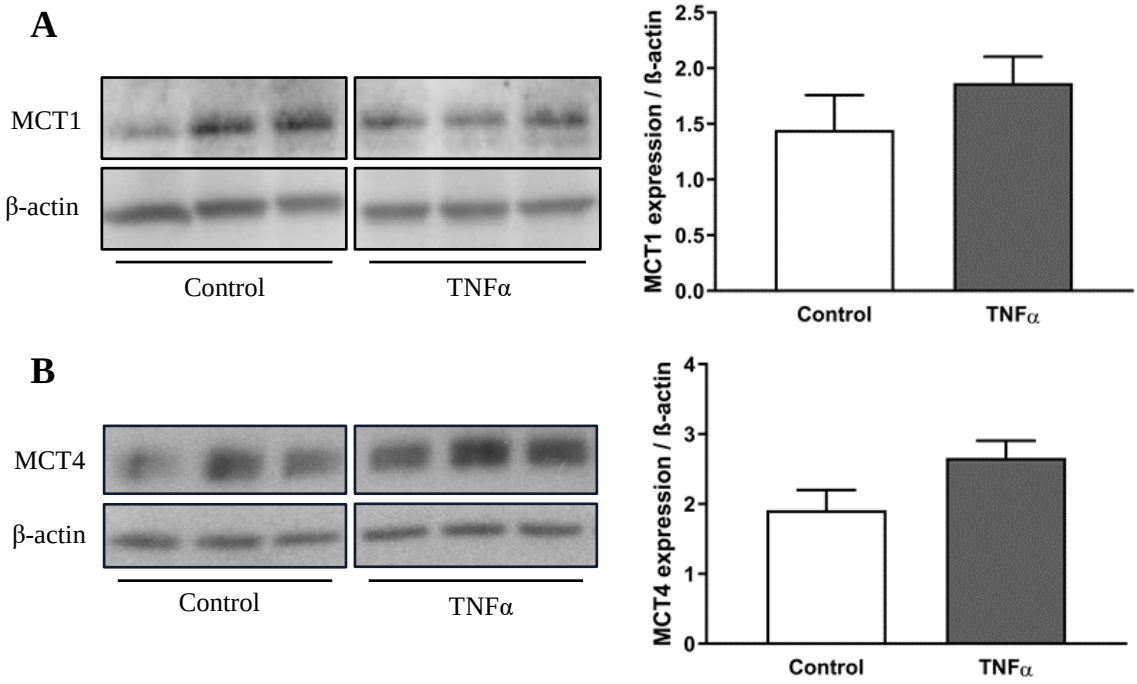

Figure 14 Protein expression of MCT1 and 4 in hPASMCs after 2 days of TNFα stimulation **A** Western Blot and quantification chart of MCT1 **B** Western Blot and quantification chart of MCT4; each condition n=3

3.1.2.4 TGFβ stimulation

After TGFβ stimulation for 2 days, MCT1 mRNA and protein levels did not significantly change in hPASMCs (figure 15, 16A). MCT4 mRNA and protein expression were significantly upregulated when compared to basal medium treated cells by independent t-test (figure 15, 16B). The mRNA expression was elevated by 1,62 (±0,19) fold.

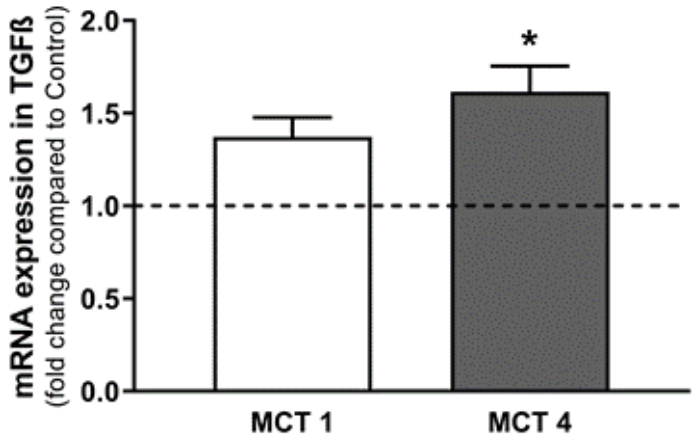

Figure 15 mRNA expression of MCT1 and 4 in hPASMCs upon TGFβ treatment for 2 days; dotted line corresponds to basal medium control, each condition n=3, independent t-test, * p<0,05

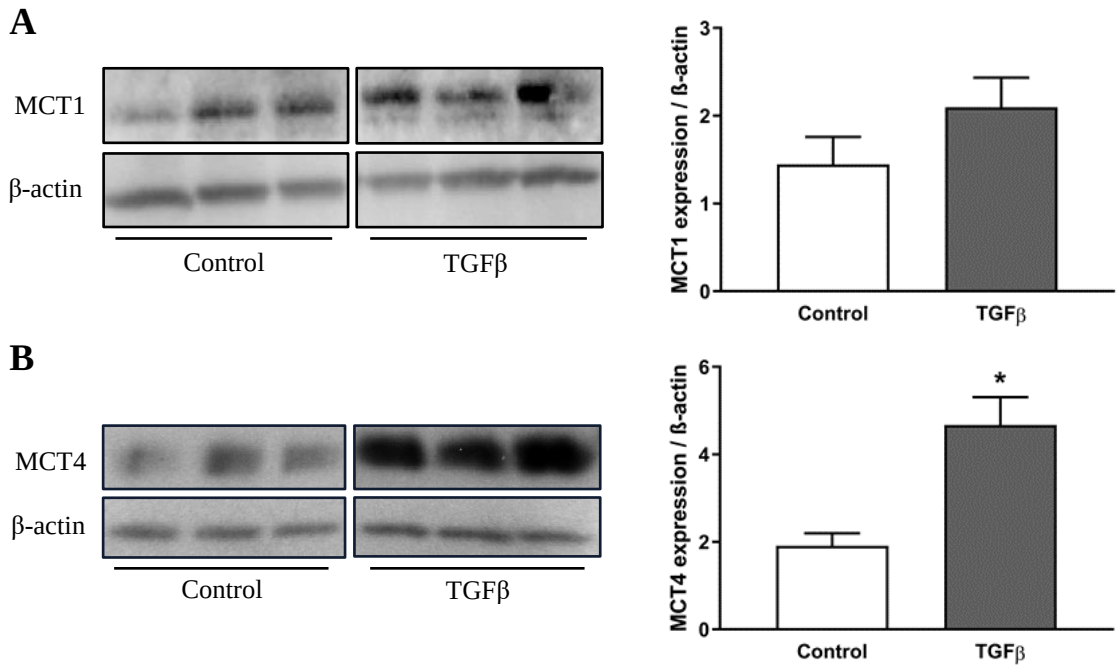

Figure 16 Protein expression of MCT1 and 4 in hPASMCs after 2 days of TGFβ stimulation **A** Western Blot and quantification chart of MCT1 **B** Western Blot and quantification chart of MCT4; each condition n=3, independent t-test, * p<0,05

3.2 Effect of MCT1 and 4 inhibition or silencing in hPASMCs

3.2.1 MCT1 and 4 silencing

The effect of MCT1 or 4 silencing on functional characteristics was investigated in hPASMCs. Silencing was achieved by siRNA transfection. The successful knockdown of MCT1 or 4 was controlled by immunoblot, which is shown in figure 17.

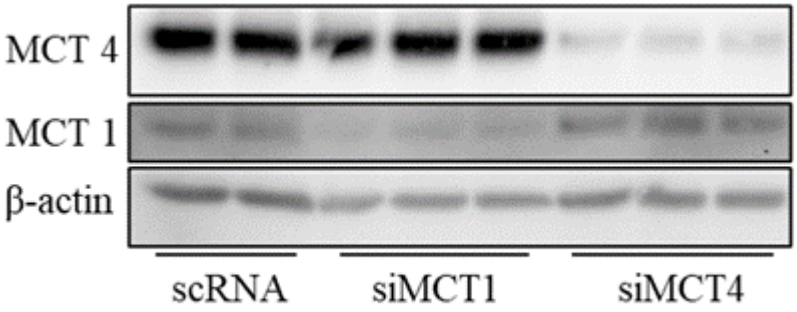

Figure 17 Successful knockdown of MCT1 and 4 shown by immunoblot

Pathological hyperproliferation was induced in hPASMCs by two different *in vitro* disease models, chronic hypoxic incubation for 3 days and PDGF stimulation for 2 days. Afterwards, the proliferation capacity of the hPASMCs was measured using BrdU incorporation assay and the effect of MCT1 or 4 silencing was investigated. Chronic hypoxic incubation of hPASMCs for 3 days significantly induced cell proliferation, indicated by increased BrdU incorporation, in scRNA treated control cells when compared to normoxic control cells. Knockdown of MCT1 or MCT4 significantly reduced or even prevented the hypoxia induced proliferation in hPASMCs. The groups were compared by one-way ANOVA followed by post-hoc Turkey HSD test. The results are shown in figure 18.

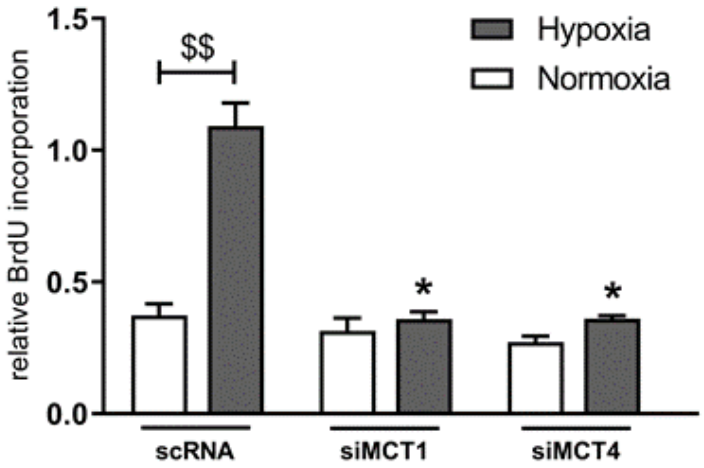

Figure 18 Effect of MCT1 and 4 silencing on hPASMC proliferation after 3 days of chronic hypoxic conditions; each condition n=4, one-way ANOVA followed by post-hoc Turkey HSD test, $$ p<0,01, * p<0,05, * compared to scRNA hypoxia

Upon PDGF treatment for 2 days, scRNA treated control hPASMCs developed a significantly increased proliferation when compared to control cells, grown in basal medium. Silencing of MCT1 or 4 did not significantly reduce the PDGF mediated hyperproliferation. The groups were compared by one-way ANOVA followed by post-hoc Turkey HSD test. The results are shown in figure 19.

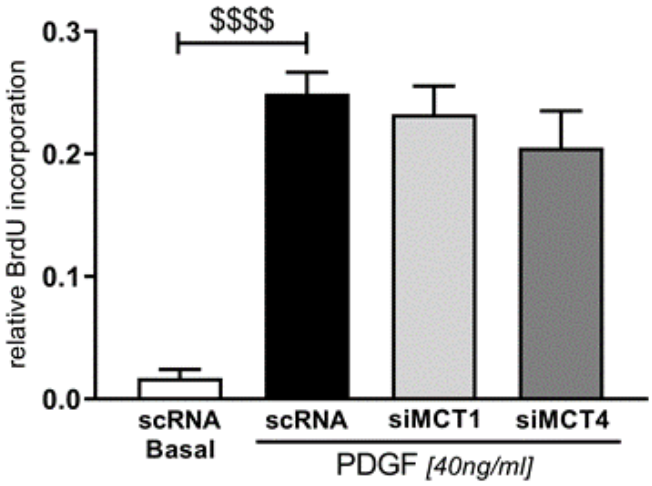

Figure 19 Effect of MCT1 and 4 silencing on hPASMC proliferation after 2 days of PDGF stimulation; each condition n=4, one-way ANOVA followed by post-hoc Turkey HSD test, $$$$ p<0,0001

3.2.2 Effect of pharmaceutical MCT1 inhibition by AZD3965

Besides silencing, the effect of pharmaceutical MCT inhibition on functional characteristics was investigated. Pharmacological MCT1 inhibition was achieved by AZD3965, a potent MCT1 inhibitor (see 1.2.3) [20].

Cell viability of hPASMCs upon AZD3965 treatment was performed to discriminate possible functional effects from toxic side-effects. Different concentrations, up to 50µM, were used. HPASMC viability was not influenced by AZD3965 treatment, when compared to DMSO treated control group by one-way ANOVA followed by post-hoc Turkey HSD test. The results are shown in figure 20.

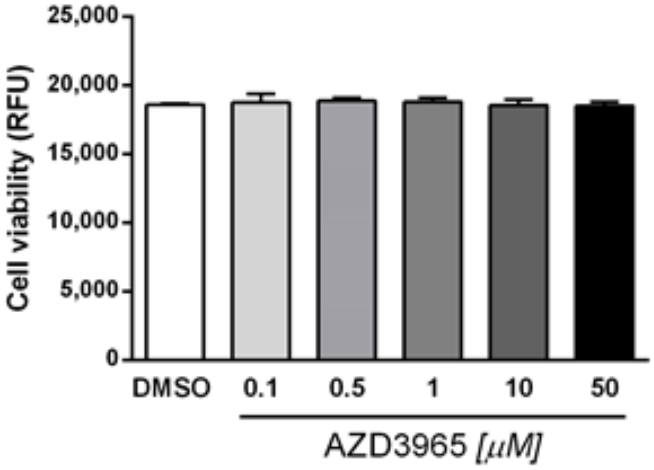

Figure 20 Dose dependent effect of AZD3965 treatment on cell viability in hPASMCs; each condition n=4

The same approach as for silencing experiments was used to investigate the effect of pharmaceutical MCT1 inhibition on induced hPASMC proliferation. Upon chronic hypoxic conditions for 3 days hPASMC proliferation was significantly induced in the DMSO treated group when compared to normoxic control cells. AZD3965 mediated pharmaceutical inhibition of MCT1 did not significantly influence the hypoxia mediated hyperproliferation of hPASMCs at any tested concentration. The groups were compared by one-way ANOVA followed by post-hoc Turkey HSD test. The results are shown in figure 21.

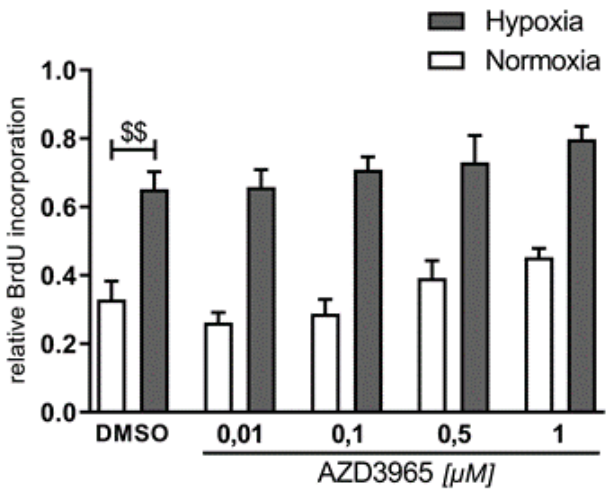

Figure 21 Effect of pharmaceutical MCT1 inhibition on hPASMCs proliferation upon 3 days of hypoxic conditions; each condition n=4, one-way ANOVA followed by post-hoc Turkey HSD test, $$ p<0,01

Treatment of hPASMCs with PDGF for 2 days significantly induced proliferation in hPASMC DMSO treated cells when compared to DMSO treated control grown in basal medium. MCT1 inhibition by AZD3965 did not decrease PDGF induced proliferation. Instead, a significantly increased proliferation was observed starting from 1 µM of AZD3965 compound, when compared to PDGF treated DMSO control. The groups were compared by one-way ANOVA followed by post-hoc Turkey HSD test. The results are shown in figure 22.

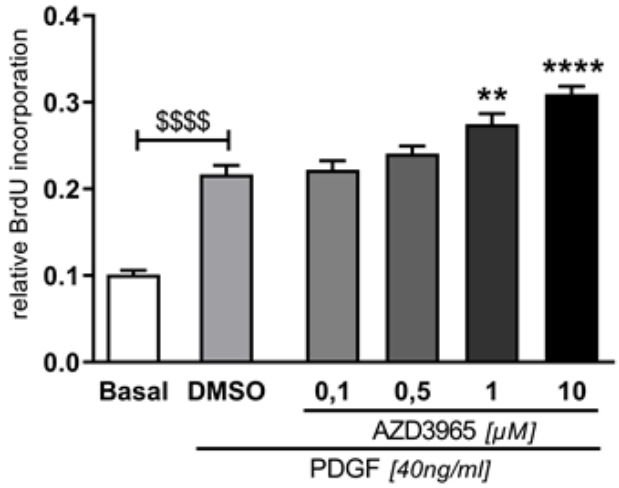

Figure 22 Effect of pharmaceutical MCT1 and 4 inhibition on hPASMC proliferation after PDGF treatment for 2 days; each condition n=4, one-way ANOVA followed by post-hoc Turkey HSD test, $$$$ p<0,0001, ** p<0,01, **** p<0,0001, * compared to DMSO treated and PDGF stimulated control

3.2.3 Effect of pharmaceutical MCT1 and 4 inhibition by syrosingopine

Syrosingopine is a compound effectively inhibiting MCT4 at low concentrations and with increasing concentrations MCT1 as well (see 1.2.3). According to the literature the IC_{50} is about 2,5 µM for MCT1 and 40 nM for MCT4 [12]. Meaning that a single MCT4 or double MCT1 and 4 inhibition can be achieved, depending on the used concentration.

Cell viability upon drug treatment was investigated to test for cytotoxic side-effects. Concentrations of up to 20 µM of syrosingopine did not significantly reduce cell viability when compared to DMSO treated cells. Staurosporine was added as a positive control and significantly diminished cell viability compared to DMSO control. The groups were compared by one-way ANOVA followed by post-hoc Turkey HSD test. The results are shown in figure 23.

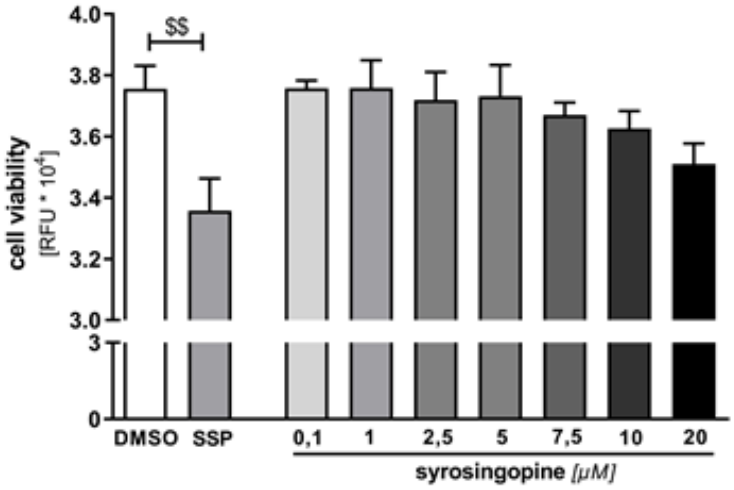

Figure 23 Dose dependent effect of syrosingopine treatment on cell viability in hPASMCs; each condition n=4, one-way ANOVA followed by post-hoc Turkey HSD test, $$ p<0,01, SSP=staurosporine

The effect of syrosingopine treatment in hPASMCs on hypoxia and PDGF induced proliferative capacity was investigated. In chronic hypoxic conditions the DMSO treated control cells acquired a significantly increased proliferative capacity compared to normoxic conditions. Syrosingopine treatment, at a concentration of 5 and 10µM, significantly reduced or even prevented the hypoxia induced proliferation in hPASMCs compared to DMSO treated control cells. The highest tested concentration of 10 µM also significantly reduced proliferative capacity in normoxic conditions compared to DMSO treated control. The results are shown in figure 24.

Upon PDGF treatment for 2 days, DMSO treated control hPASMCs acquired a hyperproliferative phenotype, shown by significantly increased BrdU incorporation in the DMSO control. HPASMCs treated with lower concentrations of syrosingopine developed a significantly increased proliferative capacity when compared to DMSO treated control cells. Whereas the highest used concentration of 10 µM significantly reduced PDGF induced proliferation. The results are shown in figure 25. In both experiments, all groups were compared by one-way ANOVA followed by post-hoc Turkey HSD test.

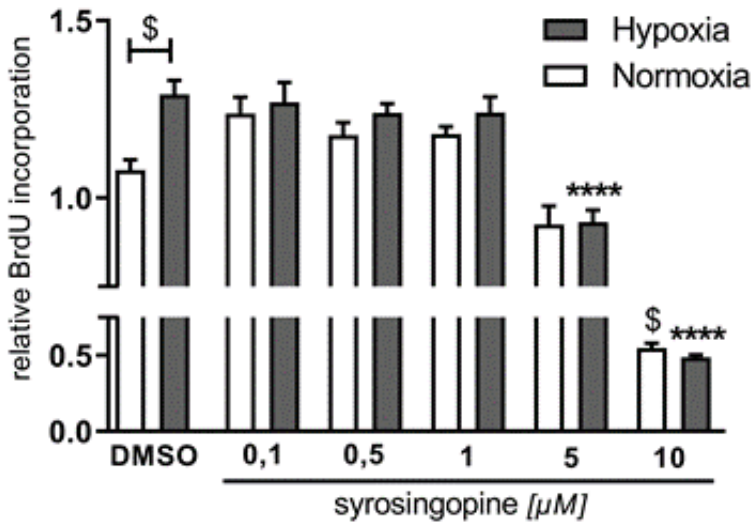

Figure 24 Effect of syrosingopine treatment on hPASMC proliferation after chronic hypoxic incubation for 3 days; each condition n=4, one-way ANOVA followed by post-hoc Turkey HSD test, $ p<0,05, **** p<0,0001, * compared to DMSO treated hypoxic control

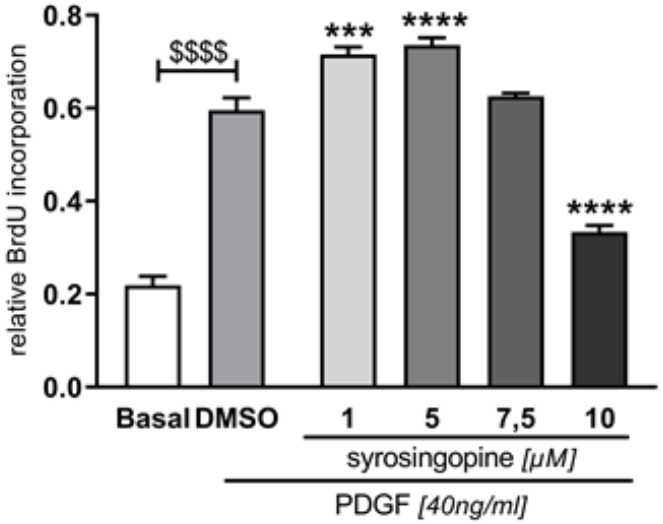

Figure 25 Effect of syrosingopine treatment on hPASMC proliferation after PDGF treatment for 2 days; each condition n=4, one-way ANOVA followed by post-hoc Turkey HSD test, $$$$ p<0,0001, *** p<0,001, **** p<0,0001, * compared to DMSO treated and PDGF stimulated control

In addition to proliferation assays, the effect of pharmacological MCT1 and 4 inhibition by syrosingopine on hPASMC migration in hypoxic conditions was investigated using a wound confluence assay. In this case a concentration of 10 µM of the compound was used. The chosen concentration is based on the reduced proliferation at this concentration from the previous experiments. In hypoxic conditions the cell migration of hPASMCs was significantly reduced after 12 hours of treatment by syrosingopine when compared to DMSO treated control cells by independent t-test. The results are shown in figure 26.

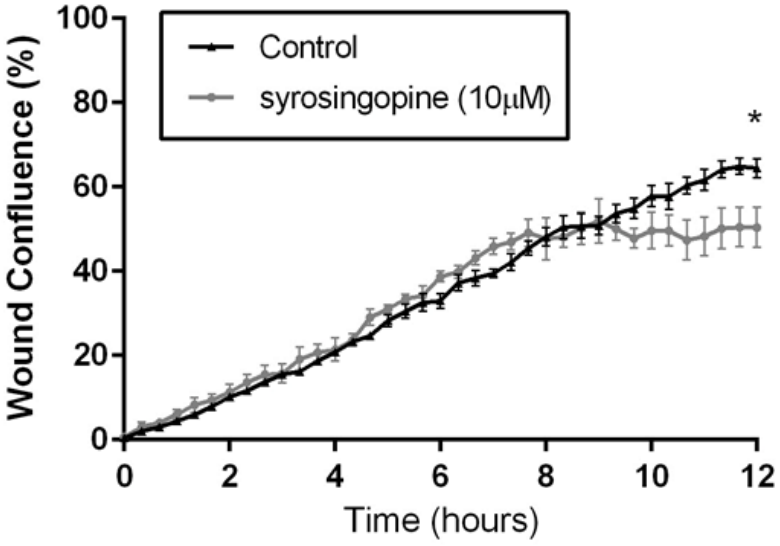

Figure 26 Effect of syrosingopine treatment in hPASMCs on cell migration in hypoxic conditions; independent t-test, * $p<0,05$

To examine whether the treatment by syrosingopine influences cell fate of hPASMCs, apoptotic cells were stained using an annexin XII based fluorescent dye. Apoptotic cells were identified at different concentrations of the compound and compared to DMSO treated hPASMCs in hypoxic conditions. In hPASMCs treated with 10 µM of syrosingopine significantly more apoptosis positive cells were found compared to DMSO treated cells by one-way ANOVA followed by post-hoc Turkey HSD test. The result is shown in figure 27.

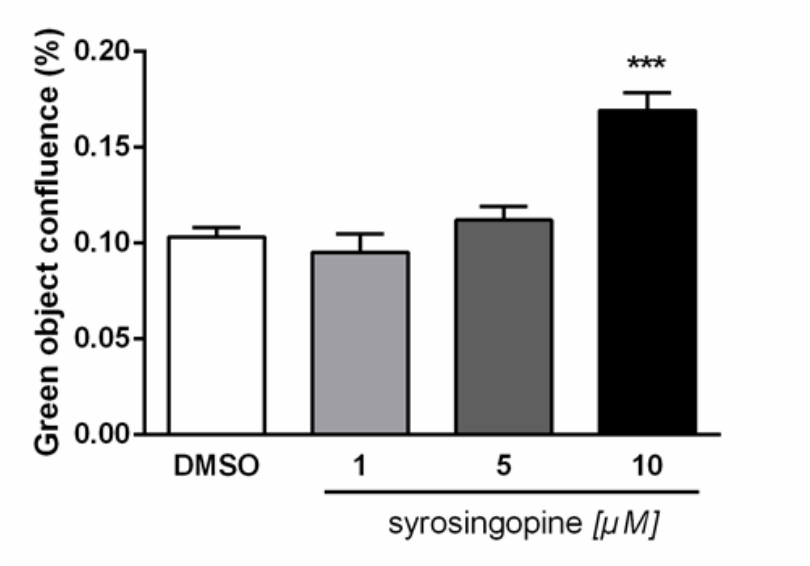

Figure 27 Effect of syrosingopine treatment on hPASMC apoptosis rate after hypoxic incubation for 12 hours; each condition n=4, one-way ANOVA followed by post-hoc Turkey HSD test, *** p<0,001, * compared to DMSO treated control

Extracellular lactate measurements were performed to associate the findings of impeded proliferation and migration and induced apoptosis rate with successful lactate transport inhibition. Therefore, hPASMCs were exposed to normoxic and hypoxic conditions for 2 days. According to the Pasteur effect, extracellular lactate concentrations were significantly increased in hypoxic conditions compared to normoxia [192]. Upon syrosingopine treatment the extracellular lactate concentration was significantly reduced at normoxic and hypoxic conditions compared to DMSO treated cells by one-way ANOVA followed by post-hoc Turkey HSD test. The results are shown in figure 28.

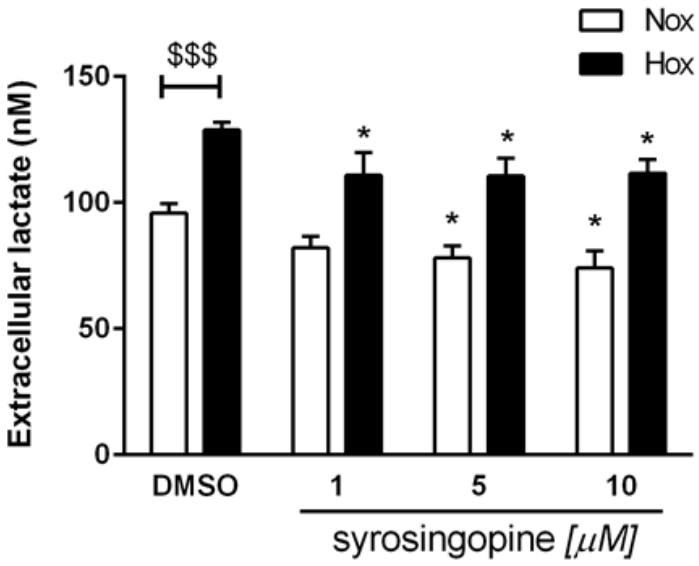

Figure 28 Effect of syrosingopine treatment in hPASMCs on extracellular lactate concentrations at normoxic and hypoxic conditions for 2 days; each condition n=4, one-way ANOVA followed by post-hoc Turkey HSD test, $$$ $p<0,001$, * $p<0,05$, * compared to respective normoxic or hypoxic DMSO control

3.3 Circulating MCT1 and 4 as a PH biomarker

3.3.1 Characterization of the patient collective

MCT1 and 4 plasma concentrations were measured in PA blood samples derived from PH and non-PH patients to investigate their suitability as disease biomarkers. The patient collective of the MCT1 and 4 biomarker study consisted of 200 patients. Of these patients 75 (37,5%) were diagnosed with IPAH, 75 (37,5%) with CTEPH and 50 (25%) patients did not suffer from pulmonary hypertension and therefore served as control group, referred to as non-PH.

Demographic (age, gender), functional (6MWD) and hemodynamic (mPAP, PVR, CI and BNP) data of these patients, derived from same hospital stay as the blood withdrawal, were collected for further analysis. An overview of the collected data is given in table 7.

Table 7 Hemodynamic and exercise data of the whole patient collective

		Mean (±SD), Median [IQR] or Count
Age [years]		65 [21]
Gender	female	116
	male	84
mPAP [mmHg]		37 [21]
PVR [dyn*s/cm5]		545,00 [630]
CI [(l/min)/m²]		2,4 [0,7]
6MWD [m]		340 (±120)
BNP [ng/l]		176 [352]
WHO-FC	1	1
	2	29
	3	105
	4	21

The patients served as control group suffered from different underlying diseases. In specific, 23 (46%) from respiratory disorders, 9 (18%) from cardiac diseases, 7 (14%) had a reported thromboembolic event and 5 (10%) a connective tissue disease. As previously defined as exclusion criteria, none of the patients had an increased mPAP or an underlying cancerous disease.

Naturally, significant differences of mPAP, PVR, CI and BNP were observed comparing non-PH to PH affected subgroups. On the other hand, 6MWD did not differ from PH

patients and the control group. Remarkably more female patients were found in IPAH subgroup.

Detailed hemodynamic and exercise data of the subgroups is displayed in table 8.

Table 8 Hemodynamic and exercise data divided by subgroups

		Group			
		non-PH	PH	IPAH	CTEPH
		Mean (±SD), Median [IQR] or Count			
Age [years]		65 [22]	64,5 [23]	58 [31]	69 [23]
Gender [f:m]		1,17:1	1,46:1	2:1	1,08:1
mPAP [mmHg]		15 [4]	43 [16]	45 [21]	39 [12]
PVR [dyn*s/cm5]		121 [96]	671 [571]	822 [609]	583 [457]
CI [(l/min)/m²]		2,9 [1,2]	2,2 [0,8]	2,1 [0,8]	2,3 [0,7]
6MWD [m]		381 (± 118)	331,44 (±119)	320,61 (±123)	343,91 (±114)
BNP [ng/l]		39 [76]	227 [427]	228 [387]	225 [333]
WHO-FC	3 / 4	8 / 3	97 / 18	41 / 11	41 / 11
Survival	Cens.	n.a.	55	25	25
	Death	n.a.	60	26	26
MCT1 [ng/ml]		4,04 [3,33]	4,87 [4,83]	5,19 [4,75]	4,81 [5,01]
MCT4 [ng/ml]		19,71 [6,62]	17,98 [7,81]	19,23 [8,16]	17,23 [7,80]

3.3.2 Cumulative survival of PH patients

Survival data of the PH patients was collected whenever possible. Only PH patients were included, resulting in a collective of 150 patients. In total 60 (40%) events occurred and 90 (60%) patients were censored at the day of last contact alive. The cumulative survival of the PH patients is shown using a Kaplan-Meier curve in figure 29. In the investigated PH collective the median survival is 7,3 (88 months) years. After 1 year 89,1%, after 3 years 72,3 % and after 5 years 62,8% were alive.

In the IPAH subgroup, 34 events occurred and 41 patients were censored. The median survival of the IPAH patients was 7,3 years (88 months). After 1 year 87,7% of the IPAH patients were still alive, after 3 years 70,5% and after 5 years 61,8%.

In the CTEPH subgroup, 26 events occurred and 49 patients were censored. The median survival was 7,1 years (85 months). The 1 year survival was 90,8%, the 3 year survival 74,6% and the 5 year survival 64,1%.

