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SHORT REPORT
Translational basic science

Exploring breath metabolomics as a non-invasive tool for detecting pulmonary vascular disease

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Aims The aim of this initial study was to explore whether prediction models based on breath metabolome profiles could detect differences between pulmonary vascular disease (PVD) patients and healthy controls. Additionally, we sought to investigate the potential to distinguish between two major subtypes of PVD—pulmonary arterial hypertension (PAH) and chronic thromboembolic pulmonary hypertension (CTEPH)—to support early detection and targeted treatment.

Methods and results We used real-time breath analysis to compare the breath profiles of patients with PVD to healthy controls, and the metabolome of patients with PAH to those with CTEPH. Pathway enrichment analysis was conducted to reveal underlying metabolic pathways. Breath profiles of 75 patients (47 (62.7%) with PAH and 28 (37.3%) with CTEPH) were analysed and compared with those of 115 healthy controls. The prediction models identified PVD with an area under the curve of 0.917 and distinguished PAH from CTEPH with an AUC of 0.764. PVD patients showed significant metabolic alterations, particularly in *de novo* fatty acid synthesis and fatty acid activation.

Conclusion Breath analysis shows potential as a non-invasive and real-time diagnostic tool by demonstrating detectable differences in breath profiles between PVD patients and healthy controls. Establishing these differences is a critical first step in assessing the feasibility of identifying breath markers for PVD and exploring further differentiation between PAH and CTEPH.

Registration Trial registration number: NCT05458934.

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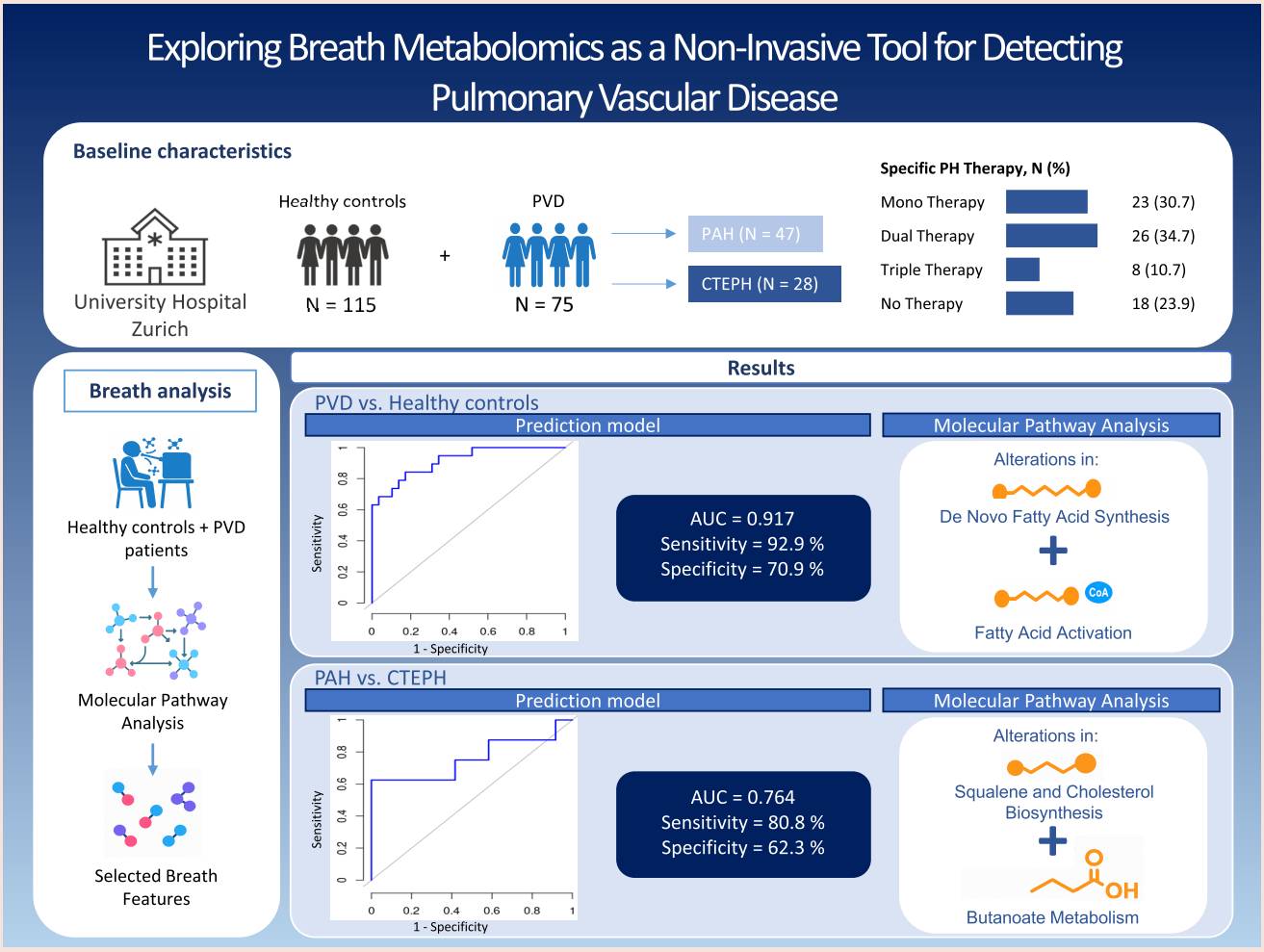
Graphical abstract



