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

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# CHAPTER 5

## DECREASED GLYCOLYSIS AS METABOLIC FINGERPRINT OF ENDOTHELIAL CELLS IN CHRONIC THROMBOEMBOLIC PULMONARY HYPERTENSION

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## ABSTRACT

Chronic thromboembolic pulmonary hypertension (CTEPH) is caused by non-resolved thrombi obliterating pulmonary arteries and peripheral vasculopathy in non-occluded arteries. We hypothesized that metabolic dysregulation of endothelial cells (EC) contributes to cellular changes that promote vascular narrowing in CTEPH. The study aimed to characterize the metabolic fingerprint of EC-CTEPH. We used EC isolated from specimens removed at pulmonary endarterectomy. Human pulmonary artery endothelial (HPAE) cells from healthy donors were used as control cells. Expression levels of metabolic enzymes were studied at mRNA and protein levels by RT-PCR and western blot, respectively. Compared to HPAE, EC-CTEPH showed lower mRNA and protein levels of hexokinase-2 and lactate dehydrogenase-A, which represent the first and last steps of the glycolytic process. mRNA levels of the glycolytic regulators 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-3 and phosphofructo-kinase-1 were reduced in EC-CTEPH. Transcript levels of pyruvate dehydrogenase kinase-1, inhibitor of pyruvate dehydrogenase, and glutamate dehydrogenase-1, involved in glutamine metabolism, were also downregulated in EC-CTEPH. Fatty acid oxidation and the pentose phosphate pathway did not differ between EC-CTEPH and HPAE. Our results show reduced glycolytic metabolism in pulmonary artery EC in patients with CTEPH. This reduced glycolytic activity could contribute to endothelial dysfunction and vascular remodeling in CTEPH.


**Keywords**: Endothelial cell – cell metabolism – glycolysis – pulmonary hypertension – chronic thromboembolic pulmonary hypertension

## INTRODUCTION

Chronic thromboembolic pulmonary hypertension (CTEPH) is a severe complication of pulmonary embolism (PE) that may lead to right heart failure and death [1]. Chronic obstruction of pulmonary arteries with unresolved thrombi is the initial trigger of CTEPH. Unresolved thrombi disturb the normal laminar blood flow of pulmonary arterial tree by limiting blood flow in affected arteries and, as a consequence, diverting it to nonoccluded arteries [1]. These hemodynamic changes disturb pulmonary endothelial cells (ECs) by altering shear stress, triggering inflammation and promoting a local imbalance of vasoactive mediators (nitric oxide (NO), prostacyclin ($PGI_2$) and endothelin-1 (ET1), among others), which leads to endothelial dysfunction [2,3]. Endothelial dysfunction is known to play a leading role in vascular remodeling via secretory imbalance of vasoactive factors and (pulmonary) vascular constriction among others [3,4]. Endothelial dysfunction induced vascular remodeling together with proximal chronic obstruction of the pulmonary vessels are thought to play a pivotal role in the development of CTEPH [1,2,5-7]. EC metabolism is currently regarded as an important co-determinant for endothelial (dys)function and vascular remodeling. Dysregulated is involved in EC proliferation, migration and energy production [8-12]. In addition, knock-down and inhibitory studies in animals and humans, respectively, showed the role of metabolic remodeling in pulmonary hypertension (PH) [13-15]. In this context, studying EC metabolism gains relevance to better understand EC behavior in CTEPH disease pathogenesis.

The gold standard treatment for CTEPH is the surgical removal of the thrombotic material along with the adhered and enlarged arterial intima by pulmonary endarterectomy (PEA) [5]. Approximately 40% of patients with CTEPH are not eligible for PEA due to thrombotic lesions not accessible to surgery or concomitant comorbidities. In addition, in about 35% of patients, pulmonary hypertension persists after surgery [16]. A deeper understanding of the molecular mechanisms leading to CTEPH is crucial to minimize persistence of PH after PEA and to find novel therapies for patients that are non-candidates to surgery.

Cellular metabolism involves glucose, fatty acids (FAs) and glutamine as main substrates for energy (ATP) and biomass production [17]. Glucose oxidation through glycolysis is the primary source of energy in endothelial cells and is linked via pyruvate dehydrogenase (PDH) to mitochondrial respiration, named oxidative phosphorylation (OXPHOS) (**Figure 1**). The pentose phosphate pathway (PPP) parallels the glycolysis pathway and generates reductive

power and macromolecules for endothelial cell viability and migration (**Figure 1**). Glutamine and FAs, through fatty acid oxidation (FAO), feed metabolic side branches of the tricarboxylic acid (TCA) cycle with additional metabolites for maintaining cellular energy and biomass production (**Figure 1**) [11,17,18].

