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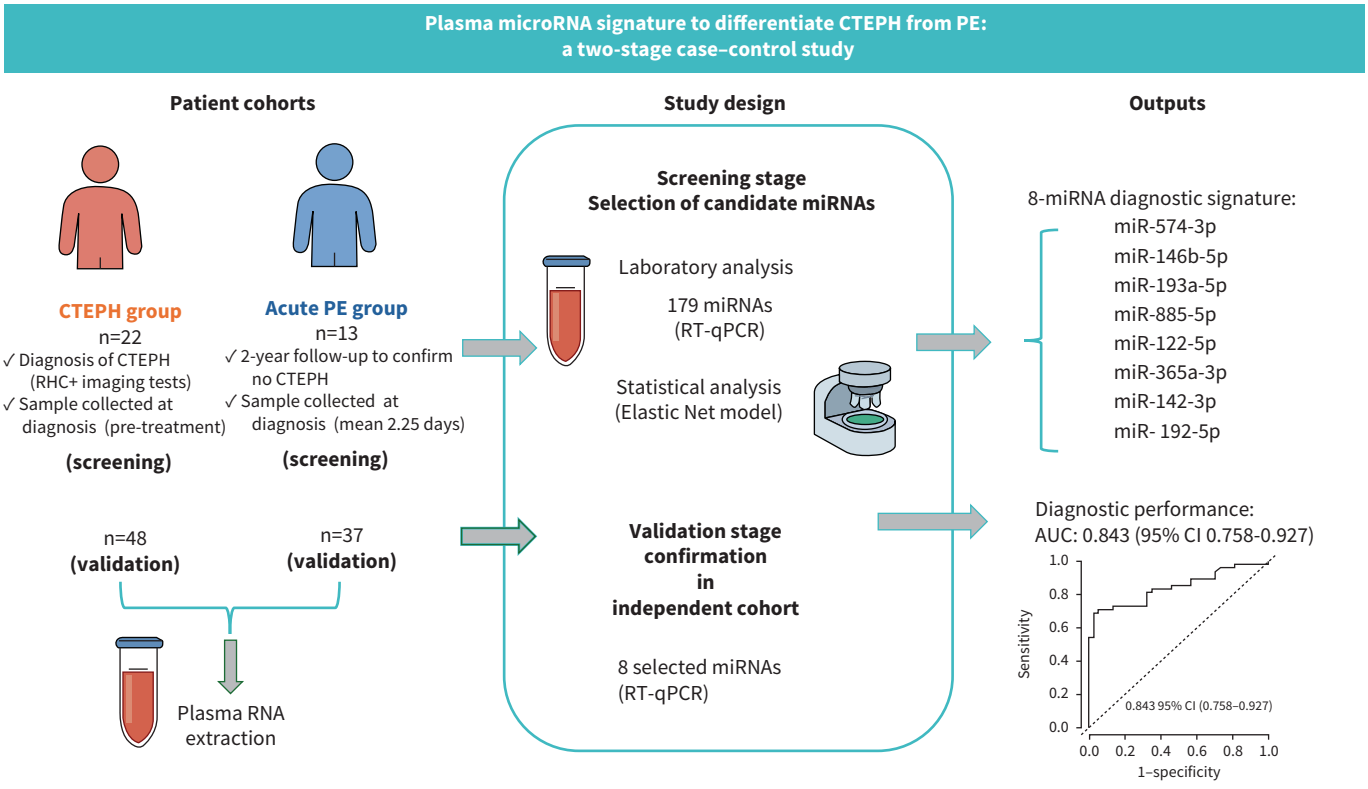

GRAPHICAL ABSTRACT Plasma miRNA signature to differentiate chronic thromboembolic pulmonary hypertension (CTEPH) from pulmonary embolism (PE): a two-stage case–control study. RHC: right heart catheterisation; miRNA: microRNA; RT-qPCR: real-time quantitative polymerase chain reaction; AUC: area under the receiver operating characteristic curve.

https://doi.org/10.1183/23120541.00340-2025

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microRNAs as epigenetic biomarkers for CTEPH: a case–control study towards personalised medicine

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A validated profile/signature of eight plasma miRNAs shows strong potential as early biomarkers to differentiate CTEPH from PE, paving the way for personalised medicine. This innovative tool could transform diagnosis improving patient outcomes. https://bit.ly/3TQ4ryN

Cite this article as: Oto J, Tura-Ceide O, Hervás D, *et al*. microRNAs as epigenetic biomarkers for CTEPH: a case–control study towards personalised medicine. *ERJ Open Res* 2026; 12: 00340-2025 [DOI: 10.1183/23120541.00340-2025].

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Received: 25 March 2025
Accepted: 13 July 2025

Abstract

Background Chronic thromboembolic pulmonary hypertension (CTEPH) is a severe complication of acute pulmonary embolism (PE) in which delayed diagnosis significantly affects patient health outcomes. This case–control study aimed to identify a set of microRNAs (miRNAs) in plasma with diagnostic potential to differentiate patients with CTEPH from those with PE.

Methods Two groups were analysed: 22 patients with confirmed CTEPH and 13 patients with PE, followed by validation in an independent cohort of 48 CTEPH and 37 PE patients.

Results Using real-time PCR, eight miRNAs were identified as significantly different between the groups: miR-574-3p, miR-146b-5p, miR-193a-5p, miR-885-5p, miR-122-5p, miR-365a-3p, miR-142-3p and miR-192-5p. These miRNAs target key biological pathways, including vascular smooth muscle contraction, apoptosis and VEGF signalling, underlying the pathophysiology of CTEPH. The miRNA panel demonstrated strong diagnostic accuracy with an area under the curve of 0.843 in the validation cohort.

Conclusions The results highlight the potential of miRNA biomarkers as a diagnostic tool for early detection of CTEPH, representing a paradigm shift in its management, but further validation in larger cohorts is necessary to confirm their applicability. These insights could pave the way for improved clinical outcomes through timely diagnosis and targeted interventions.

