Functional and metabolic characterization of endothelial cells in chronic thromboembolic pulmonary hypertension

Smolders, V.F.E.D.

CHAPTER 6

COMPARISON OF METABOLIC PROFILE IN ENDOTHELIAL CELLS OF CHRONIC THROMBOEMBOLIC PULMONARY HYPERTENSION AND PULMONARY ARTERIAL HYPERTENSION

V.F.E.D. Smolders, C. Rodríguez, X. Hu, L. Piccari, C. Morén, I. Blanco, R. Szulcek, L. Sebastian, M. Castellà, J. Osorio, M. Cascante, V. Peinado, J.A. Barberà, P. H. A. Quax, O. Tura-Ceide

Submitted

ABSTRACT

Chronic thromboembolic pulmonary hypertension (CTEPH) and pulmonary arterial hypertension (PAH) are two forms of pulmonary hypertension (PH) characterized by an obstructive vasculopathy that can be fatal when left untreated. Endothelial dysfunction along with metabolic changes towards increased glycolysis are thought to be important in PAH disease initiation and progression. Much less is known of the existence of such abnormalities in endothelial cells (ECs) from CTEPH patients. This study provides a systematic metabolic comparison of ECs derived from CTEPH and PAH patients.

Metabolic gene expression (glucose transporter 1 (GLUT1), hexokinase 2 (HK2), 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-3 (PFKFB3), pyruvate dehydrogenase kinase 1 (PDK1), lactate dehydrogenase (LDHA), pyruvate dehydrogenase (PDH), glutamate dehydrogenase 1 (GLUD1), glucose-6-phosphate-dehydrogenase (G6PD)) and HOXD gene expression was studied by the use of qPCR in cultured ECs from CTEPH and PAH patients. Western blot analyses were done for HK2, LDHA, PDH and G6PD. Basal viability and residual viability of CTEPH-EC and PAH-EC after incubation with metabolic inhibitors was measured by MTT. Additionally, migration capacity was assessed by use of a wound healing assay. Human pulmonary arterial endothelial cells (HPAEC) and human lung microvascular endothelial cells (HMVEC-L) were used as healthy control cells.

CTEPH-EC showed significant lower mRNA levels of GLUT1, HK2, LDHA and GLUD1 compared to PAH-EC. At the protein level phosphorylated PDH was found lower expressed in CTEPH-EC compared to PAH-EC. The pentose phosphate pathway was not found different between CTEPH-EC and PAH-EC. PAH-EC, CTEPH-EC and HPAEC presented similar HOXD gene expression. Viability after incubation with metabolic inhibitors was not differently affected between PAH-EC, CTEPH-EC and HPAEC. At last, CTEPH-EC and PAH-EC showed similar migration capacity and viability.

CTEPH-EC and PAH-EC show differences in glycolysis and glutamine metabolism. They have similar migration capacity and similar viability when treated with metabolic inhibitors. More studies are needed to better understand the importance of reduced glycolysis and glutamine metabolism in CTEPH-EC, and whether such differences may lead to the development of novel therapeutic approaches to treat CTEPH.

Keywords: Endothelial cell – cell metabolism – glycolysis – OXPHOS – chronic thromboembolic pulmonary hypertension – pulmonary arterial hypertension

INTRODUCTION

Pulmonary hypertension (PH) is defined as a mean pulmonary arterial pressure (mPAP) >20 mmHg[1]. Based on similar pathogenesis, clinical and hemodynamics characteristics PH is categorized into 5 clinical groups: pulmonary arterial hypertension (PAH); PH due to left heart disease; PH associated with lung disease and/or hypoxia; Chronic thromboembolic pulmonary hypertension (CTEPH) and PH with unclear or multifactorial mechanisms[2]. Although the most common cause of PH is left heart disease, PAH and CTEPH have received over the years the largest interest of researchers [2,3]. PAH and CTEPH result from remodeling of pulmonary blood vessels that causes increase in pulmonary vascular resistance, subsequent right heart failure and, ultimately death. This vascular remodeling is characterized by thickening of the endothelial and ⁄or smooth muscle layer of muscular vessels and by presence of vaso-occlusive lesions[4-6]. To date, exact molecular mechanisms and regulatory pathways behind vascular remodeling in PAH and CTEPH are not fully resolved. Overlap in the presence of pulmonary vascular remodeling between PAH and CTEPH suggest possible similar cellular and molecular disease mechanisms that might help to better understand CTEPH pathology.

The pulmonary vascular endothelium, at the interface between the blood and lung tissue, plays key roles in maintaining vessel homeostasis[7]. Under normal conditions, the endothelium maintains a quiescent state with at basal level a predominant use of glycolysis rather than oxidative phosphorylation in the mitochondria. However, upon stimulation by growth factors such as vascular endothelial growth factor (VEGF) or endothelial injury, the endothelium switches to an activated state which is associated with changes in cellular metabolism that are thought to contribute to EC dysfunction and pulmonary vascular remodeling[8,9]. It is known that, under sustained pathological conditions, EC metabolic alterations promote vascular diseases by mean of excessive cellular proliferation, increased angiogenesis and a pro-survival cellular phenotype[8,10]. Endothelial cells (ECs) originating from vascular lesions in patients with PAH are found to have a hyperproliferative and apoptosis-resistant phenotype that is supported by a metabolic switch towards glycolysis, changes in oxidative phosphorylation and increased glutaminolysis[11-13]. Increased expression of pyruvate dehydrogenase kinase (PDK) in PAH is thought to be responsible for the increased reliance on glycolysis[14,15]. Overall, those studies have been performed with PAH-EC isolated from distal small arterioles but not much is known about the involvement of macrovascular pulmonary arterial ECs (PAECs) in PAH.

Expression patterns of homeobox (HOX) gene clusters have been found to be associated with the positional fate of ECs. In mammals HOX genes appear in 4 clusters, the HOXA, HOXB, HOXC and HOXD and especially the HOXD expression pattern has shown to cluster based on the type of blood vessels[16,17]. To date, it is unknown whether macrovascular PAECs from CTEPH patients share the same metabolic characteristics as PAECs from PAH or whether metabolic modulation might be beneficial in patients with CTEPH.

ECs rely on glucose, fatty acids and amino acids as their main sources of energy and biosynthetic precursors[10]. During glycolysis, glucose is metabolized into lactate by rate-limiting glycolytic enzymes hexokinase-2 (HK2), phosphofructokinase-1 (PFK1) and lactate dehydrogenase A (LDHA). The glycolytic process is controlled by the glycolytic regulator 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-3 (PFKFB3) but also by the enzyme activity of the pyruvate dehydrogenase (PDH) complex. PDH is phosphorylated and inhibited by pyruvate dehydrogenase kinase (PDK) which blocks pyruvate entry into the mitochondria and promote pyruvate fermentation into lactate by LDHA instead [8,10,18]. The glycolytic pathway is paralleled by the pentose phosphate pathway (PPP) that uses glycolytic intermediates, mediated through the expression of glucose-6-phosphate-dehydrogenase (G6PD), but also shunts intermediates back into the glycolysis pathway when needed. The PPP is an additional pathway for nucleotide production and redox homeostasis[8]. Despite the fact that ECs mainly rely on glycolysis for ATP production, mitochondria remain fully functional and rather function as suppliers for cellular building blocks through metabolism of amino acids such as glutamine and glutamate that serve as biosynthetic precursors for the production of nucleotides and macromolecules through glutaminolysis[19].

In this study, we used isolated ECs from vascular tissue collected at pulmonary endarterectomy (PEA) and after lung transplantation from CTEPH and PAH patients, respectively. We perform a systemic metabolic comparison of PAECs from patients with CTEPH and PAH. In addition, we asses migration, basal viability and residual viability upon inhibition by metabolic regulators of glycolysis and glutamine metabolism.