Kaplan-Meier curves of the survival of IPAH and CTEPH patients are shown in the attachment (see 7.1.1).

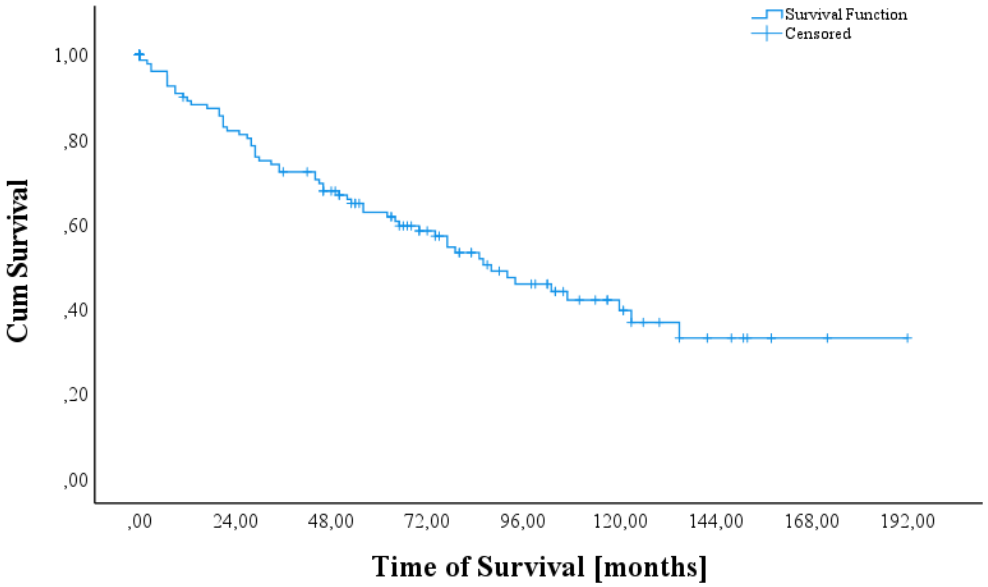

Figure 29 Cumulative survival of overall PH patients; n=150, events=60, censored=90

3.3.3 MCT1 as a PH biomarker

3.3.3.1 Distribution of MCT1 plasma concentration

The distribution of MCT1 plasma concentration within the whole collective had a median concentration of 4,80 [4,16] ng/ml. The measured concentration ranged from 0,37 ng/ml up to 19,48 ng/ml. In total 15 (7,5%) values had to be excluded, as 2 (1%) were not detectable and 13 (6,5%) were out of the detection range. The distribution is displayed in figure 30 and detailed descriptive statistics are shown in table 9.

The distribution presented right skewed. As the distribution was right skewed further statistical analyses were performed using the respective logarithmic values as described in the methods, although in this case it did not generate normally distributed data. The related descriptive data of ln_MCT1 is shown in table 9 and the distribution of ln_MCT1 within the whole collective is displayed in figure 31.

Table 9 Descriptive statistics of MCT1 and ln_MCT1 distribution

		MCT1 [ng/ml]	ln_MCT1
N	Valid	185	185
	Missing	15	15
Mean		5,5664	1,4769
Median		4,8002	1,5687
Std. Deviation		3,86388	,73196
Interquartile Range		4,16	,92
Maximum		19,85	2,99
Minimum		,37	-1,01
Kurtosis		1,894	,438
Skewness		1,373	-,473
Shapiro Wilk test	Sig.	,00	,04

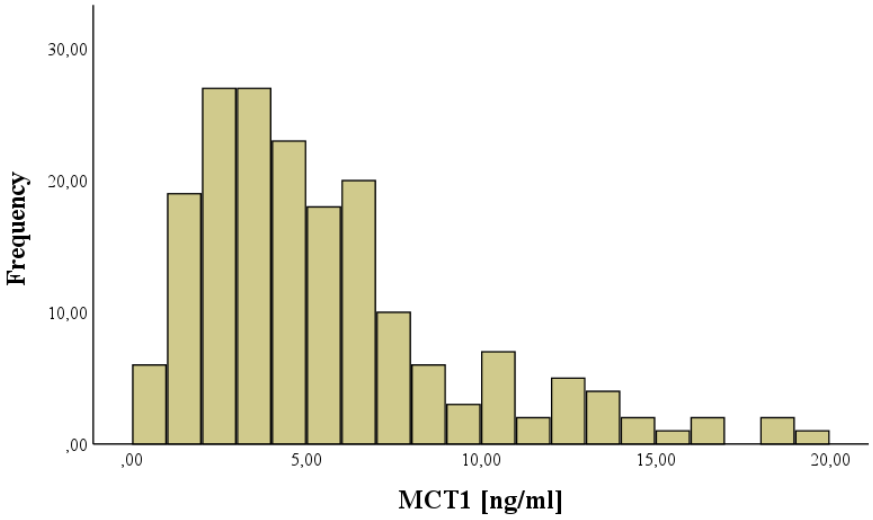

Figure 30 Histogram of MCT1 distribution

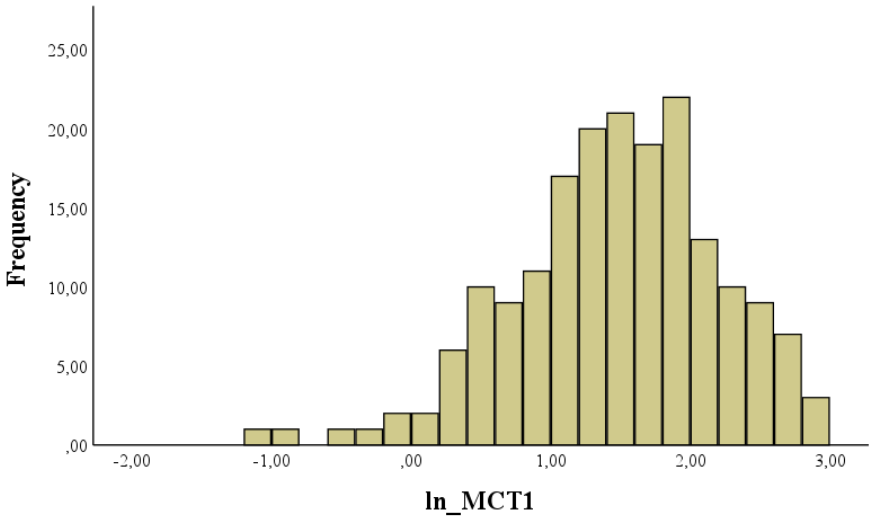

Figure 31 Histogram of ln_MCT1 distribution

3.3.3.2 MCT1 as a diagnostic biomarker

Measured plasma concentrations of MCT1 were compared from non-PH to PH patients and among the subgroups, IPAH and CTEPH. Non-PH patients had a median circulating MCT1 concentration of 4,04 [3,33] ng/ml and PH patients of 4,87 [4,83] ng/ml, which did not result in a statistically significant difference. The results are shown in figure 32.

IPAH patients had a median circulating MCT1 concentration of 5,19 [4,75] ng/ml and CTEPH patients a median of 4,81 [5,01] ng/ml. Comparing the MCT1 concentrations

between non-PH, IPAH and CTEPH patients did not reveal a significant difference. The results are shown in figure 33.

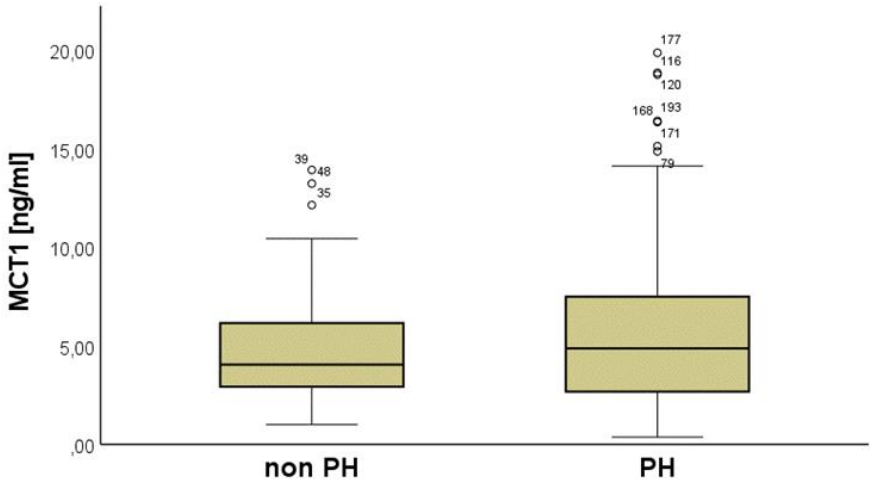

Figure 32 MCT1 plasma concentration in non-PH and PH patients; non-PH n=45, PH n=140, Mann-Whitney U test of ln_MCT1 p=0,265

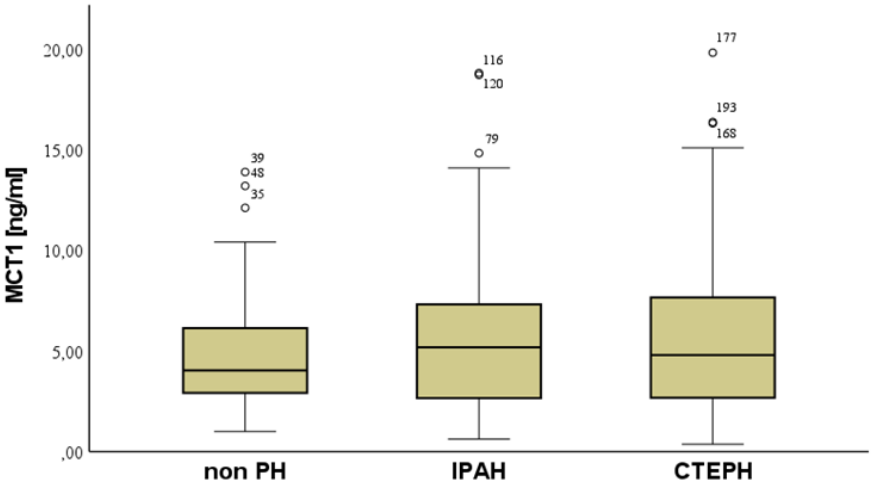

Figure 33 MCT1 plasma concentration in non-PH, IPAH and CTEPH patients; non-PH n=45, IPAH n=70, CTEPH n=70, Kruskal-Wallis test of ln_MCT1 p=0,534

3.3.3.3 MCT1 as a biomarker for severity of the disease, age and gender

It was tested whether gender or age influence circulating MCT1 concentration. Therefore, MCT1 concentration was compared in male to female patients. No difference of circulating MCT1 concentration was found in male compared to female patients. The results are shown in table 10.

The association of age and MCT1 concentration was tested by calculating Spearman correlation. No significant association was found between age and MCT1 concentration. The respective scatter plot is shown in figure 34.

Moreover, MCT1 plasma level was tested as a biomarker for the severity of the disease. Therefore, non-PH patients were excluded and the analysis was performed in PH patients only. First, the plasma concentration was compared depending on WHO-FC. No significant difference of MCT1 concentration was observed comparing the different WHO-FC in PH patients. The related results are shown in table 10.

Table 10 MCT1 concentration by gender and WHO-FC

		MCT1	
		Median [IQR]	Significance
Gender	female	4,81 [3,80]	p=0,686
	male	4,75 [4,92]	
WHO-FC	1	3,64 [0]	p=0,938
	2	4,41 [3,73]	
	3	5,33 [4,91]	
	4	4,83 [6,06]	

Gender: male n=80, female n=105, Mann-Whitney U test of ln_MCT1,
WHO-FC: I n=1, II n=26, III n=90, IV n=16, Kruskal-Wallis Test of ln_MCT1

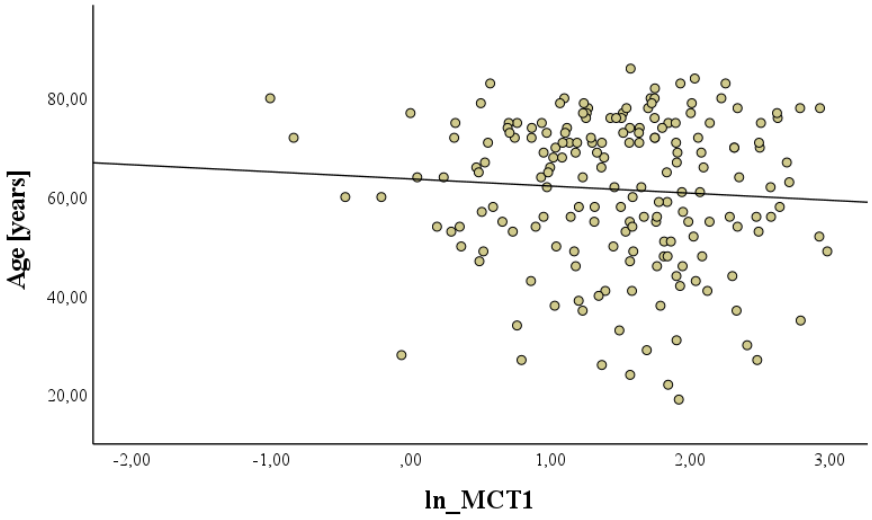

Figure 34 Spearman correlation of ln_MCT1 and age; n=185, p=0,468, r=-0,05

Different hemodynamic and functional data of the patients were available. In particular mPAP, PVR, CI, 6MWD and BNP. The association of these parameters with circulating MCT1 concentration in PH patients was calculated and in addition, compared to the only currently established biomarker in PH, BNP.

None of the parameters showed a significant association with MCT1 plasma level. On the other hand, BNP showed significant, weak to medium strong correlations with mPAP, PVR and CI. The detailed correlations and statistics are displayed in table 11.

Same analyses were performed within IPAH and CTEPH subgroup. In the subgroup analyses no significant correlations were found either. Detailed results are summarized in the attachments (see 7.1).

Table 11 Correlation of ln_MCT1 with hemodynamic data

		ln_MCT1	BNP [ng/l]
mPAP [mmHg]	Spearman Correlation	-,046	,334
	Sig. (2-tailed)	,587	,001*
	N	140	100
PVR [dyn*s/cm5]	Spearman Correlation	,031	,408
	Sig. (2-tailed)	,719	,000*
	N	140	100
CI [(l/min)/m²]	Spearman Correlation	-,024	-,479
	Sig. (2-tailed)	,777	,000*
	N	140	100
6MWD [m]	Spearman Correlation	,02	-,226
	Sig. (2-tailed)	,837	,050
	N	107	76
BNP [ng/l]	Spearman Correlation	,012	1
	Sig. (2-tailed)	,906	
	N	93	100

** indicates significance*

3.3.3.4 MCT1 as a prognostic biomarker

Plasma concentration of MCT1 was investigated as a prognostic biomarker in PH patients. Therefore, the PH patients were divided into 2 or 4 different groups according to the percentiles of their ln_MCT1 values. The generated groups were then compared by their survival time. No significant difference of the survival time was observed when compared between the different groups. The respective Kaplan-Meier plots are shown in figure 35 and 36.

Subgroup analyses were performed in IPAH and CTEPH group without any significant difference. Results are shown in the attachments (see 7.1).

.

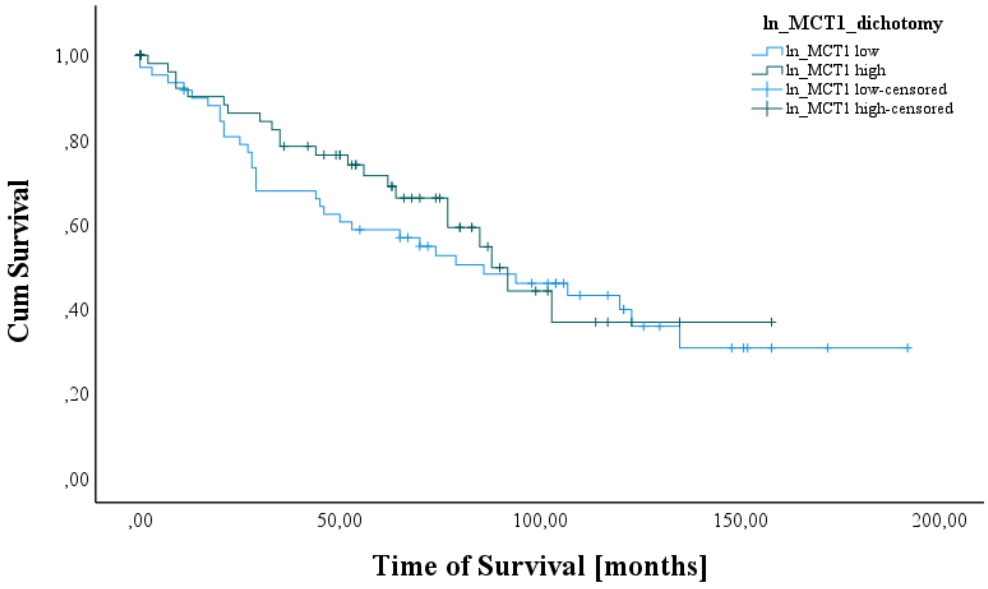

Figure 35 Survival of PH patients by dichotom ln_MCT1 separation; ln_MCT1-low: n=69, events=33, ln_MCT1-high: n=70, events=22, Log-Rank test p=0,431

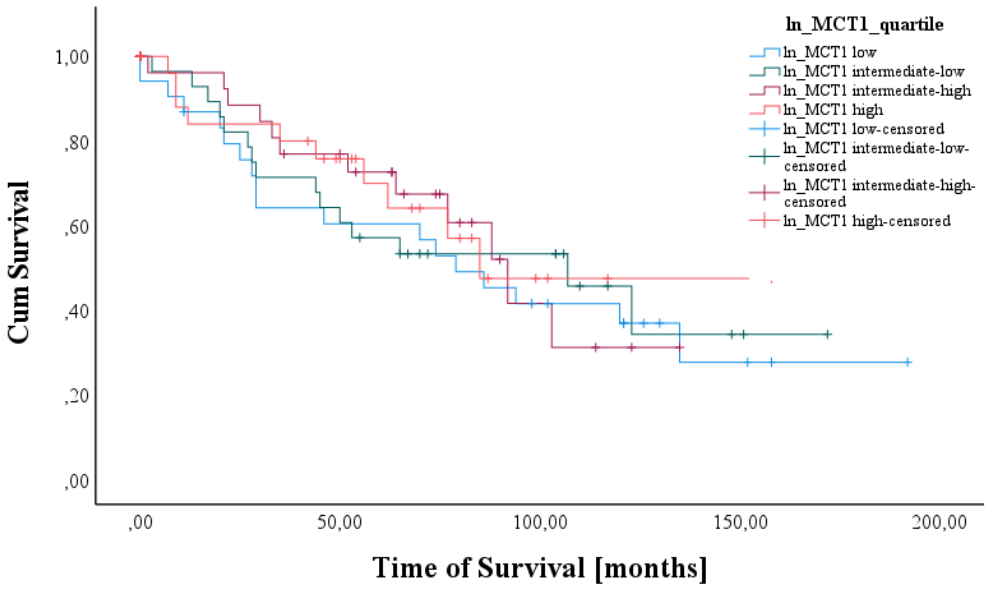

Figure 36 Survival of PH patients by quartile ln_MCT1 separation; ln_MCT1-low: n=34, events=18, ln_MCT1-intermediate-low: n=35, events=15, ln_MCT1-intermediate-high: n35, events=12, ln_MCT1-high: n=35, events=10, Log-Rank test p=0,818

3.3.4 MCT4 as a PH biomarker

3.3.4.1 Distribution of MCT4 plasma concentration

MCT4 plasma concentration within the whole collective had a median concentration of 19,61 [7,34] ng/ml. The measured concentration ranged from 7,74 ng/ml up to 40,94 ng/ml. In total 4 (2%) values were excluded, as they were out of the detection range. The distribution is displayed in figure 37 and detailed descriptive statistics are shown in table 12.

The distribution presented right skewed. Again, further statistical analyses were performed using the normally distributed, logarithmic values, as described in the methods. The descriptive data of ln_MCT4 is listed in table 12 and the histogram is shown in figure 38.

Table 12 Descriptive statistics of MCT4 and ln_MCT4 distribution

		MCT4 [ng/ml]	ln_MCT4
N	Valid	196	196
	Missing	4	4
Mean		19,6127	2,9279
Median		18,2579	2,9046
Std. Deviation		6,22804	,31166
Interquartile Range		7,34	,39
Maximum		40,94	3,71
Minimum		7,74	2,05
Skewness		,842	,000
Kurtosis		,622	-,085
Shapiro Wilk test	Sig.	,00	,90

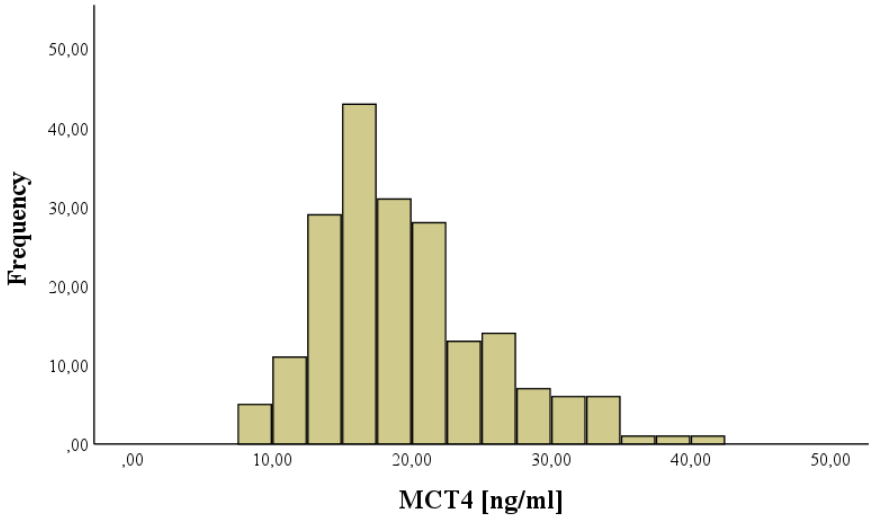

Figure 37 Histogram of MCT4 distribution

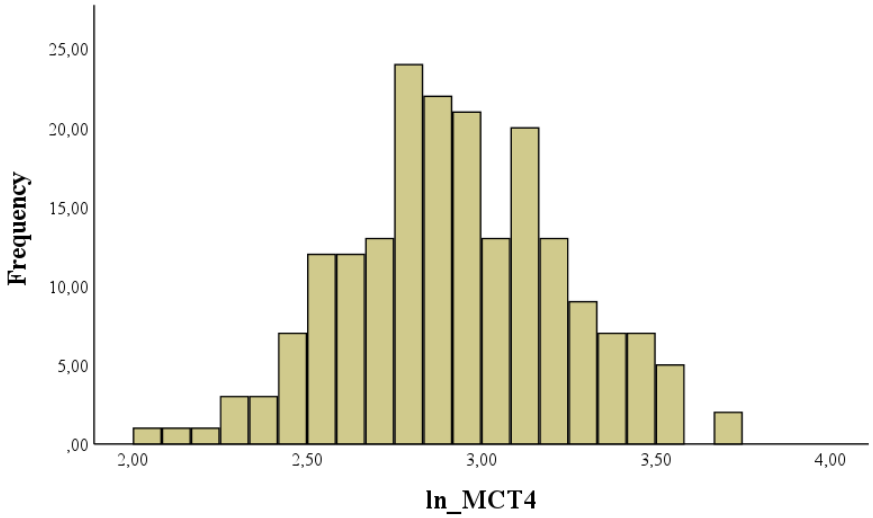

Figure 38 Histogram of ln_MCT4 distribution

3.3.4.2 MCT4 as a diagnostic biomarker

Just as for MCT1, the measured MCT4 concentration was compared among the different subgroups.

Non-PH patients had a median MCT4 concentration of 19,71 [6,62] ng/ml and PH patients a median concentration of 17,98 [7,81] ng/ml, which did not result in a statistically significant difference. The results are shown in figure 39.

IPAH patients had a median circulating MCT4 concentration of 19,27 [8,16] ng/ml and the CTEPH patients a median of 17,23 [7,80] ng/ml. There was no significant difference

in the MCT4 plasma concentrations among the different subgroups. The results are shown in figure 40.

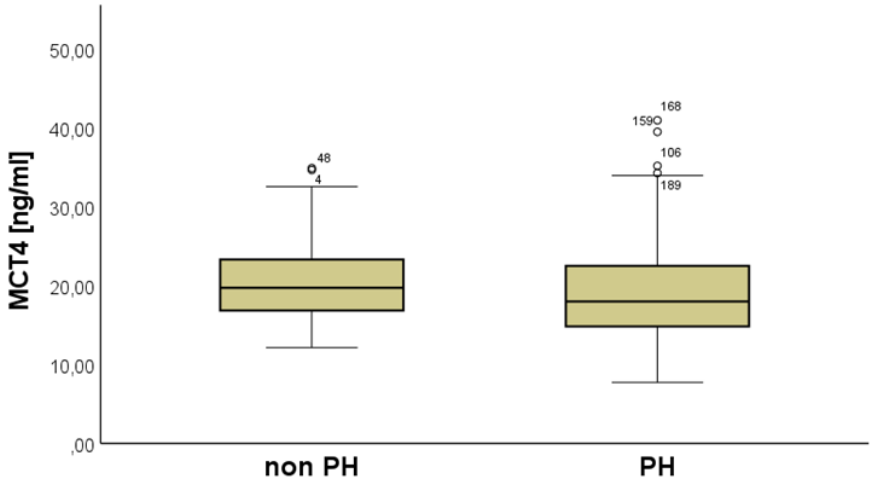

Figure 39 MCT4 plasma concentration in non-PH and PH patients; non-PH n=50, PH n=146, independent t-test of ln_MCT4 p=0,099

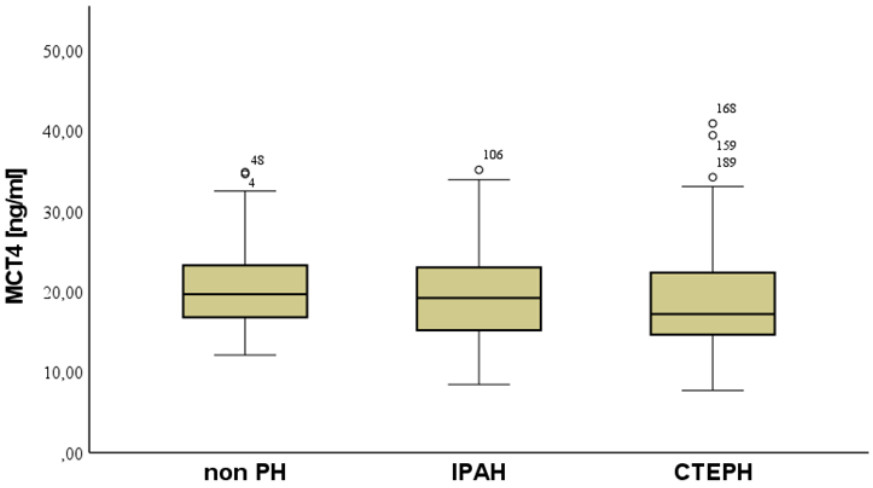

Figure 40 MCT4 plasma concentration in non-PH, IPAH and CTEPH patients; non-PH n=50, IPAH n=74, CTEPH n=72, Kruskal-Wallis test of ln_MCT4 p=0,233

3.3.4.3 MCT4 as a biomarker for severity of the disease, age and gender

It was tested whether MCT4 plasma concentration are influenced by gender or age. Therefore, MCT4 concentration was compared among the entities. No significant difference of MCT4 plasma level was found between male and female patients.

The association of age and MCT4 plasma level was evaluated by calculating Spearman correlation. No significant association could be found between age and MCT4. The respective scatter plot is shown in figure 41.

Moreover, MCT4 concentration was compared depending on WHO-FC in PH patients. Among the different WHO-FC, no significant difference of MCT4 concentration was found. The results are shown in table 13.

Table 13 MCT4 concentration by gender and WHO-FC

		MCT4	
		Median [IQR]	significance
Gender	female	19,35 [7,69]	p=0,094
	male	17,31 [8,10]	
WHO-FC	1	22,49 [0]	p=0,181
	2	20,49 [9,55]	
	3	17,33 [7,71]	
	4	17,54 [13,20]	

Gender: male n=82, female n=114, Mann-Whitney U test of ln_MCT4,
WHO-FC: I n=1, II n=25, III n=96, IV n=17, Kruskal-Wallis Test of ln_MCT4

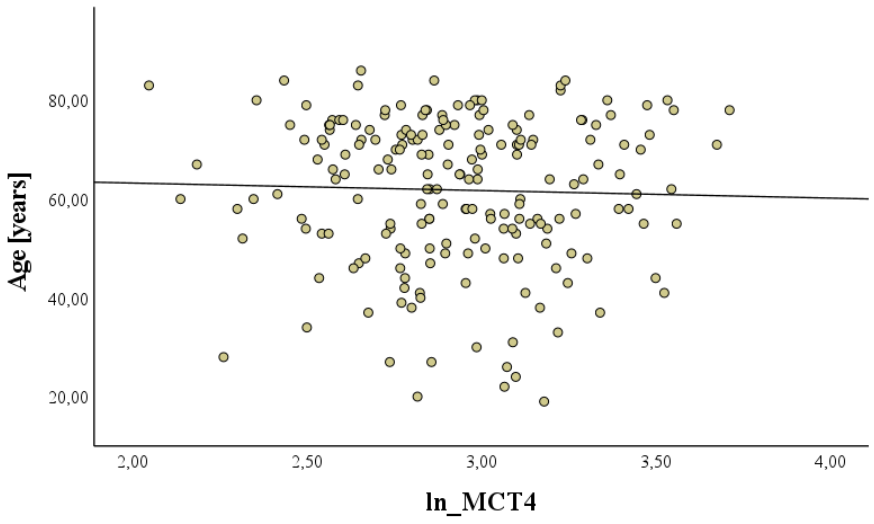

Figure 41 Spearman correlation of ln_MCT4 and age; n=196, p=0,585, r=-0,41

Spearman or Pearson correlation of MCT4 plasma level with functional and hemodynamic data was calculated to test whether the potential biomarker is associated with the severity of the disease in PH patients. Therefore, MCT4 was associated with mPAP, PVR, CI, 6MWD and BNP. Again, the correlations of BNP with the hemodynamic and functional data were calculated as a comparison. No significant association of MCT4 plasma level with any of the hemodynamic or functional data was found. Detailed correlations and statistics are shown in table 14.

Subgroup analyses within IPAH and CTEPH subgroups are shown in the attachments (see 7.1).

Table 14 Correlation of ln_MCT4 with hemodynamic data

		ln_MCT4	BNP [ng/l]
mPAP [mmHg]	Spearman Correlation	-,043	,334
	Sig. (2-tailed)	,605	,000*
	N	146	100
PVR [dyn*s/cm5]	Spearman Correlation	-,004	,408
	Sig. (2-tailed)	,964	,000*
	N	146	100
CI [(l/min)/m²]	Spearman Correlation	,006	-,479
	Sig. (2-tailed)	,945	,000*
	N	146	100
6MWD [m]	Pearson Correlation	,163	-,226
	Sig. (2-tailed)	,057	,050
	N	112	76
BNP [ng/l]	Spearman Correlation	-,000	1
	Sig. (2-tailed)	,998	
	N	97	100

** indicates significance*

3.3.4.4 MCT4 as a prognostic biomarker

The concentration of MCT4 was investigated regarding its prognostic use in PH patients. Therefore, PH patients were divided into 2 or 4 different groups based on the percentiles of ln_MCT4.

No significant difference in the survival time among the different MCT4 groups were found in PH patients. The respective Kaplan-Meier plots are shown in figure 42 and 43.