Introduction

Chronic thromboembolic pulmonary hypertension (CTEPH) is a rare complication of acute pulmonary embolism (PE), accounting for ∼20% of pulmonary hypertension (PH) cases [1, 2]. Its prevalence ranges from 3.2 per million (Spain) to 38 per million (UK) [3, 4]. The incidence of CTEPH after PE is unclear, with reports varying from 0.1% to 11.8% within 2 years post-PE [5–9]. Studies suggest an incidence of ∼3% in PE survivors, aligning with clinical experience. Many cases initially diagnosed as acute PE may actually represent the first manifestation of undetected CTEPH [10]. Notably, only 50–75% of CTEPH patients have a prior PE diagnosis [11, 12].

CTEPH involves persistent thrombotic obstruction in pulmonary arteries, causing increased pulmonary vascular resistance, right heart failure and death if untreated. Diagnosis requires evidence of unresolved PE

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and precapillary PH after ⩾3 months of anticoagulation. It includes haemodynamic confirmation *via* right heart catheterisation (RHC) and lesion localisation by imaging (computed tomography angiography, digital subtraction pulmonary angiography). Diagnosis is often delayed (14–24 months post-symptom onset) [2, 11, 12], and treatment delays worsen prognosis [13, 14]. Thus, novel, minimally invasive biomarkers could aid in early CTEPH diagnosis, especially in distinguishing it from PE.

microRNAs (miRNAs) are 19–23-nucleotide, single-stranded, non-coding RNAs that regulate the translation of their target mRNAs by binding to their 3′UTR region and preventing their translation by blocking, degradation or deadenylation [15]. They affect numerous biological processes, potentially regulating 30–90% of human genes, and act in intercellular communication *via* exosomes and microparticles. miRNAs may serve as biomarkers for early CTEPH detection.

This study aimed to identify a plasma miRNA profile with diagnostic value to distinguish PE from CTEPH.

Methods

Study design

This case-control study aimed to identify a plasma miRNA profile to differentiate CTEPH from PE. It was conducted in two stages: an initial screening to detect differentially expressed miRNAs, followed by a validation stage in an independent cohort.

Setting

The study was carried out at Hospital Clínic of Barcelona and Hospital Virgen del Rocío in Sevilla, Spain. Patient recruitment and sample collection occurred between September 2016 and July 2018. Laboratory analyses were centralised at the Health Research Institute Hospital La Fe, Valencia, Spain.

Participants

Two groups were included: 70 CTEPH patients (cases) [16] and 50 with unprovoked PE (controls) [17], matched by age and sex, with a 2-year follow-up confirming absence of sequelae or complications.

CTEPH diagnosis was established based on a combination of haemodynamic and imaging studies. RHC was performed to confirm the presence of precapillary PH, defined by a mean pulmonary arterial pressure (mPAP) ⩾20 mmHg, pulmonary artery wedge pressure ⩽15 mmHg and pulmonary vascular resistance >2 Wood units. Additionally, imaging studies such as ventilation–perfusion (V/Q) scintigraphy were used to detect mismatched perfusion defects, and computed tomography pulmonary angiography (CTPA) or conventional pulmonary angiography was employed to identify chronic thromboembolic obstructions.

The screening stage comprised 22 cases and 13 controls. The validation stage comprised an independent cohort of 48 CTEPH and 37 PE patients, also matched by age and sex. Inclusion criteria were the aforementioned clinical criteria and providing written informed consent. Risk stratification of acute PE was performed according to the 2019 ESC/ERS Guidelines, based on the presence of haemodynamic instability, right ventricular dysfunction (on echocardiography or CTPA) and elevated cardiac biomarkers.

Exclusion criteria included sequelae or complications after 2 years follow-up from PE such as neoplasm or CTEPH diagnosis. The follow-up protocol for patients with acute PE involved scheduled visits at 3, 6, 12 and 24 months, during which the presence of residual or new-onset dyspnoea was specifically assessed. In such cases, screening echocardiography for CTEPH was performed. Additionally, all asymptomatic patients underwent echocardiography at 24 months to rule out the possibility of sequelae following acute PE.

Ethical approval was obtained from the relevant institutional committees. Informed consent was obtained from all participants.

Variables

The primary outcome was differential plasma miRNA expression between groups. Predictors included levels of 179 candidate miRNAs. Confounders (age, sex, comorbidities) were recorded for adjustment in statistical analyses. Ethnicity and race were recorded by the physician based on the individual's place of birth, considering this criterion an appropriate approach for classification within the studied population.

Data collection and measurement

Fasting venous blood was drawn into sodium citrate tubes (Becton Dickinson, Plymouth, UK) and plasma was obtained by centrifugation at 2500 ×g for 10 min at 4°C and aliquots were stored at −80°C until used. Case samples were collected at CTEPH diagnosis (prior to treatment), and control samples within 10

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days post-PE diagnosis (mean 2.25±2.18 days). Only PE patients with an uneventful 2-year follow-up were included.

Plasma collection and extracellular vesicle (EV) isolation procedures are detailed in the supplementary material, as they are not essential to the core methodology of this study.

Total RNA (including miRNAs) from plasma was isolated using the miRNeasy Mini Kit (Qiagen, Hilden, Germany), following the strategy conducted in previous studies [18, 19, 20, 21] and manufacturer's instructions with several modifications [22]. During the isolation, an RNA carrier (tRNA, Ambion, Bleiswijk, The Netherlands) was included to enhance the yield, and a mixture of synthetic miRNAs (RNA Spike-in Kit for RT, Qiagen) was included to control for RNA isolation efficiency and cDNA synthesis. The concentration and purity of the RNA was assessed by spectrophotometric quantification with the NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE, USA). RNA was stored at −80°C until used. No plasma sample was haemolysed, determined at the absorbance of haemoglobin (412 nm).

miRNA quantification

The expression level of miRNAs was quantified by real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR). The miRCURY LNA RT Kit (Qiagen) was used for the retrotranscription, and the expression level of 179 candidate miRNAs mostly present in plasma was quantified with the Serum/plasma miRNA PCR Panel V5 (Qiagen) and miRCURY LNA SYBR Green Master Mix (Qiagen) as a fluorophore, according to manufacturer's indications in a LightCycler 480 II (Roche, Mannheim, Germany). Furthermore, each panel includes the following internal controls: five synthetic RNAs of the RNA Spike-in-kit aimed to monitor the RNA isolation and cDNA synthesis, and an inter-plate calibrator in triplicate and a negative control to evaluate qPCR performance.