MATERIAL AND METHODS

Study population and samples collected

This study included ECs from 12 CTEPH patients who underwent pulmonary endarterectomy at the Hospital Clinic of Barcelona, Spain. This study further included 6 patients with end-stage PAH who underwent lung transplantation or lung autopsy at the Amsterdam VU University Medical Center, The Netherlands. The study was approved by the institutional Ethics Committee of the Hospital Clínic of Barcelona and the IRB of the Amsterdam VU University Medical Center, the Netherlands. Informed consent was signed by all patients. All patients were diagnosed according to the 2015 ESC/ERS Guidelines[20].

Pulmonary endothelial cell isolation and culture

ECs isolated from endarterectomy specimens from CTEPH patients, referred to as CTEPH-EC, were isolated and cultured as previously described[21]. EC phenotype was characterized by staining the cells with antibodies against a FACS panel of endothelial and smooth muscle cell-specific markers, including endothelial nitric oxide synthase (eNOS) and alpha-smooth muscle actin (α-SMA). ECs from 2 heritable and 4 idiopathic PAH patients (referred to as PAH-EC) were isolated from lung tissues and artery rings of end-stage PAH patients obtained from lung transplantations or from autopsies and cultured as previously described[22,23]. ECs were purified by magnetic affinity cell sorting (MACS, Miltenyi Biotec) based on CD144 antibody labeling and purity was ensured by regular FACS testing. In short, these ECs were plated onto 0.2% gelatin-coated wells and grown in endothelium cell growth medium-2 (EGM-2) supplemented with EGM™-2 SingleQuots (Lonza, USA) and 10% fetal bovine serum (FBS) (GE Healthcare, USA). Patients characteristics are presented in **Supplementary Table 1**. Human pulmonary artery endothelial cells (HPAEC) (Lonza, CC-2530) were used as control cells. Human lung microvascular endothelial cells (HMVEC-L) (Lonza, CC-2527) were grown in EBM™-2 Basal medium supplemented with EGM™-2 MV microvascular endothelial cell growth medium singleQuots™ supplements (Lonza).

Gene expression analyses

The mRNA levels of metabolic genes were measured by qPCR (N=10 CTEPH-EC, N=6 PAH-EC and N=3 or 6 HPAEC). ECs were seeded at a density of 6×10^4 per 40 mm cell culture dish (pre-coated with 0.2% gelatin). Total RNA was extracted using TRIsure™ (Bioline, Germany) and concentrations were determined by spectrophotometry. Reverse transcription was

performed using reactive mix high-Capacity cDNA RT kit (Applied Biosystems, USA). For qRT-PCR, SYBR Green I (ThermoFisher Scientific, USA) was used and specific primers were used on the ViiA7 Real-Time PCR system (Applied Biosystems, USA). Relative quantification was calculated by normalizing the Ct (threshold cycle) of the gene of interest to the Ct of an endogenous control (β-actin) in the same sample, using the comparative ΔΔCt method. All primers were designed with Primer 3Plus and delivered by Integrated DNA Technologies. Primer sequences can be found in **supplementary Table 2.**

Protein expression analyses

ECs from 6 CTEPH donors, 5 PAH donors and 3 HPAEC were seeded into T75 flaks pre-coated with 0.2% gelatin, at a density of 4 x 10^5 cells per flask, and allowed to adhere and grow in complete endothelial medium till confluency was reached. Next, cells were washed twice with ice-cold PBS and treated with Pierce® RIPA buffer (ThermoFisher Scientific, USA) supplemented with a Halt protease/phosphatase inhibitor cocktail (ThermoFisher Scientific, USA). After incubation on ice, the cell lysate was obtained by centrifugation. Protein concentrations in the lysates were determined using the Pierce™ BCA protein assay kit (ThermoFisher Scientific, USA). Then, 15-25µg of total protein was loaded onto 4-12% Bis-Tris Gels (ThermoFisher Scientific, USA). The proteins were transferred to nitrocellulose membranes and blocked for 1 hour at room temperature with Casein Blocking Buffer (Sigma-Aldrich, USA). The membranes were incubated with specific primary antibodies (HK-2, LDHA, G6PD, PDHA1, phospho-PDHA1, PDK1, OXPHOS cocktail and vinculin) overnight at 4°C. Horseradish peroxidase-conjugated anti-IgG was used as the secondary antibody. Immunoreactive bands were detected by WesternBright™ Quantum™ substrate (Advansta, USA). Images were analyzed with ImageJ software. The antibodies list can be found in **supplementary Table 3.**

Functional analyses

Wound healing assay

ECs from 5 CTEPH donors, 4 PAH donors and 3 HPAEC were seeded into 24-wells pre-coated with 0.2% gelatin, at a density of 5 x 10^4 cells per well and allowed to adhere and grow in 10% FBS EGM-2 medium at 37℃ till confluency was reached. The cells were then starved for 2 hours in 2% FBS EGM-2 medium at 37℃ prior to wound healing assay. The wound healing assays were performed with the 2% FBS EGM-2 starving medium containing hydroxyurea at

a final concentration of 2mM to exclude the effect of cell proliferation. Microscopic screenshots of each well (Zeiss Axiovert 200 Microscope) were taken at 0, 5, 10, and 24-hour time points since the wound was made.

MTT

ECs from 3-6 CTEPH donors, 3-5 PAH donors and 3 HPAEC were seeded into 96-wells pre-coated with 0.2% gelatin, at a density of 5 x 10^3 cells per well and allowed to adhere and grow in 10% FBS EGM-2 medium till 80% confluency was reached.. For residual viability, cells were starved with EGM-2 medium containing only 0.5% FBS overnight (about 15 hours). Inhibition treatments were performed in starving medium (0.5% FBS EGM-2) and incubated at 37℃ for 24 hours. 3PO (15 and 30 µM), BPTES (2 and 4 µM), UK5099 (2 and 4 µM), Etomoxir (2 and 4 µM), and DCA (40 and 80mM) were the inhibitors and concentrations of choices to evaluate the effects on cell viability by blocking different biological pathways. The Vybrant® MTT Cell Proliferation Assay Kit (ThermoFisher Scientific, USA) was used for assessing basal and residual viability. For residual viability, medium was initially exchanged for 0.5% FBS EGM-2 phenol-red-free medium containing or without inhibitors depending on the experimental conditions. Basal viability was measured in 10% FBS EGM-2 phenol-red-free medium. 20mM MTT solutions were added and the plates were then incubated at 37℃ for 4 hours. SDS 0.01M HCl solution was then loaded followed by further 4-hour incubation at 37℃. The absorbance was recorded at 570nm on Synergy™ HTX Multi-Mode Microplate Reader.

Statistical analysis

Results are described as means ± standard deviation and were compared using Unpaired t-test, one-way ANOVA or two-way ANOVA. Statistical analyses were performed using GraphPad Software. P-values <0.05 were considered statistically significant.