Keywords Breath analysis • Pulmonary vascular disease • Metabolomics

Introduction

Pulmonary hypertension (PH) is a significant global health issue. Among PH, pulmonary arterial hypertension (PAH) and chronic thromboembolic hypertension (CTEPH), hereafter referred to as pulmonary vascular disease (PVD), share similar clinical presentations but differ in pathophysiology: PAH arises from vascular remodelling, whereas CTEPH results from chronic thromboembolic obstruction. Given the severity of PH, non-invasive, highly sensitive screening tools are needed to expedite diagnosis, reduce patient burden, and differentiate between PAH and CTEPH.

Volatile organic compounds (VOCs) are detectable at low concentrations in exhaled breath. Previous studies have only superficially demonstrated their potential as non-invasive biomarkers for PAH, primarily due to the limitations of the techniques used.[1,2] In contrast, secondary electrospray ionization–high resolution mass spectrometry (SESI-HRMS) provides a rapid, comprehensive and accurate analysis of exhaled breath within minutes. This study aimed to compare breath profiles of PVD patients and healthy controls using real-time SESI-HRMS.

Methods

Study design

A prospective, cross-sectional study was conducted at University Hospital Zurich between 10/2022 and 10/2023. The study was conducted as reported previously.[3] Inclusion criteria required participants to be ≥18 years old with a confirmed diagnosis of PAH or CTEPH. Exclusion criteria included inability to provide informed consent or follow the study protocol, current intensive clinical monitoring, and pregnancy. Each participant completed one study visit, which included a brief interview and exhaled breath analysis. The interview included smoking history, medical and drug history, and recent consumption of food, beverages, caffeinated drinks, cigarettes, alcohol, and cosmetics. Haemodynamic parameters from the latest right heart catheterisation were recorded, along with recent *n*-terminal prohormone of brain natriuretic peptide (NT-proBNP), the 6-min walk test, and PH-specific medications. The control group comprised healthy volunteers without known illnesses or medication usage. As this was a preliminary feasibility study, healthy controls were not matched to the PH cohort. The study was approved by the Cantonal Ethics Committee of Zurich (BASEC-No. 2021-00539), adhered to the principles of the Declaration

of Helsinki and Good Clinical Practice, and was registered at ClinicalTrials.gov (NCT05458934). All participants provided written informed consent.