To normalise the expression level of each miRNA, the best endogenous reference with the highest stability and the lowest biological variance among all samples was selected. The candidate normalisation miRNAs proposed by the Serum/plasma miRNA PCR Panel V5 were miR-423-5p, miR-425-5p, miR-93-5p, miR-191-5p and miR-103a-3p. To select the best reference, the comprehensive tool RefFinder was employed which integrates the computational programs geNorm, Normfinder, BestKeeper and the comparative delta-Ct method (https://www.ciidirsinaloa.com.mx/RefFinder-master/). miRNA expression levels were normalised by the $2^{-\Delta\Delta CT}$ method.

Once a profile of miRNAs potentially able to distinguish between CTEPH and PE patients was selected, their expression level was quantified in an independent cohort of subjects (48 CTEPH patients and 37 PE patients) by RT-qPCR in duplicate, using LNA miRNA-specific primers (Qiagen). Each duplicate measurement was considered adequate when the standard deviation (SD) was <0.5.

Target identification and pathway analysis

Validated and predicted targets of dysregulated miRNAs were identified *via* miRWalk and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways, focusing on vascular and haemodynamic processes (*e.g.*, apoptosis, muscle contraction, VEGF signalling). Protein–protein interactions were visualised with Cytoscape (v3.10.1) using STRING database (v11.5, confidence score >0.7). Core clusters and hub proteins were identified with MCODE and cytoHubba plugins.

Statistical analysis

Analysis was performed in R (v3.5.1). Numerical variables were summarised as median (interquartile range (IQR)), categorical as counts (%). An elastic net logistic regression model included clinical variables and miRNA expression. Lambda was selected *via* 500 iterations of 10-fold cross-validation. Model performance was assessed by receiver operating characteristic area under the curve (ROC AUC) (bootstrap, n=1000). Heatmaps and boxplots were generated (α=0.6, λ=0.237512). Validation ROC analysis included sensitivity/specificity curves. Significance was set at $p<0.05$.

Selection bias was minimised through age- and sex-matching; measurement bias by standardised protocols and blinded to laboratory personnel. Sample size reflected patient availability during the study period.

Results

Participant characteristics

The clinical characteristics of the participants are detailed in table 1. Cases (CTEPH patients) had a median age of 64 years (IQR: 56–72), with 50% being female, while controls (PE patients) had a median age of 63 years (IQR: 53–67), with 31% being female. 95% of the patients were of Southern European origin and

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TABLE 1 Clinical characteristics of the study subjects

Variables	Screening			Validation		
	CTEPH	PE	p-values	CTEPH	PE	p-values
Subjects, n	22	13		48	37	
Age years, median (range)	64 (56–72)	63 (53–67)	0.487	59 (36–81)	58 (36–81)	0.478
Female sex, n (%)	11 (50)	4 (31)	0.266	22 (45)	15 (40)	0.625
BMI kg·m^{-2}, median (range)	28.2 (25.5–30.2)	30.7 (28.7–35.4)	0.326	28 (19–44)	27 (19.5–44)	0.389
Ethnic and racial profile, n (%)						
Race: white (Southern European)	22 (100)	13 (100)	1.0	44 (92)	35 (94)	0.693
Race: white (Eastern European)	0	0	NA	0	2	NA
Race: North African (Maghrebi)	0	0	NA	3	0	NA
Ethnicity: Hispanic or Latino	0	0	NA	1	0	NA
Arterial hypertension, n (%)	15 (68)	7 (54)	0.000	14 (56)	14 (38)	0.399
Diabetes mellitus, n (%)	2 (9)	2 (28)	0.477	2 (12)	6 (16)	0.059
Dyslipidaemia, n (%)	10 (45)	2 (28)	0.070	4 (16)	14 (38)	0.000
Ischaemic cardiomyopathy, n (%)	2 (9)	1 (14)	0.764	2 (12)	4 (11)	0.258
Stroke history, n (%)	2 (9)	0	0.388	0	2 (5)	0.110
Renal insufficiency, n (%)	2 (9)	2 (28)	0.477	6 (28)	5 (14)	0.890
Smokers, n (%)	10 (45)	3 (43)	0.519	9 (37)	22 (59)	0.000
PE risk classification, n (%)[#]						
Low risk	--	11 (85)	NA	--	18 (49)	NA
Intermediate–low	--	2 (15)	NA	--	11 (30)	NA
Intermediate–high	--	--	NA	--	5 (13)	NA
High risk	--	--	NA	--	3 (8)	NA

CTEPH: chronic thromboembolic pulmonary hypertension; PE: pulmonary embolism; BMI: body mass index. [#]: risk classification of PE patients based on 2019 European Society of Cardiology (ESC)/European Respiratory Society (ERS) Guidelines, combining RV dysfunction (Transthoracic echocardiography (TTE) or Computed tomography pulmonary angiography), cardiac biomarkers and haemodynamic status.

identified as white. A multidisciplinary expert committee from one of the two designated National Reference Centers for Complex Pulmonary Hypertension in Spain assessed the appropriate treatment modality for each patient. A total of 61 out of 70 patients with CTEPH (87.1%) received definitive invasive treatment, either through pulmonary endarterectomy (PEA), balloon pulmonary angioplasty (BPA), or both. Specifically, 51 patients underwent PEA, and 13 underwent BPA, including three who had previously received PEA. These findings underscore that the vast majority of patients were eligible for and underwent corrective intervention. The remaining nine patients (12.9%) were managed exclusively with medical therapy, mainly due to the distal nature of the lesions or the presence of significant comorbidities.

All patients were enrolled immediately following the diagnosis of CTEPH. At the time of inclusion, 54% of patients were classified as WHO functional class III, while 40% were classified as class II. The haemodynamic parameters of the screening cohort of CTEPH patients are provided in supplementary table S1.

Among the 50 patients with acute PE included in both cohorts, 29 (58%) were classified as low-risk, 13 (26%) as intermediate–low-risk, five (10%) as intermediate–high-risk and three (6%) as high-risk PE, based on guideline-defined criteria. Significant differences were observed between the screening groups in arterial hypertension. Similarly, differences were noted between the validation groups for diabetes mellitus and smoking status. These results are detailed in table 1.