RESULTS

HOXD expression

Based on previous studies, this study particularly focused on the HOXD expression as it has been shown that the expression patterns of the HOXD gene family, and especially HOXD3, -8 and -9, allow to distinguish between the microvascular and macrovascular origin of ECs.[17] Compared to HMVEC-L, CTEPH-EC and PAH-EC showed 1.9 fold (p=0.0005) and 1.7 fold (p=0.002) lower HOXD3 expression levels, respectively, whereas HOXD8 expression levels were 1.9 fold (p<0.0001) lower in both CTEPH-EC and PAH-EC. At last, HOXD9 showed 2 fold (p=0.003) lower expression levels in both CTEPH-EC and PAH-EC compared to HMVEC-L. Expression levels of all three HOXD genes were not found differently expressed between CTEPH-EC and PAH-EC. Control HPAEC showed similar expression profiles as CTEPH-EC and PAH-EC for all HOXD genes studied (**Figure 1A**).All ECs studied present a typical cobblestone morphology (**Figure 1B**)

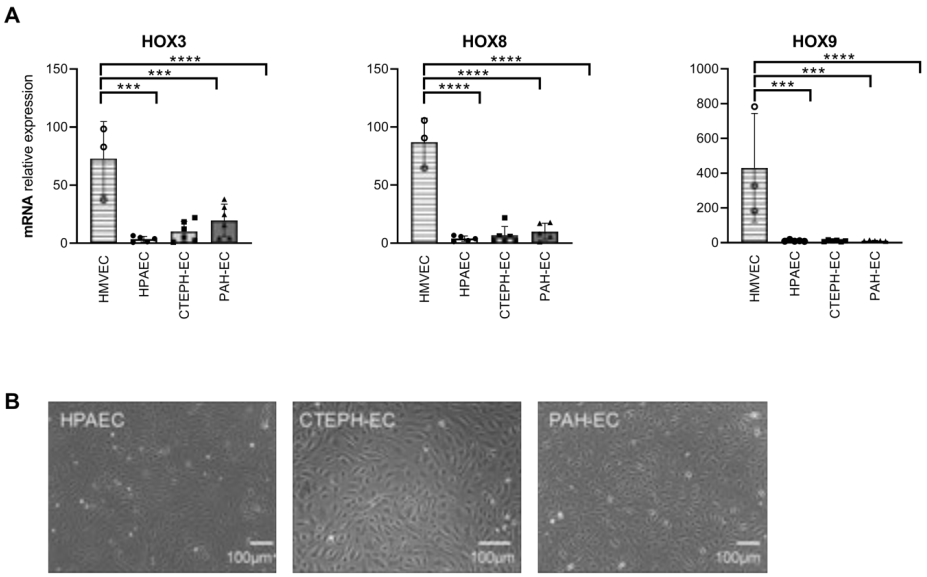

Figure 1. HOXD gene expression. A) mRNA expression levels of HOXD3, HOXD8 and HOXD9 were found significantly increased in HMVEC-L compared to HPAEC, CTEPH-EC and PAH-EC. No differences in gene expression was found between HPAEC, CTEPH-EC and PAH-EC. HMVEC-L, n=3; HPAEC, n=5; CTEPH-EC, n=6; PAH-EC, n=3; One-way ANOVA, p<0.001= ***; p<0.0001 = ****, data is expressed as mean ± SD. **B)** Pictures of cultured HPAEC, CTEPH-EC and PAH-EC. All ECs present a cobblestone morphology, typical of ECs. Scale bar =100µm

Metabolic gene expression

mRNA levels of GLUT1 in CTEPH-EC were 1.7 fold (p=0.006) lower compared to PAH-EC and 1.8 fold (p=0.01) higher in PAH-EC compared to HPAEC. mRNA levels of glycolytic rate-limiting enzyme HK-2 were 2.2 fold lower in CTEPH-EC as compared to PAH-EC (p=0.0003) and 1.5 fold higher in PAH-ECs compared to HPAEC (p=0.02). Glycolytic master regulator PFKFB3 did not show a difference at mRNA level between the three subject groups. Final glycolytic enzyme LDHA showed 1.8 fold lower mRNA levels in CTEPH-EC compared to PAH-EC (p=0.002) and 1.5 fold higher levels in PAH-EC compared to HPAEC (p=0.04) (**Figure 2A**). Mitochondrial gate-keeper enzyme pyruvate dehydrogenase (PDH) subunit E1 alpha (PDHA1) showed 1.3 fold (p=0.04) lower mRNA levels in CTEPH-EC compared to PAH-EC and 1.4 fold (p=0.02) higher levels in PAH-EC compared to HPAECs. mRNA levels of PDK1 were not found differently expressed between all three groups (**Figure 2B**). Also, mRNA levels of PPP associated G6PD were similar in the three studied groups (**Figure 2C**). Glutamate converting enzyme glutamate-dehydrogenase-1 (GLUD1) showed 1.5 fold (p=0.0001) lower mRNA levels in CTEPH-EC compared to PAH-EC. GLUD1 mRNA levels in PAH-EC were 1.2 fold (p=0.06) higher compared to HPAEC. In addition, GLUD1 mRNA levels in CTEPH-EC were 1.2 fold (p=0.06) lower compared to HPAEC. mRNA levels of glutamine converting enzyme, glutaminase 1 (GLS1), were similar in all three PAH-EC, CTEPH-EC and HPAEC (**Figure 2D**).

Metabolic protein expression

Protein levels of glycolytic rate-limiting enzymes HK-2 and LDHA were found similarly expressed between all three study groups, only for HK-2 mRNA expression levels seemed to show a tendency towards upregulation in CTEPH-EC compared to PAH-EC (**Figure 3A**). Protein levels of G6PD were also similar between CTEPH-EC, PAH-EC and HPAEC (**Figure 3B**). Protein levels of phosphorylated PDHA1 (inactive form) showed a tendency towards 1.4 fold (p=0.09) lower levels in CTEPH-EC compared to PAH-EC. No differences were found in phosphorylated PDHA1 protein levels between CTEPH-EC and HPAEC, and between PAH-EC and HPAEC. Protein levels of the non-phosphorylated form of PDHA1 (active form) showed a tendency towards lower protein levels in CTEPH-EC as compared to PAH-EC (p=0.08). The same tendency of lower levels was observed in HPAEC compared to PAH-EC (p=0.08) (**Figure 3C**). No difference was observed in protein levels of PDK1 between the three groups (**Figure 3C**). Protein levels for oxidative phosphorylation associated proteins from complex-I, complex-II

and complex-IV showed respectively 1.7 fold (p=0.06), 1.7 (p=0.04) and 1.8 (p=0.005) lower levels in CTEPH-EC compared to PAH-EC. The same 3 complexes showed respectively 3.9 fold (p=0.005), 2.6 fold (p=0.01) and 3.9 fold (p=0.0004) higher protein levels in PAH-EC compared to HPAEC. Protein levels of complexes-I, -II and -III were similar between CTEPH-EC and HPAEC. Complex-IV and -V showed similar protein levels between all three groups (**Figure 4**).