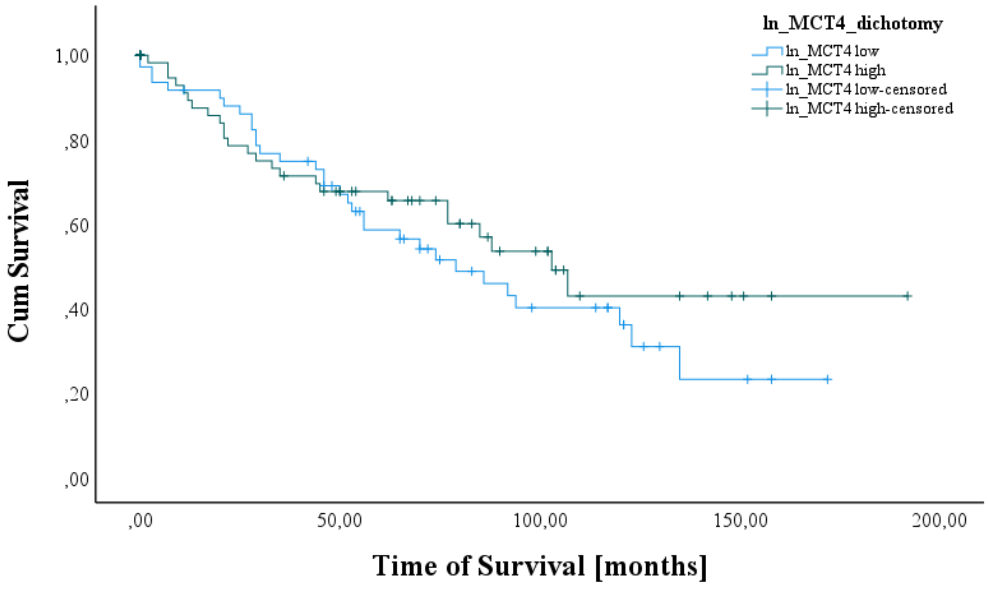

Figure 42 Survival of PH patients by dichotom ln_MCT4 separation; ln_MCT4-low: n=72, events=32, ln_MCT4-high: n=73, events=25, Log-Rank test p=0,330

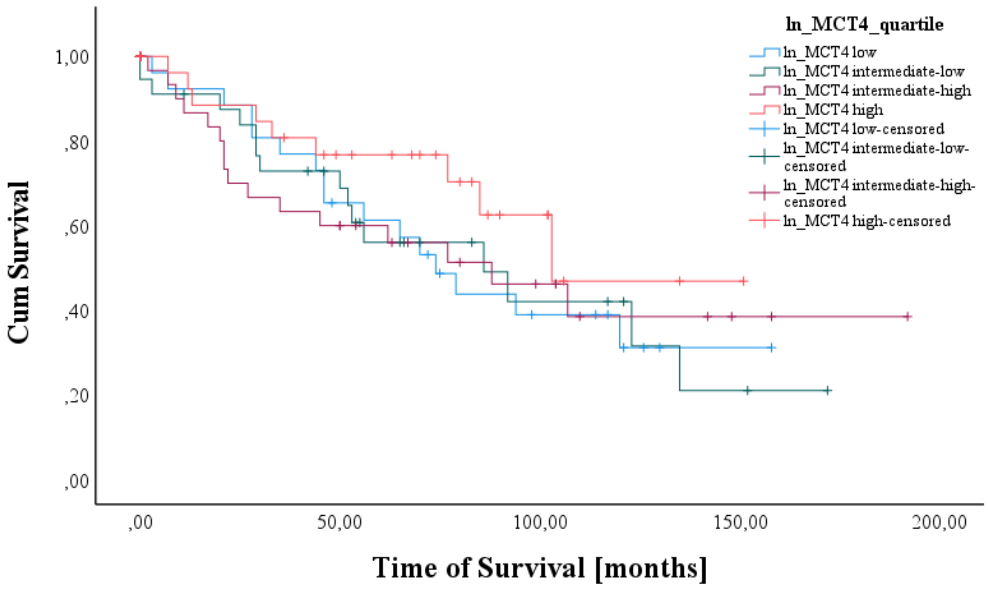

Figure 43 Survival of PH patients by quartile ln_MCT4 separation; ln_MCT4-low: n=35, events=16, ln_MCT4-intermediate-low: n=37, events=16, ln_MCT4-intermediate-high: n37, events=16, ln_MCT4-high: n=36, events=9, Log-Rank test p=0,519

Subgroup analyses were performed again. In IPAH patients there was a trend, close to significance when comparing the survival time of dichotom ln_MCT4 separation by Log Rank test. IPAH patients assigned to the group with low ln_MCT4 values had a tendency of an impeded outcome when compared to patients with higher ln_MCT4 values. Ln_MCT4-low had a median survival of 5,8 years (70 months) and ln_MCT4-high a median survival of 7,3 years (88 months). The result is shown in figure 44. Further subgroup analysis did not deliver any significant results and are shown in the attachment (see 7.1).

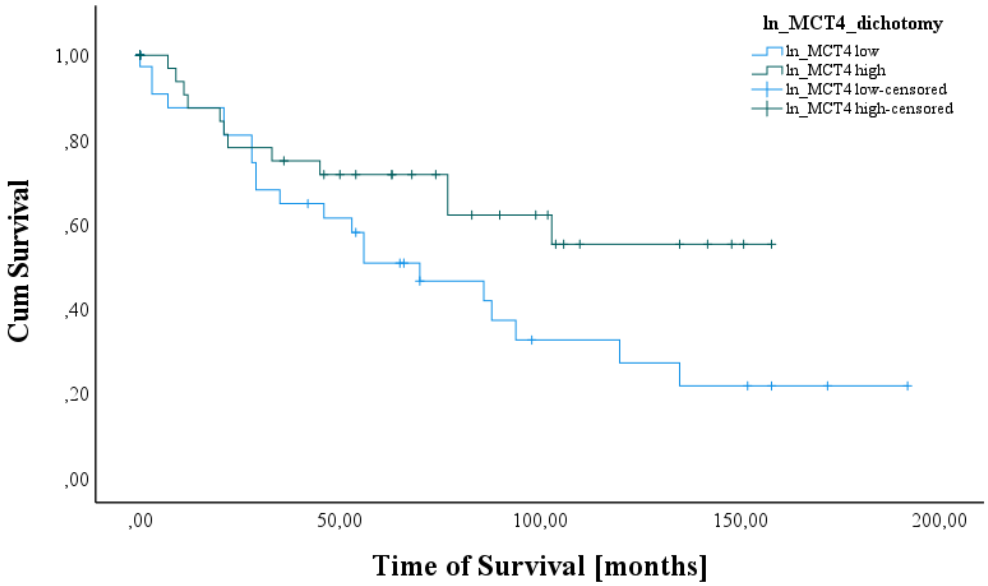

Figure 44 Survival of IPAH patients by dichotom ln_MCT4 separation; ln_MCT4-low: n=37, events=21, ln_MCT4-high: n=37, events=12, Log-Rank test p=0,052

Within the IPAH subgroup ln_MCT4 values were compared in dependance of age, PVR and WHO-FC. Performing multivariate Cox regression analysis, a significant result was found for ln_MCT4 dichotom separation. The Hazard Ratio was 2,274 (95% CI 1,105-4,678) with a p-value of 0,026. The result is shown in figure 45. Further details of the variables used in the multivariate approach are shown in the attachments (see 7.1.4). As there were no survival differences observed within the other subgroups, no further Cox regression analyses were performed.

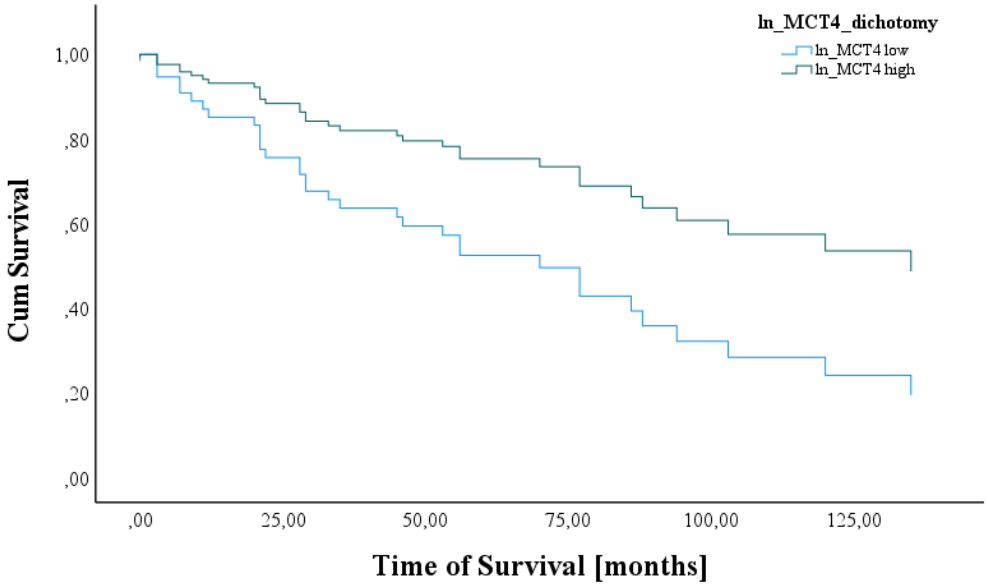

Figure 45 Cox regression of ln_MCT4, age, PVR and WHO-FC; ln_MCT4-low: n=37, events=21, ln_MCT4-high: n=37, events=12, Cox regression p=0,026, HR 2,274

3.3.5 Correlation of MCT1 and 4 plasma levels

It was investigated whether both tested potential biomarkers, MCT1 and 4, were associated with each other. Analyzed by Spearman correlation, ln_MCT1 and ln_MCT4 values were highly significant and strongly correlating with each other in PH patients. The correlation is visualized in a scatter plot in figure 46.

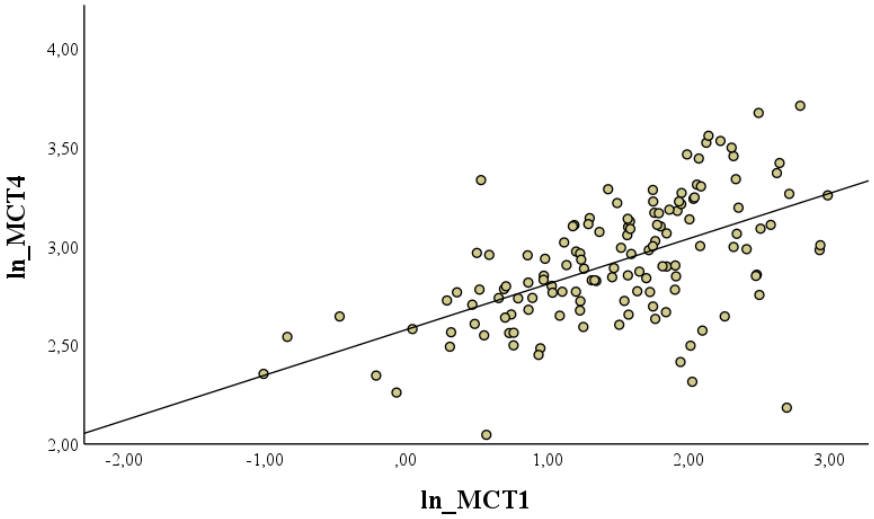

Figure 46 Spearman correlation of ln_MCT1 and ln_MCT4 in PH patients; n=137, p<0,001, r=-0,571

4 Discussion

PH affected vascular cells favor cytosolic glycolysis, instead of oxidative phosphorylation to cover their ATP demand [6,220]. A phenomena that is also known as Warburg effect [39]. Consequently resulting in an increased lactate production and release [41,54,242]. However, the knowledge about lactate transporters in PH is poor. This study is the first to target the relevance and practical use of lactate transporters in the context of PH. Different lactate transporters are described, based on theoretical considerations and knowledge originated from cancer research this study focuses on MCT1 and 4 (see 1.2.2) [50].

The aim of this study is to investigate the expression profile of MCT1 and 4 in IPAH patient lungs and in hPASMCs upon different stimulatory factors. Moreover, the therapeutic potential of MCT1 and 4 inhibition in hPASMCs is addressed and circulating MCT1 and 4 and their substrate, lactate, are investigated as a disease biomarker.

4.1 Expression profile of MCT1 and 4

So far, no comprehensive investigation of MCT1 or 4 expression in PH patients was performed. We found the expression of MCT1 and 4 is significantly increased in lung homogenates of IPAH patients (figure 7, p. 34; figure 8, p. 35). This finding is consistent on mRNA and protein level. Caruso et al. investigated the mRNA expression of MCT1 in blood outgrowth endothelial cells (BOEC) harvested from BMPR2 mutant PH and IPAH patients [28]. BOEC derive from circulating progenitor cells and further differentiate into endothelial cells *in vitro*. They are seen representative for endothelial cells [28]. Caruso et al. found MCT1 mRNA is upregulated in BOEC from BMPR2 mutant PH patients by about 2,5 fold, which is comparable to our results. But in contrast, they found MCT1 mRNA is not upregulated in BOEC derived from IPAH patients. Still, a trend towards an increased MCT1 mRNA expression was visible. As they were only analyzing 5 samples (n=7 for BMPR2 mutant) the trend might become significant with a higher number. In their study MCT1 mRNA expression was examined as an indicator for a highly glycolytic phenotype. Protein expression or the expression of MCT4 was not further pursued. Valérie et al. investigated the metabolic fingerprint of EC harvested from CTEPH patients (n=9) [229]. They found that MCT1 and 4 mRNA expression is not changed in EC derived from CTEPH patients. Indicating that the expression of MCT1 and 4 may depend on the respective PH group. Halestrap et al. stated that EC generally

only express MCT1 but no MCT4 [75]. On the other hand, MCT4 is specifically upregulated in highly glycolytic tissues (see 1.2) [80]. Therefore, it needs to be investigated whether the assertion from Halestrap is true in diseased ECs, which acquire a glycolytic phenotype [105]. Caruso et al. also found MCT1 mRNA is increased in the lungs of SUGEN-hypoxia treated rats. Suggesting a promising animal model to study the relevance of MCT1 in an *in vivo* disease model. In numerous cancer entities MCT1 and 4 are upregulated to maintain pH and lactate homeostasis [177]. PH is considered a cancer like disease, having the same metabolic changes [39]. Reviews by D'Allesandro et al. and Ruffenach et al. address the overlap of PH and cancer pathobiology [189,202]. As found in cancer research, we confirm that the expression of MCT1 and 4 is increased in IPAH patients. Thus, suggests that they are important to maintain lactate and pH homeostasis in PH and further underline the similarity of PAH and cancer pathobiology. It is reported, that some cancer entities exclusively or majorly express a single isoform of MCT1 or MCT4 [177]. We found that in IPAH patients both transporters are expressed and upregulated in disease conditions. Since we analyzed the expression profile in lung homogenates, the result is not cell specific. Future studies will have to clarify which cells are participating to the increased MCT1 and 4 expression and whether PAH EC express MCT4. As lactate production is increased in all 3 cell layers of pulmonary vasculature in PH and PH affected vasculature has significantly altered metabolomic gene profile, we assume that all cell layers rely on an increased MCT1 and 4 expression to survive [41].

The underlying factors, inducing MCT1 and 4 expression in pulmonary vasculature, were further investigated. Focusing on hPASMCs, we investigated the expression profile of MCT1 and 4 upon hypoxic and non-hypoxic stimuli. In hPASMCs, chronic hypoxic incubation significantly upregulates MCT4 mRNA and protein expression (figure 9, p. 36; figure 10, p. 37). Hypoxia mediated induction confirms previous findings showing HIF-1α dependent induction of MCT4 expression by Ullah et al. [227]. HIF-1α is a major transcription factor physiologically activated in hypoxic conditions [74]. In PH affected cells HIF-1α is even activated under normoxic conditions and one of the driving forces promoting the glycolytic shift [39]. Hypoxia mediated upregulation of MCT4 in hPASMCs suggest that hypoxic signaling, for example HIF-1α, supports the increased expression of MCT4 in IPAH lung homogenates. MCT1 expression, on the other hand, is not influenced by hypoxic stimulation (figure 9, p. 36; figure 10, p. 37). The published data about the hypoxic influence on MCT1 expression is inconsistent. Bergersen et al.

claimed MCT1 expression is induced by hypoxia. Contrary Boidot et al. showed the opposite [14,19]. They found p53 suppresses MCT1 mRNA induction by hypoxic signaling. This might explain the findings from Bergersen et al., as they worked with p53 mutant breast cancer and glioblastoma cell lines [130,230]. Our results support the findings from Boidot et al. that MCT1 is not influenced by hypoxic stimulation. To be comprehensive, divergent p53 changes and downregulation of p53 are described in PAH [105,234]. At some degree, *in vitro* experiments are limited and cannot reflect the complexity of a living organism. As we were working with p53 wildtype hPASMCs our findings are limited at this point. A possible solution to target this issue is to work with hPASMCs harvested from PAH patients. Besides HIF-1α dependent hypoxic signaling, Rattigan et al. found that high lactate concentrations induce MCT1 expression, in a NF-κB dependent manner [193]. They were working with mesenchymal cells. Although we show that lactate release in hPASMCs is significantly increased in hypoxic conditions (figure 28, p. 51), we found MCT1 expression is not influenced. In contrast to Rattigan et al. our results do not proof an interference of high lactate concentrations and MCT1 expression in hPASMCs.

As non-hypoxic stimuli, hPASMCs were stimulated with PDGF, TNFα and TGFβ. These factors are upregulated and relevant factors in PH pathobiology [107,209,222]. PDGF and TGFβ even promote the Warburg effect in PH [92,240]. Consequently, we assumed that they influence the expression of lactate transporters. However, we found MCT1 expression in hPASMCs is not influenced by PDGF, TNFα or TGFβ (figure 11-16, p. 38-41). These findings are unexpected, especially upon TGFβ stimulation. Uddin et al. previously found MCT1 mRNA and protein expression is induced upon TGFβ stimulation in a lung cancer cell line (A549 cells) [226]. Here, we cannot confirm TGFβ induction of MCT1 expression in hPASMCs. Again, wildtype hPASMCs lack mutations found in PAH and cancer. The non-hypoxic stimulating factors were selected based on their relevance in PAH pathobiology, especially for their influence on glycolytic shift. Further studies on MCT1 regulation in PH might focus on known stimulating factors of MCT1 expression. The first insight into MCT1 regulation generated from studies performed in skeletal muscle. In skeletal muscle, MCT1 expression is induced upon physical exercise [75]. This raised the idea that AMPK or calcium dependent pathways influence MCT1 expression, as they are induced in skeletal muscle upon physical exercise [22,195]. There is evidence that AMPK, induces MCT1 expression [14,81]. Halestrap et

al. showed using MCT1 promoter luciferase construct that MCT1 promoter activity is stimulated in presence of an AMPK activator [81]. But we assume AMPK is unlikely to induce MCT1 in IPAH lungs as it is downregulated in PAH patients and is rather protective for PH development [187]. Promoter sequence analyses revealed that MCT1 promoter has several NFAT binding sites and Murray et al. found that MCT1 is significantly upregulated in activated T-lymphocytes [77,145]. NFAT in turn is an important transcription factor in PAH and already has been therapeutically targeted [22,39]. High myc expression was associated with MCT1 expression in cancer cells [47,48]. Myc is a transcription factor inducing various genes that are involved in proliferation of the cells and promotes the Warburg effect in cancer [13,84]. Induced myc activity is described in PAH [36,189]. Doherty et al. found that upon stimulation of myc transcription factor in lymphoma cells, MCT1 mRNA and protein expression is significantly induced. Using chromatin immunoprecipitation (ChIP) they showed, that myc is binding to MCT1 promoter region in normal and tumor cells [48]. Limited by the use of wildtype hPASMCs, our results indicate that TNFα, TGFβ and PDGF are not influencing MCT1 expression in hPASMCs. As explained, we consider NFAT, myc and p53 as promising candidates inducing MCT1 expression in hPASMCs. However, it needs to be confirmed that MCT1 is upregulated in hPASMCs at all, as we were unable to induce MCT1 expression in hPASMCs. It is conceivable that hPASMCs primarily rely on MCT4. Nonetheless, MCT1 remains a relevant transporter at least in PAH pathophysiology, indicated by the significant overexpression of MCT1 in IPAH lung homogenates. The participating cells of MCT1 overexpression in IPAH lungs and the expression of MCT1 in hPASMCs need to be addressed in further studies.

MCT4 protein expression is significantly upregulated upon PDGF treatment in hPASMCs (figure 12, p. 39). Xia et al. showed that PDGF stimulation in PASMCs derived from rat induce a glycolytic phenotype. Accordingly, they found MCT4 protein expression is significantly increased upon PDGF stimulation [240]. They did not further investigate MCT1 expression. Working with PASMCs derived from human, we can confirm the findings from Xia et al. that MCT4 protein expression is significantly induced by PDGF stimulation. Additionally, they found that upon PDGF treatment HIF-1α protein expression is increased. HIF-1α induction by PDGF stimulation is a possible mechanism of MCT4 upregulation in hPASMCs. Interestingly, we found MCT4 mRNA is downregulated by PDGF treatment (figure 11, p. 38). Unfortunately, MCT4 mRNA

expression was not investigated by Xia et al. Decreased mRNA does not preclude upregulation of MCT4 protein expression. Post-transcriptional mechanisms of MCT4 mRNA might influence MCT4 protein expression. Halestrap et al. already suggested that this phenomena occurs with MCT1 and 2 mRNA [77]. For MCT4 mRNA this has not been confirmed yet and is an interesting issue to pursue.

TGFβ stimulation of hPASMCs clearly induces MCT4 expression. The effect was consistently visible on mRNA and protein level (figure 15, 16, p. 41). The cellular effects of TGFβ stimulation are heterogenous, depending on the effected cells. TGFβ signaling may induce inflammation, cell cycle arrest and inhibit proliferation [87]. Especially in heritable forms of PAH mutations of TGFβ signaling receptors (BMPR2) are commonly found [51]. Selimovic et al. found that plasma concentrations of TGFβ are significantly elevated in PAH patients (PAH 26,42 ng/ml, Control 7 ng/ml) [209]. As already mentioned, TGFβ influences the metabolic program in PAH and cancer cells. Accordingly, MCT4 expression was suspected to be TGFβ dependent in a review about the role of TGFβ in cancer [2]. We are able to proof that TGFβ stimulation affects MCT4 mRNA and protein expression. The underlying mechanisms remain unknown and besides our study further evidence is missing.

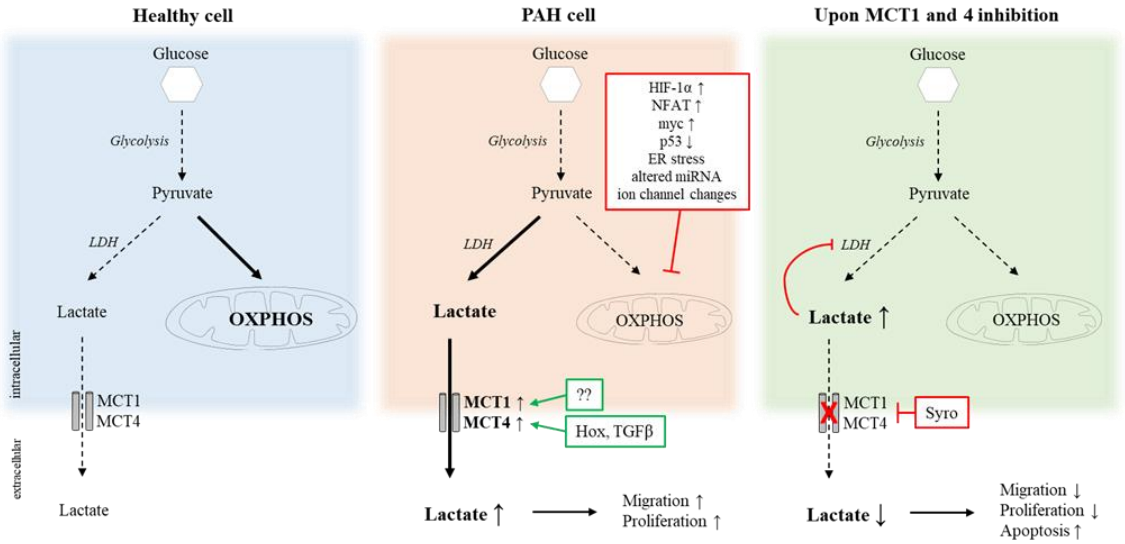

Figure 47 Summarizing scheme of the main findings. LDH – Lactate dehydrogenase, OXPHOS – Oxidative phosphorylation, MCT – Monocarboxylate Transporter, Hox – Hypoxia, TGFβ – Transforming Growth Factor β, Syro = Syrosingopine

Taken together we found MCT1 and 4 is upregulated in IPAH lung homogenates, emphasizing their relevance in the pathogenesis of the disease. MCT4 expression in

hPASMCs is influenced by hypoxia, PDGF and TGFβ dependent stimulatory pathways. Mechanisms influencing MCT1 expression could not be identified. Probably direct p53 alterations, NFAT and myc induction in PH affect MCT1 expression [22,105,234].

4.2 Therapeutic potential of MCT1 and 4 inhibition

PH is still an incurable disease, so there is an obvious demand for novel, potentially curable, therapeutic approaches [85]. Not surprisingly, metabolic alterations have been successfully targeted to treat PH, already [85,189,223]. Reports from cancer research encourage the potential use of MCT1 and 4 inhibitors as novel therapeutic targets [50,55,177]. We analyzed the functional effects of MCT1 and 4 inhibition in hPASMCs stimulated by chronic hypoxic incubation or PDGF. As PH pathobiology is complex, chronic hypoxic or PDGF stimulation obviously cannot reflect the entirety of pathological changes. By promoting the Warburg effect and inducing a hyperproliferative phenotype, both stimulators reflect common features of PAH pathophysiology [207,240]. Recently, Hernandez-Saavedra et al. published TGFβ stimulation recapitulates PH metabolic alterations, as well [92]. Additionally, we found MCT4 expression is induced by TGFβ stimulation. Thus, TGFβ stimulation, combined approaches or *in vivo* studies present possible approaches for future studies.

Using siRNA, we found that knockdown of MCT1 and knockdown of MCT4 successfully prevents hypoxia induced proliferation of hPASMCs (figure 18, p. 43). Although MCT1 expression is not influenced by hypoxic conditions, knockdown of MCT1 had a significant effect. This suggests that MCT1 function is important for hypoxia induced cell proliferation, even though it is not upregulated. The other investigated proliferation stimulus was PDGF. We found PDGF mediated proliferation is not influenced by MCT1 or 4 silencing (figure 19, p. 43). Silencing of MCT4 has no influence on PDGF induced cell proliferation although MCT4 protein expression is significantly increased upon PDGF stimulation (figure 12, p. 39). Which is contrary to the observations of MCT1 function in hypoxic conditions. The results indicate that lactate transporter function is essential for hypoxia induced cell proliferation but not necessary for PDGF induced cell proliferation. The independence of PDGF induced proliferation from lactate transporter function is unexpected, as Xiao et al. showed that PDGF promotes Warburg effect in rat PASMCs [240]. We confirmed an increased lactate release upon hypoxic stimulation but did not evaluate lactate release upon PDGF stimulation. Presumably, PDGF acts

differently in PASMCs derived from human than in rat PASMCs. This would explain their independence of lactate transporters. But as there is contrary evidence, it is more likely that the still functional lactate transporters can compensate the loss of the other isoform. Marchiq et al. state in a review about the therapeutic effect of lactate transporters in cancer, that in cells co-expressing MCT1 and 4, single inhibition is not effective due to functional redundancy [133]. Simultaneous silencing of MCT1 and 4 need to be performed to target this issue. On the other hand, the distinct visible effects in hypoxic conditions upon single MCT silencing cannot be explained by that fact and need to be confirmed by other investigators. Nevertheless, silencing of MCT1 or MCT4 has been used in cancer research already. Choi et al. found that MCT4 knockdown by siRNA inhibits cell proliferation in prostate cancer cell line [32]. Moreover, they associated a decrease in MCT4 protein expression with a decrease in tumor growth using prostate xenograft in mice. Baek et al. investigated the effect of MCT4 depletion in pancreatic adenocarcinoma [7]. They found that silencing of MCT4 resulted in intracellular lactate accumulation, leading to decreased glycolysis and restoring oxidative phosphorylation. Altered metabolism was accompanied with a compromised cell viability and reduced cell growth. As a side effect they observed increased MCT1 protein expression upon MCT4 knockdown. In our experiment, we found MCT1 protein expression is not altered by MCT4 knockdown in hPASMCs (figure 17, p. 42). Additionally, Baek et al. developed a xenograft model of pancreatic adenocarcinoma in mice. Xenografts with silenced MCT4 were significantly smaller, presented lower proliferative capacity and induced apoptosis of the tumor cells. In a breast cancer model Gallagher et al. found that silencing of MCT4 reduces cell migration [63]. Having potential and specific pharmacological inhibitors of MCT1, silencing experiments are not that common as for MCT4 (see 1.2.3) [77]. In glioblastoma patients, high MCT1 expression of the tumor is associated with an impeded survival [139]. Miranda-Gonçalves et al. were able to show that knockdown of MCT1 significantly decreases cell grow *in vitro* and improved survival of glioblastoma xenograft mice [139]. We can confirm a reduced cell proliferation by silencing of MCT1 and 4 in accordance with the reports from cancer research. Consistent with our finding, Miranda-Gonçalves et al. did not observe induced MCT4 protein expression upon silencing of MCT1 (figure 17, p. 42). However, functional effects cannot be completely derived by analyzing the expression only. Silencing of MCT1 in glioblastoma cells was associated with a decreased glucose uptake and lactate release [139]. Suggesting that the effects are due to restoration of cell metabolism. This finding is consistent with the findings of Baek

et al. that knockdown of MCT4 effects cell metabolism. If having enough glucose fuel, an advantage of the Warburg effect is, that substrates for biosynthetic pathways are abundant [103]. Impeded Warburg effect by knockdown of MCT1 or MCT4 assumably decelerate cell growth by reducing the availability of building substrates. This effect is not exclusive for knockdown experiments, it was also shown for pharmacological treatment [128,157].

Different pharmacological compounds are described to inhibit MCT1 and 4 separately or collectively (see 1.2.3). AZD3965, developed by AstraZeneca, is a compound potently inhibiting MCT1 [40]. This compound showed great potential in various *in vitro* and *in vivo* cancer studies [133]. A phase I/II trial (NCT01791595) in patients suffering from diffuse large B-cell lymphoma (DLBCL) and Burkitt lymphoma additionally highlights the potential therapeutic benefit of MCT1 inhibition [55]. First results of the trial showed promising efficacy in late state DLBCL patients and a good tolerability [82]. Encouraged by the particularly good data, we treated hPASMCs with AZD3965 to inhibit MCT1. However, pharmacological inhibition of MCT1 in hPASMC did not reduce hypoxia or PDGF induced proliferation (figure 21, 22, p. 45). Based on the silencing experiments, we expected absent inhibitory potential in PDGF treated hPASMCs. Regarding chronic hypoxia induced proliferation this result is contrary to our results from knockdown experiments. Raising the demand for an explanation of the contrary results. Le Floch et al. found, while working with another specific MCT1 inhibitor (AR-C155858) in fibroblasts, that cells co-expressing MCT4 are resistant to pharmacological MCT1 inhibition, just as previously described for silencing experiments [128]. Another study by Quanz et al. screened 246 cancer cell lines for the efficacy of a different MCT1 inhibitor (BAY-8002) [190]. Likewise, they found, as a marker for sensitivity of treatment, a lack of MCT4 expression. Curtis et al. confirmed this finding for AZD3965 using different cancer cell lines [40]. The resistance to AZD3965 treatment by functional redundancy is even supported by an upregulation of MCT4 expression upon AZD3965 treatment as Bola et al. and Doherty et al. observed [20,48]. In this work, we did not control MCT4 expression upon AZD3965 treatment. Coming back to the study, published from Miranda-Gonçalves et al., about silencing of MCT1 in glioblastoma cells, the investigated glioblastoma cells were expressing MCT4 protein, confirmed by immunoblot [139]. Still, the tumor growth was significantly reduced by MCT1 knockdown. It begs the question whether MCT4 expression is a resistance factor to pharmacological MCT1 inhibition but

not to knockdown approaches. This hypothesis is supported by our results. However, the underlying mechanisms remain unknown. In consequence of MCT1 and 4 co-expression in hPASMCs we conclude that single pharmacological inhibition of MCT1 represent no promising therapeutic approach. Whether this finding can be expanded to the other affected cell types in PAH depends on the cell specific expression of MCT1 and 4. But the overall expression profile in IPAH lung homogenates suggests that single pharmacological inhibition of MCT1 generally represents no potential therapeutic approach for PAH.

Next, we wanted to investigate the functional effects of pharmacological MCT1 and 4 inhibition. There is no specific MCT4 inhibitor commercially available. Some authors claim AstraZeneca recently developed a specific MCT4 inhibitor but it is not commercially available yet [55,133]. A study of Benjamin et al. aroused our interest. Screening 1120 compounds in murine 6.5 mast cells for synergistic therapeutic efficacy with metformin, they came up with syrosingopine having the best efficacy [11]. This compound is derived from a clinically approved antihypertensive drug, reserpin. Syrosingopine together with metformin treatment resulted in a reduced cell grow in 35 of 43 investigated cell lines accompanied by induction of apoptosis. Using an *in vivo* liver cancer xenograft model, syrosingopine and metformin treatment synergistically showed great efficacy by reducing tumor growth. Benjamin et al. were able to show that the effect of syrosingopine is due to MCT4 and MCT1 inhibition [12]. Syrosingopine blocks MCT4 with a higher affinity using low concentrations and additionally MCT1 when using high concentrations (MCT4 IC_{50} 40 nM, MCT1 IC_{50} 2,5 µM) [12]. Besides MCT inhibition, syrosingopine binds to VMAT, the reason why it was used as an antihypertensive drug. Benjamin et al. could show that the observed cellular effects are independent of VMAT inhibition [11]. Screening different cancer cell lines, they found that dual inhibition of MCT1 and 4 is most promising to kill cancer cells, which is consistent with previously mentioned data. Encouraged by their findings we investigated the therapeutic efficacy of syrosingopine using hPASMCs. We found that syrosingopine treatment significantly reduces pathologically induced proliferation by chronic hypoxic stimulation in hPASMCs (figure 24, p. 47). Moreover, even PDGF induced proliferation is significantly reduced by syrosingopine, in contrast to our previous approaches (figure 25, p. 48). We ensured that the used concentrations are not explained by cytotoxic side effects of the compound (figure 23, p. 46). Of note, higher concentrations of up to 5-10 µM are necessary to get a

profound effect. The effective concentrations indicate a dual inhibition of MCT1 and 4. Thus, confirming the finding from Benjamin et al. that dual inhibition of MCT1 and 4 is necessary to kill cancer cells. Referring to Benjamin, lower effective concentrations can be achieved when combining with one of the existing highly affinitive MCT1 inhibitor. However, as there is no previous evidence using syrosingopine in hPASMCs we decided to start with sole syrosingopine treatment. Combined pharmacological treatment represents a promising approach for future investigators. Another study by Saulle et al. showed that syrosingopine treatment *in vitro* impairs leukemic cell proliferation and is additive to chemotherapeutic agents [205]. Our results confirm the antiproliferative efficacy of syrosingopine treatment in hPASMCs. When comparing syrosingopine treatment with silencing experiments, contrary results were observed. Single knockdown of MCT4 effectively reduced cell proliferation in hypoxic conditions. However, low concentrations of syrosingopine, indicating single MCT4 inhibition, did not diminish cell growth. This observation is consistent with single MCT1 inhibition. Knockdown and pharmacological inhibition of MCT1 and 4 have divergent effects in hPASMCs. Incomplete inhibition of the lactate transporters or effects that are not depending on transport activity may contribute to the differences.