The final model, developed for both the screening and validation phases, was adjusted to account for these variables.

miRNA profile/signature development

In the screening stage, we identified miRNAs that were differentially expressed between patients with CTEPH and those with PE. A total of 179 miRNAs were initially evaluated for their expression levels. Following normalisation using miR-425-5p, selected for its stability and low biological variability across samples, an elastic net logistic regression identified a profile/signature of eight miRNAs that significantly differentiated cases and controls. These miRNAs were miR-574-3p, miR-146b-5p, miR-193a-5p, miR-885-5p, miR-122-5p, miR-365a-3p, miR-142-3p and miR-192-5p (table 2). Figure 1 shows the expression of the eight-miRNA-profile/signature with diagnostic ability for CTEPH.

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TABLE 2 Characteristics of the microRNAs (miRNAs) included in the diagnostic profile/signature for chronic thromboembolic pulmonary hypertension

miRNA name	Gen ID in NCBI	miRNA sequence
miR-574-3p	693159	CACGCUCAUGCACACACCCACA
miR-146b-5p	574447	UGAGAACUGAAUUCCAUAGGCUG
miR-193a-5p	406968	UGGGUCUUUGCGGGCGAGAUGA
miR-885-5p	100126334	UCCAUUACACUACCCUGCCUCU
miR-122-5p	406906	UGGAGUGUGACAAUGGUGUUUG
miR-365a-3p	100126355	AGGGACUUUUGGGGGCAGAUGUG
miR-142-3p	406934	UGUAGUGUUUCCUACUUUAUGGA
miR-192-5p	406967	CUGACCUAUGAAUUGACAGCC

Supplementary figure S1a shows the expression levels of each miRNA in the development clinical groups, while supplementary figure S1b displays the box plots for the same miRNAs in the validation clinical groups.

Furthermore, we estimated the predictive ability of this miRNA profile/signature. The diagnostic formula to predict the likelihood of CTEPH based on the expression level of these miRNAs is as follows:

$$\Pr(\text{CTEPH}) = \frac{e^{LP}}{1 + e^{LP}}$$

where LP is:

$$\begin{aligned} LP = \beta_0 &- 0.161 \times \text{miR} - 547 - 3\text{p} + 0.404 \times \text{miR} - 146\text{b} - 5\text{p} - 0.087 \times \text{miR} - 193\text{a} - 5\text{p} - 0.011 \\ &\times \text{miR} - 885 - 5\text{p} - 0.123 \times \text{miR} - 122 - 5\text{p} - 0.21 \times \text{miR} - 365\text{a} - 3\text{p} + 0.138 \\ &\times \text{miR} - 142 - 3\text{p} - 0.154 \times \text{miR} - 192 - 5\text{p} \end{aligned}$$

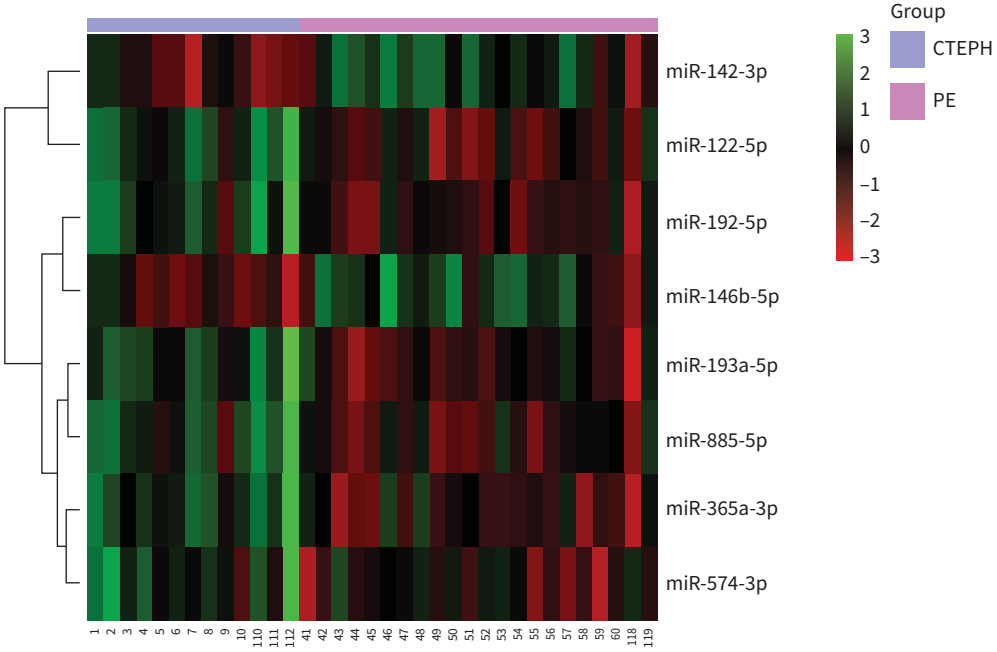

FIGURE 1 Heatmap of the dysregulated microRNAs (miRNAs) in plasma samples included in the predictive profile/signature of patients with chronic thromboembolic pulmonary hypertension (CTEPH). PE: pulmonary embolism.

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Validation of the miRNA profile/signature

The expression level of the identified miRNA profile/signature was subsequently validated in an independent cohort comprising 48 CTEPH patients and 37 PE patients. The ROC analysis yielded an AUC of 0.843 (95% confidence interval (CI): 0.758–0.927), indicating a strong diagnostic performance of the miRNA profile/signature, as illustrated in figure 2. Sensitivity and specificity profiles across various diagnostic thresholds are depicted in supplementary figure S2. This panel has been registered with the Spanish Patent and Trademark Office (OEPM) (P202231096).