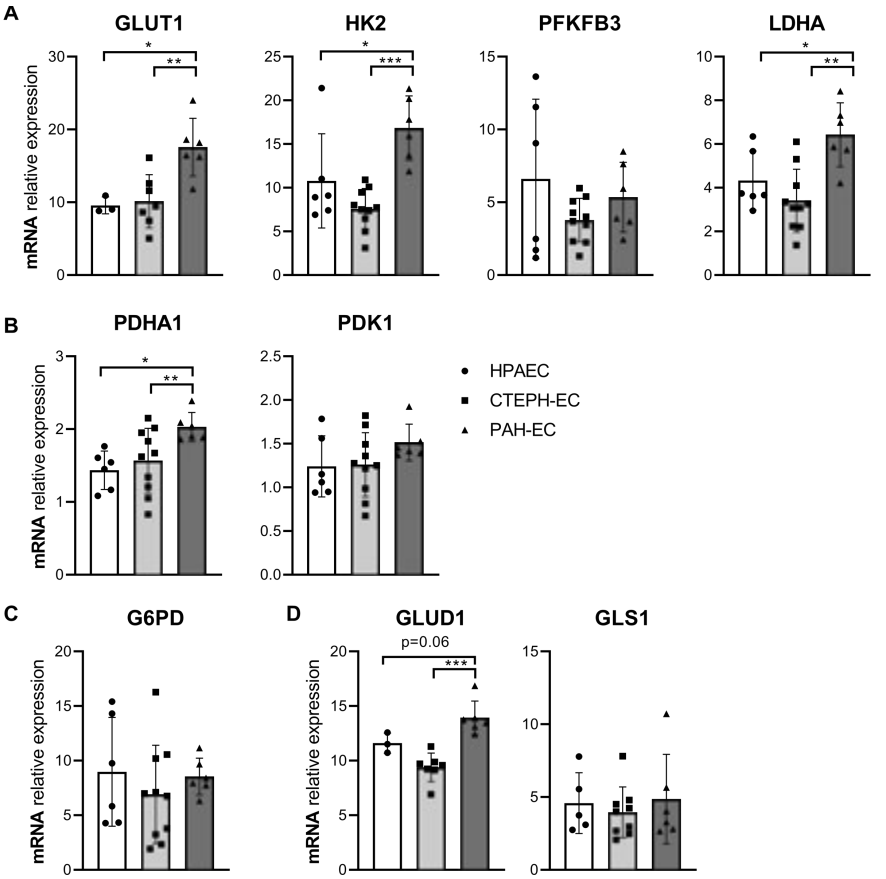

Figure 2. Gene expression of key metabolic enzymes. A) mRNA expression levels of GLUT1 were found significantly downregulated in CTEPH-EC compared to PAH-EC and, in PAH-EC compared to HPAEC. Similar mRNA levels were observed between CTEPH-EC and HPAEC. HK2 mRNA levels were found significantly downregulated in CTEPH-EC compared to PAH-EC and in PAH-EC compared to HPAEC. No difference in PFKFB3 mRNA expression was observed between all three groups. mRNA expression levels of LDHA were significantly lower in CTEPH-EC compared to PAH-EC and in PAH-EC compared to HPAEC. **B)** mRNA levels of PDHA1 were only found significantly different between CTEPH-EC and PAH-EC. mRNA levels of PDK1 were similar between all three groups. **C)** G6PD mRNA levels were found similar between all three groups. **C)** mRNA levels of GLUD1 were significantly decreased in CTEPH-EC compared to PAH-EC and a tendency to lower levels in PAH-EC compared to HPAEC was observed. mRNA levels of metabolic enzyme GLS1 was found similar between all three groups studied. CTEPH-EC, n>7; PAH-EC, n=6; HPAEC, n>3; One-way ANOVA, data is expressed as mean ± SD, p<0.05= *; p<0.01= **; p<0.001= ***.

Residual viability

Residual viability after incubation with metabolic inhibitors was assessed in all three groups. Incubation with glycolytic inhibitor 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) showed a dose-dependent reduction in viability in all three groups (p=0.0001). However, no difference in residual viability was observed between CTEPH-EC, PAH-EC and HPAEC after incubation with 3PO. Also, for glutaminase inhibitor bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)-ethyl-sulfide (BPTES) and mitochondrial pyruvate carrier blocker 2-Cyano-3-(1-phenyl-1H-indol-3-yl)-2-propenoic-acid (UK-5099) a dose-dependent reduction in residual viability was observed in all three groups (p=0.02 and p<0.0001, respectively) but no difference was found in viability between CTEPH-EC, PAH-EC and HPAEC after incubation with both inhibitors. PDK inhibitor dichloroacetate (DCA) showed a dose-dependent reduction in residual viability in all three groups (p=0.0001) but in between groups no difference in response was observed for CTEPH-EC, PAH-EC and HPAEC (**Figure 5**).

Basal viability and migration

Because of clear metabolic differences observed between CTEPH-EC and PAH-EC, basal viability and migration were assessed to investigate a relationship between the observed changes in cell metabolism and cellular functions. Basal viability was found to show a trend towards a 1.2 fold (p=0.09) decrease in CTEPH-EC compared to PAH-EC. Basal viability was similar between CTEPH-EC and HPAEC, and between PAH-EC and HPAEC (**Figure 6A**). Both CTEPH-EC and PAH-EC showed reduced wound closing speed compared to HPAEC but did not reach the statistical level (p=0.09 and p=0.07, respectively). No difference in closing speed was observed between CTEPH-EC and PAH-EC (**Figure 6B**). The results of this study are summarized in **Table 2.**

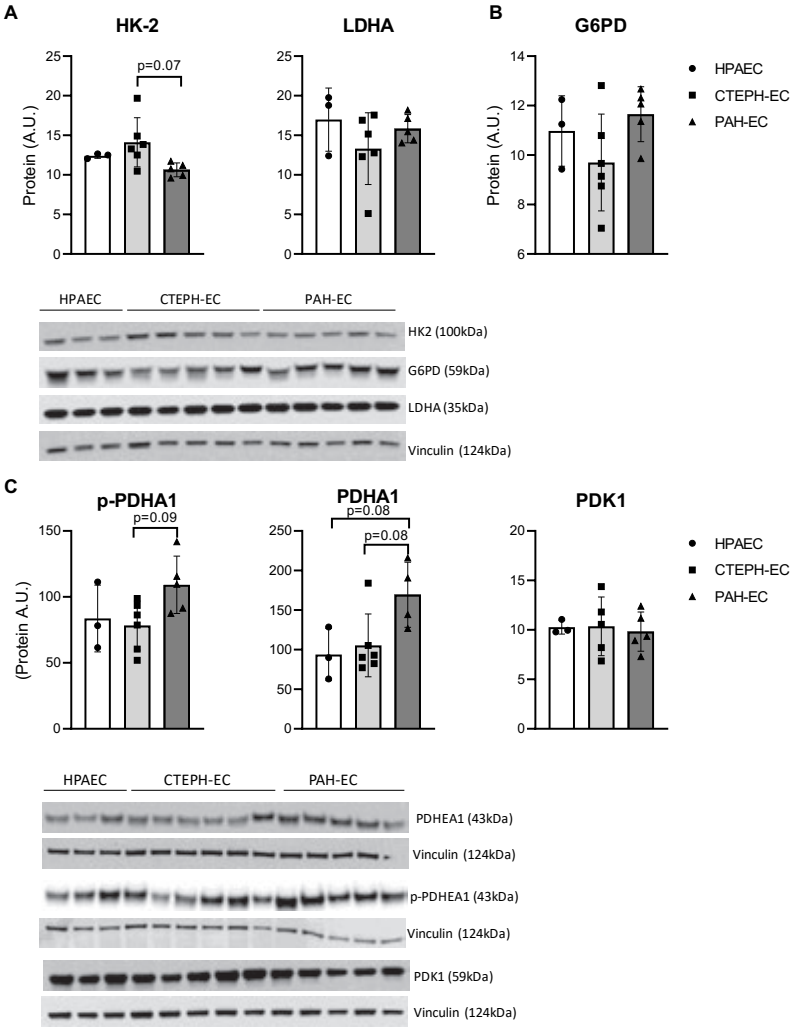

Figure 3. Protein expression of key metabolic enzymes. **A)** Protein levels of HK2 showed a tendency towards upregulation in CTEPH-EC compared to PAH-EC. **B)** Protein levels of PPP key enzyme G6PD were similar in all three groups studied **C)** Protein levels of the phosphorylated E1 alpha PDH subunit (active PDH) showed a tendency towards a decrease in CTEPH-EC compared to PAH-EC. Protein levels of the active form of PDHA1 (non-phosphorylated subunit) also showed a tendency towards a decrease in CTEPH-EC compared to PAH-EC. Protein levels of PDHA1 inhibitor, PDK1, were found similar in all three groups. CTEPH-EC, n=5-6; PAH-EC, n=5; HPAEC, n=3; One-way ANOVA, data is expressed as mean ± SD.