Based on the relationship between EC metabolism and vascular function, we hypothesize that ECs-CTEPH present metabolic alterations which might explain EC dysfunction and subsequently vascular changes occurring in the pulmonary vessels. In this study, we used EC harvested from specimens extracted in PEA to study cellular metabolic changes in CTEPH.

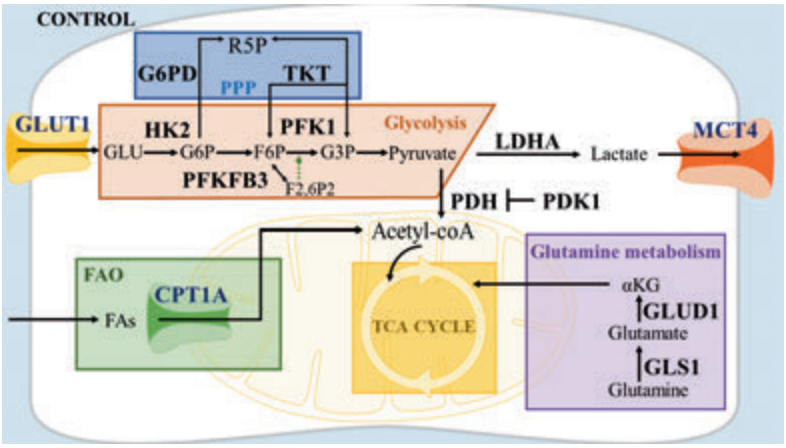


**Figure 1. Metabolic pathways in endothelial cells**. ECs use glucose, fatty acids (FAs) and glutamine as main source of energy. Glucose is converted into lactate in a multistep process during glycolysis. Glycolytic intermediate, pyruvate can also be shunted into the TCA cycle for oxidative phosphorylation. Pyruvate entry is dependent on activity of pyruvate dehydrogenase (PDH) and its inhibitor pyruvate dehydrogenase kinase-1 (PDK1). Glycolytic intermediate glucose-6-phosphate (G6P) can be shuttled into the pentose phosphate pathway (PPP) where it is converted into ribose-5-phosphate (R5P). The non-oxidative branch of the PPP generates R5P out of glycolytic intermediates, catalyzed by transketolase (TKT), this reaction is bidirectional. Both FAs and glutamine serve as precursors for the generation of TCA cycle intermediates via fatty acid oxidation (FAO) and glutamine metabolism, respectively.

# MATERIAL AND METHODS

## Study population

Endovascular material from proximal pulmonary arteries obtained in surgical PEA in 9 CTEPH patients was used as a source of pulmonary ECs. The study was approved by the institutional Ethics Committee of the Hospital Clínic of Barcelona and informed consent was obtained from all patients. All patients were diagnosed according to the 2015 ESC/ERS Guidelines, had an average age of 54 ± 6 years and a 3:6 female-male ratio. The full table can be found at the end of the document.

## EC-CTEPH cell isolation and cell culture

Endarterectomy specimens collected from patients was finely dissected immediately after PEA. The tissue section used to isolate cells was free of thrombotic material and comprised intimal and superficial medial vessel layers. Endothelial cells were isolated (referred to as EC-CTEPH) as previously described [19]. Human pulmonary artery endothelial cells (HPAE) (Lonza) were used as controls. EC-CTEPH and HPAE cells were maintained in endothelium cell growth medium-2 (EGM-2) BulletKit (Lonza) supplemented with 10% FBS (GE Healthcare) on plates pre-coated with gelatin (0.2%) at 37°C, 5% $CO_2$. Primary cultures between passage 4 and 8 were used in experiments. The endothelial nature of isolated cells was confirmed by immunocytochemistry and flow cytometry with a panel of endothelial and smooth muscle cell-specific markers, including endothelial nitric oxide synthase (eNOS) and alpha-smooth muscle actin (α-SMA) as previously shown [20].



## Expression profile mRNA

Total RNA was extracted from cultured HPAE and EC-CTEPH when 80%-90% confluent using TRIsure reagent (Bioline). Following reverse transcription (high capacity cDNA RT kit Applied Biosystems), quantitative real-time PCR experiments were performed in the presence of SYBR Green (Applied biosystems) with a ViiA 7 Real-Time PCR System (Applied Biosystems). cDNA copy numbers were normalized against endogenous control β-actin. All experiments were performed in triplicate and are expressed as $2^{-\Delta\Delta Ct}$. All primers were delivered by IDT and primer sequences can be consulted in supplementary data files.