Functional insights into miRNA targets

In silico analysis identified both validated and predicted targets of the dysregulated miRNAs, using databases such as miRWalk and KEGG pathways (table 3). Some miRNAs, such as miR-193a-5p and miR-574-3p, exhibit the highest number of target proteins, represented as the number of connections. In contrast, miR-192-5p and miR-365-3p showed a low number of target proteins. Key protein mRNA targets like *PIK3R1*, *PIK3R3*, *MAPK10* and *TPM1* exhibited significant interactions and were strongly implicated in the pathways analysed, as shown in figure 3. Among these, *MAPK10* was identified as the target for six of the eight miRNAs included in the profile/signature, while *PIK3R3*, *BRAF* and *PIK3R1* were targets of five of the eight studied miRNAs.

Protein–protein interaction network

A protein–protein interaction network was constructed to identify the most relevant protein–protein clusters and hub proteins associated with the dysregulated miRNAs. From all the protein interactions, we pinpointed the top 10 most relevant proteins based on the interactions: ITG1, CTNNB1, CTLA4, FAS, CD28, ITGB3, PIK3R1, PTK2, PIK3CA and CASP8 (figure 4). Notably, PIK3R1, a target of two highly interactive miRNAs (miR-193a-5p and miR-574-3p), emerged as a key protein in the network, affecting five out of the eight miRNAs.

Discussion

CTEPH diagnosis is frequently delayed, with a median of 13 months post-PE (IQR 5.7–34), and most patients present with advanced disease [10]. This delay negatively impacts prognosis, leading to worse pulmonary haemodynamics and increased mortality [23]. PE recovery is shaped by complex, partially unknown mechanisms, which makes early CTEPH detection particularly difficult. The complexity of the diagnostic workup also contributes to low adherence to clinical guidelines [13]. Consequently, experts have emphasised the need to integrate clinical and omics data to improve diagnosis and identify novel therapeutic targets [24].

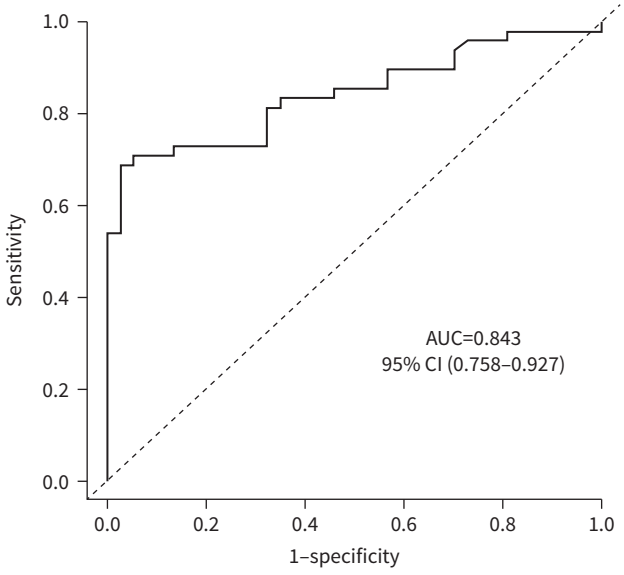

FIGURE 2 Receiver operating characteristic (ROC) curve of the miRNA profile/signature able to discriminate patients with chronic thromboembolic pulmonary hypertension from patients with pulmonary embolism. AUC: area under the ROC curve; CI: confidence interval.

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TABLE 3 Targets of the eight microRNAs (miRNAs) included in the profile/signature able to discriminate CTEPH from PE