Breath analysis

Breath analysis was performed using SESI-HRMS (Fossil Ion Technology, Spain and Thermo Fisher Scientific, Germany) coupled to a Q Exactive Plus High-Resolution Mass Spectrometry (Thermo Fisher Scientific, Germany) (SESI-HRMS) as described previously.[3,4] Participants exhaled through a sterile filter attached to a heated tube made of stainless steel and coated with Silconert that was 50 cm long and 4 mm wide. This tube was connected to the curtain AUX gas port of an Orbitrap spectrometer. In total, 12 breath measurements were conducted, six in positive mode and six in negative mode.

Data analysis

Data were pre-processed by Deep Breath Intelligence (DBI, Switzerland), yielding 1941 mass-to-charge ratios (m/z) in positive mode and 1204 in negative mode. Fold-change values were calculated by logarithmic transformation of the ratio of feature signal intensity (log2FC). Differences in feature intensity between the groups were analysed using two-sample *t*-tests. *P*-values were adjusted for multiple comparison by computing their corresponding *q* values. Prediction models were built using partial least-squares discriminant analysis (PLS-DA) to with the lowest prediction error in cross-validation (random subsets) as the criterion. An extreme gradient boosting model (XGboost) was used. Breath features with a variable importance projection score ≥ 1 in the testing model were included in the final model. A 100-fold cross-validation was computed, and the 95% confidence intervals (CI) were determined via the bootstrap method (2000 resamples). 80% of the data were used in the training sets, and 20% in the test sets. Statistical analyses were performed using R (version 4.4.1, R Core Team, Vienna, Austria). To assign exact mass features to potential molecular compounds, MetaboAnalyst v6.0 was utilized with the mummichog algorithm. All features were arranged in ascending order based on their calculated *P*-values, with the significance cut-off set at the top 10% of features. Specific ionisation adducts were used (Putative SESI-HRMS ionisation forms used for MetaboAnalyst v6.0, Ionisation adducts used: $[M+H]^+$, $[M–H_2O+H]^+$, $[M–H_2O–H]^-$, $[M+NH_4]^+$, $[M+HCOO]^-$, $[M–H]^-$, $[M(^{13}C)–H]^-$, $[M(^{13}C)+H]^+$. M: molecule; H: hydrogen; H_2O: water; NH_4: ammonium ion; $HCOO^-$: methanoate). The allowed mass tolerance was 2 ppm.

Results

Study cohort and control group characteristics

A total of 75 patients were enrolled in the study and compared with 115 healthy controls. Baseline characteristics are presented in *Table 1*.

PVD vs. healthy controls

The analysis revealed an upregulation in metabolic pathways, predominantly involving fatty acid metabolism, including *de novo* synthesis and fatty acid activation *Table 2A*. Our prediction model incorporated 1475 breath features and was based on the first seven PLS-DA components to minimize the classification error rate. The model demonstrated excellent sensitivity of 92.9% (95% CI: 87.8–97.4%) and adequate specificity of 70.9% (95% CI: 61.2–81.0%). The accuracy was 84.1% (95% CI: 78.7–88.8%), with an area under the curve (AUC) indicating excellent overall discriminative power at 0.917 (95% CI: 0.872–0.957) (*Figure 1A and B*).

PAH vs. CTEPH

In PAH significant alterations in squalene and cholesterol biosynthesis, as well as butanoate metabolism were revealed, compared with CTEPH (*Table 2B*). The prediction model including 1452 features showed a sensitivity of 80.8% (95% CI: 68.8–91.7%), a specificity of 62.3 (95% CI:

Table 1 Cohort characteristics

	Patients with pulmonary vascular disease (*n* = 75)	Healthy controls (*n* = 115)
Age, yr	62.0 (48–73)	34.0 (26–45)
Female sex, n (%)	41 (54.7)	53 (46.5)
BMI, kg/m^2	25.5 (4.1)	23.9 (4.6)
Smoking status, n (%)		
Never-smoker	43 (57.3)	87 (76.3)
Smoker	7 (9.3)	8 (7.0)
Ex-smoker	25 (33.3)	19 (16.7)
Pack years	27.5 (15.0–40.0)	5.0 (3.0–10.0)
Diagnosis, n (%)		
Pulmonary arterial hypertension	47 (62.7)	
Idiopathic	20 (26.7)	
Acute vasoresponder	1 (1.3)	
Heritable	3 (4.0)	
Connective tissue disease	12 (16.0)	
HIV infection	1 (1.3)	
Portal hypertension	5 (6.7)	
Congenital heart disease	3 (4.0)	
PAH with features of venous/capillary involvement	2 (2.7)	
Chronic thromboembolic pulmonary hypertension	28 (37.3)	
NYHA functional class, n (%)		
I	17 (22.7)	
II	37 (49.3)	
III	21 (28.0)	
IV	0 (0.0)	
6-MWD, m	513 (423–570)	
NT-pro-BNP, pg/mL	263 (95–539)	
ERS/ESC 4-strata risk score, n (%)		
1	32 (42.7)	
2	30 (40.0)	
3	11 (14.7)	
4	2 (2.6)	
Disease duration, n (%)		
< 5 years	34 (45.3)	
≥ 5 years	41 (54.7)	
mPAP, mmHg	36 (24–48)	
PVR, WU	5.4 (3.6)	
Specific PH therapy, n (%)		
Mono therapy	23 (30.7)	
Dual therapy	26 (34.7)	
Triple therapy	8 (10.7)	
No therapy	18 (23.9)	
Comorbidities, n (%)		
None	10 (13.3)	115 (100)
Heart	35 (46.7)	0 (0)
Lung	44 (58.7)	0 (0)

6-MWD, 6-minute walk distance; BMI, Body Mass Index; mPAP, mean pulmonary arterial pressure; n, Number of patients; NT-pro-BNP, n-terminal pro-brain-type natriuretic peptide; NYHA, New York Heart Association; PAH, pulmonary arterial hypertension; PH, pulmonary hypertension; PVR, pulmonary vascular resistance index.

Table 2 Most relevant altered metabolic pathways and assigned putative metabolites identified from exhaled breath profiles using SESI-HRMS