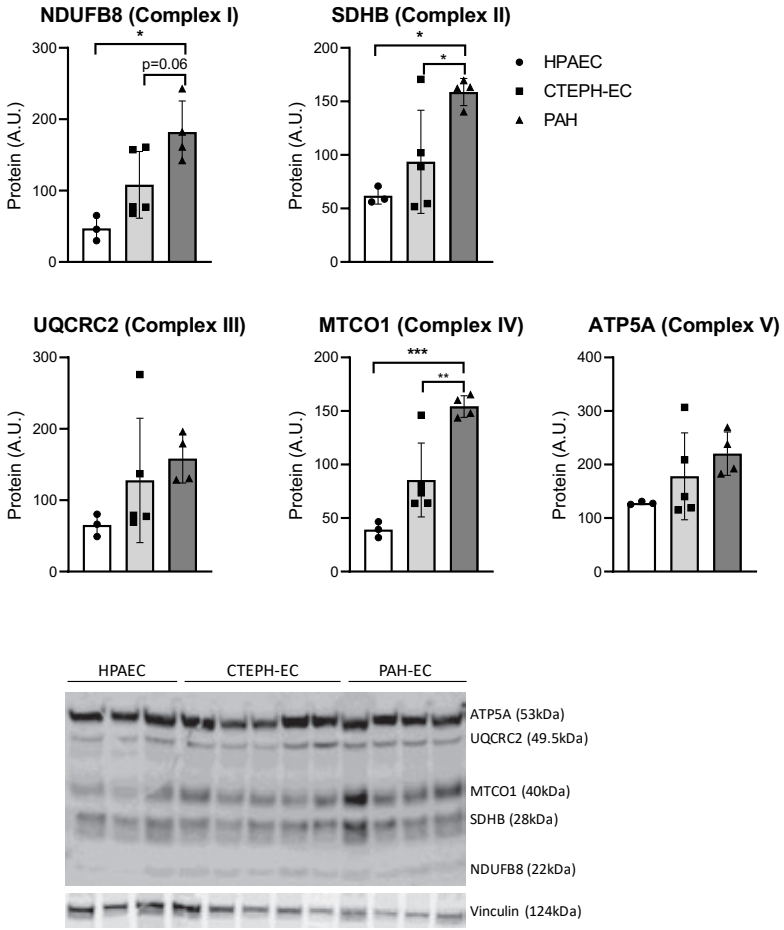

Figure 4. Oxidative associated proteins. Protein levels of complexes-I, -II and -IV were found significantly downregulated in CTEPH-EC compared to PAH-EC. All three complexes are also significantly different between PAH-EC and HPAEC. Similar levels were observed for complexes-III and -V between all three groups studied. CTEPH-EC, n=5; PAH-EC, n=4; HPAEC, n=3; One-way ANOVA, data is expressed as mean ± SD, p<0.05= *; p<0.01= **; p<0.001= ***.

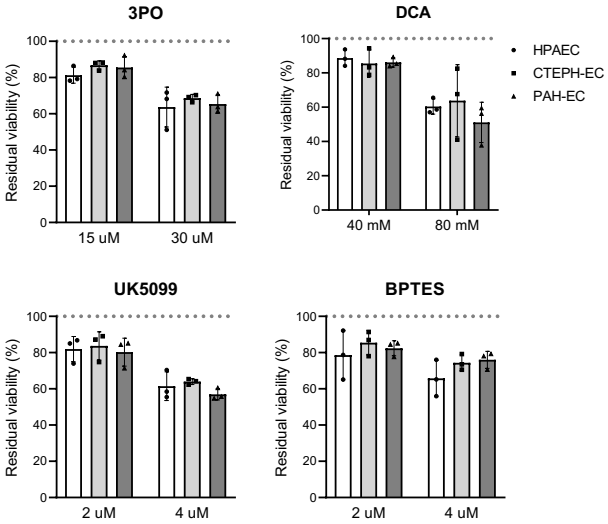

Figure 5. Residual viability upon metabolic inhibition. The effect of metabolic inhibition was evaluated by use of MTT. 3PO (glycolytic inhibitor), BPTES (glutaminase inhibitor), UK5099 (mitochondrial pyruvate carrier blocker) and DCA (PDK1 inhibitor). A dose dependent effect on the viability of all three groups studied is found. No difference in residual viability was observed between CTEPH-EC, PAH-EC and HPAEC in response to metabolic inhibitors. CTEPH-EC, n=3; PAH-EC, n=3; HPAEC, n=3; Two-way ANOVA, data is expressed as mean ± SD.

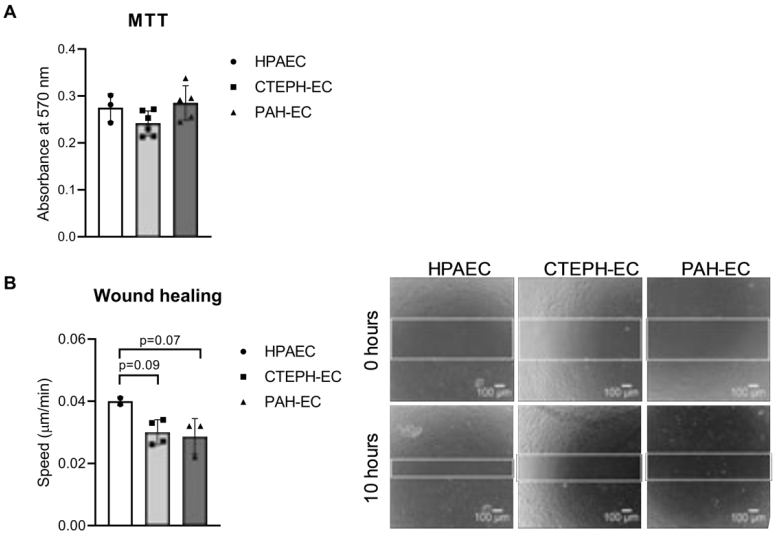

Figure 6. Basal viability and wound healing. A) CTEPH-EC showed a trend towards less viability compared to PAH-EC. **B)** Migration capacity was assessed by use of the wound healing assay and expressed as speed of closure over time (µm/min). Both CTEPH-EC and PAH-EC showed a trend towards a reduction in speed of closure compared to HPAEC. No difference in wound closure was found between CTEPH-EC and PAH-EC. CTEPH-EC, n>6; PAH-EC, n>2; HPAEC, n>2; One-way ANOVA, data is expressed as mean ± SD.

Table 1. Comparison of CTEPH-EC and PAH-EC

	CTEPH-EC
HOXD (mRNA)	=
Glycolysis (mRNA)	↓
p-PDHA1 (inactive) (protein)	↓ [a]
Glutamine metabolism (mRNA)	↓
Viability (metabolic inhibition)	=
Basal viability	↓ [a]
Migration	=

Summary of the most relevant findings of CTEPH-EC in comparison with PAH-EC.
[a] Tendency was observed

DISCUSSION

This study showed a pronounced difference in gene expression of glycolytic and glutamine-related enzymes between CTEPH-EC and PAH-EC. The decrease in glycolytic enzymes in CTEPH-EC was further accompanied by a decrease in PDHA1 mRNA levels. Furthermore, reduced phosphorylated PDHA1 protein levels in CTEPH-EC compared to PAH-EC were observed. In addition, protein levels of oxidative phosphorylation complexed I, II and IV were reduced in CTEPH-EC compared to PAH-EC. No differences were seen in PPP related enzymes both at mRNA and protein level. All ECs showed a response to metabolic inhibitors despite a distinct metabolic profile between CTEPH-EC and PAH-EC. No difference in migration capacity could be observed between CTEPH-EC and PAH-EC. Finally, a trend towards lower viability was observed in CTEPH-EC compared to PAH-EC.

In this study CTEPH-EC and PAH-EC presented a similar HOXD gene expression pattern. Toshner *et al.* described that, taking into account the HOXD expression patterns, ECs could be clustered based on the type of blood vessel that they were derived from. Their study showed that high expression of HOXD3, HOXD8 and HOXD9 was associated with ECs that are microvascular in origin[17]. The expression profiles of all 3 HOXD genes, both in CTEPH-EC and PAH-EC, were significantly lower than those in HMVEC-L. Based on this data it can be concluded that CTEPH-EC and PAH-EC origin from the same macrovascular lineage.