miRNA	Targets						
	Apoptosis pathway	Cell cycle pathway	Cell adhesion molecules pathway	Vascular smooth muscle contraction pathway	Cardiac muscle contraction pathway	Fluid shear stress and atherosclerosis pathway	VEGF signalling pathway
miR-574-3p	AIFM1, AKT2, AKT3, BAK1, BAX, BCL2, BIRC3, CASP10, CASP2, CFLAR, CSF2RB, CYCS, DFFA, DIABLO, IKBKB, ITPR1, MAP3K14, MAK1, MAPK10, PIK3R1, PIK3R10, TNFSF10, TP53, XIAP	ABL1, CCND2, CDC14B, CDC25B, CDK2, CREBBP, E2F3, GSK3B, MAD2L1, MDM2, ORC4, SKP2, SMAD4, SMC1A, TFDP1, TFDP2, TP53, YWHAG	CADM3, CD226, CD28, CD40, CD99, CNTN1, CTLA4, F11R, HLA-DPB1, ICOSLG, IGFS11, ITGA8, ITGA9, ITGAM, NCAM1, NECTIN1, NFASC, NLGN4Y, NRXN2, PTPRC, SDC1, SDC4, SPN, TIGIT	ADCY5, CALM1, GNA11, GNA13, GUCY1A2, IRAG1, ITPR1, MAPK1, MYLK, MYLK3, PLA2G12A, PLA2G4F, PLCB3, PPP1CB, PRKCA, PRKCB	ASPH, CACNA2D1, CACNB2, CACNG2, COX6B2, UQCRB	ACBR2B, AKT2, AKT3, BCL2, BMPR1B, CALM1, GSTM5, GSTO2, IKBKB, ITGB3, MAP2K7, MAPK10, MAPK13, MAPK14, MEF2A, MEF2C, MGST1, PDGFA, PDGFB, PIK3R1, PIK3R3, SDC1, SDC4, SQSTM1, SUMO3, TP53, VEGFA	AKT2, AKT3. CDC42, MAPK1, MAPK13, MAPK14, NFATC2, PIK3R1, PIK3R3, PLA2G4F, PRKCA, PRKCB, PXN, VEGFA
miR-146b-5p	CTSO, CTSS, CYCS, ERN1, IKBKB, LMNB2, MAPK10, PIK3R1, PMAIP1, RIPK1	BUB3. CCND1, CDC7, CDK4, CHEK1, ORC4, RBX1, SMAD2, SMC1A, WEE2	CADM1, CD8A, CLDN18, CLDN22, NEO1, NLGN1, PECAM1, SELE	ACTA2, ADCY2, ADCY5, CALCRL, GUCY1A1, KCNMA1, MYLK3, PPP1R12B, PRKACB, PRKCE, PRKCQ	SLC8A1, SLC8A3	BMPR2, GSTM3, GSTO2, IKBKB, MAP2K6, MAPK10, MAPK14, PECAM1, PIK3R1, SELE	MAPK14, PIK3R1, PXN
miR-193a-5p	AKT3, ATM, BCL2L11, CASP8, CFLAR, CTSC, CTSO, CYCS, DIABLO, FADD, FAS, FASLG, GADD45G, HRK, IKBKB, ITPR2, KRAS, MAP3K14, MAPK10, MAPK8, MAPK9, PDPK1, PIK3CA, PIK3CD, PIK3R1, PIK3R3, PRF1, RAF1, TP53AIP1, XIAP	ABL1, ATM, CCND2, CDC14B, CDC27, CDK6, CDKN1A, CDKN2A, CDKN2B, CHEK1, CUL1, E2F2, GADD45G, MAD2L1, MDM2, SMAD2, TFDP2, WEE1, WEE2, YWHAZ	CADM1, CD226, CD274, CD4, CD40, CD6, CD8B, CD8B2, CDH2, CLDN15, CNTN2, CNTNAP2, F11R, HLA-B, HLA-C, HLA-DOA, HLA-DPB1, HLA-DQA1, ICOSLG, ITGA4, MPZ, MPZL1, NCAM1, NCAM2, NLGN1, NRXN1, NTNG1, OCLN, PECAM1, PTPRM, PVR, SDC1, SDC4, SELP, SPN	ADCY3, ADCY6, ADRA1D, ARAF, ARHGEF1, AVPR1B, BRAF, CALCA, CALCRL, CALM3, CALML4, GNA11, GNA12, GNA13, GUCY1A1, IRAG1, ITPR2, KCNMB2, MYL9, MYLK4, PLA2G2C, PLA2G2D, PLCB1, PLCB2, PPP1CC, PRKCA, PRKCB, PRKCG, RAF1	ASPH, ATP2A2, ATP2A3, CACNA2D1, CACNA2D2, CACNA2D4, CACNB2, CACNG5, COX7A1, COX7B, MYL4, SLC8A1, SLC9A6, TPM1, TPM3, TPM4, UQCR10, UQCRB	ACVR2B, AKT3, ARHGEF2, BMPR1B, CALM3, CALML4, CYBA, GPC1, GSTM1, GSTM2, GSTM5, IKBKB, IL1R1, IL1R2, MAP2K4, MAP2K6, MAPK10, MAPK14, MAPK8, MAPK9, MEF2C, PDGFA, PDGFB, PECAM1, PIAS4, PIK3CA, PIK3CD, PIK3R1, PIK3R3, PRKCZ, PTK2, SDC1, SDC4	AKT3, CDC42, KRAS, MAPK14, PIK3CA, PIK3CD, PIK3R1, PIK3R3, PRKCA, PRKCB, PRKCG, PTK2, RAF1
miR-885-5p	BAK1, BCL2L11, BIRC5, CFLAR, CTSF, DFFB, IL3RA, MAPK10, MCL1, PIK3R1, PIK3R3, RIPK1, TNFSF10, TRADD, TRAF1, XIAP	CDC27, CREBBP, E2F4, HDAC2, MDM2, ORC4, SMAD2, SMC1A, STAG2, TFDP2, YWHAZ	CADM1, CD34, CD40, CD40LG, CD99, CDH1, CLDN22, CNTN2, HLA-DOA, IGSF11, LRRC4, MPZ, MPZL1, NEO1, NLGN4Y, NRXN3, PTPRF, TIGIT	ADCY6, ADRA1A, BRAF, CALM1, CALML4, GUCY1A2, IRAG1, KCNMB1, PLA2G3, PLA2G4D, PPP1R12A, PPP1R12B, PRKCB, PTGIR	ASPH, ATP2A3, CACNA2D2, CACNA2D4, CACNG2, CACNG3, COX6B2, SLC8A2, SLC9A6, TPM1, TPM3	ACVR2B, BMPRA1, CALM1, CALML4, CAV1, CTNNB1, GPC1, GSTO2, IL1R1, MAP2K5, MAPK10, MAPK11, MAPK13, PIK3R1, PIK3R3, PRKAA2, RAC1, SUMO2, THBD	CDC42, MAPK11, MAPK13, MAPKAPK2, PIK3R1, PIK3R3, PLA2G4D, PPP3R1, PRKCB, PXN, RAC1

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TABLE 3 Continued

miRNA	Targets						
	Apoptosis pathway	Cell cycle pathway	Cell adhesion molecules pathway	Vascular smooth muscle contraction pathway	Cardiac muscle contraction pathway	Fluid shear stress and atherosclerosis pathway	VEGF signalling pathway
miR-122-5p	ATM, CASP6, CASP9, CASP10, CTSB, CYCS, LMNB1, MAPK1, MAPK9, PDPK1, PIK3CA, PMAIP1, TNFRSF10B, TP53AIP1, TRAF1	ATM, BUB3, CCND1, CCNE1, CDC14B, CDC16, CDC25A, CDK4, CHEK1, HDAC2, MAD2L1, ORC4, SKP1, SMAD2, SMC1A, TFDP2	CD226, CD28, CD4, CDH5, CLDN16, CLDN2, CNTN1, CNTNAP2, ESAM, HLA-DPB1, ITGA4, ITGA9, ITGAL, ITGB1, JAM3, NCAM1, NCAM2, NLGN1, OCLN, SPN	ADCY1, BRAF, CACNA1C, GNA13, MAPK1, MYLK, MYLK3, MYLK4, PLCB2, PPP1R12B, PRKCQ, ROCK1, ROCK2	ATP1A2, ATP1B2, ATP2A1, CACNA1C, CACNB4, CACNG8, COX5B, SLC8A1, SLC9A6, TPM1, TPM4, TRDN	CDH5, DUSP1, KLF2, MAP2K6, MAP2K7, MAPK9, MAPK14, MEF2A, MGST2, PDGFA, PDGFB, PIK3CA	CASP9, MAPK1, MAPK14, PIK3CA, PPP3R2
miR-365a-3p	EIF2AK3, MAPK10, MCL1, PIK3R3, RIPK1, XIAP	CDKN1A, HDAC2, SKP2, SMAD2, TFDP2	CD40, CLDN19, CNTN1, MPZL1, TIGIT, VCAN	ADCY9, BRAF, CACNA1C, CALM3, GNAQ	CACNA1C, TPM3, TRDN	CALM3, MAP2K6, MAPK10, PIK3R3	PIK3R3
miR-142-3p	AKT3, ERN1, MAPK3, MAPK10	CCNH, CDKN2B, E2F5, MYC, SKP1, SKP2, SMAD2, SMAD4, SMC1A, TFGB1	CD226, CD8A, CD8B, CNTN2, HLA-DOA, ICAM1, ICOS, JAM3, NECTIN3, NLGN4X, SDC3	CALCRL, GUCY1A1, IRAG1, KCNMA1, MAPK3, MYH11, MYLK4, PPP1R12B, PRKACA, PRKACB	TRDN, UQCR10, UQCRB	AKT3, ASS1, CAV3, ICAM1, MAPK10, MEF2C	AKT3, MAPK3, MAPKAPK3
miR-192-5p	APAF1, BCL2L11, CTSK, MAPK9, MCL1, PIK3R1, PIK3R3, TP53, TRAF1	RB1, TP53	CADM1, CLDN9, MPZ, NLGN1, TIGIT	BRAF, GNA12, GUCY1A2, KCNMB4, PRKG1	CACNG5, CACNG8, TPM1, TPM3, TRDN	GSTO2, MAPK9, MGST2, PIK3R1, PIK3R3, TP53	CDC42, PIK3R1, PIK3R3