## Western blotting

Protein was isolated from HPAE and EC-CTEPH at 80%-90% confluency. To isolate protein, cells were treated with cold RIPA lysis and extraction buffer (Pierce) supplemented with Halt

protease and phosphatase inhibitor cocktail (ThermoFisher scientific). After centrifugation, lysate supernatant was collected and stored at -80°C. Protein concentrations were determined using BCA protein assay kit (Pierce). Equal amounts of protein lysates were separated by NuPAGE™ 4-12% Bis-Tris Protein Gels (Invitrogen) and transferred onto nitrocellulose membranes using iBlot Transfer Device (Invitrogen). After transfer, membranes were blocked in Casein Blocking buffer (Sigma-Aldrich). The membranes were incubated with primary antibodies overnight at 4°C with the following antibodies: GLUT1 (sc-377228, 1:500), HK2 (sc-374091, 1:800), LDHA (sc-137243, 1:800), PFKFB3 (sab-1402305, 1:1000) and G6PD (ab-993, 1:10000). After washing with TBS-Tween, the blots were incubated with secondary horseradish peroxidase-conjugated antibodies. Signal was detected using WesternBright Quantum substrate. The intensity of the individual bands in the blots was quantified using Image Lab (Bio-rad). All experiments were performed in triplicate and are expressed as relative expression normalized by β-actin (NB600-503) protein levels.

### Statistical analysis

To evaluate the significance of differences between patient derived samples and non-diseased controls, Mann-Whitney test was used. Results are expressed as mean ± SEM. Differences were considered significant when $p < 0.05$ (*), $0.001 < p < 0.01$ (**), $p < 0.001$ (***). Statistical tests were undertaken using GraphPad software.

# RESULTS

## Glycolytic flux in EC-CTEPH

We first explored the mRNA expression of glycolytic related enzymes in EC-CTEPH compared to control human pulmonary artery endothelial cells (HPAE). Compared with control cells, EC-CTEPH showed 40% and 22% lower levels of hexokinase-2 (HK2) (p=0.0007) and phosphofructokinase-1 (PFK1) (p=0.006), respectively, which represent the first and second rate-limiting enzyme of the glycolytic pathway. mRNA levels of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-3 (PFKFB3), master regulating factor of the downstream glycolytic pathway were 42% lower in EC-CTEPH compared to control cells (p=0.002). Lactate-dehydrogenase-subunit-A (LDHA), the final glycolytic enzyme, showed 37% lower levels in EC-CTEPH compared to HPAE (p=0.004) (**Figure 2 A-D**). Overall, these results show a significant reduction in the expression levels of all glycolytic enzymes studied in EC-CTEPH.

The expression levels of glycolytic transporters of the first and final steps of the glycolysis pathway, such as glucose transporter 1 (GLUT1) and lactate monocarboxylate transporter 1 and 4 (MCT1 and MCT4, respectively), did not differ between EC-CTEPH and HPAE (**Figure 2E-F**).