It is known that, in PAH, vascular ECs have adopted several metabolic changes that are associated with vascular hyperproliferation and resistance to apoptosis. PAH-EC are found to increase glycolysis in order to assure proliferation[12,26]. Not much is known about metabolic alterations in CTEPH-EC, but, based on few recent *in vitro* and *in vivo* studies from our group, CTEPH-EC seem to be associated with impairments in their metabolism that point towards lower glycolysis and glutamine metabolism in CTEPH-EC compared to healthy ECs[27,28]. In accordance with those observations, our study showed the downregulation of glycolytic genes GLUT1, HK2 and LDHA in CTEPH-EC compared to PAH-EC, accompanied by a tendency towards higher viability of PAH-EC. Glycolytic regulator PFKFB3 was not found differently expressed between all ECs types studied. An explanation could be that PFKFB3 activity is dependent on post-translational modifications [29,30]. The current study indicates an unexpected difference in the reliance on glycolysis between CTEPH-EC and PAH-EC that could possibly explain the difference in viability between CTEPH-EC and PAH-EC. Another important key feature of metabolic adaptations in ECs from PAH patients is increased inhibition of PDHA1 by PDK1. PDK1 phosphorylates PDHA1, which blocks mitochondrial oxidative phosphorylation and further promotes glycolysis [31,32]. The present study showed increased gene expression of PDHA1 in PAH-EC compared to CTEPH-EC but not difference in gene expression of PDK1. More importantly, protein levels of phosphorylated (inactive) PDHA1 were higher expressed in PAH-EC compared to CTEPH-EC. No differences were found at the protein level for PDK1. Although this study could not show an increased expression of PDK1 in PAH-EC, the increase in phosphorylated PDHA1 is in line with the increase use of glycolysis by PAH-EC. Next to increased levels of phosphorylated (inactive) PDHA1, protein levels of active PDHA1 seemed also higher in PAH-EC compared to CTEPH-EC, and, even though it seems unexpected, higher levels of oxidative phosphorylation associated proteins in PAH-EC is indicative for functional mitochondrial respiration and could explain the increase in active PDHA1. Besides glycolysis, glutamine metabolism is thought to be involved in PAH pathology and has shown to be altered in CTEPH-EC[27,33]. Glutamine metabolism is essential in EC proliferation and is driven by the expression of GLS1 and GLUD1[19]. The current study showed increased gene expression of GLUD1 but not GLS1 in PAH-EC compared to CTEPH-EC. This observation implies a role of glutamine metabolism in PAH-EC but, also, further confirms a difference in metabolism between PAH-EC and CTEPH-EC that needs deeper attention. At last, the oxidative arm of the PPP, important for maintaining cell viability under high rates of

proliferation[34], was not found different between CTEPH-EC and PAH-EC, neither between PAH-EC and HPAEC. Despite similar vasculopathy between both diseases, a different metabolic profile at the level of glycolysis, oxidative phosphorylation and glutamine metabolism is present in CTEPH-EC compared to PAH-EC and suggests differences in molecular mechanisms and regulatory pathways that could be important in disease pathology.

Based on the different EC metabolic profile between CTEPH and PAH, ECs were treated with metabolic inhibitors to see whether differences in metabolism could be translated into differences in viability upon inhibition. All ECs studied showed a dose-dependent reduction in viability after incubation with metabolic inhibitors 3PO, DCA, BPTES and UK5099 but, no significant differences in response could be found between PAH-EC, CTEPH-EC and HPAEC. The lack of significant response to glycolytic inhibitor 3PO and DCA, and oxidative phosphorylation inhibitor UK5099 in PAH-EC compared to CTEPH-EC is unexpected. Not only because this study showed an increase in glycolysis in PAH-EC, but, also, because previous studies have shown beneficial effects of DCA in both human PAH and experimental PAH[14,15]. Both studies have reported mainly effect of DCA on pulmonary artery smooth muscles, and together with the existence of non-responders due to genetic alterations, this could be an explanation for similar responses between PAH-EC and CTEPH-EC. Nonetheless, regardless their metabolism, ECs of all three groups did show a reduction in residual viability after incubation with metabolic inhibitors which could imply lack of selective inhibition of diseased ECs. Moreover, based on the results of this study, blocking glycolysis may not be beneficial in CTEPH-EC and could even further compromise CTEPH-EC viability. Overall, those observations show a clear response to several metabolic inhibitors in all three groups studied but no differences in response to those inhibitors between the three groups.

Besides proliferation and viability also migration is controlled by metabolic pathways such as glycolysis, glutamine metabolism and PPP [35,36]. Interestingly, both CTEPH-EC and PAH-EC showed a reduction in migration speed compared to control HPAEC, but no difference was observed between CTEPH-EC and PAH-EC despite the significant differences in cell metabolism observed. These results of reduced migration are in line with previous studies in ECs from CTEPH and PAH patients[21,37], and indicate that both CTEPH-EC and PAH-EC present inherent endothelial dysfunctionalities compared to healthy ECs. A possible explanation for a similar reduction in migration could be the existence of alterations in other pathways that, in

parallel to cell metabolism, control migratory behavior such as VEGF and Notch signaling pathways [38].

This study compared CTEPH-EC and PAH-EC that present a similar HOXD gene profile. CTEPH-EC and PAH-EC show differences in glycolysis and glutamine metabolism that could support higher viability of PAH-EC. Nevertheless, no difference in response to metabolic inhibitors is observed between CTEPH-EC and PAH-EC. Moreover, reduced migration is observed in both CTEPH-EC and PAH-EC compared to control ECs despite differences in cell metabolism. More studies are needed to better understand the importance of reduced glycolysis and glutamine metabolism in CTEPH-EC, and whether such differences may lead to the development of novel therapeutic approaches to treat CTEPH.

LIMITATIONS

This study has several limitations. Patients with non-operable disease or increased risk factors (e.g. high BMI or PVR > 900 dyn·s·m^{-5}) for surgery are not included in this study, which can cause a certain selection bias of CTEPH patients with a more moderate-to-severe disease state compared to the PAH population group. However, CTEPH patients still show hemodynamic severity (mean mPAP >40 mmHg, low cardiac index and PVR > 500 dyn·s·m^{-5}). The PAH patients cohort is comprised of 6 individuals, therefore these results should be verified with a bigger PAH group. Also, some functional data is lacking in this group (we could only trace one 6-minute-walking-distance and four evaluations of NYHA functional class at baseline, however, the values we found for NYHA FC are similar to those found in previous literature[39]). Differences in disease severity could potentially contribute to the differences in metabolism and viability between endothelial cells isolated from PAH and CTEPH patients.

REFERENCES

1 Simonneau, G. *et al.* Haemodynamic definitions and updated clinical classification of pulmonary hypertension. *The European respiratory journal* **53**, doi:10.1183/13993003.01913-2018 (2019).

2 Hoeper, M. M. *et al.* A global view of pulmonary hypertension. *The Lancet. Respiratory medicine* **4**, 306-322, doi:10.1016/s2213-2600(15)00543-3 (2016).

3 Mann, G. M. F. a. D. L. *Heart failure: A companion to braunwald's heart disease* 4edn, 617-630 (Elsevier 2020).

4 Pietra, G. G. *et al.* Pathologic assessment of vasculopathies in pulmonary hypertension. *Journal of the American College of Cardiology* **43**, 25s-32s, doi:10.1016/j.jacc.2004.02.033 (2004).

5 Humbert, M. Pulmonary arterial hypertension and chronic thromboembolic pulmonary hypertension: pathophysiology. *European respiratory review : an official journal of the European Respiratory Society* **19**, 59-63, doi:10.1183/09059180.00007309 (2010).