Besides cell proliferation further functional effects of syrosingopine treatment in hPASMCs were investigated. We found that upon syrosingopine treatment cell migration is significantly reduced in hypoxic conditions (figure 26, p. 49). Proper MCT4 function was previously described to be essential for cancer cell migration [47]. Our results confirm findings from Gallagher et al. that knockdown of MCT4 reduces cell migration [63]. Heightened concentration of extracellular lactate leads to an increased cell migration in various cancer cell lines, as reviewed by Hirschhaeuser et al. [96]. Droui et al. found that increased migration is promoted via lactate dependent induction of NF-κB and VEGF [50]. We found syrosingopine treatment leads to a significant reduction of extracellular lactate concentrations (figure 28, p. 51). It is important to recognize lactate as more than the end-product of glycolysis. The multiple effects of lactate in cancer are reviewed by Dhup et al. [45]. Hashimoto et al. found lactate to induce 673 genes in a rat myoblast cell lines [86]. Our results indicate that reduced cell migration upon syrosingopine treatment is achieved by successful inhibition of lactate transport, further supported by impeded paracrine effects of lactate. Additionally, the effect of syrosingopine exposure on cell fate was investigated. We found that syrosingopine

treatment results in a significantly induced apoptosis of hPASMCs in hypoxic conditions (figure 27, p. 50). Increased apoptosis upon syrosingopine treatment was described in cancer cells by Benjamin et al. [11]. Saulle et al. confirmed the increased apoptosis upon syrosingopine treatment [205]. In the already cited work from Baek et al., single knockdown of MCT4 induced apoptosis in pancreatic cancer cells [7]. In contrast, Polański et al. found single MCT1 inhibition by AZD3965 induced cell death in AZD3965 sensitive cells, but in a non-apoptotic and non-autophagic manner [181]. They assumed a necrotic cell death, whereas Saulle et al. observed autophagic cell death while necrosis was absent upon syrosingopine treatment [205]. Previous observations consistently attribute an effect on cell fate upon MCT1 or 4 inhibition. We confirm altered cell fate by dual inhibition of MCT1 and 4 via induction of apoptosis. Based on the cytotoxicity experiments we consider necrotic cell death for unlikely (figure 20, p. 44; figure 23, p. 46).

Benjamin et al. state that mere lactate transport inhibition is only cytostatic [12]. Affected cells prevent from intracellular lactate accumulation as a consequence of inhibited lactate export by restoring oxidative phosphorylation. The cells thereby can escape from certain death. Benjamin et al. confirmed this by inhibiting oxidative phosphorylation using metformin or other ETC inhibitors. Inhibition of oxidative phosphorylation significantly improves efficacy of syrosingopine treatment [11,12]. The same escape mechanism is described by Baek et al. [7]. By no means the syrosingopine treatment is thereby futile, but possibly it’s not curative. Indeed, our experiments represent a preventive approach and thereby confirm a preventative effect of syrosingopine treatment. To clarify, if syrosingopine treatment can reverse pathologic alterations and eventually cure the disease, other experimental set-ups are necessary. Anyway, Benjamin et al. already give the solution with metformin representing a ready to use combination therapy.

Taken together we found that at least one functional lactate transporter, MCT1 or 4, is essential for hPASMC proliferation in disease mimicking conditions. High concentrations of syrosingopine, indicating dual inhibition, effectively reduce pathologic cell proliferation and migration via lactate transport inhibition, inducing apoptosis and preventing paracrine effects of lactate. Of note, dual inhibition of MCT1 and 4 is essential, confirming previous publications claiming, that the isoforms can compensate for the loss of the other. Based on our *in vitro* findings, we conclude that syrosingopine is a promising therapeutic compound to treat PAH.

4.3 Circulating MCT1 and 4 as a PH biomarker

Discovery of novel biomarkers with diagnostic or prognostic power is of big clinical interest [15,129]. As we found MCT1 and 4 expression is upregulated in the lungs of IPAH patients, we measured plasma concentrations of MCT1 and 4 in blood samples derived from non-PH and PH patients to investigate their potential use as disease biomarkers. In contrast to the increased protein expression of lung homogenates, we found circulating plasma concentrations of MCT1 and 4 are not elevated in patients suffering from PH (figure 32, p. 57; figure 39, p. 64). The result is consistent comparing the PH subgroups. Circulating MCT1 and 4 does not differ among the PH groups (figure 33, p. 57; figure 40, p. 64). We ensured that age or gender are not influencing MCT1 or 4 plasma concentration (figure 34, table 10, p. 58; figure 41, table 13, p. 65). The non-PH patients, serving as control group, were suffering from different underlying diseases. Objectified by WHO-FC and 6MWD, the non-PH patients were obviously not healthy (table 8, p. 53). Otherwise, they would not be suspicious for PH and undergo RHC. Possibly, a significant difference in circulating MCT1 or 4 is thereby concealed. Although there is only limited data about the plasma concentrations of MCT1 and 4, the tissue expression in various cancer entities is correlating with the severity of the disease and the prognosis of the patients, which is comprehensively reviewed by Pinheiro et al. [177]. Instead of investigating tissue expression, we consciously decided to evaluate plasma concentrations, as our aim was to discover a clinically useful, non-invasive biomarker. Iizuka et al. discovered MCT1 in human circulation [113]. They found skeletal muscle to release MCT4 into the cell medium upon stimulation [112]. Additionally, studying microglia in brain, Potolicchio et al. found MCT1 is secreted in exosomes by microglia upon activation [183]. The transition from plasma bound to circulating MCTs is still unknown. We assumed that cells from pulmonary vasculature, stimulated by the disease conditions, increasingly release MCT into systemic circulation.

Unfortunately, Iizuka et al. did not determine the concentration of the detected MCT1 and in literature there are no published reference values about physiological circulating plasma concentrations of MCT1 or 4. The only comparative values are given by the supplier of the ELISA kit (mybiosource). They measured circulating MCT1 and 4 in peripheral blood samples derived from apparently healthy subjects. No further information about their health status is known. The values are shown in table 15.

Table 15 MCT1 and 4 plasma concentrations in the studies and supplier collective

	study collective				mybiosource collective	
	non-PH		PH		Healthy individuals	
	Mean	Range	Mean	Range	Mean	Range
MCT1 [ng/ml]	4,88	1,0–13,91	5,79	0,37-19,85	3,62	1,34-5,23
MCT4 [ng/ml]	20,68	12,14-34,88	19,25	7,74-40,94	7,68	3,56-12,13

The reported concentrations from the company are lower than the ones we measured. Particularly, we observed a broader range. This suggests that when comparing PH patients to a truly healthy control group a significant difference might be found. Even though plasma concentrations of healthy patients are interesting, our demand on a diagnostic PH biomarker is to distinguish PH patients from patients who are suspicious for PH. Therefore, we still consider our result as reliable and conclude that circulating MCT1 and 4 represent no promising diagnostic biomarker. Further factors might potentially influence the measured plasma concentrations. The use of central venous blood samples in comparison to peripheral blood samples, as the supplier tested, has to be considered. Moreover, erythrocytes express MCT1 [76]. It is essential to prevent hemolysis. We cannot exclude presence of hemolysis in the investigated samples. Hemolysis in some samples could account for the outliers and the broad range of MCT1 distribution.

In addition to a diagnostic use, the potential as a biomarker for the severity of the disease was investigated. Therefore, MCT1 and 4 plasma levels were analyzed in dependence of functional data like (WHO-FC, 6WMD) and hemodynamic data (mPAP, PVR, PAWP, CI) derived from the same hospital stay. In none of the cases a significant association with MCT1 or 4 plasma concentrations were found (table 11, p. 60; table 14, p. 66). Whereas, compared to MCT1 and 4, BNP plasma levels were superiorly associated with severity indicating parameters. We found that MCT1 and 4 plasma concentrations are not reflecting the severity of the disease. Moreover, our results indicate MCT1 and 4 are inferior to the currently used biomarker BNP and do not deliver additional information value.

The prognostic usage of circulating MCT1 and 4 was investigated as well. Therefore, survival data of all PH patients were surveyed, whenever possible. In the investigated patient collective IPAH patients had a 5 year survival of 61,8% and CTEPH patients of 64,1% (figure 48, 49, p. 90). Gall et al. report the survival of IPAH and CTEPH patients

from the “The Giessen Pulmonary Hypertension Registry” [62]. In IPAH patients they observed a 5 year survival of 65,7% and CTEPH patients of 66,7%. We did not observe a strikingly different survival. However, the IPAH patients included in our study were having a worse outcome. The patient recruitment timepoints differed (Giessen Registry: 1993 to 2011, our collective: 2008 to 2017). You would rather expect an improved outcome in the novel cohort with improving therapeutic possibilities. Obviously, this is not the case in our observed cohort. Previous cohorts may include non-IPAH patients from today’s point-of-view, as diagnosis and definitions are getting more accurate. On the other hand, the published confirms an improved outcome of IPAH patients over time [60]. Probably the smaller size of our collective causes the observed survival differences, as Gall et al. investigated a bigger population with 294 IPAH and 459 CTEPH patients. Therefore, we still consider our survival data as representative. We found the survival of the overall PH patients did not differ depending on MCT1 or MCT4 concentration (figure 35, 36, p. 61; figure 42, 43, p. 67). Only when evaluating survival depending on MCT4 within IPAH subgroup a trend, close to significance, is visible (figure 44, p. 68). Indicating that patients with low circulating MCT4 values have an impeded survival, which is contrary to our hypothesis of an induced MCT4 secretion by pulmonary vasculopathy. This finding should not be overrated, as statistic evaluation is not significant and the effect is only visible in one particular analysis (dichotomy MCT4 separation in IPAH subgroup). Taken together, we do not consider circulating plasma concentration of MCT1 and 4 as promising prognostic biomarker. In contrast tissue expression of MCT1 and 4, analyzed by immunohistochemistry, is associated with patient prognosis as found in various cancer entities. To give some examples, high MCT1 or 4 expression predicts poor prognosis in pancreatic [7], prostate [32,172] or lung carcinoma [52,181,199]. The findings are comprehensively reviewed in the already cited work by Pinheiro et al. [177]. Again, the knowledge is based on work derived from cancer research, but it seems that high expression of tissue bound MCT1 and 4 is associated with a poor prognosis, while circulating concentration is not. Based on the trend of an impeded survival of IPAH patients with low circulating MCT4 level (figure 44, p. 68), it is conceivable that MCT4 is rather kept at the cell membrane and less released into systemic circulation.

Another explanation for our results are the uncertainties of measuring circulating MCT1 and 4. A general issue when measuring MCT1 and 4 is the conserved and thereby similar

structure of the isoforms [77]. Only the N- and C-terminal ends, as well as the loop region between transmembrane domain 6 and 7, notably distinguish [80]. The challenge is that the differing structures are found intracellular [80]. According to the supplier, the antibodies, used in MCT1 and 4 ELISA kits, recognize an epitope that is localized at the C-terminal end. We cannot distinguish at which structure, shredded or membrane bound, MCT1 and 4 were measured in systemic circulation. Whether the circulating epitope is accessible for the antibodies in circulating MCT1 and 4 is an issue that needs to be addressed. We found MCT1 and 4 plasma concentrations are strongly associated with each other (figure 46, p. 70). This may indicate the simultaneous expression of MCT1 and 4 is a consequence of the glycolytic phenotype. The simultaneous upregulation of MCT1 and 4 is supported by our finding that MCT1 and 4 are both upregulated in IPAH lung homogenates (figure 7, p. 34; figure 8, p. 35)

Limited by the issue of recognized epitope and the unknown mechanisms leading to the release into systemic circulation, a lot more work is necessary to evaluate the usage of MCT1 and 4 as biomarker. From our present findings, we conclude that circulating MCT1 and 4 are no promising diagnostic or prognostic biomarkers for IPAH or CTEPH patients.

4.4 Limitations

During the discussion some limitations of the study were already addressed. At this point they will be recapitulated.

Lung homogenates were used to evaluate MCT1 and 4 expression. Working with homogenates results in a cell mixture. By qPCR and western blot, the overall expression across the entirety of homogenized cells is evaluated. It still needs to be confirmed that MCT1 and 4 are upregulated in the pulmonary vasculature, as we assume. For example, by using immunohistochemistry. Besides differentiation of pulmonary vessels and lung interstitial tissue, a closer look at the vasculature building cells is desirable. It needs to be clarified whether all cells have an increased MCT1 and 4 expression.

Our *in vitro* studies were focusing on hPASMCs. Certainly, hPASMCs are important in PH pathophysiology and one cell type needs to be first being investigated. Further studies are needed to address the expression and functional effects of MCT1 and 4 inhibition in endothelial cells, fibroblasts and immune cells. Of course, *in vitro* experiments are always limited in reflecting *in vivo* diseases. The use of wildtype hPASMCs neglects some

potentially important alterations, like p53 mutation. More *in vitro* evidence must be generated, especially before heading for *in vivo* experiments. Additionally, the promising effect using syrosingopine in hPASMCs must be confirmed using different stimuli. We worked with hypoxic and PDGF stimulation to induce abnormal proliferation and metabolic alterations. Other cell types and stimuli will aid to confirm the therapeutic principle. Moreover, the changes regarding MCT1 and 4 expression might be time dependent. Significant induction of MCT1 or 4 expression was possibly missed, as the expression was analyzed at a certain timepoint

The study design of the biomarker experiments was retrospective and single-center, resulting in a relevant limitation of this study. The patients undergoing RHC with exclusion of PH served as control group. These patients surely do not represent a healthy control group as they were suspicious for PH and suffered from other underlying diseases. Probably this is the reason why our measured values are higher than the values measured by the supplier in apparently healthy individuals. Including truly healthy individuals as control group is a possible approach. Although patients suffering from cancer were excluded from the experiment, other currently known or still unknown diseases rely on MCT1 and 4 and may influence the results [55]. Presence of hemolysis or insufficient separation of blood plasma can also influence the results, as erythrocytes express MCT1 at their membrane and white blood cells express MCT4 [76,79]. We cannot exclude presence of hemolysis in the evaluated samples, for example due to difficult blood withdrawal. Ultimately, collection of central venous blood is a limitation. The concentrations may differ in peripheral blood samples although this has not been tested. Additionally, collection of central venous blood always relies on an invasive process. Practical blood biomarkers should be easily accessible. Beside pre-analytic issues, the measurement itself by the ELISA kit can cause difficulties. The recognized epitope of the used ELISA antibodies are localized intracellular [80]. It remains unclear in which extend these epitopes are accessible or whether the measured MCT1 and 4 protein really originates from pulmonary vasculature. Another issue might be that prescribed medication influences MCT1 and 4 concentration in the circulation. No presorting of the patients according to their home medication happened. To give an example, it is conceivable, although not proven, that metformin influences MCT1 and 4 in systemic circulation by interacting with oxidative phosphorylation [11,55].

5 Summary

Background: In Pulmonary Hypertension (PH), pulmonary vascular cells favor cytosolic glycolysis instead of oxidative phosphorylation, to cover their energy demand. This feature is also known as Warburg effect. The metabolic change is accompanied by a distinct lactate production. Still, the expression and functional relevance of lactate transporters in PH have not yet been investigated. Studies originated from cancer research highlighted the relevance of the lactate transporters, Monocarboxylate Transporter (MCT) 1 and 4, in disease pathobiology. In this study, the role of MCT1 and 4 in the context of PH is addressed.

Methods: MCT1 and 4 expression was investigated in lung tissue homogenates derived from IPAH and donor patients. *In vitro* experiments were performed in human pulmonary arterial smooth muscle cells (hPASMCs). In hPASMCs the expression profile upon hypoxic and non-hypoxic stimuli was investigated. The effect of MCT1 and/or 4 knockdown or pharmaceutical inhibition, using AZD3965 or syrosingopine, on functional characteristics in hPASMCs were investigated. For this purpose, proliferative capacity, cell migration, apoptosis rate, cell viability and extracellular lactate concentration were determined. The potential of MCT1 and 4 as disease biomarkers were evaluated by measuring the circulating concentration in plasma samples, derived from non-PH and PH patients.

Results: The mRNA and protein expression of MCT1 and 4 were increased in the lungs of IPAH patients compared to donor lungs. Chronic hypoxia, PDGF and TGFβ stimulation induced MCT4 expression in hPASMCs, whereas MCT1 was not influenced by any of the investigated stimuli. Dual inhibition of MCT1 and 4 by syrosingopine reduced lactate export, induced apoptosis and decreased cell proliferation and migration with absent cytotoxic side-effects, in hypoxic conditions. Circulating MCT1 and 4 did not reveal a diagnostic or prognostic benefit as a disease biomarker.

Conclusion: MCT1 and 4 play an important role in PH pathobiology. At least one functional transporter is necessary for disease progression. Dual inhibition of MCT1 and 4 represent a promising therapeutic approach. Circulating MCT1 and 4 are no suitable PH biomarkers.

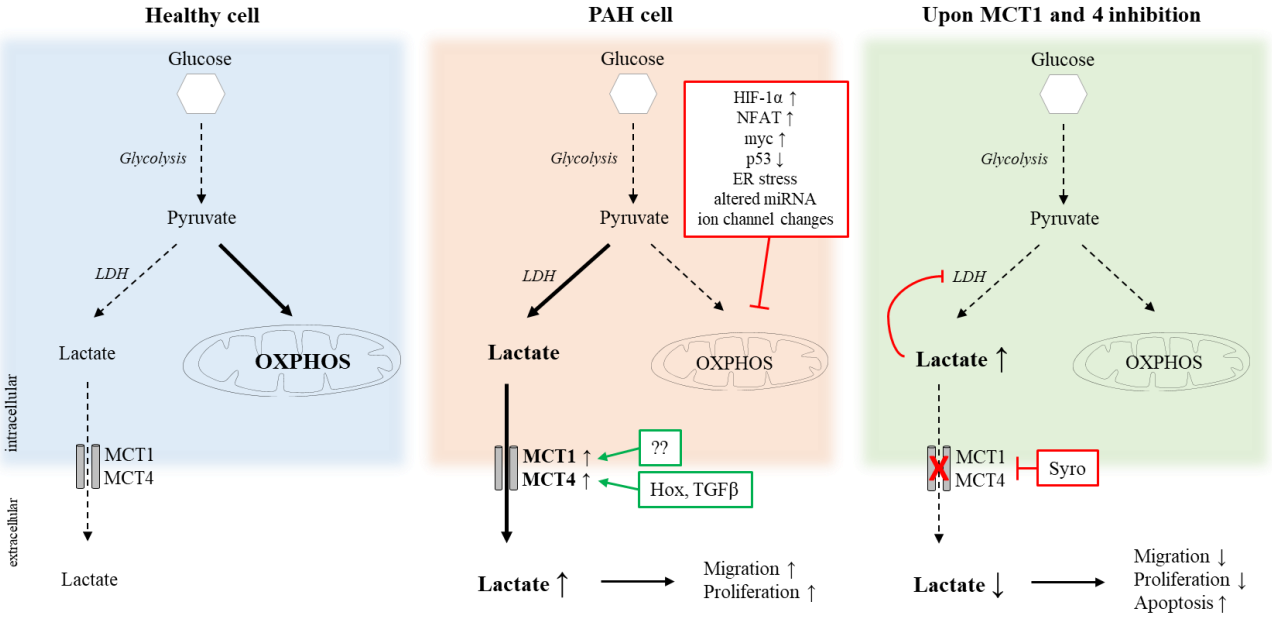

Figure 47 Summarizing scheme of the main findings. LDH – Lactate dehydrogenase, OXPHOS – Oxidative phosphorylation, MCT – Monocarboxylate Transporter, Hox – Hypoxia, TGFβ – Transforming Growth Factor β, Syro = Syrosingopine

6 Zusammenfassung

Hintergrund: Im Rahmen der pulmonalen Hypertonie (PH) generieren die pulmonalen Gefäßzellen ihre Energie vornehmlich durch anaerobe Glykolyse, anstatt durch oxidative Phosphorylierung. Dieses Phänomen ist auch bekannt als Warburg Effekt. Damit geht eine vermehrte Laktatproduktion einher. Dennoch wurde die Expression und funktionelle Relevanz von Laktatransportern im Kontext der PH bisher nicht untersucht. Aus der Krebsforschung ist hingegen die Wichtigkeit der Laktattransporter, Monocarboxylat Transporter (MCT) 1 und 4, bekannt. In dieser Arbeit wird die Rolle von MCT1 und 4 im Kontext der PH genauer beleuchtet.

Methoden: Die Expression von MCT1 und 4 wurde in Lungengewebe von IPAH und Kontrollpatienten bestimmt. Die *in vitro* Experimente wurden in humanen glatten Muskelzellen aus pulmonalen Arterien (hPASMCs) durchgeführt. Die Expression nach hypoxischen und nicht-hypoxischen Stimuli wurde bestimmt. Es erfolgte ein Knockdown von MCT1 und/oder 4 oder eine medikamentöse Inhibition, mittels AZD3965 oder Syrosingopin und der Effekt auf funktionelle Charakteristika wurde untersucht. Dazu wurde die Zellproliferation, -migration, -viabilität, sowie Apoptoserate und die extrazelluläre Laktatkonzentration bestimmt. Die Plasmakonzentrationen von MCT1 und 4 wurden bestimmt, um deren Potential als Biomarker für die Erkrankung zu untersuchen.

Ergebnisse: Die mRNA- und Proteinexpression war in den Lungengeweben von IPAH Patienten erhöht. Chronische Hypoxie, PDGF und TGFβ Stimulation induzierten die Expression von MCT4 in hPASMCs. Wohingegen die Expression von MCT1 durch keine der Stimuli beeinflusst wurde. Eine duale Hemmung von MCT1 und 4 reduzierte den zellulären Laktatexport, steigerte die Apoptoserate und hemmte die Zellproliferation und -migration, ohne zytotoxische Effekte, unter hypoxischen Bedingungen. Die Plasmawerte von MCT1 und 4 zeigten keinen diagnostischen oder prognostischen Nutzen.

Fazit: MCT1 und 4 spielen eine entscheidende Rolle in der Pathobiologie von PH. Für den Krankheitsprogress ist wenigstens ein funktionierender Transporter notwendig. Die duale Hemmung von MCT1 und 4 ist ein vielversprechender Therapieansatz. Die Plasmakonzentrationen von MCT1 und 4 sind keine geeigneten PH Biomarker.

7 Attachment

7.1 Circulating MCT1 and 4 as a PH biomarker

7.1.1 Cumulative survival of IPAH and CTEPH patients

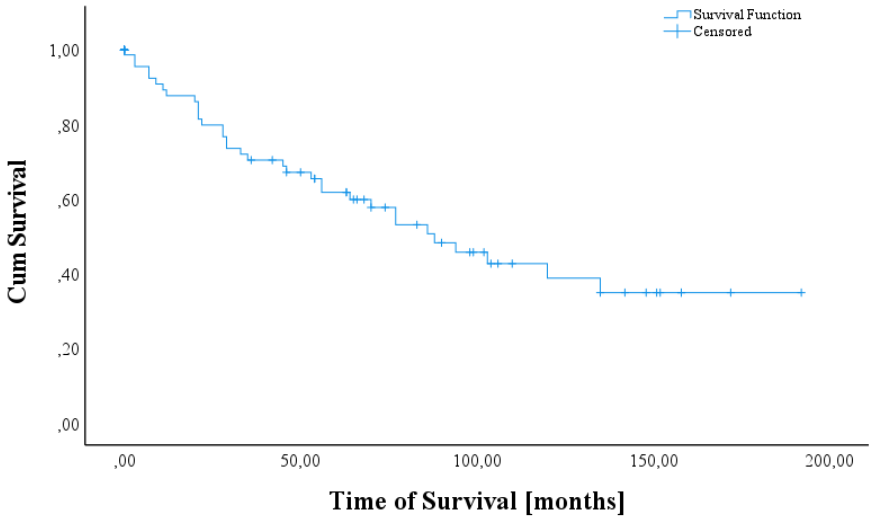

Figure 48 Cumulative survival of IPAH patients; n=75, events=34, censored=41

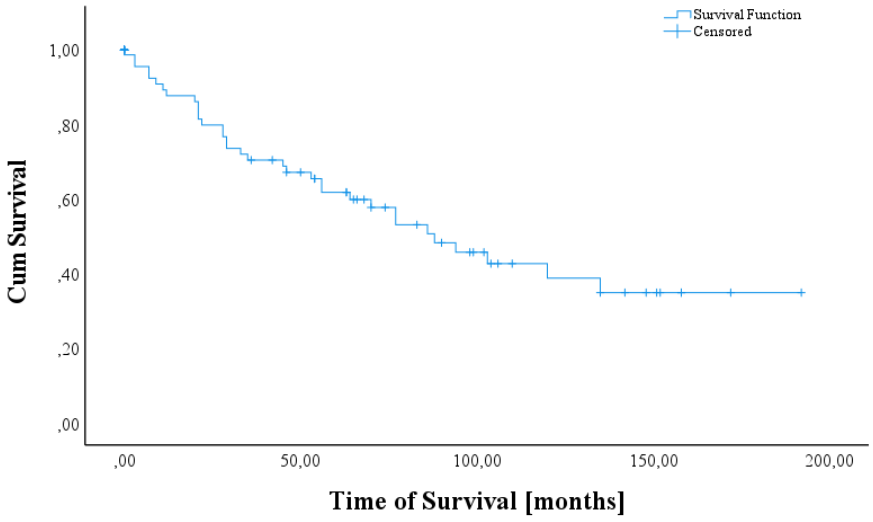

Figure 49 Cumulative survival of CTEPH patients; n=75, events=26, censored=49

7.1.2 MCT1 as a biomarker in IPAH subgroup

7.1.2.1 MCT1 as a biomarker for severity of the disease, age and gender

Table 16 MCT1 concentration by gender and WHO-FC in IPAH subgroup

IPAH		MCT1 [ng/ml]	
		Median [IQR]	Significance
Gender	female	4,89 [3,78]	p=0,536
	male	5,85 [7,42]	
WHO-FC	2	3,44 [3,18]	p=0,322
	3	5,75 [4,80]	
	4	5,59 [6,42]	

Gender: male n=23, female n=47, Mann-Whitney U test of ln_MCT1,
WHO-FC: II n=12, III n=51, IV n=7, Kruskal-Wallis Test of ln_MCT1

Table 17 Correlation of ln_MCT1 in IPAH subgroup

IPAH		ln_MCT1	BNP [ng/l]
Age [years]	Spearman Correlation	,071	,205
	Sig. (2-tailed)	,561	,120
	N	70	59
mPAP [mmHg]	Spearman Correlation	-,169	,290
	Sig. (2-tailed)	,161	,026*
	N	70	59
PVR [dyn*s/cm5]	Spearman Correlation	-,044	,352
	Sig. (2-tailed)	,715	,006*
	N	70	59
CI [l/min)/m²]	Spearman Correlation	,026	-,449
	Sig. (2-tailed)	,829	,000*
	N	70	59
6MWD [m]	Spearman Correlation	-,079	-,210
	Sig. (2-tailed)	,560	,153
	N	57	48
BNP [ng/l]	Spearman Correlation	-,070	1
	Sig. (2-tailed)	,607	.
	N	56	59

** indicates significance*

7.1.2.2 MCT1 as a prognostic biomarker

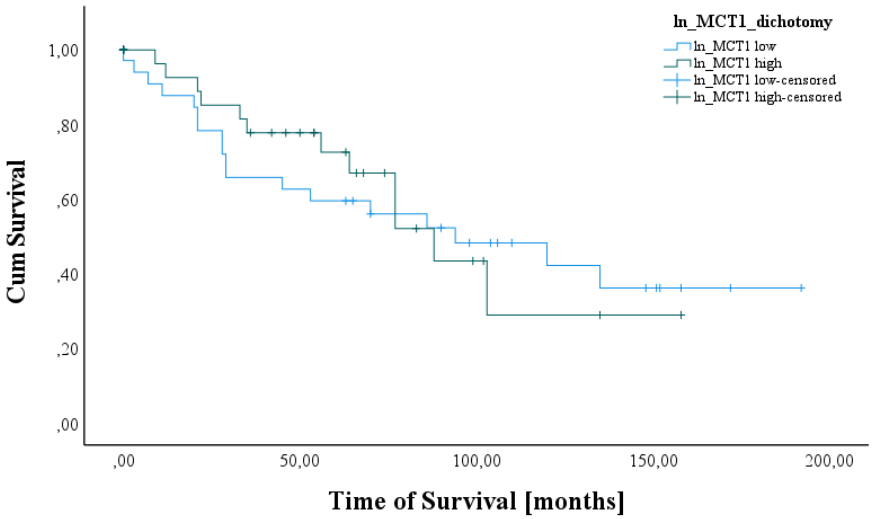

Figure 50 Survival of IPAH patients by dichotom ln_MCT1 separation; ln_MCT1-low: n=35, events=18, ln_MCT1-high: n=35, events=12, Log-Rank test p=0,795

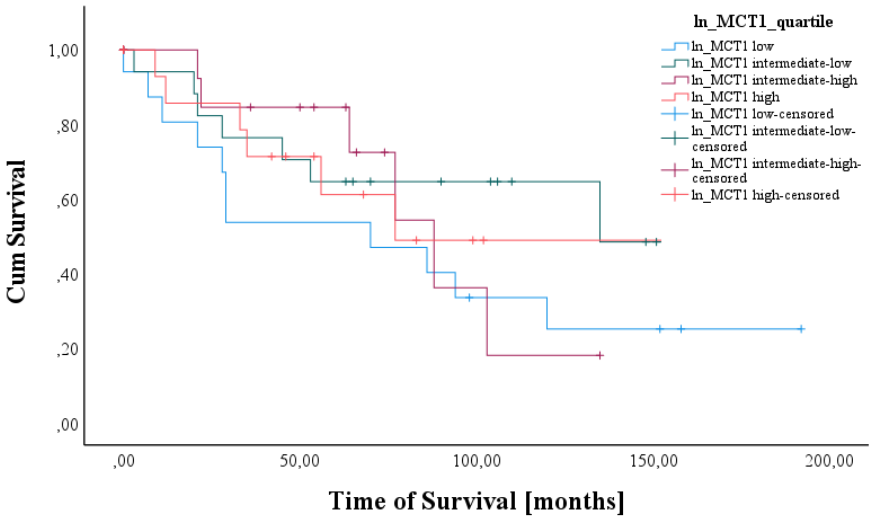

Figure 51 Survival of IPAH patients by quartile ln_MCT1 separation; ln_MCT1-low 1: n=17, events=11, ln_MCT1-intermediate-low: n=18, events=7, ln_MCT1-intermediate-high: n=18, events=6, ln_MCT1-high: n=17, events=6, Log-Rank test p=0,503

7.1.3 MCT1 as a biomarker in CTEPH subgroup

7.1.3.1 MCT1 as a biomarker for severity of the disease, age and gender

Table 18 MCT1 concentration by gender and WHO-FC in CTEPH subgroup

CTEPH		MCT1 [ng/ml]	
		Median [IQR]	Significance
Gender	female	4,81 [4,86]	p=0,828
	male	4,8 [5,44]	
WHO-FC	2	4,85 [6,81]	p=0,322
	3	4,81 [5,57]	
	4	4,52 [4,61]	

Gender: male n=35, female n=35, Mann-Whitney U test of ln_MCT1,
WHO-FC: I n=1, II n=14, III n=39, IV n=9, Kruskal-Wallis Test of ln_MCT1

Table 19 Correlation of ln_MCT1 in CTEPH subgroup

CTEPH		ln_MCT1	BNP [ng/l]
Age [years]	Spearman Correlation	-,262	,098
	Sig. (2-tailed)	,029*	,540
	N	70	41
mPAP [mmHg]	Spearman Correlation	,065	,387
	Sig. (2-tailed)	,591	,012*
	N	70	41
PVR [dyn*s/cm5]	Spearman Correlation	,104	,516
	Sig. (2-tailed)	,393	,001*
	N	70	41
CI [l/min)/m²]	Spearman Correlation	-,049	-,509
	Sig. (2-tailed)	,688	,001*
	N	70	41
6MWD [m]	Spearman Correlation	,127	-,265
	Sig. (2-tailed)	,378	,172
	N	50	28
BNP [ng/l]	Spearman Correlation	,139	1
	Sig. (2-tailed)	,412	.
	N	37	41