A. Differences between PVD patients and healthy controls.

Putative metabolic pathways	Putative molecular name	m/z	Log2FC	*P*-value	*q*-value	Putative molecular formula	Ionisation
De novo fatty acids	Octadecenoic acid	−284.26761	1.67	0.00200	0.03690	C18H36O2	M(C13)−H [1−]
		267.26824	1.52	0.00001	0.00191		M-H2O + H [1+]
	(9Z)-Octadecenoic acid	265.25259	1.43	0.00086	0.02405	C18H34O2	M-H2O + H [1+]
		283.26316	1.17	0.01114	0.08016		M + H [1+]
	Tetradecanoic acid	211.20564	1.19	0.01046	0.07793	C14H28O2	M-H2O + H [1+]
		−228.20502	1.68	0.00115	0.02788		M(C13)−H [1−]
	Hexadecanoic acid	−256.23631	1.67	0.00312	0.04809	C16H32O2	M(C13)−H [1−]
		239.23694	1.56	0.00001	0.00231		M-H2O + H [1+]
	Dodecanoic acid	202.18824	1.71	0.01219	0.08381	C12H24O2	M(C13)+H [1+]
		−199.17036	1.42	0.00156	0.03154		M-H [1−]
	Linoleate	−279.23294	2.29	0.00333	0.04840	C18H32O2	M-H [1−]
		281.24751	0.01	0.00787	0.06816		M + H [1+]
Fatty acid metabolism	Dodecanoic acid	202.18824	1.71	0.01219	0.08381	C12H24O2	M(C13)+H [1+]
		−199.17036	1.42	0.00156	0.03154		M-H [1−]
	Tetradecanoic acid	−228.20502	1.68	0.00115	0.02788	C14H28O2	M(C13)−H [1−]
		211.20564	1.92	0.01046	0.07793		M-H2O + H [1+]
	Hexadecanoic acid	−256.23631	1.67	0.00312	0.04809	C16H32O2	M(C13)−H [1−]
		239.23694	1.56	0.00001	0.00231		M−H2O + H [1+]
	Linoleate	281.24751	1.47	3.40E-05	0.06816	C18H32O1	M + H [1+]
		−279.23294	2.29	1.60E-05	0.04840		M−H [1−]
	Octadecanoic acid	267.26824	1.52	0.00001	0.00191	C18H36O2	M-H2O + H [1+]
		−284.26761	1.67	0.00200	0.03690		M(C13)−H [1−]
Fatty acid activation	9-Octadecenoic acid	−281.2486	1.63	0.00112	0.02788	C18H34O2	M-H [1−]
		−282.25196	1.67	0.00208	0.03736		M(C13)−H [1−]
		283.26316	0.99	0.01114	0.08016		M + H [1+]
	Tetradecanoic acid	211.20564	0.92	0.01046	0.07793	C14H28O2	M-H2O + H [1+]
		−228.20502	1.33	0.00115	0.02788		M(C13)−H [1−]
		−227.20165	0.75	0.00602	0.05899		M-H [1−]
	Hexadecanoic acid	239.23694	1.01	0.00001	0.00231	C16H32O2	M-H2O + H [1+]
		257.24749	0.97	0.00003	0.00418		M + H [1+]
		258.25086	0.97	0.00221	0.03801		M(C13)+H [1+]
		−256.23631	0.37	0.00312	0.04809		M(C13)−H [1-]
	Linoleate	281.24751	1.01	0.00787	0.06816	C18H32O2	M + H [1+]
		263.23694	0.97	0.00865	0.07011		M-H2O + H [1+]
		−279.23294	2.93	0.00333	0.04840		M-H [1−]
	Octadecanoic acid	267.26824	0.98	0.00001	0.00191	C18H36O2	M-H2O + H [1+]
		285.27881	1.03	0.00001	0.00191		M + H [1+]
		−284.26761	1.92	0.00200	0.03690		M(C13)−H [1−]
		−283.26425	0.56	0.00688	0.06463		M-H [1−]

B. Differences between PAH and CTEPH patients.

Butanoate metabolism	2-Oxobutyric acid	85.02841	0.63	0.00848	0.12729	C4H6O3	M-H2O + H [1+]
	6-Acetamido-2-oxohexanoate	188.09173	2.16	0.00958	0.13153	C8H13NO4	M + H [1+]
	3-Butynoate	85.02841	0.63	0.00848	0.12729	C4H4O2	M + H [1+]
	5-Acetamidopentanoate	160.09682	2.34	0.01867	0.17349	C7H13NO3	M + H [1+]
	Succinate semialdehyde	85.02841	1.14	0.00848	0.12729	C4H6O3	M-H2O + H [1+]
	GABA	104.0706	1.66	0.00856	0.12729	C4H9NO2	M + H [1+]
	Butyrate	71.04914	0.42	0.00117	0.09636	C4H8O2	M-H2O + H [1+]
		89.0597	0.35	0.00348	0.09838		M + H [1+]
	Acetoacetate	85.02841	0.63	0.00848	0.12729	C4H6O3	M-H2O + H [1+]
Squalene and cholesterol	Mevalonic acid	149.08086	2.40	0.00366	0.09927	C4H6O3	M + H [1+]
		166.10738	2.54	0.00124	0.09636		M + NH4 [1+]
	Acetoacetate	*85.02841*	*0.63*	*0.00848*	*0.12729*	*C4H6O3*	*M-H2O + H [1+]*

Log2FC, logarithm of 2 of the fold-change; m/z, mass-to-charge ratio.