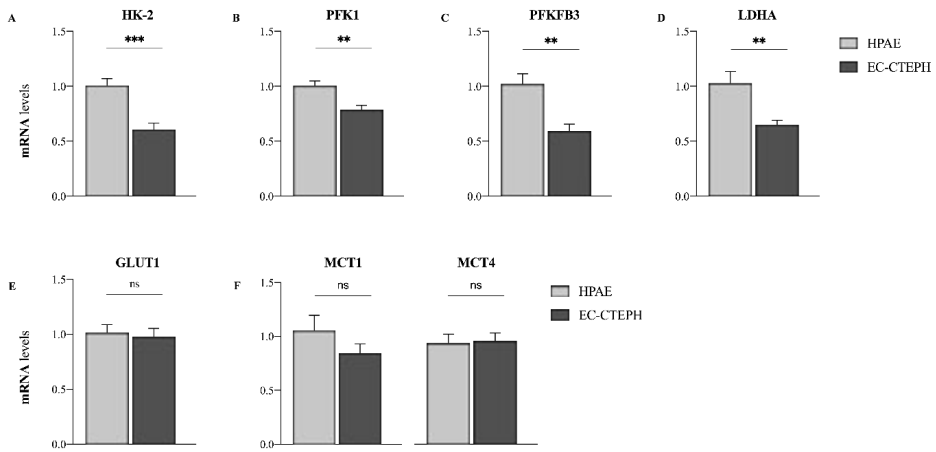


**Figure 2. Glycolytic flux in EC-CTEPH.** mRNA expression of glycolytic enzymes in EC-CTEPH compared to control cells. **(A-D)** HK2, PFK1, PFKFB3 and LDHA expression was reduced in EC-CTEPH compared to HPAE (p=0.0007; p=0.006; p=0.002; p=0.004). **(E-F)** GLUT1 expression was similar in EC-CTEPH and HPAE (p=0.6) and also lactate transporters MCT1 and MCT4 were similar in EC-CTEPH and HPAE (p=0.1 and p=0.9, respectively). Results are expressed as mean ± SEM. N=9 (EC-CTEPH), N=3 (HPAE)

To confirm these findings at the protein level, we performed western blot analyses . Protein levels of HK2 and LDHA showed 58% and 60% lower levels, respectively, in EC-CTEPH (p=0.0063 and p<0.0001 respectively) (**Figure 3A**). PFKFB3 protein levels were numerically lower in EC-CTEPH but not statistically significant (p=0.2) (**Figure 3A**). GLUT1 protein levels were similar in EC-CTEPH compared to control cells, (p=0.4) (**Figure 3B**).

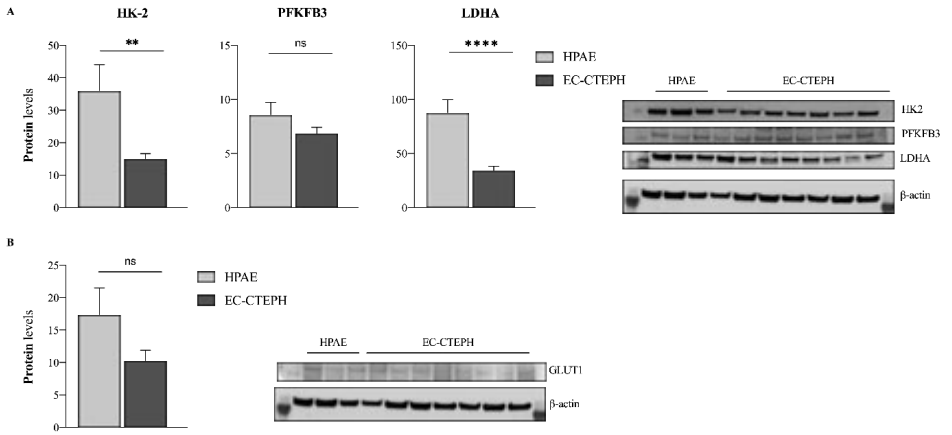


**Figure 3. Glycolytic flux in EC-CTEPH.** Protein levels of glycolytic enzymes in EC-CTEPH compared to control cells. **(A)** HK2 protein levels were reduced in EC-CTEPH compared to HPAE (p=0.006). LDHA protein levels were reduced in EC-CTEPH compared to HPAE (p<0.0001). PFKFB3 showed a reduction at protein levels in EC-CTEPH compared to HPAE but did not reach significance (p=0.2). **(B)** GLUT1 protein levels were similar in EC-CTEPH and HPAE (p=0.4). Corresponding blots of proteins studied in EC-CTEPH compared against endogenous control β-actin are shown. Results are expressed as mean ± SEM. N=9 (EC-CTEPH), N=3 (HPAE)

## Other metabolic pathways in EC-CTEPH

We additionally investigated the involvement of other metabolic pathways in CTEPH development by analyzing mRNA expression of enzymes involved in mitochondrial OXPHOS, PPP, FAO and glutamine metabolism. Compared to control cells , EC-CTEPH had were 34% lower levels of the PDH inhibitor pyruvate-dehydrogenase-kinase (PDK1) (p=0.02) (**Figure 4A).** mRNA levels of its target, pyruvate dehydrogenase subunit E1 alpha 1 (PDHA1) and promotor of pyruvate entry into the mitochondria,, did not differ between EC-CTEPH and control cells (p=0.2) (**Figure 4A**).

mRNA levels of TCA cycle associated enzymes isocitrate dehydrogenase 1 (IDH1) did not differ in EC-CTEPH compared to HPAE (p=0.4) (Figure 4B). Although, subunit alpha of IDH isoform 3 (IDH3A) showed a trend towards 14% lower levels in EC-CTEPH compared to controls (p=0.09).