Target proteins were identified in miRWalk and were combined with the results from the Kyoto Encyclopedia of Genes and Genomes. Validated targets are defined as those that have been experimentally proven to be regulated by a miRNA. Predicted targets are defined as those that have been theoretically identified based on the free binding energy between the miRNA and the presumed target mRNA sequence. The underlined targets have been experimentally validated. CTEPH: chronic thromboembolic pulmonary hypertension; PE: pulmonary embolism.

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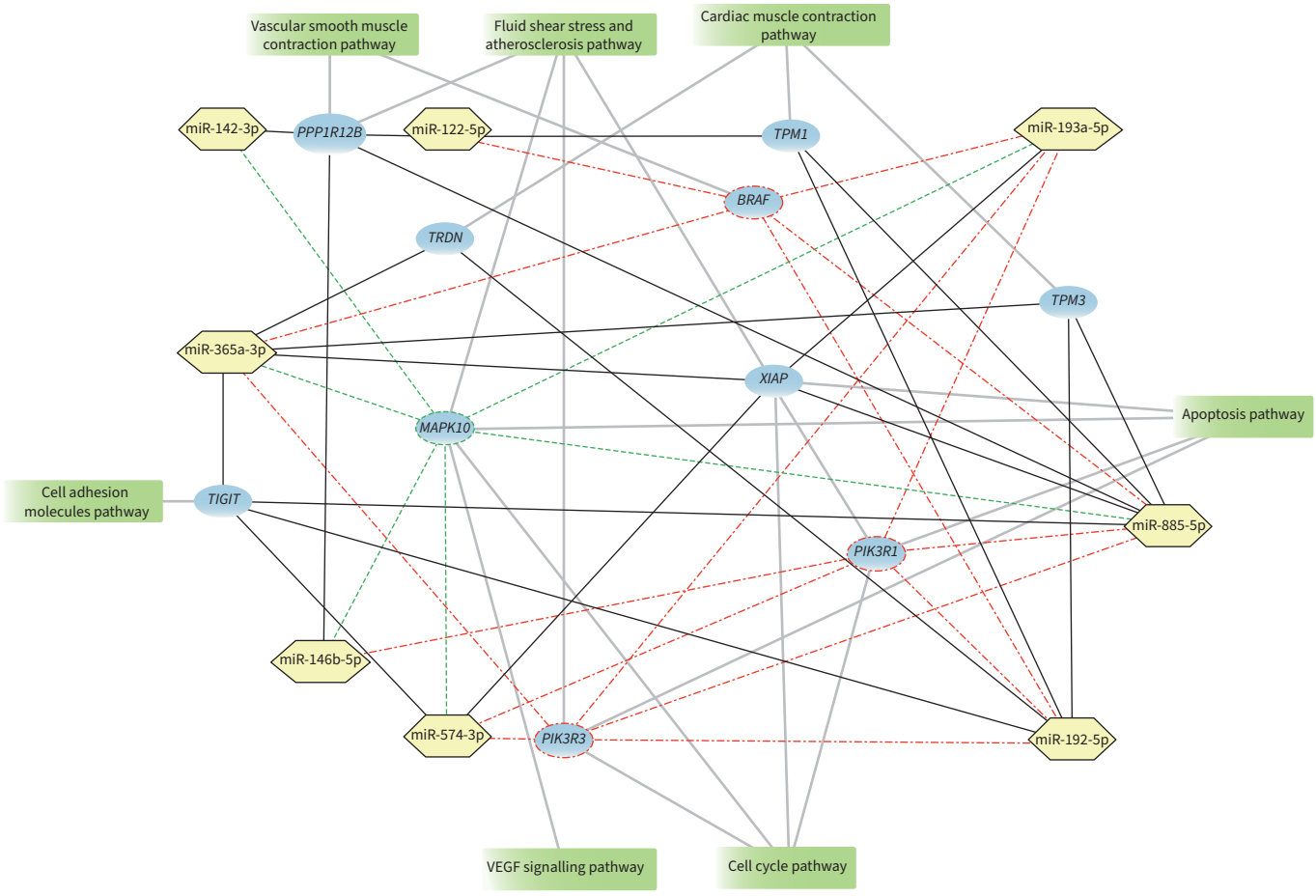

FIGURE 3 Main interactions among the target proteins regulated by the eight microRNAs (miRNAs) included in the profile/signature able to discriminate chronic thromboembolic pulmonary hypertension from pulmonary embolism patients. KEGG pathways involved with the target genes are also represented and linked to each gene. The gene with the highest number of interactions is highlighted in red, along with its interaction lines. Genes with the second highest number of interactions are highlighted in green. KEGG: Kyoto Encyclopedia of Genes and Genomes.