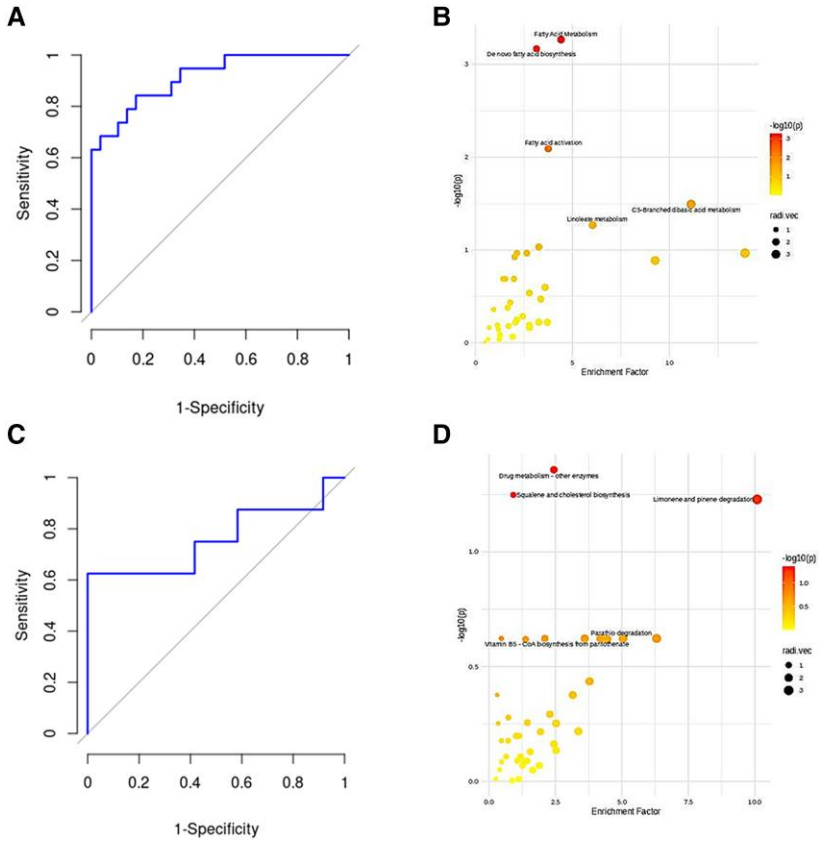


Figure 1 Discriminative analysis of breath biomarkers for the diagnosis and subclassification of pulmonary vascular disease. (*A*) Receiver operating characteristic curve for distinguishing pulmonary vascular disease patients from healthy controls. The model achieved a sensitivity of 92.9%, specificity of 70.9%, and an area under the curve of 0.917. (*B*) Metabolic pathway enrichment analysis of exhaled breath profiles in pulmonary vascular disease patients compared with healthy controls. Volatile organic compounds were analysed using secondary electrospray ionization–high resolution mass spectrometry. The *x*-axis indicates enrichment factor and the *y*-axis $-\log_{10}$(*P*-value). Dot size represents pathway impact, and colour intensity reflects statistical significance, with red indicating higher significance. Enriched pathways included fatty acid metabolism, *de novo* fatty acid biosynthesis, and fatty acid activation. (*C*) Receiver operating characteristic curve for differentiating pulmonary arterial hypertension from chronic thromboembolic pulmonary hypertension, with a sensitivity of 80.8%, specificity of 62.3%, and an area under the curve of 0.764. (*D*) Metabolic pathway enrichment analysis comparing pulmonary arterial hypertension and chronic thromboembolic pulmonary hypertension breath profiles, based on secondary electrospray ionization–high resolution mass spectrometry data. Notably enriched pathways included drug metabolism (other enzymes) and squalene and cholesterol biosynthesis, highlighting distinct metabolic signatures between pulmonary arterial hypertension and chronic thromboembolic pulmonary hypertension.