** indicates significance*

7.1.3.2 MCT1 as a prognostic biomarker

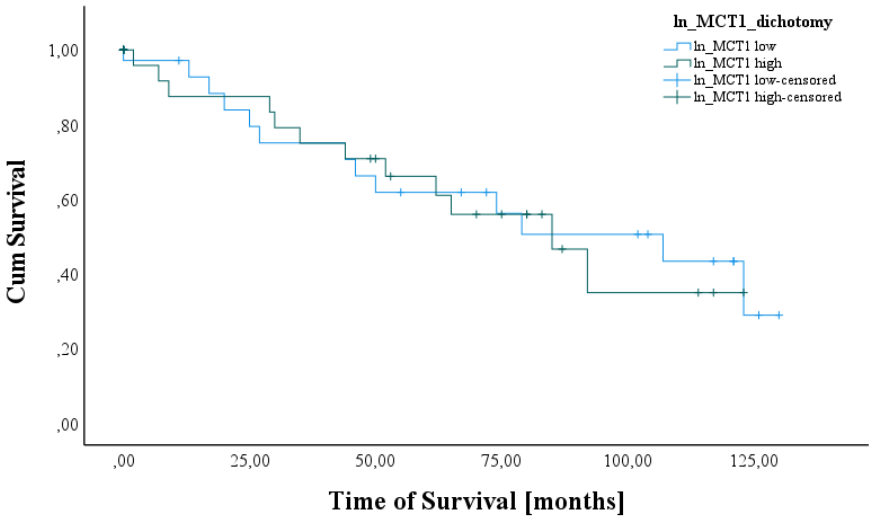

Figure 52 Survival of CTEPH patients by dichotom ln_MCT1 separation; ln_MCT1-low: n=35, events=13, ln_MCT1-high: n=35, events=12, Log-Rank test p=0,984

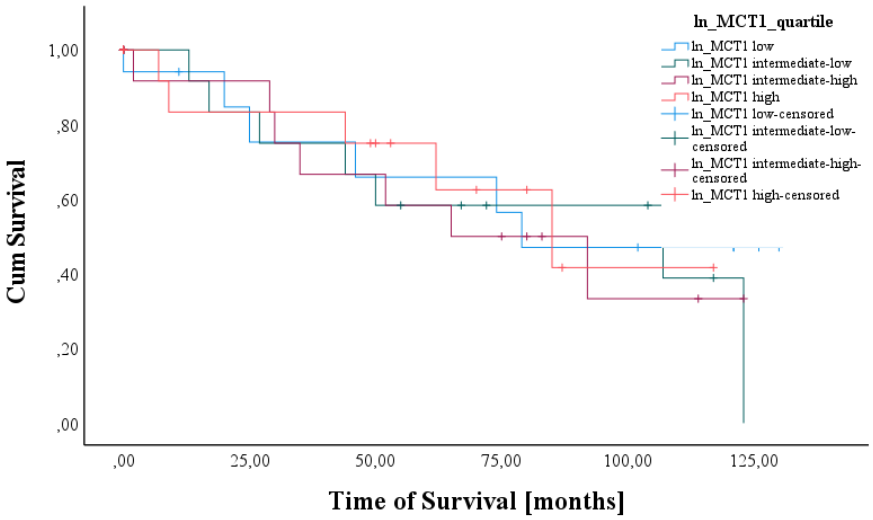

Figure 53 Survival of CTEPH patients by quartile ln_MCT1 separation; ln_MCT1-low: n=17, events=6, ln_MCT1-intermediate-low: n=18, events=7, ln_MCT1-intermediate-high: n=18, events=7, ln_MCT1-high: n=17, events=5, Log-Rank test p=0,963

7.1.4 MCT4 as a biomarker in IPAH subgroup

7.1.4.1 MCT4 as a biomarker for severity of the disease and age and gender

Table 20 MCT4 concentration by gender and WHO-FC in IPAH subgroup

IPAH		MCT4 [ng/ml]	
		Median [IQR]	Significance
Gender	female	19,57 [8,77]	p=0,130
	male	16,13 [7,34]	
WHO-FC	2	19,36 [9,75]	
	3	18,15 [8,31]	p=0,253
	4	22,17 [18,67]	

Gender: male n=25, female n=49, Mann-Whitney U test of ln_MCT4, WHO-FC: II n=12, III n=55, IV n=7, Kruskal-Wallis Test of ln_MCT4

Table 21 Correlation of ln_MCT4 in IPAH subgroup

IPAH		ln_MCT4	BNP [ng/l]
Age [years]	Spearman Correlation	-,023	,205
	Sig. (2-tailed)	,844	,120
	N	74	59
mPAP [mmHg]	Spearman Correlation	-,139	,290
	Sig. (2-tailed)	,237	,026*
	N	74	59
PVR [dyn*s/cm5]	Spearman Correlation	-,042	,352
	Sig. (2-tailed)	,725	,006*
	N	74	59
CI [l/min)/m²]	Spearman Correlation	,045	-,449
	Sig. (2-tailed)	,703	,000*
	N	74	59
6MWD [m]	Pearson Correlation	,085	-,210
	Sig. (2-tailed)	,517	,153
	N	60	48
BNP [ng/l]	Spearman Correlation	-,184	1
	Sig. (2-tailed)	,167	.
	N	58	59

** indicates significance*

7.1.4.2 MCT4 as a prognostic biomarker

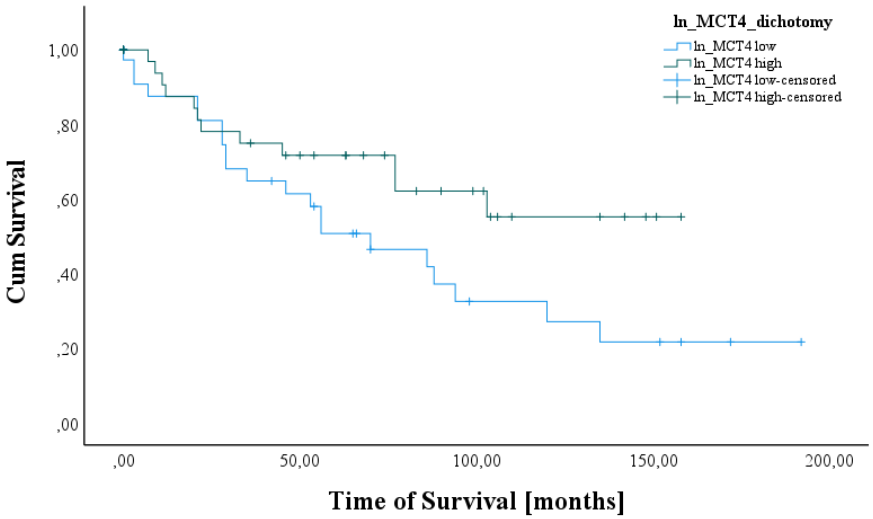

Figure 54 Survival of IPAH patients by dichotom ln_MCT4 separation; ln_MCT4-low: n=37, events=21, ln_MCT4-high: n=37, events=12, Log-Rank test p=0,052

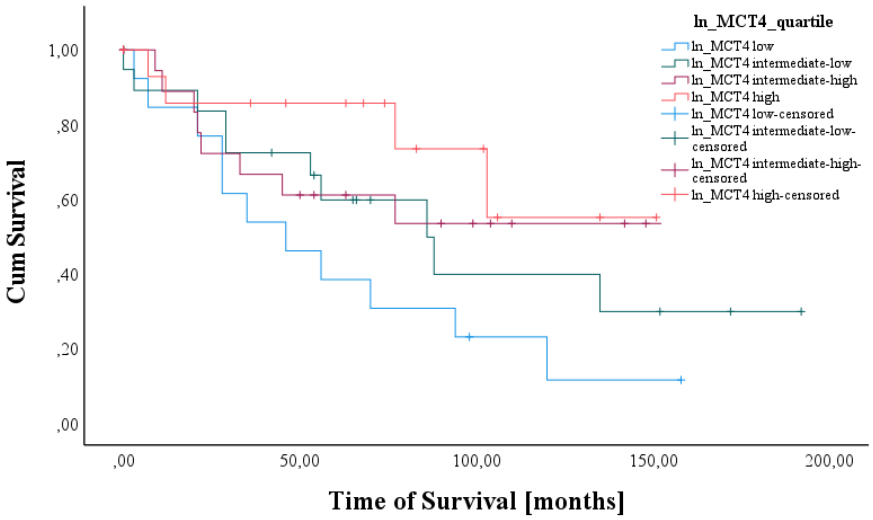

Figure 55 Survival of IPAH patients by quartile ln_MCT4 separation; ln_MCT4-low: n=18, events=11, ln_MCT4-intermediate-low: n=19, events=10, ln_MCT4-intermediate-high: n=19, events=8, ln_MCT4-high: n=18, events=4, Log-Rank test p=0,093

Table 22 Multivariate Cox Regression within IPAH subgroup

			95,0% CI for Exp(B)	
	Sig.	Exp(B)	Lower	Upper
ln_MCT4_dichotomy	,026	2,274	1,105	4,678
Age	,016	1,036	1,007	1,065
PVR	,232	1,001	1,000	1,002
WHO-FC	,085	1,997	,909	4,383

7.1.5 MCT4 as a biomarker in CTEPH subgroup

7.1.5.1 MCT4 as a biomarker for severity of the disease and age and gender

Table 23 MCT4 concentration by gender and WHO-FC in CTEPH subgroup

CTEPH		MCT4 [ng/ml]	
		Median [IQR]	Significance
Gender	female	16,81 [10,11]	p=0,457
	male	18,12 [8,25]	
WHO-FC	2	21,41 [10,11]	p=0,103
	3	16,72 [6,05]	
	4	16,33 [9,97]	

Gender: male n=38, female n=34, Mann-Whitney U test of ln_MCT4,
WHO-FC: I=1, II n=13, III n=41, IV n=10, Kruskal-Wallis Test of ln_MCT4

Table 24 Correlation of ln_MCT4 in CTEPH subgroup

CTEPH		ln_MCT4	BNP [ng/l]
Age [years]	Spearman Correlation	-,168	,098
	Sig. (2-tailed)	,158	,540
	N	72	41
mPAP [mmHg]	Spearman Correlation	,071	,387
	Sig. (2-tailed)	,551	,012*
	N	72	41
PVR [dyn*s/cm5]	Spearman Correlation	,029	,516
	Sig. (2-tailed)	,812	,001*
	N	72	41
CI [l/min)/m²]	Spearman Correlation	-,035	-,509
	Sig. (2-tailed)	,767	,001*
	N	72	41
6MWD [m]	Pearson Correlation	,229	-,265
	Sig. (2-tailed)	,102	,172
	N	52	28
BNP [ng/l]	Spearman Correlation	,277	1
	Sig. (2-tailed)	,088	.
	N	39	41

** indicates significance*

7.1.5.2 MCT4 as a prognostic biomarker

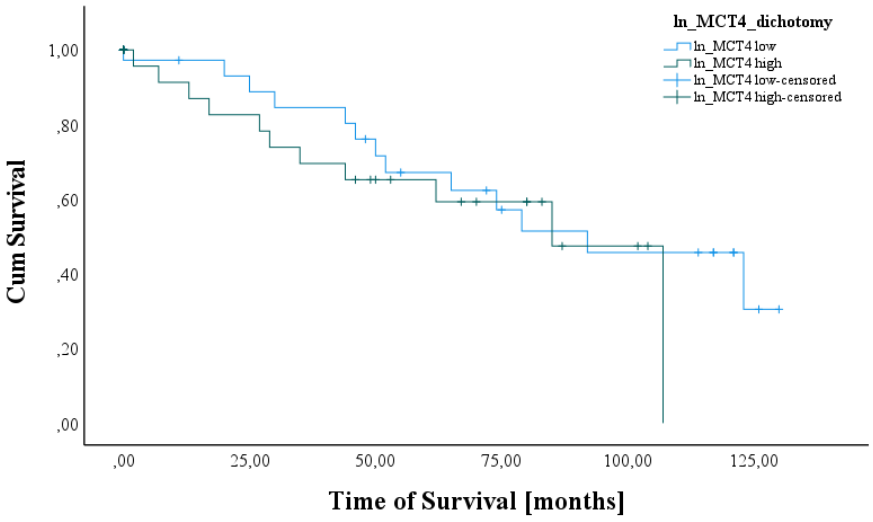

Figure 56 Survival of CTEPH patients by dichotom ln_MCT4 separation; ln_MCT4-low: n=36, events=13, ln_MCT4-high: n=36, events=11, Log-Rank test p=0,601

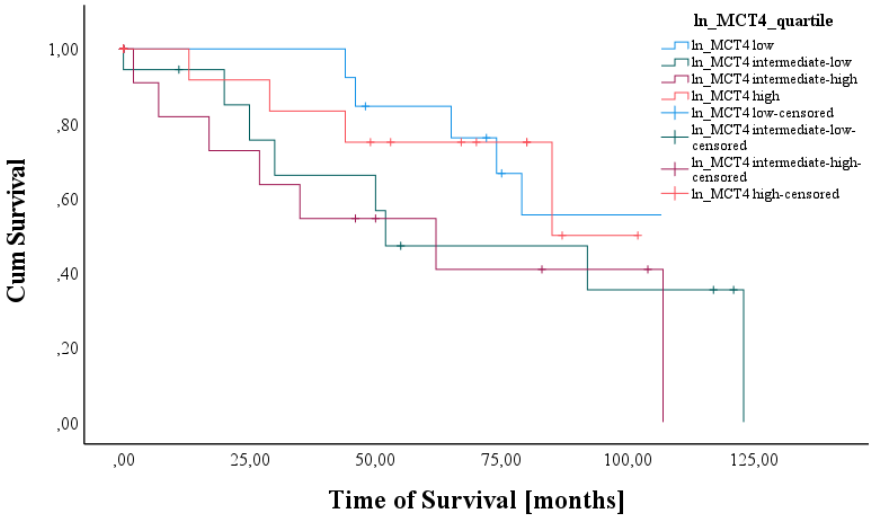

Figure 57 Survival of CTEPH patients by quartile ln_MCT4 separation; ln_MCT4-low: n=18, events=5, ln_MCT4-intermediate-low: n=18, events=8, ln_MCT4-intermediate-high: n=18, events=7, ln_MCT4-high: n=18, events=4, Log-Rank test p=0,149

8 List of abbreviations

6MWD	6 minute walk distance
AMP	Adenosine monophosphat
AMP-K	AMP activated protein kinase
ATP	Adenosine triphosphate
BMPR2	Bone Morphogenetic Protein Receptor type II
BNP	Brain Natriuretic Peptide
BOEC	Blood Outgrowth Endothelial Cells
BrdU	Bromdesoxyuridin
CCB	Calcium Channel Blocker
cGMP	cyclic Guanosine Monophosphate
CHC	α-cyano-4-hydroxycinnamate
ChIP	Chromatin Immunoprecipitation
CO	Cardiac Output
COPD	Chronic Obstructive Pulmonary Disease
CPFE	Combined Pulmonary Fibrosis and Emphysema
Ct	Cycle of threshold
CTEPH	Chronic Thromboembolic Pulmonary Hypertension
DCA	Dichloroacetate
DIDS	diisothiocyanostilbene-2.2'-disulphonate
DLBCL	Diffuse Large B-cell Lymphoma
DMSO	Dimethylsulfoxid
DNA	deoxyribonucleic acid
DPG	Diastolic Pressure Gradient
EC	Endothelial Cell
EDTA	Ethylendiamintetraacetate
EGF	Epidermal Growth Factor
ELISA	Enzyme-linked Immunosorbent Assay
eNOS	Endothelial Nitric Oxide Synthase
ERA	Endothelin Receptor Antagonists
ERC	European Reseach Council
ERS	European Respiratory Society
FC	Functional Class
FDG	18F-fluordeoxyglucose
FGF	Fibroblast Growth Factor
Hb	Hemoglobin
HIF-1α	Hypoxia inducible Factor 1α
Hox	Hypoxic incubation
HPAH	Heritable Pulmonary Arterial Hypertension

hPASMC	human Pulmonary Arterial Smooth Muscle Cells
HRP	Horseradish peroxidase
IL	Interleukin
ILD	Interstitial Lung Disease
IPAH	Idiopathic Pulmonary Arterial Hypertension
IQR	Interquartile Range
K_m	michaelis constant
K_v	Voltage-gated potassium channels
LDH	Lactate dehydrogenase
LHD	left heart disease
MCT	Monocarboxylate Transporter
miRNA	microRNA
mPAP	mean Pulmonary Arterial Pressure
NCBI	National Center for Biotechnology Information
NFAT	Nuclear Factor of activated T-cells
NF-κB	Nuclear Factor kappa-light-chain-Enhancer of activated B-cells
NO	Nitric Oxide
Nox	Normoxic incubation
OXPHOS	Oxidative phosphorylation
PA	Pulmonary Artery
PAH	Pulmonary Arterial Hypertension
PASMC	Pulmonary Arterial Smooth Muscle Cell
PAWP	Pulmonary Arterial Wedge Pressure
PBGD	Porphobilinogen deaminase
PBS	Phosphate buffered saline solution
PCR	Polymerize Chain Reaction
PDE-5	Phosphodiesterase-5
PDGF	Platelet-Derived Growth Factor
PDK	Pyruvate dehydrogenase kinase
PEA	Pulmonary Endarteriectomy
PH	Pulmonary Hypertension
PH-LHD	Pulmonary Hypertension due to Left Heart Disease
PMSF	Phenylmethylsulfonylfluorid
PRA	Prostacyclin Receptor Agonists
PVDF	Polyvinylidene difluoride
PVR	Pulmonary Vascular Resistence
RA	Right Atrium
RAP	Right Atrial Pressure
RFU	Relative Fluorescence Units
RHC	Right Heart Chátheter

RIPA	Radioimmunoprecipitation assay
RPE	Retinal Pigment Epithelium
RV	Right Ventricle
scRNA	scramble siRNA
SD	Standard deviation
SDS PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
sGC	soluble Guanylate Cyclase
siRNA	small interfering RNA
SLC16	Solute Carrier 16
SMCT	Sodium coupled Monocarboxylate Transporter
SOD2	Superoxide dismutase 2
sPAP	systolic Pulmonary Arterial Pressure
SSP	Staurosporine
TAE	TRIS-Acetate-EDTA
TAPSE	Tricuspid Annular Plane Systolic Excursion
TAT1	t-type amino acid transporter
TGF-β	Transforming Growth Factor-β
TM	Transmembrane
TNFα	Tumor Necrosis Factor α
TPG	Transpulmonary Pressure Gradient
TRV	Tricuspid Regurgitation Velocity
TTE	Transthoracic Echocardiography
V/Q	Ventilation/Perfusion
VEGF	Vascular Endothelial Growth Factor
VMAT	Vesicular Monoamine Transporter
WHO	World Health Organisation
WU	Wood Units

9 List of figures

10 List of tables

11 References

1. Altenberg B, Greulich KO. Genes of glycolysis are ubiquitously overexpressed in 24 cancer classes. *Genomics*. 2004;84:1014–1020.
2. Angioni R, Sánchez-Rodríguez R, Viola A, Molon B. TGF-β in Cancer: Metabolic Driver of the Tolerogenic Crosstalk in the Tumor Microenvironment. *Cancers (Basel)*. 2021;13.
3. Anwar A, Ruffenach G, Mahajan A, Eghbali M, Umar S. Novel biomarkers for pulmonary arterial hypertension. *Respir Res*. 2016;17:88.
4. Archer SL, Marsboom G, Kim GH, Zhang HJ, Toth PT, Svensson EC, Dyck JRB, Gomberg-Maitland M, Thébaud B, Husain AN, Cipriani N, Rehman J. Epigenetic attenuation of mitochondrial superoxide dismutase 2 in pulmonary arterial hypertension: A basis for excessive cell proliferation and a new therapeutic target. *Circulation*. 2010;121:2661–2671.
5. Archer SL, Weir EK, Wilkins MR. Basic science of pulmonary arterial hypertension for clinicians: new concepts and experimental therapies. *Circulation*. 2010;121:2045–2066.
6. Assad TR, Hemnes AR. Metabolic Dysfunction in Pulmonary Arterial Hypertension. *Curr Hypertens Rep*. 2015;17:20.
7. Baek G, Tse YF, Hu Z, Cox D, Buboltz N, McCue P, Yeo CJ, White MA, DeBerardinis RJ, Knudsen ES, Witkiewicz AK. MCT4 defines a glycolytic subtype of pancreatic cancer with poor prognosis and unique metabolic dependencies. *Cell Rep*. 2014;9:2233–2249.
8. Barst RJ, McGoon M, Torbicki A, Sitbon O, Krowka MJ, Olschewski H, Gaine S. Diagnosis and differential assessment of pulmonary arterial hypertension. *J Am Coll Cardiol*. 2004;43:40S-47S.
9. Baumann F, Leukel P, Doerfelt A, Beier CP, Dettmer K, Oefner PJ, Kastenberger M, Kreutz M, Nickl-Jockschat T, Bogdahn U, Bosserhoff A-K, Hau P. Lactate promotes glioma migration by TGF-beta2-dependent regulation of matrix metalloproteinase-2. *Neuro-oncology*. 2009;11:368–380.
10. Beckert S, Farrahi F, Aslam RS, Scheuenstuhl H, Königsrainer A, Hussain MZ, Hunt TK. Lactate stimulates endothelial cell migration. *Wound Repair Regen*. 2006;14:321–324.
11. Benjamin D, Colombi M, Hindupur SK, Betz C, Lane HA, El-Shemerly MYM, Lu M, Quagliata L, Terracciano L, Moes S, Sharpe T, Wodnar-Filipowicz A, Moroni C, Hall MN. Syrosingopine sensitizes cancer cells to killing by metformin. *Sci Adv*. 2016;2:e1601756.
12. Benjamin D, Robay D, Hindupur SK, Pohlmann J, Colombi M, El-Shemerly MY, Maira S-M, Moroni C, Lane HA, Hall MN. Dual Inhibition of the Lactate Transporters MCT1 and MCT4 Is Synthetic Lethal with Metformin due to NAD+ Depletion in Cancer Cells. *Cell Rep*. 2018;25:3047-3058.e4.
13. Bensinger SJ, Christofk HR. New aspects of the Warburg effect in cancer cell biology. *Semin Cell Dev Biol*. 2012;23:352–361.
14. Bergersen LH. Lactate Transport and Signaling in the Brain: Potential Therapeutic Targets and Roles in Body—Brain Interaction. *J Cereb Blood Flow Metab*. 2015;35:176–185.
15. Bernus A, Wagner BD, Accurso F, Doran A, Kaess H, Ivy DD. Brain natriuretic peptide levels in managing pediatric patients with pulmonary arterial hypertension. *Chest*. 2009;135:745–751.
16. Biomarkers Definitions Working Group. Biomarkers and surrogate endpoints: Preferred definitions and conceptual framework. *Clin Pharmacol Ther*. 2001;69:89–95.
17. Bisetto S, Whitaker-Menezes D, Wilski NA, Tuluc M, Curry J, Zhan T, Snyder CM, Martinez-Outschoorn UE, Philp NJ. Monocarboxylate Transporter 4 (MCT4) Knockout Mice Have Attenuated 4NQO Induced Carcinogenesis; A Role for MCT4 in Driving Oral Squamous Cell Cancer. *Front Oncol*. 2018;8:324.
18. Blyth KG, Groenning BA, Mark PB, Martin TN, Foster JE, Steedman T, Morton JJ, Dargie HJ, Peacock AJ. NT-proBNP can be used to detect right ventricular systolic dysfunction in pulmonary hypertension. *Eur Respir J*. 2007;29:737–744.

19. Boidot R, Végran F, Meulle A, Le Breton A, Dessy C, Sonveaux P, Lizard-Nacol S, Feron O. Regulation of monocarboxylate transporter MCT1 expression by p53 mediates inward and outward lactate fluxes in tumors. *Cancer Res*. 2012;72:939–948.
20. Bola BM, Chadwick AL, Michopoulos F, Blount KG, Telfer BA, Williams KJ, Smith PD, Critchlow SE, Stratford IJ. Inhibition of monocarboxylate transporter-1 (MCT1) by AZD3965 enhances radiosensitivity by reducing lactate transport. *Mol Cancer Ther*. 2014;13:2805–2816.
21. Bonderman D, Wilkens H, Wakounig S, Schäfers H-J, Jansa P, Lindner J, Simkova I, Martischnig AM, Dudczak J, Sadushi R, Skoro-Sajer N, Klepetko W, Lang IM. Risk factors for chronic thromboembolic pulmonary hypertension. *Eur Respir J*. 2009;33:325–331.
22. Bonnet S, Rochefort G, Sutendra G, Archer SL, Haromy A, Webster L, Hashimoto K, Bonnet SN, Michelakis ED. The nuclear factor of activated T cells in pulmonary arterial hypertension can be therapeutically targeted. *Proc Natl Acad Sci U S A*. 2007;104:11418–11423.
23. Boomsma F, van der Meiracker, A. H. Plasma A- and B-type natriuretic peptides: physiology, methodology and clinical use. *Cardiovascular Research*;2001:442–449.
24. Brooks GA. Cell-cell and intracellular lactate shuttles. *J Physiol (Lond)*. 2009;587:5591–5600.
25. Brooks GA. The Science and Translation of Lactate Shuttle Theory. *Cell Metab*. 2018;27:757–785.
26. Burke A, Virmani R. Evaluation of pulmonary hypertension in biopsies of the lung. *Current Diagnostic Pathology*. 1996;3:14–26.
27. Carpenter L, Halestrap AP. The kinetics, substrate and inhibitor specificity of the lactate transporter of Ehrlich-Lettre tumour cells studied with the intracellular pH indicator BCECF. *Biochem. J.* 1994;304 (Pt 3):751–760.
28. Caruso P, Dunmore BJ, Schlosser K, Schoors S, Dos Santos C, Perez-Iratxeta C, Lavoie JR, Zhang H, Long L, Flockton AR, Frid MG, Upton PD, D'Alessandro A, Hadinnapola C, Kiskin FN, Taha M, Hurst LA, Ormiston ML, Hata A, Stenmark KR, Carmeliet P, Stewart DJ, Morrell NW. Identification of MicroRNA-124 as a Major Regulator of Enhanced Endothelial Cell Glycolysis in Pulmonary Arterial Hypertension via PTBP1 (Polypyrimidine Tract Binding Protein) and Pyruvate Kinase M2. *Circulation*. 2017;136:2451–2467.
29. Casey JR, Grinstein S, Orlowski J. Sensors and regulators of intracellular pH. *Nat Rev Mol Cell Biol*. 2010;11:50–61.
30. Chen H, Wang L, Beretov J, Hao J, Xiao W, Li Y. Co-expression of CD147/EMMPRIN with monocarboxylate transporters and multiple drug resistance proteins is associated with epithelial ovarian cancer progression. *Clin Exp Metastasis*. 2010;27:557–569.
31. Cheng C, Edin NFJ, Lauritzen KH, Aspmodal I, Christoffersen S, Jian L, Rasmussen LJ, Pettersen EO, Xiaoqun G, Bergersen LH. Alterations of monocarboxylate transporter densities during hypoxia in brain and breast tumour cells. *Cell Oncol (Dordr)*. 2012;35:217–227.
32. Choi SYC, Xue H, Wu R, Fazli L, Lin D, Collins CC, Gleave ME, Gout PW, Wang Y. The MCT4 Gene: A Novel, Potential Target for Therapy of Advanced Prostate Cancer. *Clin Cancer Res*. 2016;22:2721–2733.
33. Christman Brian W., McPherson Charles D., Newman John H., King Gayle A., Bernard Gordon R., Groves Bertron M., Loyd James E. An Imbalance between the Excretion of Thromboxane and Prostacyclin Metabolites in Pulmonary Hypertension. [Epub ahead of print].
34. Colen CB, Shen Y, Ghoddoussi F, Yu P, Francis TB, Koch BJ, Monterey MD, Galloway MP, Sloan AE, Mathupala SP. Metabolic targeting of lactate efflux by malignant glioma inhibits invasiveness and induces necrosis: an in vivo study. *Neoplasia*. 2011;13:620–632.
35. Coller HA, Grandori C, Tamayo P, Colbert T, Lander ES, Eisenman RN, Golub TR. Expression analysis with oligonucleotide microarrays reveals that MYC regulates genes involved in growth, cell cycle, signaling, and adhesion. *Proc Natl Acad Sci U S A*. 2000;97:3260–3265.
36. Cool CD, Kuebler WM, Bogaard HJ, Spiekerkoetter E, Nicolls MR, Voelkel NF. The hallmarks of severe pulmonary arterial hypertension: the cancer hypothesis-ten years later. *Am J Physiol Lung Cell Mol Physiol*. 2020;318:L1115-L1130.

37. Cool CD, Stewart JS, Werahera P, Miller GJ, Williams RL, Voelkel NF, Tuder RM. Three-Dimensional Reconstruction of Pulmonary Arteries in Plexiform Pulmonary Hypertension Using Cell-Specific Markers. *Am J Pathol*. 1999;155:411–419.
38. Corporation P. Lactate-Glo™ Assay Technical Manual, TM493. [Epub ahead of print].
39. Cottrill KA, Chan SY. Metabolic dysfunction in pulmonary hypertension: The expanding relevance of the Warburg effect. *Eur J Clin Invest*. 2013;43:855–865.
40. Curtis NJ, Mooney L, Hopcroft L, Michopoulos F, Whalley N, Zhong H, Murray C, Logie A, Revill M, Byth KF, Benjamin AD, Firth MA, Green S, Smith PD, Critchlow SE. Pre-clinical pharmacology of AZD3965, a selective inhibitor of MCT1: DLBCL, NHL and Burkitt's lymphoma anti-tumor activity. *Oncotarget*. 2017;8:69219–69236.
41. D'Alessandro A, El Kasmi KC, Plecitá-Hlavatá L, Ježek P, Li M, Zhang H, Gupte SA, Stenmark KR. Hallmarks of Pulmonary Hypertension: Mesenchymal and Inflammatory Cell Metabolic Reprogramming. *Antioxid Redox Signal*. 2018;28:230–250.
42. D'Alonzo GE. Survival in Patients with Primary Pulmonary Hypertension. *Ann Intern Med*. 1991;115:343.
43. D'Andrea A, Naeije R, Grünig E, Caso P, D'Alto M, Di Palma E, Nunziata L, Riegler L, Scarafile R, Cocchia R, Vriz O, Citro R, Calabrò R, Russo MG, Bossone E. Echocardiography of the pulmonary circulation and right ventricular function: Exploring the physiologic spectrum in 1,480 normal subjects. *Chest*. 2014;145:1071–1078.
44. Devaraj A, Wells AU, Meister MG, Corte TJ, Wort SJ, Hansell DM. Detection of pulmonary hypertension with multidetector CT and echocardiography alone and in combination. *Radiology*. 2010;254:609–616.
45. Dhup S, Dadhich RK, Porporato PE, Sonveaux P. Multiple biological activities of lactic acid in cancer: influences on tumor growth, angiogenesis and metastasis. *Curr Pharm Des*. 2012;18:1319–1330.
46. Diehl K, Dinges L-A, Helm O, Ammar N, Plundrich D, Arlt A, Röcken C, Sebens S, Schäfer H. Nuclear factor E2-related factor-2 has a differential impact on MCT1 and MCT4 lactate carrier expression in colonic epithelial cells: A condition favoring metabolic symbiosis between colorectal cancer and stromal cells. *Oncogene*. 2018;37:39–51.
47. Doherty JR, Cleveland JL. Targeting lactate metabolism for cancer therapeutics. *J Clin Invest*. 2013;123:3685–3692.
48. Doherty JR, Yang C, Scott KEN, Cameron MD, Fallahi M, Li W, Hall MA, Amelio AL, Mishra JK, Li F, Tortosa M, Genau HM, Rounbehler RJ, Lu Y, Dang CV, Kumar KG, Butler AA, Bannister TD, Hooper AT, Unsal-Kacmaz K, Roush WR, Cleveland JL. Blocking lactate export by inhibiting the Myc target MCT1 Disables glycolysis and glutathione synthesis. *Cancer Res*. 2014;74:908–920.
49. Dorfmüller P, Humbert M. Progress in pulmonary arterial hypertension pathology: relighting a torch inside the tunnel. *Am J Respir Crit Care Med*. 2012;186:210–212.
50. Draoui N, Feron O. Lactate shuttles at a glance: from physiological paradigms to anti-cancer treatments. *Dis Model Mech*. 2011;4:727–732.
51. Eickelberg O, Seeger W. Pulmonary hypertension: pathophysiology, genetics and functional genomics. *Internist*:759–768.
52. Eilertsen M, Andersen S, Al-Saad S, Kiselev Y, Donnem T, Stenvold H, Pettersen I, Al-Shibli K, Richardsen E, Busund L-T, Bremnes RM. Monocarboxylate transporters 1-4 in NSCLC: MCT1 is an independent prognostic marker for survival. *PLoS ONE*. 2014;9:e105038.
53. Fang JC, DeMarco T, Givertz MM, Borlaug BA, Lewis GD, Rame JE, Gomberg-Maitland M, Murali S, Frantz RP, McGlothlin D, Horn EM, Benza RL. World Health Organization Pulmonary Hypertension group 2: pulmonary hypertension due to left heart disease in the adult--a summary statement from the Pulmonary Hypertension Council of the International Society for Heart and Lung Transplantation. *J Heart Lung Transplant*. 2012;31:913–933.