No difference was found in mRNA expression levels of subunit gamma of isoform 3 (IDH3G) between EC-CTEPH and HPAE (p=0.9) (**Figure 4B**). Additionally, subunit B of the succinate dehydrogenase (SDH) complex (SDHB) showed 12% lower levels in EC-CTEPH compared to HPAE (p=0.05). Both subunits A and C of the SDH complex (SDHA and SDHC, respectively) were similar in EC-CTEPH and HPAE (p=0.2 and p=0.6, respectively) (**Figure 4C**).

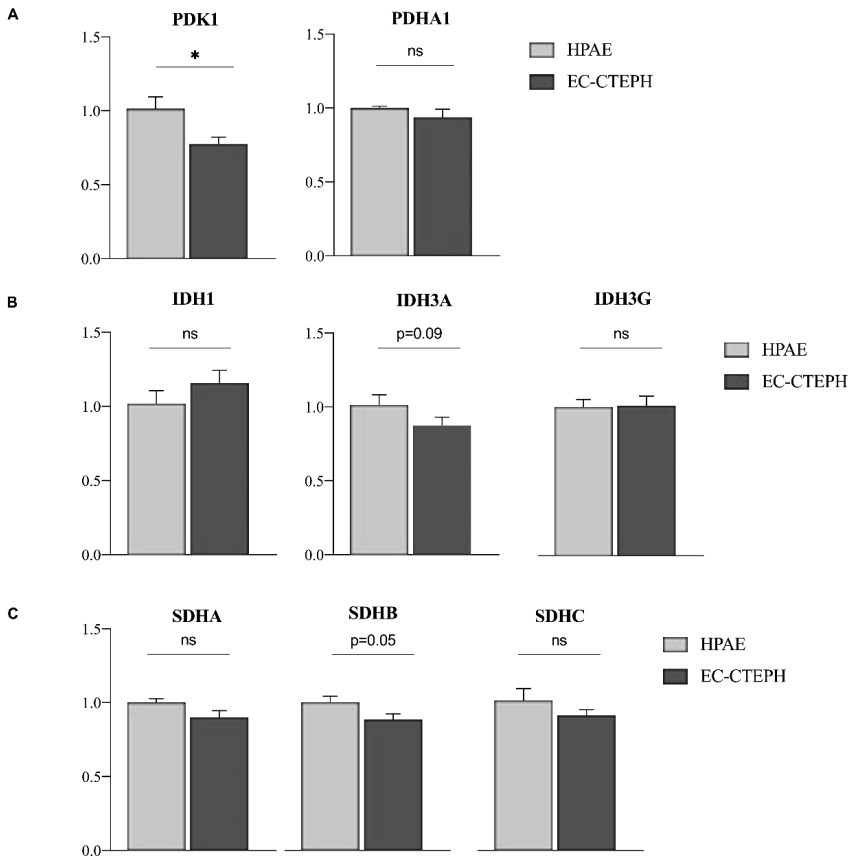


**Figure 4. Oxidative phosphorylation in EC-CTEPH.** (**A)** mRNA levels of PDH inhibitor PFK1 were reduced in EC-CTEPH compared to HPAE (p=0.02), whereas PDHA1 itself did not show a difference between EC-CTEPH and HPAEC (p=0.2). **(B)** Gene expression of both isoforms 1 and 3 from isocitrate dehydrogenase (IDH) were not found different in EC-CTEPH compared to HPAE with p=0.4 for IDH1, p=0.09 for IDH3A and p=0.9 for IDH3G. **(C)** Only subunit B of the succinate dehydrogenase (SDHB) complex showed reduced gene expression in EC-CTEPH compared to HPAE (p=0.05). Subunit A and C were similar in EC-CTEPH and HPAE (p=0.2 and p=0.6, respectively) Results are expressed as mean ± SEM. N=9 (EC-CTEPH), N=3 (HPAE)

The PPP showed 22% lower transcript levels in transketolase (TKT), regulator of the non-oxidative branch, in EC-CTEPH compared to HPAE (p=0.05) (**Figure 5A**). Glucose-6-phosphate-dehydrogenase (G6PD) expression, rate-limiting enzyme of the oxidative branch, was similar in the two cell lines at both mRNA (p=0.2) and protein level (p=0.3) (**Figure 5A** and **Supplement 1**).