6 Simonneau, G., Torbicki, A., Dorfmuller, P. & Kim, N. The pathophysiology of chronic thromboembolic pulmonary hypertension. *European respiratory review : an official journal of the European Respiratory Society* **26**, doi:10.1183/16000617.0112-2016 (2017).

7 Huertas, A. *et al.* Pulmonary vascular endothelium: the orchestra conductor in respiratory diseases: Highlights from basic research to therapy. *The European respiratory journal* **51**, doi:10.1183/13993003.00745-2017 (2018).

8 Rohlenova, K., Veys, K., Miranda-Santos, I., De Bock, K. & Carmeliet, P. Endothelial Cell Metabolism in Health and Disease. *Trends in cell biology* **28**, 224-236, doi:10.1016/j.tcb.2017.10.010 (2018).

9 Budhiraja, R., Tuder, R. M. & Hassoun, P. M. Endothelial dysfunction in pulmonary hypertension. *Circulation* **109**, 159-165, doi:10.1161/01.Cir.0000102381.57477.50 (2004).

10 Eelen, G. *et al.* Endothelial Cell Metabolism. *Physiological reviews* **98**, 3-58, doi:10.1152/physrev.00001.2017 (2018).

11 Masri, F. A. *et al.* Hyperproliferative apoptosis-resistant endothelial cells in idiopathic pulmonary arterial hypertension. *American journal of physiology. Lung cellular and molecular physiology* **293**, L548-554, doi:10.1152/ajplung.00428.2006 (2007).

12 Xu, W. *et al.* Alterations of cellular bioenergetics in pulmonary artery endothelial cells. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 1342-1347, doi:10.1073/pnas.0605080104 (2007).

13 Bertero, T. *et al.* Vascular stiffness mechanoactivates YAP/TAZ-dependent glutaminolysis to drive pulmonary hypertension. *The Journal of clinical investigation* **126**, 3313-3335, doi:10.1172/jci86387 (2016).

14 Michelakis, E. D. *et al.* Dichloroacetate, a metabolic modulator, prevents and reverses chronic hypoxic pulmonary hypertension in rats: role of increased expression and activity of voltage-gated potassium channels. *Circulation* **105**, 244-250, doi:10.1161/hc0202.101974 (2002).

15 Michelakis, E. D. *et al.* Inhibition of pyruvate dehydrogenase kinase improves pulmonary arterial hypertension in genetically susceptible patients. *Science translational medicine* **9**, doi:10.1126/scitranslmed.aao4583 (2017).

16 Akbas, G. E. & Taylor, H. S. HOXC and HOXD gene expression in human endometrium: lack of redundancy with HOXA paralogs. *Biology of reproduction* **70**, 39-45, doi:10.1095/biolreprod.102.014969 (2004).

17 Toshner, M. *et al.* Transcript analysis reveals a specific HOX signature associated with positional identity of human endothelial cells. *PloS one* **9**, e91334, doi:10.1371/journal.pone.0091334 (2014).

18 Spinelli, J. B. & Haigis, M. C. The multifaceted contributions of mitochondria to cellular metabolism. *Nature cell biology* **20**, 745-754, doi:10.1038/s41556-018-0124-1 (2018).

19 Kim, B., Li, J., Jang, C. & Arany, Z. Glutamine fuels proliferation but not migration of endothelial cells. *The EMBO journal* **36**, 2321-2333, doi:10.15252/embj.201796436 (2017).

20 Galiè, N. *et al.* 2015 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension: The Joint Task Force for the Diagnosis and Treatment of Pulmonary Hypertension of the European Society of Cardiology (ESC) and the European Respiratory Society (ERS): Endorsed by: Association for European Paediatric and Congenital Cardiology (AEPC), International Society for Heart and Lung Transplantation (ISHLT). *Eur Heart J* **37**, 67-119, doi:10.1093/eurheartj/ehv317 (2016).

21 Tura-Ceide, O. *et al.* Derivation and characterisation of endothelial cells from patients with chronic thromboembolic pulmonary hypertension. *European Respiratory Journal* **44**, P2327 (2014).

22 Szulcek, R. *et al.* Delayed Microvascular Shear Adaptation in Pulmonary Arterial Hypertension. Role of Platelet Endothelial Cell Adhesion Molecule-1 Cleavage. *American journal of respiratory and critical care medicine* **193**, 1410-1420, doi:10.1164/rccm.201506-1231OC (2016).

23 van der Heijden, M. *et al.* Opposing effects of the angiopoietins on the thrombin-induced permeability of human pulmonary microvascular endothelial cells. *PloS one* **6**, e23448, doi:10.1371/journal.pone.0023448 (2011).

24 Batton, K. A. *et al.* Sex differences in pulmonary arterial hypertension: role of infection and autoimmunity in the pathogenesis of disease. *Biol Sex Differ* **9**, 15, doi:10.1186/s13293-018-0176-8 (2018).

25 Hoeper, M. M., Boucly, A. & Sitbon, O. Age, risk and outcomes in idiopathic pulmonary arterial hypertension. *The European respiratory journal* **51**, doi:10.1183/13993003.00629-2018 (2018).

26 Archer, S. L. Pyruvate Kinase and Warburg Metabolism in Pulmonary Arterial Hypertension: Uncoupled Glycolysis and the Cancer-Like Phenotype of Pulmonary Arterial Hypertension. *Circulation* **136**, 2486-2490, doi:10.1161/circulationaha.117.031655 (2017).

27 Smolders, V. F. *et al.* Decreased glycolysis as metabolic footprint of endothelial cells in chronic thromboembolic pulmonary hypertension. *European Respiratory Journal* **54**, OA5167, doi:10.1183/13993003.congress-2019.OA5167 (2019).

28 Osorio Trujillo, J. *et al.* Heterogeneity in lung 18F-FDG uptake in precapillary pulmonary hypertension. *European Respiratory Journal* **54**, PA4755, doi:10.1183/13993003.congress-2019.PA4755 (2019).

29 Shi, L., Pan, H., Liu, Z., Xie, J. & Han, W. Roles of PFKFB3 in cancer. *Signal transduction and targeted therapy* **2**, 17044, doi:10.1038/sigtrans.2017.44 (2017).

30 Li, F. L. *et al.* Acetylation accumulates PFKFB3 in cytoplasm to promote glycolysis and protects cells from cisplatin-induced apoptosis. *Nature communications* **9**, 508, doi:10.1038/s41467-018-02950-5 (2018).

31 Di, R., Yang, Z., Xu, P. & Xu, Y. Silencing PDK1 limits hypoxia-induced pulmonary arterial hypertension in mice via the Akt/p70S6K signaling pathway. *Experimental and therapeutic medicine* **18**, 699-704, doi:10.3892/etm.2019.7627 (2019).

32 Paulin, R. & Michelakis, E. D. The metabolic theory of pulmonary arterial hypertension. *Circulation research* **115**, 148-164, doi:10.1161/circresaha.115.301130 (2014).

33 Egnatchik, R. A. *et al.* Dysfunctional BMPR2 signaling drives an abnormal endothelial requirement for glutamine in pulmonary arterial hypertension. *Pulmonary circulation* **7**, 186-199, doi:10.1086/690236 (2017).

34 Alamri, A., Burzangi, A. S., Coats, P. & Watson, D. G. Untargeted Metabolic Profiling Cell-Based Approach of Pulmonary Artery Smooth Muscle Cells in Response to High Glucose and the Effect of the Antioxidant Vitamins D and E. *Metabolites* **8**, doi:10.3390/metabo8040087 (2018).