54. Fijalkowska I, Xu W, Comhair SAA, Janocha AJ, Mavrakis LA, Krishnamachary B, Zhen L, Mao T, Richter A, Erzurum SC, Tuder RM. Hypoxia inducible-factor1alpha regulates the metabolic shift of pulmonary hypertensive endothelial cells. *Am J Pathol*. 2010;176:1130–1138.
55. Fisel P, Schaeffeler E, Schwab M. Clinical and Functional Relevance of the Monocarboxylate Transporter Family in Disease Pathophysiology and Drug Therapy. *Clin Transl Sci*. 2018;11:352–364.
56. Friesema ECH, Ganguly S, Abdalla A, Manning Fox JE, Halestrap AP, Visser TJ. Identification of monocarboxylate transporter 8 as a specific thyroid hormone transporter. *J Biol Chem*. 2003;278:40128–40135.
57. Frost A, Badesch D, Gibbs JSR, Gopalan D, Khanna D, Manes A, Oudiz R, Satoh T, Torres F, Torbicki A. Diagnosis of pulmonary hypertension. *Eur Respir J*. 2019;53.
58. Futagi Y, Kobayashi M, Narumi K, Furugen A, Iseki K. Identification of a selective inhibitor of human monocarboxylate transporter 4. *Biochem Biophys Res Commun*. 2018;495:427–432.
59. Galiè N, Channick RN, Frantz RP, Grünig E, Jing ZC, Moiseeva O, Preston IR, Pulido T, Safdar Z, Tamura Y, McLaughlin VV. Risk stratification and medical therapy of pulmonary arterial hypertension. *Eur Respir J*. 2019;53.
60. Galiè N, Corris PA, Frost A, Girgis RE, Granton J, Jing ZC, Klepetko W, McGoon MD, McLaughlin VV, Preston IR, Rubin LJ, Sandoval J, Seeger W, Keogh A. Updated treatment algorithm of pulmonary arterial hypertension. *J Am Coll Cardiol*. 2013;62:D60-72.
61. Galiè N, Kim NHS. Pulmonary microvascular disease in chronic thromboembolic pulmonary hypertension. *Proc Am Thorac Soc*. 2006;3:571–576.
62. Gall H, Felix JF, Schneck FK, Milger K, Sommer N, Voswinckel R, Franco OH, Hofman A, Schermuly RT, Weissmann N, Grimminger F, Seeger W, Ghofrani HA. The Giessen Pulmonary Hypertension Registry: Survival in pulmonary hypertension subgroups. *J Heart Lung Transplant*. 2017;36:957–967.
63. Gallagher SM, Castorino JJ, Wang D, Philp NJ. Monocarboxylate transporter 4 regulates maturation and trafficking of CD147 to the plasma membrane in the metastatic breast cancer cell line MDA-MB-231. *Cancer Res*. 2007;67:4182–4189.
64. Ganapathy V, Thangaraju M, Gopal E, Martin PM, Itagaki S, Miyauchi S, Prasad PD. Sodium-coupled monocarboxylate transporters in normal tissues and in cancer. *AAPS J*. 2008;10:193–199.
65. Ghofrani HA, Morrell NW, Hoeper MM, Olschewski H, Peacock AJ, Barst RJ, Shapiro S, Golpon H, Toshner M, Grimminger F, Pascoe S. Imatinib in pulmonary arterial hypertension patients with inadequate response to established therapy. *Am J Respir Crit Care Med*. 2010;182:1171–1177.
66. Giaid Adel, Saleh Dina. Reduced Expression of Endothelial Nitric Oxide Synthase in the Lungs of Patients with Pulmonary Hypertension. [Epub ahead of print].
67. Giaid Adel, Yanagisawa Masashi, Langleben David, Michel Rene P., Levy Robert, Shennib Hani, Kimura Sadao, Masaki Tomoh, Duguid William P., Stewart Duncan J. Expression of Endothelin-1 in the Lungs of Patients with Pulmonary Hypertension. [Epub ahead of print].
68. Gomberg-Maitland M, Bull TM, Saggar R, Barst RJ, Elgazayerly A, Fleming TR, Grimminger F, Rainisio M, Stewart DJ, Stockbridge N, Ventura C, Ghofrani AH, Rubin LJ. New trial designs and potential therapies for pulmonary artery hypertension. *J Am Coll Cardiol*. 2013;62:D82-91.
69. Gottfried E, Kreutz M, Mackensen A. Tumor metabolism as modulator of immune response and tumor progression. *Semin Cancer Biol*. 2012;22:335–341.
70. Greiner S, Jud A, Aurich M, Hess A, Hilbel T, Hardt S, Katus HA, Mereles D. Reliability of noninvasive assessment of systolic pulmonary artery pressure by Doppler echocardiography compared to right heart catheterization: Analysis in a large patient population. *J Am Heart Assoc*. 2014;3.
71. Grünig E, Benjamin N, Krüger U, Kaemmerer H, Harutyunova S, Olsson KM, Ulrich S, Gerhardt F, Neurohr C, Sablotzki A, Halank M, Marra AM, Kabitz H-J, Thimm G, Fliegel K-G, Klose H. General measures and supportive therapy for pulmonary arterial hypertension: Updated recommendations from the Cologne Consensus Conference 2018. *Int J Cardiol*. 2018;272S:30–36.

72. Guignabert C, Dorfmuller P. Pathology and pathobiology of pulmonary hypertension. *Semin Respir Crit Care Med*. 2013;34:551–559.
73. Guignabert C, Tu L, Girerd B, Ricard N, Huertas A, Montani D, Humbert M. New molecular targets of pulmonary vascular remodeling in pulmonary arterial hypertension: Importance of endothelial communication. *Chest*. 2015;147:529–537.
74. Hägg M, Wennström S. Activation of hypoxia-induced transcription in normoxia. *Exp Cell Res*. 2005;306:180–191.
75. Halestrap A. Monocarboxylate transporter 1. *AfCS-Nature Molecule Pages*. 2009. [Epub ahead of print].
76. Halestrap AP. The monocarboxylate transporter family--Structure and functional characterization. *IUBMB Life*. 2012;64:1–9.
77. Halestrap AP. The SLC16 gene family - structure, role and regulation in health and disease. *Mol Aspects Med*. 2013;34:337–349.
78. Halestrap AP, Denton RM. Specific inhibition of pyruvate transport in rat liver mitochondria and human erythrocytes by alpha-cyano-4-hydroxycinnamate. *Biochem. J.* 1974;138:313–316.
79. Halestrap AP, Meredith D. The SLC16 gene family-from monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond. *Pflugers Arch*. 2004;447:619–628.
80. Halestrap AP, Price NT. The proton-linked monocarboxylate transporter (MCT) family: Structure, function and regulation. *Biochem J*. 1999;343 Pt 2:281–299.
81. Halestrap AP, Wilson MC. The monocarboxylate transporter family--role and regulation. *IUBMB Life*. 2012;64:109–119.
82. Halford SER, Walter H, McKay P, Townsend W, Linton K, Heinzmann K, Dragoni I, Brotherton L, Veal G, Siskos A, Keun HC, Bacon C, Wedge S, Dyer MJS, Plummer ER. Phase I expansion study of the first-in-class monocarboxylate transporter 1 (MCT1) inhibitor AZD3965 in patients with diffuse large B-cell lymphoma (DLBCL) and Burkitt lymphoma (BL). *JCO*. 2021;39:3115.
83. Hall C. Essential biochemistry and physiology of (NT-pro)BNP. *Eur J Heart Fail*. 2004;6:257–260.
84. Hanahan D, Weinberg RA. Hallmarks of cancer: The next generation. *Cell*. 2011;144:646–674.
85. Harvey LD, Chan SY. Emerging Metabolic Therapies in Pulmonary Arterial Hypertension. *J Clin Med*. 2017;6.
86. Hashimoto T, Hussien R, Oommen S, Gohil K, Brooks GA. Lactate sensitive transcription factor network in L6 cells: activation of MCT1 and mitochondrial biogenesis. *FASEB J*. 2007;21:2602–2612.
87. Hatton N, Frech T, Smith B, Sawitzke A, Scholand MB, Markewitz B. Transforming growth factor signalling: A common pathway in pulmonary arterial hypertension and systemic sclerosis. *Int J Clin Pract Suppl*. 2011:35–43.
88. HEATH D, EDWARDS JE. The pathology of hypertensive pulmonary vascular disease; a description of six grades of structural changes in the pulmonary arteries with special reference to congenital cardiac septal defects. *Circulation*. 1958;18:533–547.
89. Hemnes AR, Brittain EL, Trammell AW, Fessel JP, Austin ED, Penner N, Maynard KB, Gleaves L, Talati M, Absi T, Disalvo T, West J. Evidence for right ventricular lipotoxicity in heritable pulmonary arterial hypertension. *Am J Respir Crit Care Med*. 2014;189:325–334.
90. Hemnes AR, Humbert M. Pathobiology of pulmonary arterial hypertension: Understanding the roads less travelled. *Eur Respir Rev*. 2017;26.
91. Heresi GA. Clinical perspective: Biomarkers in pulmonary arterial hypertension. *Int J Clin Pract Suppl*. 2011:5–7.
92. Hernandez-Saavedra D, Sanders L, Freeman S, Reisz JA, Lee MH, Mickael C, Kumar R, Kassa B, Gu S, Alessandro A d', Stenmark KR, Tuder RM, Graham BB. Stable isotope metabolomics of pulmonary artery smooth muscle and endothelial cells in pulmonary hypertension and with TGF-beta treatment. *Sci Rep*. 2020;10:413.

93. Herve P, Lau EM, Sitbon O, Savale L, Montani D, Godinas L, Lador F, Jaïs X, Parent F, Günther S, Humbert M, Simonneau G, Chemla D. Criteria for diagnosis of exercise pulmonary hypertension. *Eur Respir J*. 2015;46:728–737.
94. Hervé P, Launay J-M, Scrobohaci M-L, Brenot F, Simonneau G, Petitpretz P, Poubeau P, Cerrina J, Duroux P, Drouet L. Increased plasma serotonin in primary pulmonary hypertension. *The American Journal of Medicine*. 1995;99:249–254.
95. Hervé Dubouchaud, Gail E. Butterfield, Eugene E. Wolfel, Bryan C. Bergman, and George A. Brooks. Endurance training, expression, and physiology of LDH, MCT1, and MCT4 in human skeletal muscle. [Epub ahead of print].
96. Hirschhaeuser F, Sattler UGA, Mueller-Klieser W. Lactate: a metabolic key player in cancer. *Cancer Res*. 2011;71:6921–6925.
97. Hlatky MA, Greenland P, Arnett DK, Ballantyne CM, Criqui MH, Elkind MSV, Go AS, Harrell FE, Hong Y, Howard BV, Howard VJ, Hsue PY, Kramer CM, McConnell JP, Normand S-LT, O'Donnell CJ, Smith SC, Wilson PWF. Criteria for evaluation of novel markers of cardiovascular risk: A scientific statement from the American Heart Association. *Circulation*. 2009;119:2408–2416.
98. Hoeper MM, Apitz C, Grünig E, Halank M, Ewert R, Kaemmerer H, Kabitz H-J, Kähler C, Klose H, Leuchte H, Ulrich S, Olsson KM, Distler O, Rosenkranz S, Ghofrani HA. Targeted therapy of pulmonary arterial hypertension: Updated recommendations from the Cologne Consensus Conference 2018. *Int J Cardiol*. 2018;272S:37–45.
99. Hoeper MM, Benza RL, Corris P, Perrot M de, Fadel E, Keogh AM, Kühn C, Savale L, Klepetko W. Intensive care, right ventricular support and lung transplantation in patients with pulmonary hypertension. *Eur Respir J*. 2019;53.
100. Hoeper MM, Bogaard HJ, Condliffe R, Frantz R, Khanna D, Kurzyna M, Langleben D, Manes A, Satoh T, Torres F, Wilkins MR, Badesch DB. Definitions and diagnosis of pulmonary hypertension. *J Am Coll Cardiol*. 2013;62:D42-50.
101. Hoeper MM, Ghofrani H-A, Grünig E, Klose H, Olschewski H, Rosenkranz S. Pulmonary Hypertension. *Dtsch Arztebl Int*. 2017;114:73–84.
102. Hoeper MM, Humbert M, Souza R, Idrees M, Kawut SM, Sliwa-Hahnle K, Jing Z-C, Gibbs JSR. A global view of pulmonary hypertension. *The Lancet Respiratory Medicine*. 2016;4:306–322.
103. Hsu PP, Sabatini DM. Cancer cell metabolism: Warburg and beyond. *Cell*. 2008;134:703–707.
104. Humbert M. Pulmonary arterial hypertension and chronic thromboembolic pulmonary hypertension: Pathophysiology. *Eur Respir Rev*. 2010;19:59–63.
105. Humbert M, Guignabert C, Bonnet S, Dorfmüller P, Klinger JR, Nicolls MR, Olschewski AJ, Pullamsetti SS, Schermuly RT, Stenmark KR, Rabinovitch M. Pathology and pathobiology of pulmonary hypertension: state of the art and research perspectives. *Eur Respir J*. 2019;53.
106. Humbert M, Kovacs G, Hoeper MM, Badagliacca R, Berger RMF, Brida M, Carlsen J, Coats AJS, Escribano-Subias P, Ferrari P, Ferreira DS, Ghofrani HA, Giannakoulas G, Kiely DG, Mayer E, Meszaros G, Nagavci B, Olsson KM, Pepke-Zaba J, Quint JK, Rådegran G, Simonneau G, Sitbon O, Tonia T, Toshner M, Vachiery J-L, Vonk Noordegraaf A, Delcroix M, Rosenkranz S. 2022 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension. *Eur Respir J*. 2022. [Epub ahead of print].
107. Humbert M, Morrell NW, Archer SL, Stenmark KR, MacLean MR, Lang IM, Christman BW, Weir EK, Eickelberg O, Voelkel NF, Rabinovitch M. Cellular and molecular pathobiology of pulmonary arterial hypertension. *J Am Coll Cardiol*. 2004;43:13S-24S.
108. Humbert M, Sitbon O, Chaouat A, Bertocchi M, Habib G, Gressin V, Yaici A, Weitzenblum E, Cordier J-F, Chabot F, Dromer C, Pison C, Reynaud-Gaubert M, Haloun A, Laurent M, Hachulla E, Simonneau G. Pulmonary arterial hypertension in France: results from a national registry. *Am J Respir Crit Care Med*. 2006;173:1023–1030.
109. Humbert Marc, Sitbon Olivier, Simonneau Gérald. Treatment of Pulmonary Arterial Hypertension. [Epub ahead of print].

110. Huppelsberg J, Walter K, Huckstorf C, Gusta M, Gusta P. *Kurzlehrbuch Physiologie: 42 Tabellen ; [Medi-Learn Gütesiegel]*. 3rd ed. Stuttgart: Thieme; 2009.
111. ibidi GmbH. *Wound Healing and Migration Assays*. 2019. Available at: https://ibidi.com/img/cms/support/AN/AN21_Wound_Healing_Assay.pdf.
112. Iizuka K, Machida T, Hirafuji M. Extracellular MCT4 is a possible indicator for skeletal muscle MHC fiber type change. *Ann Clin Lab Sci*. 2014;44:272–276.
113. Iizuka K, Morita N, Nagai T, Hanada A, Okita K, Yonezawa K, Murakami T, Kitabatake A, Kawaguchi H. A 44-kDa of protein identical to the N-terminal amino acid sequence of MCT1 in human circulation. *Mol Cell Biochem*. 2003;248:217–223.
114. Jones DA, Benjamin CW, Linseman DA. Activation of thromboxane and prostacyclin receptors elicits opposing effects on vascular smooth muscle cell growth and mitogen-activated protein kinase signaling cascades. *Mol Pharmacol*. 1995;48:890–896.
115. Jonigk D, Golpon H, Bockmeyer CL, Maegel L, Hoeper MM, Gottlieb J, Nickel N, Hussein K, Maus U, Lehmann U, Janciauskiene S, Welte T, Haverich A, Rische J, Kreipe H, Laenger F. Plexiform lesions in pulmonary arterial hypertension composition, architecture, and microenvironment. *Am J Pathol*. 2011;179:167–179.
116. Jonigk D, Hoeper MM, Kreipe H, Länger F. Histopathologische Aspekte der pulmonalen Hypertonie. *Pathologe*. 2012;33:183–191.
117. Kaelin WG, Ratcliffe PJ. Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. *Mol Cell*. 2008;30:393–402.
118. Kambayashi Y, Nakao K, Mukoyama M, Saito Y, Ogawa Y, Shiono S, Inouye K, Yoshida N, Imura H. Isolation and sequence determination of human brain natriuretic peptide in human atrium. *FEBS Letters*. 1990;259:341–345.
119. Kato Y, Ozawa S, Miyamoto C, Maehata Y, Suzuki A, Maeda T, Baba Y. Acidic extracellular microenvironment and cancer. *Cancer Cell Int*. 2013;13:89.
120. Kim J-w, Tchernyshyov I, Semenza GL, Dang CV. HIF-1-mediated expression of pyruvate dehydrogenase kinase: A metabolic switch required for cellular adaptation to hypoxia. *Cell Metab*. 2006;3:177–185.
121. Kim NH, Delcroix M, Jais X, Madani MM, Matsubara H, Mayer E, Ogo T, Tapson VF, Ghofrani H-A, Jenkins DP. Chronic thromboembolic pulmonary hypertension. *Eur Respir J*. 2019;53.
122. Kim NH, Delcroix M, Jenkins DP, Channick R, Dartevelle P, Jansa P, Lang I, Madani MM, Ogino H, Pengo V, Mayer E. Chronic thromboembolic pulmonary hypertension. *J Am Coll Cardiol*. 2013;62:D92-9.
123. Kirk P, Wilson MC, Heddle C, Brown MH, Barclay AN, Halestrap AP. CD147 is tightly associated with lactate transporters MCT1 and MCT4 and facilitates their cell surface expression. *EMBO J*. 2000;19:3896–3904.
124. Kovacs G, Berghold A, Scheidl S, Olschewski H. Pulmonary arterial pressure during rest and exercise in healthy subjects: A systematic review. *Eur Respir J*. 2009;34:888–894.
125. Kovacs G, Dumitrescu D, Barner A, Greiner S, Grünig E, Hager A, Köhler T, Kozlik-Feldmann R, Kruck I, Lammers AE, Mereles D, Meyer A, Meyer J, Pabst S, Seyfarth H-J, Sinning C, Sorichter S, Stähler G, Wilkens H, Held M. Definition, clinical classification and initial diagnosis of pulmonary hypertension: Updated recommendations from the Cologne Consensus Conference 2018. *Int J Cardiol*. 2018;272S:11–19.
126. Kovacs G, Olschewski A, Berghold A, Olschewski H. Pulmonary vascular resistances during exercise in normal subjects: a systematic review. *Eur Respir J*. 2012;39:319–328.
127. Latif A, Chadwick AL, Kitson SJ, Gregson HJ, Sivalingam VN, Bolton J, McVey RJ, Roberts SA, Marshall KM, Williams KJ, Stratford IJ, Crosbie EJ. Monocarboxylate Transporter 1 (MCT1) is an independent prognostic biomarker in endometrial cancer. *BMC Clin Pathol*. 2017;17:27.
128. Le Floch R, Chiche J, Marchiq I, Naiken T, Naïken T, Ilc K, Ilk K, Murray CM, Critchlow SE, Roux D, Simon M-P, Pouysségur J. CD147 subunit of lactate/H+ symporters MCT1 and hypoxia-inducible MCT4 is critical for energetics and growth of glycolytic tumors. *Proc Natl Acad Sci U S A*. 2011;108:16663–16668.

129. Leuchte HH, Freyhaus H ten, Gall H, Halank M, Hoeper MM, Kaemmerer H, Kähler C, Riemekasten G, Ulrich S, Schwaiblmair M, Ewert R. Risk stratification strategy and assessment of disease progression in patients with pulmonary arterial hypertension: Updated Recommendations from the Cologne Consensus Conference 2018. *Int J Cardiol*. 2018;272S:20–29.
130. Lim LY, Vidnovic N, Ellisen LW, Leong C-O. Mutant p53 mediates survival of breast cancer cells. *Br J Cancer*. 2009;101:1606–1612.
131. Machado RF, Londhe Nerkar M-V, Dweik RA, Hammel J, Janocha A, Pyle J, Laskowski D, Jennings C, Arroliga AC, Erzurum SC. Nitric oxide and pulmonary arterial pressures in pulmonary hypertension. *Free Radic Biol Med*. 2004;37:1010–1017.
132. Marchiq I, Le Floch R, Roux D, Simon M-P, Pouyssegur J. Genetic disruption of lactate/H+ symporters (MCTs) and their subunit CD147/BASIGIN sensitizes glycolytic tumor cells to phenformin. *Cancer Res*. 2015;75:171–180.
133. Marchiq I, Pouysségur J. Hypoxia, cancer metabolism and the therapeutic benefit of targeting lactate/H(+) symporters. *J Mol Med*. 2016;94:155–171.
134. Mariotta L, Ramadan T, Singer D, Guetg A, Herzog B, Stoeger C, Palacín M, Lahoutte T, Camargo SMR, Verrey F. T-type amino acid transporter TAT1 (Slc16a10) is essential for extracellular aromatic amino acid homeostasis control. *J Physiol (Lond)*. 2012;590:6413–6424.
135. Marsboom G, Wietholt C, Haney CR, Toth PT, Ryan JJ, Morrow E, Thenappan T, Bache-Wiig P, Piao L, Paul J, Chen C-T, Archer SL. Lung ^{18}F-fluorodeoxyglucose positron emission tomography for diagnosis and monitoring of pulmonary arterial hypertension. *Am J Respir Crit Care Med*. 2012;185:670–679.
136. McGoon MD, Benza RL, Escribano-Subias P, Jiang X, Miller DP, Peacock AJ, Pepke-Zaba J, Pulido T, Rich S, Rosenkranz S, Suissa S, Humbert M. Pulmonary arterial hypertension: epidemiology and registries. *J Am Coll Cardiol*. 2013;62:D51-9.
137. McLaughlin VV, Gaine SP, Howard LS, Leuchte HH, Mathier MA, Mehta S, Palazzini M, Park MH, Tapson VF, Sitbon O. Treatment goals of pulmonary hypertension. *J Am Coll Cardiol*. 2013;62:D73-81.
138. McMurtry MS, Bonnet S, Wu X, Dyck JRB, Haromy A, Hashimoto K, Michelakis ED. Dichloroacetate prevents and reverses pulmonary hypertension by inducing pulmonary artery smooth muscle cell apoptosis. *Circ Res*. 2004;95:830–840.
139. Miranda-Gonçalves V, Gonçalves CS, Granja S, Vieira de Castro J, Reis RM, Costa BM, Baltazar F. MCT1 Is a New Prognostic Biomarker and Its Therapeutic Inhibition Boosts Response to Temozolomide in Human Glioblastoma. *Cancers (Basel)*. 2021;13.
140. Miranda-Gonçalves V, Honavar M, Pinheiro C, Martinho O, Pires MM, Pinheiro C, Cordeiro M, Bebiano G, Costa P, Palmeirim I, Reis RM, Baltazar F. Monocarboxylate transporters (MCTs) in gliomas: expression and exploitation as therapeutic targets. *Neuro-oncology*. 2013;15:172–188.
141. Mitchell JA, Ahmetaj-Shala B, Kirkby NS, Wright WR, Mackenzie LS, Reed DM, Mohamed N. Role of prostacyclin in pulmonary hypertension. *Glob Cardiol Sci Pract*. 2014;2014:382–393.
142. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. *N Engl J Med*. 1993;329:2002–2012.
143. Moraes DL, Colucci WS, Givertz MM. Secondary pulmonary hypertension in chronic heart failure: the role of the endothelium in pathophysiology and management. *Circulation*. 2000;102:1718–1723.
144. Mueller-Mottet S, Stricker H, Domenighetti G, Domeninghetti G, Azzola A, Geiser T, Schwerzmann M, Weilenmann D, Schoch O, Fellrath J-M, Rochat T, Lador F, Beghetti M, Nicod L, Aubert J-D, Popov V, Speich R, Keusch S, Hasler E, Huber LC, Grendelmeier P, Tamm M, Ulrich S. Long-term data from the Swiss pulmonary hypertension registry. *Respiration*. 2015;89:127–140.
145. Murray CM, Hutchinson R, Bantick JR, Belfield GP, Benjamin AD, Brazma D, Bundick RV, Cook ID, Craggs RI, Edwards S, Evans LR, Harrison R, Holness E, Jackson AP, Jackson CG, Kingston LP, Perry MWD, Ross ARJ, Rugman PA, Sidhu SS, Sullivan M, Taylor-Fishwick DA, Walker PC, Whitehead YM, Wilkinson DJ, Wright A, Donald DK. Monocarboxylate transporter MCT1 is a target for immunosuppression. *Nat Chem Biol*. 2005;1:371–376.

146. Naeije R. Physiology of the pulmonary circulation and the right heart. *Curr Hypertens Rep*. 2013;15:623–631.
147. Naeije R, Chesler N. Pulmonary circulation at exercise. *Compr Physiol*. 2012;2:711–741.
148. Naeije R, Chin K. Differentiating Precapillary From Postcapillary Pulmonary Hypertension. *Circulation*. 2019;140:712–714.
149. Naeije R, Vachiery J-L, Yerly P, Vanderpool R. The transpulmonary pressure gradient for the diagnosis of pulmonary vascular disease. *Eur Respir J*. 2013;41:217–223.
150. Nagaya N, Nishikimi T, Uematsu M, Satoh T, Kyotani S, Sakamaki F, Kakishita M, Fukushima K, Okano Y, Nakanishi N, Miyatake K, Kangawa K. Plasma Brain Natriuretic Peptide as a Prognostic Indicator in Patients With Primary Pulmonary Hypertension. *Circulation*. 2000;102:865–870.
151. Nakayama Y, Torigoe T, Inoue Y, Minagawa N, Izumi H, Kohno K, Yamaguchi K. Prognostic significance of monocarboxylate transporter 4 expression in patients with colorectal cancer. *Exp Ther Med*. 2012;3:25–30.
152. Nancy J. Philp, Heeyong Yoon, and Lorraine Lombardi. Mouse MCT3 gene is expressed preferentially in retinal pigment and choroid plexus epithelia. [Epub ahead of print].
153. Nazzareno Galiè, Marc Humbert, Jean-Luc Vachiery, Simon Gibbs, Irene Lang, Adam Torbicki, Gérald Simonneau, Andrew Peacock, Anton Vonk Noordegraaf, Maurice Beghetti, Ardeschir Ghofrani, Miguel Angel Gomez Sanchez, Georg Hansmann, Walter Klepetko, Patrizio Lancellotti, Marco Matucci, Theresa McDonagh, Luc A. Pierard, Pedro T. Trindade, Maurizio Zompatori, Marius Hoeper. 2015 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension. [Epub ahead of print].
154. Newman JH. Pulmonary hypertension. *Am J Respir Crit Care Med*. 2005;172:1072–1077.
155. Ni J-R, Yan P-J, Liu S-D, Hu Y, Yang K-H, Song B, Lei J-Q. Diagnostic accuracy of transthoracic echocardiography for pulmonary hypertension: a systematic review and meta-analysis. *BMJ Open*. 2019;9:e033084.
156. Njagi EN, Rizopoulos D, Molenberghs G, Dendale P, Willekens K. A joint survival-longitudinal modelling approach for the dynamic prediction of rehospitalization in telemonitored chronic heart failure patients. *Statistical Modelling*. 2013;13:179–198.
157. Noble RA, Bell N, Blair H, Sikka A, Thomas H, Phillips N, Nakjang S, Miwa S, Crossland R, Rand V, Televantou D, Long A, Keun HC, Bacon CM, Bomken S, Critchlow SE, Wedge SR. Inhibition of monocarboxyate transporter 1 by AZD3965 as a novel therapeutic approach for diffuse large B-cell lymphoma and Burkitt lymphoma. *Haematologica*. 2017;102:1247–1257.
158. Olschewski A, Berghausen EM, Eichstaedt CA, Fleischmann BK, Grünig E, Grünig G, Hansmann G, Harbaum L, Hennigs JK, Jonigk D, Kübler WM, Kwapiszewska G, Pullamsetti SS, Stacher E, Weissmann N, Wenzel D, Schermuly RT. Pathobiologie, Pathologie und Genetik der pulmonalen Hypertonie: Empfehlungen der Kölner Konsensus-Konferenz 2016. *Dtsch Med Wochenschr*. 2016;141:S4-S9.
159. Olschewski H, Behr J, Bremer H, Claussen M, Douschan P, Halank M, Held M, Hoeper MM, Holt S, Klose H, Krüger S, Lange TJ, Reichenberger F, Skowasch D, Ulrich S, Wilkens H, Seeger W. Pulmonale Hypertonie bei Lungenkrankheiten: Empfehlungen der Kölner Konsensus-Konferenz 2016. *Dtsch Med Wochenschr*. 2016;141:S57-S61.
160. Olschewski H, Behr J, Bremer H, Claussen M, Douschan P, Halank M, Held M, Hoeper MM, Holt S, Klose H, Krüger S, Lange TJ, Reichenberger F, Skowasch D, Ulrich S, Wilkens H, Seeger W. Pulmonary hypertension due to lung diseases: Updated recommendations from the Cologne Consensus Conference 2018. *Int J Cardiol*. 2018;272S:63–68.
161. Olsson KM, Nickel NP, Tongers J, Hoeper MM. Atrial flutter and fibrillation in patients with pulmonary hypertension. *Int J Cardiol*. 2013;167:2300–2305.
162. Opitz CF, Hoeper MM, Gibbs JSR, Kaemmerer H, Pepke-Zaba J, Coghlan JG, Scelsi L, D'Alto M, Olsson KM, Ulrich S, Scholtz W, Schulz U, Grünig E, Vizza CD, Staehler G, Bruch L, Huscher D, Pittrow D, Rosenkranz S. Pre-Capillary, Combined, and Post-Capillary Pulmonary Hypertension: A Pathophysiological Continuum. *J Am Coll Cardiol*. 2016;68:368–378.

163. Oswald-Mammosser M, Weitzenblum E, Quoix E, Moser G, Chaouat A, Charpentier C, Kessler R. Prognostic factors in COPD patients receiving long-term oxygen therapy. Importance of pulmonary artery pressure. *Chest*. 1995;107:1193–1198.
164. Ovens MJ, Davies AJ, Wilson MC, Murray CM, Halestrap AP. AR-C155858 is a potent inhibitor of monocarboxylate transporters MCT1 and MCT2 that binds to an intracellular site involving transmembrane helices 7-10. *Biochem J*. 2010;425:523–530.
165. Park I, Larson PEZ, Zierhut ML, Hu S, Bok R, Ozawa T, Kurhanewicz J, Vigneron DB, Vandenberg SR, James CD, Nelson SJ. Hyperpolarized 13C magnetic resonance metabolic imaging: application to brain tumors. *Neuro-oncology*. 2010;12:133–144.
166. Patel H, Zaghloul N, Lin K, Liu SF, Miller EJ, Ahmed M. Hypoxia-induced activation of specific members of the NF-kB family and its relevance to pulmonary vascular remodeling. *Int J Biochem Cell Biol*. 2017;92:141–147.
167. Paulin R, Michelakis ED. The metabolic theory of pulmonary arterial hypertension. *Circ Res*. 2014;115:148–164.
168. Peacock A, Simonneau G, Rubin L. Controversies, uncertainties and future research on the treatment of chronic thromboembolic pulmonary hypertension. *Proc Am Thorac Soc*. 2006;3:608–614.
169. Peacock AJ, Murphy NF, McMurray JJV, Caballero L, Stewart S. An epidemiological study of pulmonary arterial hypertension. *Eur Respir J*. 2007;30:104–109.
170. Pengo Vittorio, Lensing Anthonie W.A., Prins Martin H., Marchiori Antonio, Davidson Bruce L., Tiozzo Francesca, Albanese Paolo, Biasiolo Alessandra, Pegoraro Cinzia, Iliceto Sabino, Prandoni Paolo. Incidence of Chronic Thromboembolic Pulmonary Hypertension after Pulmonary Embolism. [Epub ahead of print].
171. Pérez-Escuredo J, van Hée VF, Sboarina M, Falces J, Payen VL, Pellerin L, Sonveaux P. Monocarboxylate transporters in the brain and in cancer. *Biochim Biophys Acta*. 2016;1863:2481–2497.
172. Pértega-Gomes N, Vizcaíno JR, Miranda-Gonçalves V, Pinheiro C, Silva J, Pereira H, Monteiro P, Henrique RM, Reis RM, Lopes C, Baltazar F. Monocarboxylate transporter 4 (MCT4) and CD147 overexpression is associated with poor prognosis in prostate cancer. *BMC Cancer*. 2011;11:312.
173. Pezzuto B, Badagliacca R, Poscia R, Ghio S, D'Alto M, Vitulo P, Mulè M, Albera C, Volterrani M, Fedele F, Vizza CD. Circulating biomarkers in pulmonary arterial hypertension: update and future direction. *J Heart Lung Transplant*. 2015;34:282–305.
174. Piao L, Fang Y-H, Cadete VJJ, Wietholt C, Urboniene D, Toth PT, Marsboom G, Zhang HJ, Haber I, Rehman J, Lopaschuk GD, Archer SL. The inhibition of pyruvate dehydrogenase kinase improves impaired cardiac function and electrical remodeling in two models of right ventricular hypertrophy: resuscitating the hibernating right ventricle. *J Mol Med*. 2010;88:47–60.
175. Piao L, Marsboom G, Archer SL. Mitochondrial metabolic adaptation in right ventricular hypertrophy and failure. *J Mol Med*. 2010;88:1011–1020.
176. Pinheiro C, Albergaria A, Paredes J, Sousa B, Dufloth R, Vieira D, Schmitt F, Baltazar F. Monocarboxylate transporter 1 is up-regulated in basal-like breast carcinoma. *Histopathology*. 2010;56:860–867.
177. Pinheiro C, Longatto-Filho A, Azevedo-Silva J, Casal M, Schmitt FC, Baltazar F. Role of monocarboxylate transporters in human cancers: State of the art. *J Bioenerg Biomembr*. 2012;44:127–139.
178. Pinheiro C, Longatto-Filho A, Scapulatempo C, Ferreira L, Martins S, Pellerin L, Rodrigues M, Alves VAF, Schmitt F, Baltazar F. Increased expression of monocarboxylate transporters 1, 2, and 4 in colorectal carcinomas. *Virchows Arch*. 2008;452:139–146.
179. Pinheiro C, Longatto-Filho A, Simões K, Jacob CE, Bresciani CJC, Zilberstein B, Cecconello I, Alves VAF, Schmitt F, Baltazar F. The prognostic value of CD147/EMMPRIN is associated with monocarboxylate transporter 1 co-expression in gastric cancer. *Eur J Cancer*. 2009;45:2418–2424.