The mitochondrial FA transporter carnitine palmitoyl-transferase-1 isoform A (CPT1A) was not differently expressed in EC-CTEPH (p=0.1) (**Figure 5B**). Additionally, glutamate-dehydrogenase-1 (GLUD1) (p=0.05) but not glutaminase (GLS1) (p=0.6) showed 24% lower mRNA levels in EC-CTEPH compared to HPAE (**Figure 5C**). An overview of results found in EC-CTEPH are summarized in **Figure 6.**

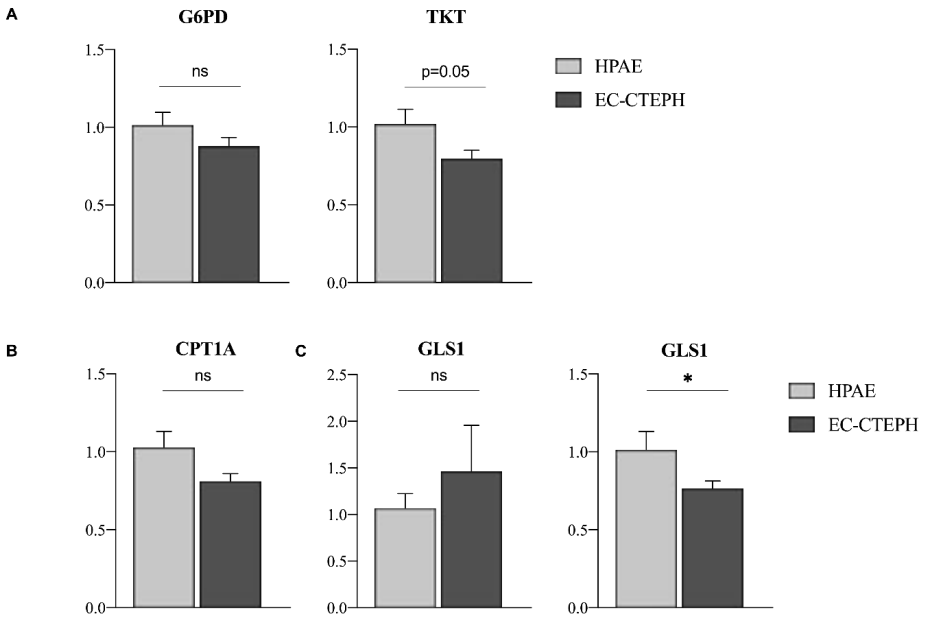


**Figure 5. Additional metabolic pathways in EC-CTEPH. (A)** mRNA levels of G6PD from the oxidative branch of the PPP was not different in EC-CTEPH compared to HPAE (p=0.2). mRNA levels of TKT from the non-oxidative branch of the PPP were lower in EC-CTEPH compared to HPAE (p=0.05) **(B)**. Gene expression of fatty acid transporter CPT1A was similar in EC-CTEPH and HPAE (p=0.1) (C). Gene expression of GLS1 was not different in EC-CTEPH and HPAE (p=0.6), whereas GLUD1 was lower in EC-CTEPH compared to HPAE (p=0.05). Results are expressed as mean ± SEM. N=9 (EC-CTEPH), N=3 (HPAE)

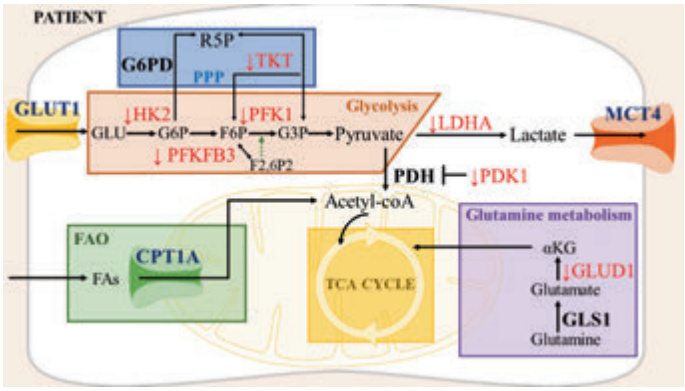


**Figure 6. Metabolic pathways in endothelial cells from CTEPH patients**. The key and rate-limiting enzymes of the glycolytic flux are downregulated in EC-CTEPH. Also the enzymes TKT and GLUD1 from the PPP and glutamine metabolism, respectively, are downregulated in EC-CTEPH. Enzymes from the metabolic pathways FAO and oxidative phosphorylation were not found differently expressed in EC-CTEPH compared to HPAE.