35 Eelen, G., Cruys, B., Welti, J., De Bock, K. & Carmeliet, P. Control of vessel sprouting by genetic and metabolic determinants. *Trends in endocrinology and metabolism: TEM* **24**, 589-596, doi:10.1016/j.tem.2013.08.006 (2013).

36 Huang, H. *et al.* Role of glutamine and interlinked asparagine metabolism in vessel formation. *The EMBO journal* **36**, 2334-2352, doi:10.15252/embj.201695518 (2017).

37 Ventetuolo, C. E. *et al.* Culture of pulmonary artery endothelial cells from pulmonary artery catheter balloon tips: considerations for use in pulmonary vascular disease. *The European respiratory journal* **55**, doi:10.1183/13993003.01313-2019 (2020).

38 Hasan, S. S. & Siekmann, A. F. The same but different: signaling pathways in control of endothelial cell migration. *Current opinion in cell biology* **36**, 86-92, doi:10.1016/j.ceb.2015.07.009 (2015).

39 Gall, H. *et al.* The Giessen Pulmonary Hypertension Registry: Survival in pulmonary hypertension subgroups. *J Heart Lung Transplant* **36**, 957-967, doi:10.1016/j.healun.2017.02.016 (2017).

SUPPLEMENTAL

Clinical data

Clinical data is summarized in **supplementary table 1**. ECs were isolated from a total of 18 PH patients, 12 patients with CTEPH and 6 with PAH. Gender was equally disturbed in the CTEPH patient group but in the PAH patient group women accounted for 83% of the patient population. CTEPH patients were significantly older than PAH patients (58.02±7.62 vs 37.17±10.72, p=0.0007). Body mass index (BMI) was equally distributed in both groups. mPAP was severely elevated in both groups. mPAP was significantly higher in PAH patients than in CTEPH patients (68.17±24.21 vs 40.67±8.86, p=0.01), as well as pulmonary vascular resistance (PVR) (1070.00±464.78 vs 607.00±246.46, p=0.05). Mean cardiac index (CI) was reduced in CTEPH but not in PAH patients, however there was no statistical difference between the groups. Pulmonary artery occlusion pressure (PAOP) was normal but not different between both groups. Right arterial pressure was elevated but not different between both groups. Both groups showed a diminished mixed oxygen blood saturation (SvO2). Both groups showed increased brain natriuretic peptide (BNP), with PAH patients presenting significantly higher levels than CTEPH patients (6021.00±4939.87 vs 166.02±300.68, p=0.006), reflecting their worse hemodynamic condition. All PAH patients were in NYHA class III/IV, whereas CTEPH patients were in NYHA class I/II (25%) and class III/IV (75%). Some patients received PH-targeted therapy either as treatment of the disease (PAH), or as a bridge to the intervention (hemodynamic values are those of the baseline, before drug treatment) (CTEPH). In PAH patients combination therapy prevailed with endothelin antagonists (ERAs) plus phosphodiesterase type 5 inhibitors (PDE5i) and/or plus prostanoids, whereas in CTEPH patients received either monotherapy (ERAs/PDE5i/Riociguat) (33%), combination therapy (ERA plus PDE5i or Riociguat (33%)) or no therapy (25%) (data of one patients was not available).

Supplementary Table 1. Clinical features and hemodynamic parameters

	CTEPH (n=12)	PAH (n=6)
Female/male	6/6	5/1
Age years	58.02 ± 7.62	37.17 ± 10.72 *
BMI kg·m^{-2}	26.69 ± 4.09	27.83 ± 16.53
mPAP mmHg	40.67 ± 8.86	68.17 ± 24.21 *
PVR dyn·s·m^{-5}	607.00 ± 246.46	1070.00 ± 464.78 $^{p=0.05}$
PAOP mmHg	9.75 ± 4.41	12.33 ± 6.66
Cardiac index L·min^{-1}·m^{-2}	2.38 ± 0.49	2.56 ± 0.82
Right atrial pressure mmHg	7.08 ± 5.23	13.83 ± 7.36
SvO2 %	61.82 ± 7.61	52.00 ± 4.97 $^{p=0.07}$
BNP pg·mL^{-1}	166.02 ± 300.68	6021.00 ± 4939.87 *
WHO FC [b]		
I	0	0
II	3	0
III	9	3
IV	0	1

Data are presented as n or mean ± SD. CTEPH: chronic thromboembolic pulmonary hypertension; PAH: pulmonary arterial hypertension; BMI: body mass index; mPAP: mean pulmonary artery pressure; PVR: pulmonary vascular resistance; PAOP: pulmonary artery occlusion pressure; SvO2: mixed venous oxygen blood saturation; BNP: brain natriuretic peptide; WHO FC: world health organization functional class. [b] Data from 2 PAH patients are not available. * p< 0.05, unpaired t-test, data expressed as mean ± SD.

PAH patients were significantly younger and mostly female which is in line with the observed female predominance of PAH[24] and the earlier onset of disease[25]. The older age of CTEPH patients can be explained by the fact that CTEPH results from a pulmonary embolism after which it can take years before patients show clinical signs of CTEPH. Furthermore, older patients might have more comorbidities and treatments, which could increase the risk of pulmonary embolism. The significant increase in hemodynamic parameters in PAH patients compared to CTEPH patients could be explained by the more severe disease state of PAH patients included in this study. In addition, this study only used ECs from CTEPH patients that

underwent pulmonary endarterectomy, and patients with persistently elevated levels of PVR are not eligible for surgery.

Material and methods

Supplementary Table 2. Primers sequences

Gene name	Forward (5'-3')	Reverse (5'-3')
G6PD	CCAAGCCCATCCCCTATATTT	CCACTTGTAGGTGCCCTCAT
GLS1	GCTGTGCTCCATTGAAGTGACT	TTGGGCAGAAACCACCATTAG
GLUD1	GGGATTCTAACTACCACTTGCTCA	AACTCTGCCGTGGGTACAAT
GLUT1	GGTTGTGCCATACTCATGACC	CAGATAGGACATCCAGGGTAGC
HK2	TCCCCTGCCACCAGACTA	TGGACTTGAATCCCTTGGTC
LDHA	GCAGATTTGGCAGAGAGTATAATG	GACATCATCCTTTATTCCGTAAAGA
PDHA1	CCTGACTTTATATGGCGATGG	CTGCCATGTTGTAAGCTTCG
PDK1	GGTTACGGGACAGATGCAGT	CGTGGTTGGTGTTGTAATGC
PFKFB3	CCTACCTGAAATGCCCTCTTC	GTCCCTTCTTTGCATCCTCTG
HOXD3	CGTAAGGATTGCATCGGACT	TCCTAAGCTCGGCTGGATAA
HOXD8	TAAACCAGCTTGCTGTGTGC	GTGAGGCTATCGCTTTCCTG
HOXD9	CCTGCTCCATTGGTTCCTTA	TCAGAAACATGGGGGACATT

Supplementary Table 3. Antibody list

Name	Dilution	Company	Code	Characteristics	Host	Reactivity	kDa
HK2	1/800	santa cruz	sc-374091	monoclonal	mouse	human	100
LDHA	1/800	santa cruz	sc-137243	monoclonal	mouse	human	35
G6PD	1/10000	abcam	ab993	polyclonal	rabbit	human	59
PDH-E1α	1/1000	santa cruz	sc-377092	monoclonal	mouse	human	43
p-PDH-E1α	1 µg/mL	abcam	ab92696	polyclonal	rabbit	human	43
Total OXPHOS	1/250	abcam	ab110413	monoclonal	mouse	human	/[a]
PDK1	1/2000	abcam	ab207450	monoclonal	rabbit	human	49
β-actin	1/1000	novus biologicals	NB600-503	polyclonal	rabbit	human	37
Vinculin	1/10000	abcam	ab129002	monoclonal	rabbit	human	124

[a] This kit contains 5 antibodies. The kDa for each antibody is indicated in figure 4