180. Plecitá-Hlavatá L, Tauber J, Li M, Zhang H, Flockton AR, Pullamsetti SS, Chelladurai P, D'Alessandro A, El Kasmi KC, Ježek P, Stenmark KR. Constitutive Reprogramming of Fibroblast Mitochondrial Metabolism in Pulmonary Hypertension. *Am J Respir Cell Mol Biol*. 2016;55:47–57.
181. Polański R, Hodgkinson CL, Fusi A, Nonaka D, Priest L, Kelly P, Trapani F, Bishop PW, White A, Critchlow SE, Smith PD, Blackhall F, Dive C, Morrow CJ. Activity of the monocarboxylate transporter 1 inhibitor AZD3965 in small cell lung cancer. *Clin Cancer Res*. 2014;20:926–937.
182. Poole RC, Halestrap AP. Transport of lactate and other monocarboxylates across mammalian plasma membranes. *Am J Physiol*. 1993;264:C761-82.
183. Potolicchio I, Carven GJ, Xu X, Stipp C, Riese RJ, Stern LJ, Santambrogio L. Proteomic analysis of microglia-derived exosomes: metabolic role of the aminopeptidase CD13 in neuropeptide catabolism. *J Immunol*. 2005;175:2237–2243.
184. Potter M, Newport E, Morten KJ. The Warburg effect: 80 years on. *Biochem Soc Trans*. 2016;44:1499–1505.
185. Price LC, Wort SJ, Perros F, Dorfmüller P, Huertas A, Montani D, Cohen-Kaminsky S, Humbert M. Inflammation in pulmonary arterial hypertension. *Chest*. 2012;141:210–221.
186. PRICE TN, JACKSON NV, HALESTRAP PA. Cloning and sequencing of four new mammalian monocarboxylate transporter (MCT) homologues confirms the existence of a transporter family with an ancient past. *Biochem. J.* 1998;329:321–328.
187. Protective Roles of Endothelial AMP-Activated Protein Kinase Against Hypoxia-Induced Pulmonary Hypertension in Mice. [Epub ahead of print].
188. Pruszczyk P. N-Terminal Pro-Brain Natriuretic Peptide as an Indicator of Right Ventricular Dysfunction. *Journal of Cardiac Failure*. 2005;11:S65-S69.
189. Pullamsetti SS, Savai R, Seeger W, Goncharova EA. Translational Advances in the Field of Pulmonary Hypertension. From Cancer Biology to New Pulmonary Arterial Hypertension Therapeutics. Targeting Cell Growth and Proliferation Signaling Hubs. *Am J Respir Crit Care Med*. 2017;195:425–437.
190. Quanz M, Bender E, Kopitz C, Grünewald S, Schlicker A, Schwede W, Eheim A, Toschi L, Neuhaus R, Richter C, Toedling J, Merz C, Lesche R, Kamburov A, Siebeneicher H, Bauser M, Hägebarth A. Preclinical Efficacy of the Novel Monocarboxylate Transporter 1 Inhibitor BAY-8002 and Associated Markers of Resistance. *Mol Cancer Ther*. 2018;17:2285–2296.
191. Rabinovitch M. Molecular pathogenesis of pulmonary arterial hypertension. *J Clin Invest*. 2012;122:4306–4313.
192. Racker E. History of the Pasteur effect and its pathobiology. *Mol Cell Biochem*. 1974;5:17–23.
193. Rattigan YI, Patel BB, Ackerstaff E, Sukenick G, Koutcher JA, Glod JW, Banerjee D. Lactate is a mediator of metabolic cooperation between stromal carcinoma associated fibroblasts and glycolytic tumor cells in the tumor microenvironment. *Exp Cell Res*. 2012;318:326–335.
194. Rich Stuart, Kaufmann Elizabeth, Levy Paul S. The Effect of High Doses of Calcium-Channel Blockers on Survival in Primary Pulmonary Hypertension. [Epub ahead of print].
195. Richter EA, Ruderman NB. AMPK and the biochemistry of exercise: implications for human health and disease. *Biochem J*. 2009;418:261–275.
196. Rosenkranz S, Gibbs JSR, Wachter R, Marco T de, Vonk-Noordegraaf A, Vachiéry J-L. Left ventricular heart failure and pulmonary hypertension. *Eur Heart J*. 2016;37:942–954.
197. Rosenkranz S, Lang IM, Blindt R, Bonderman D, Bruch L, Diller GP, Felgendreher R, Gerges C, Hohenforst-Schmidt W, Holt S, Jung C, Kindermann I, Kramer T, Kübler WM, Mitrovic V, Riedel A, Rieth A, Schmeisser A, Wachter R, Weil J, Opitz C. Pulmonale Hypertonie bei Linksherzerkrankungen: Empfehlungen der Kölner Konsensus-Konferenz 2016. *Dtsch Med Wochenschr*. 2016;141:S48-S56.
198. Rosenkranz S, Lang IM, Blindt R, Bonderman D, Bruch L, Diller GP, Felgendreher R, Gerges C, Hohenforst-Schmidt W, Holt S, Jung C, Kindermann I, Kramer T, Kübler WM, Mitrovic V, Riedel A, Rieth A, Schmeisser A, Wachter R, Weil J, Opitz CF. Pulmonary hypertension associated with left heart disease: Updated Recommendations of the Cologne Consensus Conference 2018. *Int J Cardiol*. 2018;272S:53–62.

199. Ruan Y, Zeng F, Cheng Z, Zhao X, Fu P, Chen H. High expression of monocarboxylate transporter 4 predicts poor prognosis in patients with lung adenocarcinoma. *Oncol Lett*. 2017;14:5727–5734.
200. RUBIN M. TUDER, CARLYNE D. COOL, MARK W. GERACI, JUN WANG, STEVEN H. ABMAN, LAUREL WRIGHT, DAVID BADESCH, and NORBERT F. VOELKEL. Prostacyclin Synthase Expression Is Decreased in Lungs from Patients with Severe Pulmonary Hypertension. [Epub ahead of print].
201. Rudski LG, Lai WW, Afilalo J, Hua L, Handschumacher MD, Chandrasekaran K, Solomon SD, Louie EK, Schiller NB. Guidelines for the echocardiographic assessment of the right heart in adults: A report from the American Society of Echocardiography endorsed by the European Association of Echocardiography, a registered branch of the European Society of Cardiology, and the Canadian Society of Echocardiography. *J Am Soc Echocardiogr*. 2010;23:685-713; quiz 786-8.
202. Ruffenach G, Bonnet S, Rousseaux S, Khochbin S, Provencher S, Perros F. Identity crisis in pulmonary arterial hypertension. *Pulm Circ*. 2018;8:2045893217746054.
203. Ryan JJ, Archer SL. Emerging concepts in the molecular basis of pulmonary arterial hypertension: Part I: metabolic plasticity and mitochondrial dynamics in the pulmonary circulation and right ventricle in pulmonary arterial hypertension. *Circulation*. 2015;131:1691–1702.
204. Saito T, Hasegawa Y, Ishigaki Y, Yamada T, Gao J, Imai J, Uno K, Kaneko K, Ogihara T, Shimosawa T, Asano T, Fujita T, Oka Y, Katagiri H. Importance of endothelial NF-κB signalling in vascular remodelling and aortic aneurysm formation. *Cardiovascular Research*. 2013;97:106–114.
205. Saulle E, Spinello I, Quaranta MT, Pasquini L, Pelosi E, Iorio E, Castelli G, Chirico M, Pisanu ME, Ottone T, Voso MT, Testa U, Labbaye C. Targeting Lactate Metabolism by Inhibiting MCT1 or MCT4 Impairs Leukemic Cell Proliferation, Induces Two Different Related Death-Pathways and Increases Chemotherapeutic Sensitivity of Acute Myeloid Leukemia Cells. *Front Oncol*. 2020;10:621458.
206. Schermuly RT, Dony E, Ghofrani HA, Pullamsetti S, Savai R, Roth M, Sydykov A, Lai YJ, Weissmann N, Seeger W, Grimminger F. Reversal of experimental pulmonary hypertension by PDGF inhibition. *J Clin Invest*. 2005;115:2811–2821.
207. Schermuly RT, Ghofrani HA, Wilkins MR, Grimminger F. Mechanisms of disease: Pulmonary arterial hypertension. *Nat Rev Cardiol*. 2011;8:443–455.
208. Schumann C, Lepper PM, Frank H, Schneiderbauer R, Wibmer T, Kropf C, Stoiber KM, Rüdiger S, Kruska L, Krahn T, Kramer F. Circulating biomarkers of tissue remodelling in pulmonary hypertension. *Biomarkers*. 2010;15:523–532.
209. Selimovic N, Bergh C-H, Andersson B, Sakiniene E, Carlsten H, Rundqvist B. Growth factors and interleukin-6 across the lung circulation in pulmonary hypertension. *Eur Respir J*. 2009;34:662–668.
210. Shao D, Park JES, Wort SJ. The role of endothelin-1 in the pathogenesis of pulmonary arterial hypertension. *Pharmacol Res*. 2011;63:504–511.
211. Shimoda LA, Semenza GL. HIF and the lung: role of hypoxia-inducible factors in pulmonary development and disease. *Am J Respir Crit Care Med*. 2011;183:152–156.
212. Simonneau G, Gatzoulis MA, Adatia I, Celermajer D, Denton C, Ghofrani A, Gomez Sanchez MA, Krishna Kumar R, Landzberg M, Machado RF, Olschewski H, Robbins IM, Souza R. Updated clinical classification of pulmonary hypertension. *J Am Coll Cardiol*. 2013;62:D34-41.
213. Simonneau G, Montani D, Celermajer DS, Denton CP, Gatzoulis MA, Krowka M, Williams PG, Souza R. Haemodynamic definitions and updated clinical classification of pulmonary hypertension. *Eur Respir J*. 2019;53.
214. Sitbon O, Humbert M, Jaïs X, Ioos V, Hamid AM, Provencher S, Garcia G, Parent F, Hervé P, Simonneau G. Long-term response to calcium channel blockers in idiopathic pulmonary arterial hypertension. *Circulation*. 2005;111:3105–3111.
215. Sommer N, Hüttemann M, Pak O, Scheibe S, Knoepp F, Sinkler C, Malczyk M, Gierhardt M, Esfandiary A, Kraut S, Jonas F, Veith C, Aras S, Sydykov A, Alebrahimdehkordi N, Giehl K, Hecker M, Brandes RP, Seeger W, Grimminger F, Ghofrani HA, Schermuly RT, Grossman LI,

Weissmann N. Mitochondrial Complex IV Subunit 4 Isoform 2 Is Essential for Acute Pulmonary Oxygen Sensing. *Circ Res*. 2017;121:424–438.

216. Sonveaux P, Copetti T, Saedeleer CJ de, Végran F, Verrax J, Kennedy KM, Moon EJ, Dhup S, Danhier P, Frérart F, Gallez B, Ribeiro A, Michiels C, Dewhirst MW, Feron O. Targeting the lactate transporter MCT1 in endothelial cells inhibits lactate-induced HIF-1 activation and tumor angiogenesis. *PLoS ONE*. 2012;7:e33418.
217. Stefan Walenta, Michael Wetterling, Michael Lehrke, Georg Schwickert, Kolbein Sundfør,Walenta, Stefan, Wetterling M, Lehrke M, Schwickert G, Sundfør K, Rofstad EK, Mueller-Klieser, Wolfgang Einar K. Rofstad, and Wolfgang Mueller-Klieser2. High Lactate Levels Predict Likelihood of Metastases, Tumor Recurrence, and Restricted Patient Survival in Human Cervical Cancers. *Cancer Res*. 1999;2000.
218. Stenmark KR, Frid M, Perros F. Endothelial-to-Mesenchymal Transition: An Evolving Paradigm and a Promising Therapeutic Target in PAH. *Circulation*. 2016;133:1734–1737.
219. Stenmark KR, Meyrick B, Galie N, Mooi WJ, McMurtry IF. Animal models of pulmonary arterial hypertension: the hope for etiological discovery and pharmacological cure. *Am J Physiol Lung Cell Mol Physiol*. 2009;297:L1013-32.
220. Sutendra G, Michelakis ED. The metabolic basis of pulmonary arterial hypertension. *Cell Metab*. 2014;19:558–573.
221. Tonelli AR, Arelli V, Minai OA, Newman J, Bair N, Heresi GA, Dweik RA. Causes and circumstances of death in pulmonary arterial hypertension. *Am J Respir Crit Care Med*. 2013;188:365–369.
222. Tuder RM, Archer SL, Dorfmüller P, Erzurum SC, Guignabert C, Michelakis E, Rabinovitch M, Schermuly R, Stenmark KR, Morrell NW. Relevant issues in the pathology and pathobiology of pulmonary hypertension. *J Am Coll Cardiol*. 2013;62:D4-12.
223. Tuder RM, Davis LA, Graham BB. Targeting Energetic Metabolism. *Am J Respir Crit Care Med*. 2012;185:260–266.
224. Tuder RM, Stacher E, Robinson J, Kumar R, Graham BB. Pathology of pulmonary hypertension. *Clin Chest Med*. 2013;34:639–650.
225. Tunariu N, Gibbs SJR, Win Z, Gin-Sing W, Graham A, Gishen P, Al-Nahhas A. Ventilation-perfusion scintigraphy is more sensitive than multidetector CTPA in detecting chronic thromboembolic pulmonary disease as a treatable cause of pulmonary hypertension. *J Nucl Med*. 2007;48:680–684.
226. Uddin M, Kawami M, Yumoto R, Takano M. Effect of transforming growth factor-β1 on functional expression of monocarboxylate transporter 1 in alveolar epithelial A549 cells. *Naunyn Schmiedebergs Arch Pharmacol*. 2020;393:889–896.
227. Ullah MS, Davies AJ, Halestrap AP. The plasma membrane lactate transporter MCT4, but not MCT1, is up-regulated by hypoxia through a HIF-1alpha-dependent mechanism. *J Biol Chem*. 2006;281:9030–9037.
228. Vaillancourt M, Ruffenach G, Meloche J, Bonnet S. Adaptation and remodelling of the pulmonary circulation in pulmonary hypertension. *Can J Cardiol*. 2015;31:407–415.
229. Valérie F. E. D. Smolders, Cristina Rodríguez, Constanza Morén, Isabel Blanco, Jeisson Osorio, Lucilla Piccari, Cristina Bonjoch, Paul H. A. Quax, Victor I. Peinado, Manuel Castellà, Joan Albert Barberà, Marta Cascante, and Olga Tura-Ceide. Decreased Glycolysis as Metabolic Fingerprint of Endothelial Cells in Chronic Thromboembolic Pulmonary Hypertension. [Epub ahead of print].
230. van Meir EG, Kikuchi T, Tada M, Li H, Diserens AC, Wojcik BE, Huang HJ, Friedmann T, Tribolet N de, Cavenee WK. Analysis of the p53 gene and its expression in human glioblastoma cells. *Cancer Res*. 1994;54:649–652.
231. Visser WE, Friesema ECH, Visser TJ. Minireview: Thyroid hormone transporters: the knowns and the unknowns. *Mol Endocrinol*. 2011;25:1–14.
232. Vonk-Noordegraaf A, Haddad F, Chin KM, Forfia PR, Kawut SM, Lumens J, Naeije R, Newman J, Oudiz RJ, Provencher S, Torbicki A, Voelkel NF, Hassoun PM. Right heart adaptation to

pulmonary arterial hypertension: Physiology and pathobiology. *J Am Coll Cardiol*. 2013;62:D22-33.

233. Walenta S, Wetterling M, Lehrke M, Schwickert G, Sundfør K, Rofstad EK, Mueller-Klieser W. High lactate levels predict likelihood of metastases, tumor recurrence, and restricted patient survival in human cervical cancers. *Cancer Res*. 2000;60:916–921.
234. Wang Z, Yang K, Zheng Q, Zhang C, Tang H, Babicheva A, Jiang Q, Li M, Chen Y, Carr SG, Wu K, Zhang Q, Balistrieri A, Wang C, Song S, Ayon RJ, Desai AA, Black SM, Garcia JGN, Makino A, Yuan JX-J, Lu W, Wang J. Divergent changes of p53 in pulmonary arterial endothelial and smooth muscle cells involved in the development of pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol*. 2019;316:L216-L228.
235. Warburg O, Posener K, Negelein E. Über den Stoffwechsel der Carcinomzelle. *Biochem Z*. 1924:309–344.
236. Wilkens H, Konstantinides S, Lang I, Bunck AC, Gerges M, Gerhardt F, Grgic A, Grohé C, Guth S, Held M, Hinrichs J, Hoeper MM, Klepetko W, Kramm T, Krüger U, Lankeit M, Meyer BC, Olsson KM, Schäfers H-J, Schmidt M, Seyfarth HJ, Ulrich S, Wiedenroth CB, Mayer E. Chronisch thromboembolische pulmonale Hypertonie: Empfehlungen der Kölner Konsensus Konferenz 2016. *Dtsch Med Wochenschr*. 2016;141:S62-S69.
237. Wilkens H, Konstantinides S, Lang IM, Bunck AC, Gerges M, Gerhardt F, Grgic A, Grohé C, Guth S, Held M, Hinrichs JB, Hoeper MM, Klepetko W, Kramm T, Krüger U, Lankeit M, Meyer BC, Olsson KM, Schäfers H-J, Schmidt M, Seyfarth H-J, Ulrich S, Wiedenroth CB, Mayer E. Chronic thromboembolic pulmonary hypertension (CTEPH): Updated Recommendations from the Cologne Consensus Conference 2018. *Int J Cardiol*. 2018;272S:69–78.
238. Wilson MC, Meredith D, Bunnun C, Sessions RB, Halestrap AP. Studies on the DIDS-binding site of monocarboxylate transporter 1 suggest a homology model of the open conformation and a plausible translocation cycle. *J Biol Chem*. 2009;284:20011–20021.
239. Wilson MC, Meredith D, Halestrap AP. Fluorescence resonance energy transfer studies on the interaction between the lactate transporter MCT1 and CD147 provide information on the topology and stoichiometry of the complex in situ. *J Biol Chem*. 2002;277:3666–3672.
240. Xiao Y, Peng H, Hong C, Chen Z, Deng X, Wang A, Yang F, Yang L, Chen C, Qin X. PDGF Promotes the Warburg Effect in Pulmonary Arterial Smooth Muscle Cells via Activation of the PI3K/AKT/mTOR/HIF-1α Signaling Pathway. *Cell Physiol Biochem*. 2017;42:1603–1613.
241. Xu W, Kaneko FT, Zheng S, Comhair SAA, Janocha AJ, Goggans T, Thunnissen FBJM, Farver C, Hazen SL, Jennings C, Dweik RA, Arroliga AC, Erzurum SC. Increased arginase II and decreased NO synthesis in endothelial cells of patients with pulmonary arterial hypertension. *FASEB J*. 2004;18:1746–1748.
242. Xu W, Koeck T, Lara AR, Neumann D, DiFilippo FP, Koo M, Janocha AJ, Masri FA, Arroliga AC, Jennings C, Dweik RA, Tuder RM, Stuehr DJ, Erzurum SC. *Alterations of cellular bioenergetics in pulmonary artery endothelial cells*. 2007. Available at: https://www.pnas.org/content/104/4/1342. Accessed February 6, 2020.
243. Yokota H, Guo J, Matoba M, Higashi K, Tonami H, Nagao Y. Lactate, choline, and creatine levels measured by vitro 1H-MRS as prognostic parameters in patients with non-small-cell lung cancer. *J Magn Reson Imaging*. 2007;25:992–999.
244. Yuan X-J, Wang J, Juhaszova M, Gaine SP, Rubin LJ. Attenuated K+ channel gene transcription in primary pulmonary hypertension. *The Lancet*. 1998;351:726–727.
245. Zhao L, Ashek A, Wang L, Fang W, Dabral S, Dubois O, Cupitt J, Pullamsetti SS, Cotroneo E, Jones H, Tomasi G, Nguyen Q-D, Aboagye EO, El-Bahrawy MA, Barnes G, Howard LS, Gibbs JSR, Gsell W, He J-G, Wilkins MR. Heterogeneity in lung (18)FDG uptake in pulmonary arterial hypertension: potential of dynamic (18)FDG positron emission tomography with kinetic analysis as a bridging biomarker for pulmonary vascular remodeling targeted treatments. *Circulation*. 2013;128:1214–1224.
246. Zhao Y, Peng J, Lu C, Hsin M, Mura M, Wu L, Chu L, Zamel R, Machuca T, Waddell T, Liu M, Keshavjee S, Granton J, Perrot M de. Metabolomic heterogeneity of pulmonary arterial hypertension. *PLoS ONE*. 2014;9:e88727.

247. Zheng J. Energy metabolism of cancer: Glycolysis versus oxidative phosphorylation (Review). *Oncol Lett*. 2012;4:1151–1157.
248. Ziebart T, Walenta S, Kunkel M, Reichert TE, Wagner W, Mueller-Klieser W. Metabolic and proteomic differentials in head and neck squamous cell carcinomas and normal gingival tissue. *J Cancer Res Clin Oncol*. 2011;137:193–199.

12 Ethics

12.1 MCT1 and 4 biomarker measurements

JUSTUS-LIEBIG UNIVERSITÄT GIESSEN

FACHBEREICH 11 MEDIZIN

Ethik-Kommission, Klinikstr. 29 (Alte Chirurgie), D-35385 Gießen

Priv.-Doz. Dr. Dr. H. Gall
Med. Klinik II
Pneumologie
Klinikstr. 33
35385 Gießen

ETHIK-KOMMISSION
am Fachbereich Medizin
Vorsitz: Prof. H. Tillmanns

Klinikstr. 29 (Alte Chirurgie)
D-35385 Gießen
Tel.: (0641)99-42470 / 47660
ethik.kommission@pharma.med.uni-giessen.de

Gießen, 23. März 2020
Dr. Kr./

AZ.: **100/13**

Titel: *Biomarker für die pulmonale Hypertonie.*

Amendment vom 23.März 2020 bzgl. Messung weiterer potentieller Biomarker, hier „Monocarboxylat-Transporter-1 und -4"-Bestimmung aus Restblut

Sehr geehrter Herr Priv.-Doz. Dr. Dr. Gall, Liebe Herr Gall,

nach Durchsicht der eingereichten Unterlagen (email von Herrn Daniel Bermes, Doktorand) bestehen keine Einwände der Ethikkommission gegen die zusätzliche Bestimmung der Monocarboxylat-Transporter-1 und -4. Die Ethik-Kommission stimmt dem Amendment zu.

Mit freundlichen Grüßen

Prof. Dr. H. Tillmanns
Vorsitzender der Ethik-Kommission

24 MAR 2020
H. Gall

12.2 MCT1 and 4 measurement in lung tissue samples

JUSTUS-LIEBIG UNIVERSITÄT GIESSEN

FACHBEREICH 11 MEDIZIN

Ethik-Kommission, Klinikstr. 33, D-35385 Gießen

Prof. Dr. rer. nat. Norbert Weißmann
Excellence Cluster Cardiopulmonary System (ECCPS)
Aulweg 130
35392 Gießen

ETHIK-KOMMISSION
des Fachbereichs Medizin

Vorsitz: Prof. Dr. H. Tillmanns

(Alte Frauenklinik) Klinikstr. 33
D-35385 Gießen

Tel.: (0641)99 42470 (AB) /47660
Fax: (0641)99 42479

Gießen, den 26. November 2019
Az.: Dr. Kr./

Verwendung von Proben der "UGMLC *Biobank*", *der "DZL-Biobank" und der "Bio-Datenbank für CTEPH"* im Rahmen der geplanten Fortsetzungsperiode des SFB 1213:

Pulmonary Hypertension and Cor Pulmonale (2nd Funding Period), Speaker Prof. Dr. rer. nat. Norbert Weißmann

Bestätigung der Ethikkommission

Sehr geehrter Herr Prof. Dr. Weißmann,

die Einzelprojekte der oben genannten geplanten Fortsetzungsperiode des SFB (Antrag liegt uns vor) benötigen humane Proben aus den genannten Biobanken. Alle Biobanken wurden von der Ethik-Kommission des Fachbereichs Medizin behandelt und positiv begutachtet.
Im Einzelnen:

DZL-Biobank:
AZ 58/15: ***Antrag auf Erteilung eines Votums zur Implementierung des Data Warehouses des Deutschen Zentrums für Lungenforschung (DZL) sowie zur Umwidmung des Deutschen DPLD-Registers / Biobank (GOLDnet) in die UGMLC Giessen Biobank (Pneumologisches Patientenregister und Biomaterial-Sammlung) als Teil der zentralisierten Biobank des DZL***

UGMLC-Biobank:
AZ 10/06: ***Untersuchung von humanem Lungengewebe (molekularbiologische und zellbiologische Untersuchungen) zur Aufklärung der Pathogenese der Lungenfibrose, der pulmonalen Hypertonie sowie der chronisch obstruktiven Lungenerkrankung (COPD). Ergänzung zu Antrag 31/93.***

CTEPH-Bio-Datenbank:
AZ 44/14: ***Reverse Remodeling des rechten Ventrikels: Bio-Datenbank für Patienten mit chronisch thromboembolischer pulmonaler Hypertonie (CTEPH).***

Die geplante Nutzung der Proben entspricht den Zielen beider Biobanken. Es bestehen seitens der Ethikkommission daher keine Bedenken gegen die Verwendung von Proben beider Biobanken im Rahmen der geplanten Fortsetzungsperiode des SFB 1213.Wir wünschen weiterhin viel Erfolg.

Mit freundlichen Grüßen

Prof. Dr. H. Tillmanns
Vorsitzender der Ethik-Kommission

12.3 DZL Einverständniserklärung

UGMLC Gießen Biobank
am Zentrum für Innere Medizin, Med. Klinik 2
Justus-Liebig Universität Gießen
Aulweg 132
35392 Giessen

www.dzl.de

Sehr geehrte Patientin, sehr geehrter Patient, wir bitten Sie um Ihre Unterstützung für die Lungenforschung!

Das **Deutsche Zentrum für Lungenforschung (DZL)** hat sich zum Ziel gesetzt, Ursachen von Lungenerkrankungen besser zu verstehen, um sie zuverlässiger und schneller erkennen zu können und neue Therapien zu entwickeln.
Zu diesem Zweck werden in einer Biobank **Bioproben** gesammelt **und zugehörige Patientendaten** in einer Datenbank gespeichert.
Sie können uns darin aktiv unterstützen!

In Kürze die wichtigsten Punkte:

- jeder Patient muss über Biomaterialspende und Datenspeicherung aufgeklärt werden und zustimmen
- es wird nur sog. Restmaterial gesammelt, also z.B. Blutproben, die im Rahmen der aktuellen Untersuchung entnommen aber nicht mehr weiter benötigt werden
- kein zusätzliches gesundheitliches Risiko bei der Biomaterialspende
- langfristige Aufbewahrung der Bioproben über mehrere Jahrzehnte
- Verwendung für aktuelle und zukünftige medizinisch-wissenschaftliche Fragestellungen
- beinhaltet auch genetische Untersuchungen, auch des gesamten Erbgutes (Genom)
- personenbezogene Daten werden doppelt verschlüsselt, wodurch kein Rückschluss auf die Identität des Spenders möglich ist
- keine Weitergabe der personenbezogenen Daten an Forscher oder unberechtigte Dritte (z.B. Versicherungsunternehmen oder Arbeitgeber)
- Proben und Daten werden für Forschungszwecke auf Antrag weitergegeben, hierfür ist eine zusätzliche Bewertung durch eine Ethikkommission notwendig
- Zustimmung zur Biomaterialspende gilt für diesen, sowie auch für künftige Krankenhausbesuche
- Teilnahme ist völlig freiwillig und kann jederzeit ohne Angabe von Gründen widerrufen werden

In der nachfolgenden Patienteninformation werden alle juristischen, ethischen und datenschutzrechtlichen Aspekte der Biomaterialspende nochmals detailliert erläutert. Bitte lesen Sie diese sorgfältig durch. Sollten Sie etwas nicht verstehen oder weitere Informationen benötigen, fragen Sie bitte Ihre/n behandelnde/n Ärztin/Arzt.
Über eine Zustimmung zur Biomaterialspende würden wir uns sehr freuen, helfen Sie uns forschen!

PATIENTENINFORMATION

Sehr geehrte Patientin, sehr geehrter Patient!

Sie werden gegenwärtig als Patient* am Universitätsklinikum Gießen und Marburg in Gießen, an der Kerckhoff-Klinik Bad Nauheim oder am Evangelischen Krankenhaus Mittelhessen mit den Standorten Greifenstein (Pneumologische Abteilung) und Gießen (Ev. Krhs.) oder einem Krankenhaus, das mit diesen Kliniken kooperiert, ärztlich behandelt. Unsere Einrichtungen sind Mitglied des „*University of Giessen and Marburg Lung Center*" (UGMLC) und, hierüber, auch des Deutschen Zentrums für Lungenforschung (DZL). Das DZL ist eine vom Bundesministerium für Bildung und Forschung unterstützte Einrichtung, die sich die weitere Erforschung und die Entwicklung neuer Therapieverfahren von und für Lungenerkrankungen zum Ziel gemacht hat. Als Teil dieser Bemühungen werden bestimmte Körpermaterialien, wie z.B. Blut, Gewebe, Sputum, oder bronchoalveoläre Lavagen in einer sogenannten Biobank,

* Im Rahmen dieses Textes schließt die männliche Bezeichnung stets die weibliche Bezeichnung mit ein.

UGMLC Gießen Biobank
am Zentrum für Innere Medizin, Med. Klinik 2
Justus-Liebig Universität Gießen
Aulweg 132
35392 Giessen

der „UGMLC Giessen Biobank", gesammelt. Ausgewählte, und mit den Proben verknüpfte, medizinische Daten werden in einer Datenbank gespeichert.
In einem zweiten Schritt werden die lokal im Register der UGMLC Giessen Biobank enthaltenen Daten in die DZL Datenbank (sogenanntes DZL-Data Warehouse, http://www.dzl.de/index.php/de/forschung/plattformen/biobank) überführt, und mit den Biomaterialien zusammen zentral verwaltet und ausgewertet. Dabei werden die lokal in der UGMLC Giessen Biobank gelagerten Biomaterialien auf Anfrage der DZL Biobank für wissenschaftliche Untersuchungen weitergegeben.

Die Untersuchung von menschlichen Biomaterialien und die Analyse der daraus gewonnenen oder zu gewinnenden Daten sind zu einem wichtigen Instrument medizinischer Forschung geworden. **Deshalb fragen wir unsere Patienten und daher auch Sie, ob sie bereit sind, uns bestimmte Körpermaterialien und Daten für die Forschung zur Verfügung zu stellen. Ihre Einwilligung in eine Verwendung von Biomaterialien und zugehörige Daten ist freiwillig. Soweit Sie sich nicht beteiligen möchten oder Ihre Zustimmung später widerrufen möchten, erwachsen Ihnen daraus keine Nachteile.**

Im Folgenden informieren wir Sie über die Ziele der UGMLC Giessen Biobank, welche Mitglied der zentralen Biobank des DZL ist, sowie über die Verfahrensweisen und die Maßnahmen zum Schutz Ihrer personenbezogenen Daten, damit Sie sich auf dieser Grundlage Ihre eigene Meinung bilden und eine Entscheidung treffen können.

1. Ziele der Biobank

Die UGMLC Giessen Biobank dient als Mitglied der DZL-Biobank der Förderung medizinischer Forschung. In der Biobank werden Biomaterialien und ausgewählte medizinische Daten langfristig aufbewahrt und für die Erforschung von Lungenerkrankungen zur Verfügung gestellt, um die Vorbeugung, Erkennung und Behandlung von Lungenerkrankungen zu verbessern. **Das Ziel dieser Forschung ist nicht, bei Ihnen oder anderen einzelnen Personen eine Diagnose zu erstellen oder krankheitsauslösende Veranlagungen nachzuweisen.**

2. Um welche Art von Biomaterialien und Daten handelt es sich?

In der DZL Biobank werden Biomaterialien wie z.B. Blut, Lungengewebe (Biopsien sowie chirurgisch entnommenes Lungengewebe), Sputum, Spülflüssigkeit der Atemwege (sog. bronchoalveoläre Lavagen), exhalative Atemkondensate, Urin, Zellen, Abstriche und Luftproben gesammelt.
Bei dem Biomaterial handelt es sich um Gewebe und Körperflüssigkeiten, die im Laufe Ihres früheren, derzeitigen oder zukünftigen Krankenhausaufenthaltes

a) **zum Zweck der Untersuchung/Behandlung** entnommen, dafür jedoch nicht mehr benötigt werden und daher ansonsten vernichtet würden (Reste von Blutproben, Spülflüssigkeiten, Gewebeproben etc.).

b) **zusätzlich bei der Routinediagnostik** entnommen werden. Hierzu werden im Rahmen der Routine-Blutentnahme bis zu 25 ml Blut (etwa 5 Teelöffel) zusätzlich entnommen.