## DISCUSSION

In this study, we aimed to examine EC metabolism to address a potential relationship between EC metabolism, EC dysfunction and subsequent vascular remodeling occurring in pulmonary arteries surrounding the thrombi in CTEPH. an interesting metabolic fingerprint in human EC-CTEPH isolated from PEA specimens. In contrast to the increased glycolytic profile observed in PAH [11,21], we observed a decreased glycolytic profile in EC-CTEPH based on the reduction in mRNA levels of main glycolytic enzymes HK2, PFKFB3, PFK1 and LDHA and a similar reduction in the protein levels of HK2 and LDHA. In addition, this work showed both a downregulation of PDH inhibitor PDK1 and the glutamine converting enzyme GLUD1 in EC-CTEPH. PPP and FAO associated key metabolic enzymes were not found different in EC-CTEPH compared to control cells. These data indicate a clear altered metabolic profile in EC-CTEPH compared to healthy endothelial cells.

ECs-CTEPH showed an interesting metabolic fingerprint: downregulation of controlling glycolytic enzymes PFK1 and PFKFB3 and a downregulation of PDH inhibitor PDK1. The role of metabolic alterations in the development of cardiovascular diseases such as pulmonary arterial hypertension (PAH) points out the importance of a better understanding of the EC metabolism in CTEPH [13-15,22-24]. Activating events such as shear stress, growth factors and inflammation promote endothelial dysfunction, which causes the endothelium to switch to an angiogenic state promoting vascular remodeling [3,18]. In such conditions, ECs reprogram their

metabolism towards increased glycolysis for fast production of ATP whereby upregulating key glycolytic enzymes such as GLUT1, HK2, PFK1, and PFKFB3 and suppressing OXPHOS through upregulation of PDK1 [17,18]. PDK1 shifts pyruvate away from mitochondria towards fermentation into lactate by LDHA. Increasing lactate levels are subsequently exported outside the cell by MCT4 where it acts as a pro-angiogenic factor among others to promote a sustained activated state of the endothelium [17,25,26]. In our study, the downregulation of the controlling glycolytic enzymes PFK1 and PFKFB3 and a reduction of PDH inhibitor PDK1 suggest a decreased metabolic flux towards lactate. This decreased metabolic flux is further supported by a downregulation of LDHA and no upregulation of lactate transporters MCT1 and MCT4 in ECs-CTEPH. Although EC dysfunction and EC metabolism are well studied in vascular diseases such as PAH [13,15,23,24], underlying mechanisms regarding vascular remodeling are still not completely understood, pointing out the complexity of processes involved in vascular remodeling [21,27]. As endothelial metabolism is closely linked to EC function [8-12], decreased glycolysis in EC-CTEPH might be a trigger in the development of endothelial dysfunction which impairs angiogenic capacities of the endothelium.

EC-CTEPH are isolated from larger vessels surrounding the thromboembolic lesions and might be involved in the lack of thrombus recanalization and resolution. Nonresolution of thrombi might result from underlying mechanisms such as inflammation, fibrinogen abnormalities, platelet dysfunction and impaired angiogenesis [1,28] In this study, patient derived EC-CTEPH showed a downregulation of the glycolytic flux and glutamine metabolism which might result in impaired angiogenic and/or vascular repair capacity of EC-CTEPH. PFKFB3-driven glycolysis is the key driver of the angiogenic process but also glutamine metabolism and FAO are indispensable for vessel sprouting [9,10,17,29]. Animal studies showed the importance of angiogenesis in recanalization of the thrombus [1,30]. In addition, absence of infiltrating capillaries in human CTEPH thrombi support the idea of impaired angiogenesis and its contribution to thrombus persistence followed by occlusive vascular remodeling after PE [30]. We believe that a decrease in glycolysis and impaired glutamine metabolism in EC-CTEPH, isolated from vessels surrounding the thromboembolic lesions, could provide insights into the lack of thrombus recanalization and resolution occurring in CTEPH.

Our results also showed a reduction in GLUD1 expression in EC-CTEPH, indicating that less glutamine enters the TCA cycle. Glutamine is an important metabolic substrate in activated

ECs to replenish the TCA cycle with carbons and nitrogen since the majority (>90%) of glucose-derived carbons exit the cell in the form of lactate during the glycolytic process [8,11]. As our data indicates a reduced metabolic flux towards lactate and because of that an increased amount of the total glucose-derived-pyruvate enters the TCA cycle, this results in the fact that less glutamine will be oxidized through the TCA cycle.