45.5–80.0%), and an accuracy of 73.6% (95% CI: 64.0–82.7%) with an AUC of 0.764 (95% CI: 0.636–0.872) (*Figure 1C* and *D*).

Discussion

This first study utilising real-time SESI-HRMS to assess VOCs of PVD patients successfully distinguished PVD from healthy controls (sensitivity: 92.9%, AUC: 0.917) and differentiated PAH from CTEPH (sensitivity: 80.8%, AUC: 0.764). Advances in mass spectrometry, particularly SESI-HRMS, enable precise VOC detection and quantification, which presents a promising approach for identifying PVD-specific biomarkers and underlying metabolic pathways. The most distinguishing metabolites were linked to fatty acid metabolism, including *de novo* fatty acid metabolism and fatty acid activation pathways.

In PVD, mitochondrial dysfunction, characterized by impaired β-oxidation and increased fatty acid synthase activity in pulmonary endothelial cells and cardiac myocytes, leads to intracellular accumulation of long-chain fatty acids.[5] We observed consistently lower levels of related metabolites in PVD samples, likely reflecting reduced release into circulation and alveolar space. Decreased levels of fatty acid activation products, dependent on enzymes like carnitine palmitoyltransferase 1, further support impaired mitochondrial uptake and oxidation, contributing to intracellular lipid retention and reduced exhaled levels.

Given the complexity and heterogeneity of PAH and CTEPH, we investigated molecular pathways to identify differences between the two conditions. In our study, we found that breath features corresponding to mevalonic acid were significantly elevated in PAH patients compared with CTEPH patients. The mevalonate pathway is crucial for the *de novo* synthesis of cholesterol.[6] Rho kinases, which are part of this pathway, have been explored as drug targets for PAH treatment. We also identified multiple downregulated breath features of the butanoate pathway in PAH, essential for fatty acid metabolism and energy production. An association that has been described previously in PAH lung tissue.[7] The downregulation of butanoate metabolism observed in PAH may signify impaired mitochondrial fatty acid oxidation

and energy production, a metabolic shift that contributes to right ventricular dysfunction and vascular remodelling. This pathway also plays a role in maintaining anti-inflammatory homeostasis, suggesting that its disruption could further exacerbate disease progression.

Limitations of this study include a reduced accuracy of the used database matching of measured accurate masses within 2 ppm, which provides a lower level of chemical identification confidence compared with Ultra-performance liquid chromatography-mass spectrometry. Furthermore, substantial differences in age and smoking status between the PVD cohort and healthy controls potentially influencing the detected breath features.

In conclusion, breath analysis shows promise as a non-invasive and real-time diagnostic tool for detecting PVD and potentially differentiating between PAH and CTEPH. Breath analysis may become a useful initial screening tool for dyspnoeic patients in clinical practice, aiding in the early detection of PVD and potentially uncovering new metabolic pathways associated with its subtypes.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Authors' contribution

Conceptualisation: M.L., S.U., and M.K.; methodology: M.L., S.U., M.K., and P.S.; formal analysis and investigation: S.B., K.F., N.A.S., A.A., F.S., J.H., and D.M.B.; draft of the manuscript: K.F., S.B.; critical revision of the manuscript for important intellectual content: N.A.S, A.A., F.S., J.H., D.M.B., P.S., M.K., S.U., and M.L.; funding acquisition: M.L.; graphical abstract: K.F. and M.L.

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Conflict of interest: S.U. reports personal fees from MSD, Janssen, Novartis, Orpha Swiss, and Gebro SA. M.K. reports consulting fees from Novartis and GSK and is co- founder of DBI AG, a company that provides services in the field of breath analysis. F.S. is a part-time employee of DBI AG. None declared (S.B., K.F., N.A.S., A.A., J.H., D.M.B., and M.L.).

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