Erhebung personenbezogener Daten:
Die erhobenen Daten umfassen ausgewählte Informationen zu Ihrer Person. Dabei werden sowohl medizinische Daten (z.B. Alter, Geschlecht, verordnete Medikamente, familiäre Vorbelastung für bestimmte Erkrankungen, bekannte genetische Veränderungen) als auch Untersuchungsbefunde ihres früheren, derzeitigen oder zukünftigen Krankenhausaufenthaltes erfasst (z.B. Röntgenbilder, Ultraschallbefunde, Laborwerte, Lungenfunktionen).
Die UGMLC-Giessen Biobank und das DZL streben grundsätzlich an, an mehreren Zeitpunkten die oben erwähnten Biomaterialien und begleitende medizinischen Daten zu

UGMLC Gießen Biobank
am Zentrum für Innere Medizin, Med. Klinik 2
Justus-Liebig Universität Gießen
Aulweg 132
35392 Giessen

erheben. Wir werden daher auch bei späteren Vorstellungen in unserem Haus die o.g. Proben und erhobenen medizinischen Daten sammeln.

3. Wie werden die Biomaterialien und Daten verwendet?

Wir fragen Sie nach einer sehr breit gefassten Erlaubnis zur Verwendung Ihrer Biomaterialien und Daten. Diese werden **ausschließlich für die medizinische Forschung** bereitgestellt, die die Vorbeugung, Erkennung und Behandlung von Erkrankungen verbessern soll. Sie sollen im Sinne eines möglichst großen Nutzens für die Allgemeinheit für viele verschiedene medizinische Forschungszwecke verwendet werden. Informationen über die Aktivitäten des DZL auf dem Gebiet der Lungenforschung und die medizinischen Forschungsprojekte mit Biomaterialien aus den DZL-Biobanken finden Sie auf der DZL-Homepage unter www.dzl.de. Sollten Sie hierunter Forschungsvorhaben finden, deren Inhalte nicht Ihre Zustimmung finden, können Sie jederzeit ihre Einwilligung widerrufen

Aus einem kleinen Stück Ihres Restgewebes (z.B. nach Operation bzw. endoskopischer Biopsie und histologischer Begutachtung) oder aus Ihrem Blut, sollen nach Eignungsprüfung gegebenenfalls auch Gewebe und/oder Zellkulturen angezüchtet werden. Nach erfolgreicher Kultivierung sollen verschiedene Zellfunktionen wie Wachstum und Widerstandsfähigkeit sowie die Bildung wichtiger Proteine untersucht werden, um deren Bedeutung für die Steuerung verschiedener Zellfunktionen besser zu verstehen.
Die angezüchteten Zellen sollen für spätere Versuche gelagert werden, um auch zukünftige Fragestellungen beantworten zu können. Ziel aller Untersuchungen ist es, ein besseres Verständnis über molekularbiologische Vorgänge bei Entstehung, Wachstum und Ausbreitung von Lungenerkrankungen zu erreichen und um ggf. neue Therapieansätze zu finden.

Zum derzeitigen Zeitpunkt können noch nicht alle zukünftigen medizinischen Forschungsziele beschrieben werden. Diese können sich sowohl auf Lungenerkrankungen als auch auf heute zum Teil noch unbekannte Krankheiten und genetische Zusammenhänge, auch anderer Organsysteme, beziehen. Weil sich in der Forschung immer wieder neue Fragen ergeben, kann es also sein, dass Ihre Proben und Daten auch für medizinische Forschungsfragen verwendet werden, die man heute noch nicht absehen kann. Deshalb werden an Ihren Biomaterialien möglicherweise auch genetische Untersuchungen, also Untersuchungen der Erbsubstanz, durchgeführt, und zwar unter Umständen auch eine Untersuchung Ihres gesamten Genoms. Aus Genomdaten kann möglicherweise ein Bezug zu Ihrer Person abgeleitet werden. Wir stellen jedoch technisch und organisatorisch sicher, dass Ihre Genomdaten nicht missbräuchlich verwendet werden.

Die Biomaterialien und Daten sollen langfristig aufbewahrt und für die medizinische Forschung, wie auf der Homepage des DZL (www.dzl.de) dargestellt, bereitgestellt werden. Die Dauer der Lagerung von Biomaterialien/Speicherung von Daten richtet sich dabei an die wissenschaftliche Erforderlichkeit und die tatsächliche Verwendbarkeit der Probe. Nach dem jetzigen Stand der Wissenschaft und der Technik erscheint eine Lagerung von flüssigen Biomaterialien wie Serum, Plasma oder Lungenspülflüssigkeit nur für einen Zeitraum von etwa 80 Jahren sinnvoll. DNA-haltige Biomaterialien wie Zellen, Gewebestücke oder Paraffinschnitte können allerdings auch noch nach Jahrtausenden zuverlässig analysiert werden und wertvolle Beiträge zu wissenschaftlichen Fragestellungen leisten. Solange also die entsprechenden kollaborativen Wissenschaftsstrukturen wie das DZL (oder Folgeeinrichtungen) bestehen und aktiv an den gesammelten Daten oder Proben wissenschaftlich arbeiten, werden zumindest DNA-haltige Proben samt der dazu gehörigen

klinischen Daten langfristig aufbewahrt werden. Ähnliches gilt auch flüssige Biomaterialien, wenn sich der Stand der Technik weiterentwickelt und mit neuen Analyseverfahren auch über 80 Jahre alte Proben zuverlässig untersucht werden können.

UGMLC Gießen Biobank
am Zentrum für Innere Medizin, Med. Klinik 2
Justus-Liebig Universität Gießen
Aulweg 132
35392 Giessen

Aus logistischen Gründen ist es der Biobank nicht möglich, individuelle Eingrenzungen (z.B. Ausschluss bestimmter Forschung, Ausschluss der Weitergabe der Materialien an Dritte) vorzunehmen. Wenn Sie mit der oben beschriebenen Art und Dauer der Nutzung nicht in vollem Umfang einverstanden sind, werden Ihre Biomaterialien und Daten nicht in die UGMLC Giessen Biobank aufgenommen.

Mit der Koordinierung bzw. dem Management der UGMLC Giessen Biobank wie auch der DZL Biobank sind aktuell beauftragt:
Prof. Dr. Andreas Günther / Dr. Clemens Ruppert,
Zentrum für Innere Medizin, Medizinische Klinik und Poliklinik II
Justus-Liebig-Universität Giessen
Klinikstrasse 36
35392 Giessen
Telefon: 0641/985/42502, Telefax: 0641/985/42508
Email: Andreas.Guenther@innere.med.uni-giessen.de
Clemens.Ruppert@innere.med.uni-giessen.de

4. Welche Risiken sind mit Ihrer Spende verbunden?

a. Gesundheitliche Risiken:

Da wir für die Biobank von Patienten lediglich Körpermaterial verwenden wollen, das im Rahmen der bei Ihnen vorgesehenen diagnostischen oder therapeutischen Maßnahmen ohnehin entnommen wird und als Restmaterial normalerweise vernichtet würde, ist die Spende für Sie mit keinem zusätzlichen gesundheitlichen Risiko verbunden.
Falls im Rahmen einer diagnostischen Routine-Maßnahme, z.B. bei einer Blutentnahme *zusätzlich* bis zu 25ml Blut (das entspricht etwa 5 Teelöffeln) abgenommen wird, ist diese Entnahme nach Einschätzung des behandelnden Arztes für Sie mit keinem zusätzlichen gesundheitlichen Risiko verbunden.

b. Weitere Risiken:

Bei jeder Erhebung, Speicherung und Übermittlung von Daten aus Ihren Biomaterialien im Rahmen von Forschungsprojekten bestehen Vertraulichkeitsrisiken (z.B. die Möglichkeit, Sie zu identifizieren), insbesondere im Hinblick auf die Information zu Ihrer Erbsubstanz. Diese Risiken lassen sich nicht völlig ausschließen und steigen, je mehr Daten miteinander verknüpft werden können, insbesondere auch dann, wenn Sie selbst (z.B. zur Ahnenforschung) genetische Daten im Internet veröffentlichen.
Die UGMLC-Giessen Biobank versichert Ihnen, alles nach dem Stand der Technik Mögliche zum Schutz Ihrer Privatsphäre zu tun. Näheres finden Sie unter Punkt 7: „Wer hat Zugang zu Ihren Biomaterialien und Daten?"

5. Welcher Nutzen ergibt sich für Sie persönlich?

Persönlich können Sie für Ihre Gesundheit keinen unmittelbaren Vorteil oder Nutzen aus der Spende Ihrer Proben und Daten erwarten. Deren Auswertung dient ausschließlich Forschungszwecken und nicht dazu, Rückschlüsse auf Ihre Gesundheit zu ziehen.
Eine Rückmeldung individueller Forschungsergebnisse an Sie erfolgt nicht.

6. Welcher Nutzen ergibt sich für unsere Gesellschaft?

Alle derzeit durchgeführten, wie auch künftige medizinisch-wissenschaftliche Forschungsvorhaben, zielen auf eine Verbesserung unseres Verständnisses für die Krankheitsentstehung und die Diagnosestellung und auf dieser Basis auf die Neuentwicklung von verbesserten Behandlungs-und Vorbeugungsmaßnahmen. Informationen über die Aktivitäten des DZL und der DZL-Biobanken auf dem Gebiet der Lungenforschung finden Sie auf der DZL-Homepage unter www.dzl.de.

UGMLC Gießen Biobank
am Zentrum für Innere Medizin, Med. Klinik 2
Justus-Liebig Universität Gießen
Aulweg 132
35392 Giessen

7. Wer hat Zugang zu Ihren Biomaterialien und Daten und wie werden sie geschützt?

a. Kodierung Ihrer Biomaterialien und Daten

Alle Daten, die Ihre Person unmittelbar identifizieren (Name, Geburtsdatum, Anschrift etc.) werden unverzüglich nach Gewinnung der Biomaterialien durch die UGMLC Giessen Biobank durch einen Code ersetzt (pseudonymisiert, verschlüsselt). Bei der Überführung der Datensätze und Proben an die DZL Biobank wird der Datensatz nochmals neu kodiert und gespeichert. Diese doppelte Kodierung schließt eine Identifizierung Ihrer Person durch Unbefugte nach heutiger Kenntnis weitestgehend aus. Erst in dieser Form werden die Biomaterialien und Daten für Forschungszwecke innerhalb des DZL zur Verfügung gestellt.

b. Weitergabe von Biomaterialien und Daten

Die Sie unmittelbar identifizierenden Daten bleiben in der Einrichtung, in der die Proben und Daten gewonnen wurden, und werden dort getrennt von den Biomaterialien und medizinischen Daten gespeichert. Dabei werden technische und organisatorische Maßnahmen (z.B. geschützter Serverbereich, Firewall, Datenhosting an getrennten universitären Rechenzentren, Kodierung der Daten nach dem Prinzip der Public-Key-Kryptographie) getroffen, um einen missbräuchlichen Zugriff auf die Daten zu verhindern. Ein Zugriff auf sie erfolgt nur, um entweder weitere oder fehlende medizinische Daten aus Ihren Krankenunterlagen zu ergänzen oder erneut mit Ihnen in Kontakt zu treten, falls Sie der Kontaktaufnahme zugestimmt haben (s. unten, Punkt 9). Eine Weitergabe der personenbezogenen Daten an Forscher oder andere unberechtigte Dritte, etwa Versicherungsunternehmen oder Arbeitgeber, erfolgt nicht.

Die doppelt verschlüsselten Biomaterialien und medizinischen Daten können für medizinische genauer bestimmte Forschungszwecke auf Antrag nach zuvor festgelegten Regeln an andere Einrichtungen wie Universitäten, Forschungsinstitute und forschende Unternehmen innerhalb der EU weitergegeben werden. Dabei werden die Daten unter Umständen auch mit medizinischen Datensätzen in anderen Datenbanken verknüpft, sofern die gesetzlichen Voraussetzungen hierfür erfüllt sind.

Biomaterialien und Daten, die an Forscher herausgegeben wurden, dürfen nur für den beantragten Forschungszweck verwendet und vom Empfänger nicht zu anderen Zwecken weitergegeben werden. Nicht verbrauchtes Material wird an die Biobank zurückgegeben oder vernichtet.

c. Weitergabe in Länder außerhalb der Europäischen Union

Ihre Proben und Daten können auch an Forschungspartner in Ländern außerhalb der EU weitergegeben werden, wenn eine der folgenden Voraussetzungen erfüllt ist:

- Die Europäische Kommission hat bei dem Land ein angemessenes gesetzliches Datenschutzniveau festgestellt,
  oder, wenn dies nicht erfolgt ist,
- die UGMLC Giessen Biobank vereinbart mit den Forschungspartnern vertragliche Datenschutzklauseln, die von der Europäischen Kommission oder der zuständigen Aufsichtsbehörde beschlossen oder genehmigt wurden. Sie können bei der UGMLC Giessen Biobank eine Kopie dieser Datenschutzklauseln erhalten.

Darüber hinaus kann es aber auch vorkommen, dass Proben und Daten an Forschungspartner in Drittländern weitergegeben werden sollen, für die keine dieser beiden Voraussetzungen erfüllt ist. Diese Länder haben möglicherweise ein niedrigeres Datenschutzniveau als die EU. Die UGMLC Giessen Biobank sichert zu, auch in diesen Fällen die Forschungspartner vertraglich, soweit rechtlich möglich, zur Einhaltung des EU-Datenschutz-Niveaus zu verpflichten. Dennoch besteht das Risiko, dass staatliche oder private Stellen auf Ihre Daten zugreifen, obwohl dies nach dem europäischen Datenschutzrecht nicht zulässig wäre. Zudem kann es sein, dass Ihnen dort weniger oder schlechter durchsetzbare Betroffenenrechte zustehen und es keine unabhängige Aufsichtsbehörde gibt, die Sie bei der Wahrnehmung ihrer

UGMLC Gießen Biobank
am Zentrum für Innere Medizin, Med. Klinik 2
Justus-Liebig Universität Gießen
Aulweg 132
35392 Giessen

Rechte unterstützen könnte. Eine Weitergabe Ihrer Proben und Daten kann in diesem Fall nur erfolgen, wenn Sie dem ausdrücklich zugestimmt haben. Dazu können Sie in der Einwilligungserklärung das entsprechende Kästchen ankreuzen.

d. Bewertung durch die Ethik-Kommission

Voraussetzung für die Verwendung der Biomaterialien und Daten für ein konkretes medizinisches Forschungsprojekt innerhalb oder ausserhalb des DZL ist, dass das Forschungsvorhaben inhaltlich durch entsprechende unabhängige Gremien (z.B. eine Ethikkommission) bewertet wurde. Die Ethikkommission prüft die ethischen und rechtlichen Aspekte des Forschungsprojekts. Nur wenn diese keine Einwände oder ethisch-rechtliche Bedenken dagegen hat, wird das konkrete Forschungsvorhaben auch durchgeführt.

e. Veröffentlichungen

Wissenschaftliche Veröffentlichungen von Ergebnissen erfolgen ausschließlich anonymisiert, also in einer Form, die keine Rückschlüsse auf Ihre Person zulässt. Das gilt insbesondere auch für genetische Informationen. Möglich ist allerdings eine Aufnahme genetischer Informationen in besonders geschützte wissenschaftliche Datenbanken, die für die Allgemeinheit nicht zugänglich sind.

8. Erlangen Sie einen finanziellen Vorteil aus der Nutzung Ihrer Biomaterialien und Daten?

Mit der Überlassung der Biomaterialien an die UGMLC Giessen Biobank, die lokal die DZL Biobank vertritt, werden diese Eigentum der UGMLC Giessen Biobank. Für die Überlassung Ihrer Biomaterialien und Daten erhalten Sie kein Entgelt. Sollte aus der Forschung ein kommerzieller Nutzen erzielt werden, werden Sie daran nicht beteiligt.
Die Biobank verwendet Ihre Biomaterialien und Daten ausschließlich für wissenschaftliche Zwecke. Die Proben und Daten werden nicht verkauft. Für die Bereitstellung der Proben und Daten kann die Biobank jedoch von den Nutzern eine Aufwandsentschädigung erheben.

9. Erfolgt eine erneute Kontaktaufnahme mit Ihnen?

Zur Erhebung von weiteren Verlaufsdaten kann es sinnvoll werden, zu einem späteren Zeitpunkt erneut Kontakt mit Ihnen aufzunehmen, um ergänzende Informationen und/oder

Biomaterialien von Ihnen zu erbitten. Zudem kann die erneute Kontaktaufnahme genutzt werden, um z. B. Ihre Einwilligung zum Abgleich mit anderen Datenbanken einzuholen.
Sollte eine solche Kontaktaufnahme von Ihnen erwünscht sein, wird dies durch unsere Klinik erfolgen.
Kreuzen Sie in der Einwilligungserklärung bitte an, ob Sie eine erneute Kontaktaufnahme in diesen Fällen wünschen oder nicht.

10. Was beinhaltet Ihr Widerrufsrecht?

Sie können Ihre Einwilligung zur Verwendung Ihrer Biomaterialien und Daten jederzeit ohne Angabe von Gründen und ohne nachteilige Folgen für Sie widerrufen. Die Rechtmäßigkeit der bis zum Widerruf erfolgten Nutzung der Proben und Daten bleibt davon jedoch unberührt.

Im Falle eines Widerrufs können Sie entscheiden, ob Ihre Biomaterialien vernichtet und die dazu gehörenden Daten gelöscht werden sollen. Eine Datenlöschung kann allerdings nur erfolgen, soweit dies mit zumutbarem technischem Aufwand möglich ist. Zudem können Daten aus bereits durchgeführten Analysen nicht mehr entfernt werden.
Statt der Vernichtung bzw. Löschung können Sie auch zustimmen, dass die Biomaterialien und Daten in anonymisierter Form für weitere Forschungsvorhaben verwendet werden dürfen. Anonymisierung bedeutet, dass der Identifizierungscode gelöscht wird, über den ermittelt

werden kann, von welcher Person die Probe stammt. Sobald der Bezug der Biomaterialien und der übrigen Daten zu Ihrer Person gelöscht wurde, ist gezielte eine Vernichtung aufgrund Ihrer Entscheidung jedoch nicht mehr möglich. Zudem können bereits für Forschungsprojekte ausgegebene Proben nicht zurückgeholt und Daten aus bereits durchgeführten Analysen nicht mehr entfernt werden. Trotz Widerrufs kann eine spätere Zuordnung des genetischen Materials zu Ihrer Person über andere Quellen niemals ausgeschlossen werden.

Wenden Sie sich für einen Widerruf bitte an die unter Punkt 3 genannte Leitung der Biobank- mit den dort angegebenen Kontaktdaten. Sie werden ihren Widerruf an die zuständige Datentreuhänderstelle weiterleiten.

11. Welche weiteren Datenschutzrechte haben Sie?

Rechtsgrundlage für die Datenverarbeitung ist Ihre Einwilligung gemäß Art. 6 Abs. 1 Buchst. a und Art. 9 Abs. 2 Buchst. a der EU-Datenschutz-Grundverordnung (EU-DSGVO).
Nach Art. 13 ff, EU-DSGVO haben Sie das Recht vom Verantwortlichen für die Erhebung personenbezogener Daten Auskunft über die von Ihnen gespeicherten personenbezogenen Daten zu verlangen. Ebenfalls können Sie die Berichtigung falscher Daten, eine Übertragung der von Ihnen zur Verfügung gestellten Daten sowie eine Löschung der Daten oder Einschränkung ihrer Verarbeitung verlangen.

Ihre Daten werden im Falle der UGMLC Giessen Biobank durch die Justus-Liebig-Universität (www.uni-giessen.de; Präsident aktuell Prof. Dr. J. Mukherjee), im Falle der DZL Biobank durch das Deutsche Zentrum für Lungenforschung e.V. (www.DZL.de; Koordinator aktuell Prof. Dr. W. Seeger), beide mit Sitz in Giessen, verarbeitet, die auch entsprechend verantwortliche Personen für die Erhebung personenbezogener Daten sind.

Die Justus-Liebig Universität Gießen hat einen Datenschutzbeauftragten bestellt. An ihn können Sie sich mit allen Anliegen rund um Ihre Daten wenden oder auch mit einer Beschwerde über Datenschutzverstöße. Seine Kontaktdaten lauten wie folgt:
Datenschutzbeauftragter der Justus-Liebig Universität Gießen
Dezernat B, Ludwigstraße 23, 35390 Gießen
datenschutz@uni-giessen.de, Telefon: 0641 / 99-12230, Fax: 0641 99-12229

Zudem haben Sie das Recht auf Beschwerde bei der Datenschutzaufsichtsbehörde, wenn Sie meinen, dass die Verarbeitung Ihrer Daten datenschutzrechtlich nicht zulässig ist (Art. 77 DSGVO). In Ihrem Falle handelt es sich dabei um folgende Institution:
Der Hessische Datenschutzbeauftragte
Postfach 3163, 65021 Wiesbaden
Poststelle@datenschutz.hessen.de, Tel. 0611/1408-0, Fax 0611/1408-900

12. Wo kann ich weitere Informationen erhalten?

Sollte Ihnen etwas unklar sein, fragen Sie bitte Ihren behandelnden Arzt bzw. Ihren Studienarzt, bevor Sie Ihre Zustimmung erteilen. Sie können sich wegen Rückfragen auch zu einem späteren Zeitpunkt an _____ wenden.
Name Arzt

UGMLC Gießen Biobank
am Zentrum für Innere Medizin, Med. Klinik 2
Justus-Liebig Universität Gießen
Aulweg 132
35392 Giessen

www.dzl.de

Bitte lesen Sie die folgende Einwilligungserklärung aufmerksam durch, kreuzen Sie Zutreffendes an und unterschreiben Sie anschließend am Ende dieser Einwilligungserklärung, sofern Sie einverstanden sind.

EINWILLIGUNGSERKLÄRUNG

EXEMPLAR FÜR DEN PATIENTEN

Name	Vorname	Geburtsdatum	Geburtsort

Ich bin damit einverstanden, dass meine Biomaterialien und ausgewählte klinische Daten, wie in der Patienteninformation unter Punkt 2 beschrieben, an die UGMLC Giessen Biobank bzw. die DZL Biobank gegeben und dort für medizinische Forschungszwecke aufbewahrt und verwendet werden.
Dies betrifft unter Umständen auch die (doppelt kodierte) Weitergabe für Forschungsprojekte oder an Forschungseinrichtungen im Ausland mit möglicherweise niedrigerem Datenschutzniveau. Es werden nur medizinische, jedoch keine meine Person identifizierenden Daten weitergegeben.

Ich stimme der Entnahme von Blut-, Gewebe bzw. der unter Punkt 2 angegebenen Biomaterialien zu. Ich stimme zu, dass aus meinen Biomaterialproben gegebenenfalls Zellkulturen kultiviert werden. Ich stimme zu, dass meine Biomaterialproben und die darin enthaltenen Bestandteile wie DNA, RNA und Proteine, über molekularbiologische Methoden analysiert werden, einschließlich einer kompletten Analyse des Erbgutes.

Das Eigentum an den Biomaterialien übertrage ich an die lokal mit der Biomaterial Sammlung beauftragten UGMLC Giessen Biobank. Ich weiß, dass ich über die Teilnahme an der Biobank keinen kommerziellen Nutzen oder persönliche Vorteile ziehen werde. Ich verzichte zugleich auf Entgeltzahlungen und urheber- oder patentrechtliche Ansprüche, die z.B. die mit der Entwicklung neuer Therapien oder Diagnostika verbunden sind.

Ich habe die Patienteninformation gelesen und hatte die Gelegenheit, Fragen zu stellen.

Ich weiß, dass meine Teilnahme freiwillig ist und ich meine Einwilligung jederzeit ohne Angabe von Gründen widerrufen kann, ohne dass mir daraus irgendwelche Nachteile entstehen. Bei Widerruf werden meine personenbezogenen Daten anonymisiert. Die Datenverarbeitung bis zum Zeitpunkt meines Widerrufs bleibt rechtmäßig.

Ich bin damit einverstanden, dass ich bzw. mein Hausarzt evtl. zu einem späteren Zeitpunkt erneut kontaktiert werde (falls nicht gewünscht bitte „**nein**" ankreuzen):

- zum Zweck der Gewinnung weiterer Informationen / Biomaterialien, □ **nein**
- zum Zweck der Einwilligung in den Abgleich mit anderen medizinischen Forschungsdatenbanken □ **nein**

Diese Rückmeldung soll erfolgen über die Einrichtung, in der meine Biomaterialien / Daten gewonnen wurden oder über folgenden Arzt (falls gewünscht, bitte angeben):

Name und Anschrift des Arztes: .....

UGMLC Gießen Biobank
am Zentrum für Innere Medizin, Med. Klinik 2
Justus-Liebig Universität Gießen
Aulweg 132
35392 Giessen

Einwilligung zur Erhebung, Verarbeitung und Nutzung personenbezogener Daten:

Ich erkläre mich damit einverstanden, dass die UGMLC Giessen Biobank und die DZL Biobank wie in der Patienteninformation beschrieben

- personenbezogene Daten, wie z.B. Alter, Geschlecht, Diagnose oder bekannte genetische Veränderungen, von mir erhebt und speichert,
- weitere ausgewählte Angaben über meine Gesundheit (wie z.B. Laborwerte, Ultraschallbefunde, Lungenfunktionswerte, Röntgenbilder) aus meinen Krankenunterlagen entnimmt,
- und die Daten gemeinsam mit meinen Biomaterialien pseudonymisiert (das heißt kodiert) speichert bzw aufbewahrt.

Die Biomaterialien und Daten dürfen langfristig für medizinische Forschungsvorhaben verwendet werden. Eine Übersicht über die Forschungsvorhaben unter Verwendung von Biomaterialien finde ich auf der Homepage des DZL unter www.dzl.de.

Sie dürfen pseudonymisiert nach Prüfung und Beratung durch die zuständige öffentlich rechtliche Ethikkommission an Universitäten, Forschungsinstitute und forschende Unternehmen, ggf. auch im Ausland, zu Zwecken medizinischer Forschung weitergegeben werden. Meine Person identifizierende Daten werden nicht weitergegeben.

Meine Einwilligung umfasst auch die Übermittlung meiner pseudonymisierten, d.h. verschlüsselten Patientendaten für medizinische Forschungszwecke in Länder, bei denen von der Europäischen Kommission kein angemessenes Datenschutzniveau festgestellt wurde. Dies ist generell zulässig, wenn ein Angemessenheitsbeschluss der Europäischen Kommission vorliegt oder behördlich genehmigte Datenschutzklauseln angewendet werden.

Darüber hinaus willige ich in die Weitergabe meiner Biomaterialien und Daten in Länder außerhalb der EU auch in den Fällen ein, in denen kein Angemessenheitsbeschluss der Europäischen Kommission vorliegt und keine behördlich genehmigten Datenschutzklauseln angewendet werden. Über die möglichen Risiken einer solchen Weitergabe bin ich aufgeklärt worden (Ziff. 7c in der Information).

☐ Ja ☐ Nein

Ich bin darüber aufgeklärt worden, dass unter Umständen auch Analysen des kompletten Erbgutes (Genom) durchgeführt werden und aus Genomdaten grundsätzlich ein Bezug zu meiner Person hergestellt werden könnte, dass das DZL aber technische und organisatorische Maßnahmen gegen eine missbräuchliche Verwendung getroffen hat.

Ich bin darüber aufgeklärt worden, dass ich meine Einwilligung gegenüber der UGMLC Giessen Biobank bzw. der DZL Biobank ohne Angabe von Gründen jederzeit widerrufen kann. Beim Widerruf werden auf mein Verlangen die verbliebenen Biomaterialien vernichtet und die erhobenen Daten gelöscht oder anonymisiert. Daten aus bereits durchgeführten Analysen können nicht mehr entfernt oder gelöscht werden.

Ich habe das Recht, Auskunft (einschließlich unentgeltlicher Überlassung einer Kopie) über die mich betreffenden personenbezogenen Daten zu erhalten sowie ggf. deren Berichtigung zu verlangen.
Bei Vorliegen der gesetzlichen Voraussetzungen kann ich die Löschung personenbezogener Daten, die Einschränkung der Verarbeitung oder die Datenübertragung verlangen, sowie Widerspruch gegen die Verarbeitung einlegen. Sollten Sie diese Rechte gegenüber der Datenverarbeitenden Stelle geltend machen wird geprüft, ob die gesetzlichen Voraussetzungen erfüllt sind.

Eine Kopie der Patienteninformation und Einwilligungserklärung habe ich erhalten. Das Original verbleibt bei der UGMLC Giessen Biobank.

Name des Patienten in Druckbuchstaben	***Ort, Datum***	***Unterschrift des Patienten***

Ich habe das Aufklärungsgespräch geführt und die Einwilligung des Patienten eingeholt:

Ort, Datum	***Unterschrift des Arztes/Biobank Mitarbeiter***

13 Ehrenwörtliche Erklärung

„Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nichtveröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten sowie ethische, datenschutzrechtliche und tierschutzrechtliche Grundsätze befolgt. Ich versichere, dass Dritte von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen, und dass die vorgelegte Arbeit weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zweck einer Promotion oder eines anderen Prüfungsverfahrens vorgelegt wurde. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht. Insbesondere wurden alle Personen genannt, die direkt und indirekt an der Entstehung der vorliegenden Arbeit beteiligt waren. Mit der Überprüfung meiner Arbeit durch eine Plagiatserkennungssoftware bzw. ein internetbasiertes Softwareprogramm erkläre ich mich einverstanden.“

_____ _____

Ort/Datum Unterschrift

14 Publikationsverzeichnis

Poster

Annual Retreat of the International Graduate Program Molecular Biology and Medicine of the Lung, 3.-5. Juli 2019

Vortrag

Annual Retreat of the International Graduate Program Molecular Biology and Medicine of the Lung, 11.-13. Oktober 2022

Publikationen

Sydykov, A.; Petrovic, A.; Maripov, A.M.; Gredic, M.; ***Bermes, D.G****.; Kushubakova, N.; Muratali Uulu, K.; Pilz, C.; Cholponbaeva, M.; Duishobaev, M.; Satybaldyev, S.; Satieva, N.; Mamazhakypov, A.; Sartmyrzaeva, M.; Omurzakova, N.; Kerimbekova, Z.; Baktybek, N.; Kulchoroeva, C.; Pak, O.; Zhao, L.; Weissmann, N.; Avdeev, S.; Maslov, L.N.; Ghofrani, H.A.; Schermuly, R.T.; Sarybaev, A.S.; Kosanovic, D.* Circulating Microparticles Are Differentially Increased in Lowlanders and Highlanders with High Altitude Induced Pulmonary Hypertension during the Cold Season. Cells 2022, 11, 2932. https://doi.org/10.3390/cells11192932

15 Danksagung

Schaut man auf das Deckblatt erscheint nur mein Name. Das entspricht aber nicht der ganzen Wahrheit. Viele Menschen haben mich, bei dieser Arbeit und darüber hinaus, begleitet und unterstützt. Ich hatte viel zu lernen und ohne diese Menschen hätte ich diese Arbeit nicht schreiben können. An dieser Stelle habe ich die Möglichkeit mich zu bedanken.

Prof. Dr. Dr. Henning Gall für die Überlassung des Dissertationsthemas und die exzellente Betreuung.

Prof. Dr. Ralph T. Schermuly in dessen Arbeitsgruppe die Experimente stattfanden. Vielen Dank für die Unterstützung während der Promotion.

Aleksandar Petrovic, der mir alles im Labor beibrachte. Er und Dr. Djuro Kosanovic haben mich gefördert und gefordert.

Dem restlichen Team im Cardio-Pulmonary Institute in Gießen. Besonders Christina Vroom und Ingrid Breitenborn-Müller, für die gute Zusammenarbeit.

Dem Team der PH-Ambulanz. Speziell Susanne Wissgott, die mir alles über Rechtsherzkatheter beibrachte.

Der Rettungswache Bad Münstereifel. Vor allem Michael Schmeling und Henning Hesse, die mir die Freude und das Interesse an der Medizin vermittelt haben.

Ebenso Danke ich meinen Freunden mit denen ich eine unvergessliche Zeit in Gießen verbringen durfte.

Ein besonderer Dank gilt meiner gesamten Familie, die mich auf meinem Weg immer unterstützt und begleitet hat. Im Rahmen der Dissertation speziell meinem Bruder, der mir ein wissenschaftliches Vorbild ist, mir so manchen Rat geben konnte und dessen Begeisterungsfähigkeit scheinbar grenzenlos ist.

Danke auch an Lisa, weil du dein Leben mit mir teilst und damit meines bereicherst.