FAO and CPT1A are indispensable for TCA cycle replenishment during elevated cell proliferation [10]. A reduced metabolic flux towards lactate in EC-CTEPH is also here suggestive for a decreased need of FAO in EC-CTEPH which can explain no difference in CPT1A expression in EC-CTEPH compared to HPAE.

EC-CTEPH showed no change in the PPP as G6PD was not found significantly different in EC-CTEPH compared to HPAE. The PPP is a side pathway paralleling glycolysis through which NAPDH and/or ribose-5-phosphate (R5P) is generated [17,18]. R5P is mainly produced via oxidation of glycolytic intermediate glucose-6-phosphate (G6P) by G6PD in the oxidative branch of the PPP, generating at the same time reductive power in the form of NAPDH. TKT, an enzyme of the non-oxidative branch, on the other hand, recycles excess of pentose phosphates back to glycolytic intermediates fructose-6-phosphate (F6P) and glyceraldehyde-3-phosphate (G3P). Low expression of TKT indicates a low glycolytic flux [17,18,31]. In EC-CTEPH all main glycolytic enzymes were downregulated together with a downregulation of PDK1. A low flux through glycolysis together with a relative higher amount of pyruvate shunted into TCA cycle could be a reason for a low flow of metabolites into the PPP.



It has been previously shown that isolated pulmonary arterial endothelial cells (PAECs) from patients with pulmonary arterial hypertension (PAH) show metabolic reprogramming towards glycolysis together with a suppression of glucose oxidation in the mitochondria, an increase in fatty acid and glutamine metabolism while suppressing glucose oxidation in the mitochondria [32-34]. Despite histological resemblances between CTEPH and PAH [35,36], our study showed a decrease in glycolysis and no additional upregulation of fatty acid and glutamine metabolism in patient derived EC-CTEPH compared to non-diseased pulmonary endothelial cells, pointing to altered molecular mechanisms in EC-CTEPH. However, it cannot be excluded that anatomic specific hemodynamic features on pulmonary endothelial cells can also contribute to the difference in results obtained between cells isolated from CTEPH patients compared to PAH patients. PAH is a vascular disease at the level of the microvascular

endothelium compared to CTEPH with thrombotic lesions and remodeling mainly in surgically accessible large (~3cm) and medium sized arties (≥ 2mm) [22,37,38]. A different location in the pulmonary vasculature tree exposes ECs to different blood pressures and blood flow which has an influence on the endothelium and cellular behavior of ECs studied [39]. So endothelial cells derived from PEA specimen are from a different anatomic position in the pulmonary vasculature tree compared to PAECs that are isolated from PAH recipient lungs [3,32,33]. A better understanding of metabolic changes in EC-CTEPH is a crucial step in improving our understanding of CTEPH pathogenesis. Taken together, our data indicate an altered metabolic profile in human EC isolated from CTEPH PEA specimens, compared to healthy ECs, that might diminish EC angiogenic capacities and might play a role in thrombus nonresolution, an important disease trigger in CTEPH development.

## CONCLUSION

Human EC isolated from CTEPH pulmonary endarterectomy specimens present an altered metabolic fingerprint. EC-CTEPH showed a reduced glycolytic metabolism compared to healthy EC. In addition, PDK1 and glutamine metabolism through the TCA cycle were downregulated. No difference has been found in FAO or PPP pathway. The altered glycolytic activity of EC-CTEPH could provide insights into the lack of thrombus recanalization and resolution occurring in CTEPH.

## LIMITATIONS

This study has some limitations. We used commercially available HPAE as control cells which might be not as optimal as EC isolated from healthy transplant lung via the same procedures we used, but these lung samples were not available to us. To minimize this limitation, all cells were extensively characterized in the laboratory. Nevertheless, future studies should verify our findings in control cells from healthy transplant lungs. The number of cells needed to perform the experiments also did not allow us to use control ECs freshly isolated from patients. To minimize differences induced by the culture process, all experiments were carried out with HPAE and EC-CTEPH at the same passage (four to eight). In the current brief report we have not been able to describe findings between EC function and alterations in metabolism. The lack of protein measurements of PDK1 could be considered as a limitation of this study. Despite extensive attempts to detect PDK1 at the protein level using several

antibodies, we were not successful to perform adequate protein levels detection and WB analysis. Studies characterizing cell function of EC-CTEPH regarding angiogenesis, migration and proliferation that might help to resolve molecular insights in CTEPH disease development, are under way. For some parts of our study larger sample sizes would be ideal but this a challenge as the number of surgeries/year are limited in our institution and endothelial cells are not always successfully derived.

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