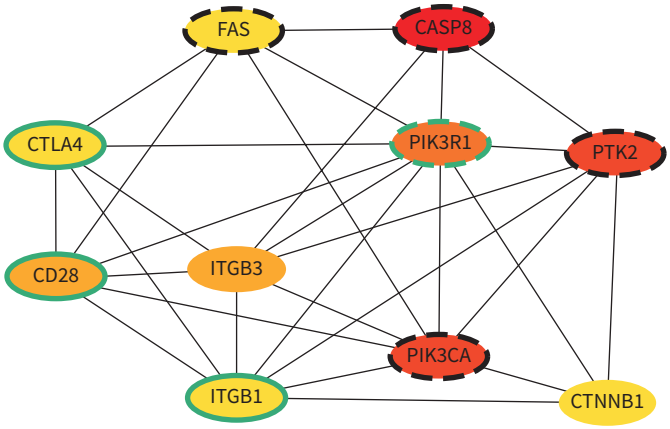

FIGURE 4 Hub protein from the protein–protein interaction network, displaying a cluster of interest and representing the top 10 most interactive proteins from the cluster. Green borders indicate target proteins from miR-574-3p, while dashed borders represent target proteins from miR-193a-5p. Red colour indicates the proteins with the highest interaction degree.

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Previous studies have suggested a role for miRNAs in predicting CTEPH. Kennewig *et al.* [25] reported elevated let-7a levels at PE diagnosis in patients who later developed CTEPH, with a 12.8-fold increased risk above a certain threshold. However, the low number of CTEPH cases (n=6) limited statistical significance. A recent systematic review identified 16 dysregulated miRNAs in CTEPH compared to controls [26], but none overlapped with our eight-miRNA panel. Differences in study design, populations and high risk of bias in the included studies (67%) may explain this discrepancy. Our study cohort, exclusively of European descent, also eliminates certain ethnic variability that may influence miRNA expression.

More recently [27], emerging evidence suggests that miRNA dysregulation may also occur early during the acute phase of venous thromboembolism. A recent study identified reduced circulating levels of miR-150-5p in patients with acute PE compared to patients with deep vein thrombosis, and lower miR-150-5p levels were associated with an increased risk of developing PAH during follow-up. It highlights the potential role of early miRNA alterations following PE in the subsequent development of pulmonary vascular disease, further supporting the relevance of miRNA-based biomarkers in the complex pathophysiology linking venous thromboembolism and PH.

In our study, expression profiling of 179 plasma miRNAs in a screening cohort (22 CTEPH, 13 PE) identified eight miRNAs with significant predictive value: miR-574-3p, miR-146b-5p, miR-193a-5p, miR-885-5p, miR-122-5p, miR-365a-3p, miR-142-3p and miR-192-5p. Validation in an independent cohort (48 CTEPH, 37 PE) confirmed strong diagnostic performance (AUC=0.843, 95% CI: 0.758–0.927). Supplementary figure S2 shows sensitivity and specificity across thresholds, supporting potential use for both screening and diagnosis depending on clinical context.

It is important to emphasise that all CTEPH cases in this study were diagnosed and managed in national surgical reference centres by expert multidisciplinary teams. Diagnosis was based on haemodynamic confirmation *via* RHC, imaging evidence of bilateral perfusion defects and anatomical characterisation of lesions through angiographic studies. The high proportion of patients receiving invasive treatment (87.1%) through PEA and/or BPA reflects the comprehensive management approach applied at our centre. This high rate of surgical or BPA treatment underscores the clinical utility of timely and accurate diagnosis, which enables early referral to expert centres and prevents disease progression towards inoperability.

No association was found between miRNA levels and CTEPH severity, but this requires confirmation in larger cohorts. Ethnicity and race were inferred from place of birth, which, although appropriate in this cohort (95% Spanish), may not accurately reflect ancestry in more diverse populations. Additionally, whether these miRNAs reflect vascular remodeling or haemodynamic dysfunction remains uncertain. An exploratory analysis comparing PE, proximal CTEPH and distal CTEPH revealed 13 differentially expressed miRNAs (supplementary figure S4), but our main analysis focused on PE *versus* CTEPH due to its greater robustness and clinical applicability.

To assess whether our miRNA signature reflects CTEPH-specific biology or general PH severity, we compared CTEPH with PAH patients using both plasma and EV fractions. Only miR-574-3p arose as significantly dysregulated in both our panel and PAH comparisons, and in the EV fraction. The remaining seven miRNAs were not differentially expressed. This partial overlap suggests that while miR-574-3p may be related to shared processes of vascular remodeling, the overall panel primarily captures mechanisms specific to CTEPH. These findings support the use of the miRNA signature as a diagnostic tool in the clinical setting where CTEPH is already suspected, for instance, in patients with a history of PE and ongoing unexplained dyspnoea or echocardiographic abnormalities, rather than as a predictive marker immediately after acute PE. Prospective studies with longitudinal sampling before and after therapeutic interventions (PEA/BPA) are underway to further refine the clinical applicability and temporal dynamics of this panel. To further illustrate the exploratory comparison between CTEPH and PAH patients at the level of EV-associated miRNAs, we plotted a heatmap of the six miRNAs identified in the less restrictive model (supplementary figure S5). This supports the relative specificity of the panel for CTEPH. However, the lack of a formal PAH comparator group in the primary study design represents a limitation.

Target prediction and functional analysis revealed that the eight miRNAs regulate genes involved in key CTEPH-related pathways, including apoptosis, cell cycle, vascular remodeling and cardiac function. All had experimentally validated targets of biological relevance: miR-574-3p targets SMAD4, a TGF-β pathway component; miR-146b-5p, PMAIP1 (NOXA); miR-193a-5p, PIK3R3 and TFDP2; miR-885-5p, RAC1 and CTNNB1; miR-122-5p, MAPK1, CDK4, TFDP2, NCAM1 and ATP1A2; miR-365a-3p, CDKN1A (p21); miR-142-3p, SKP2; and miR-192-5p, BCL2L11, CADM1 and KCNMB4. These targets are associated with vascular smooth muscle proliferation, endothelial dysfunction, apoptosis and

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thrombosis, highlighting the mechanistic relevance of the identified signature and its potential translational impact [27–36].

Recently, Lawrie *et al.* [28] and Errington *et al.* [29]. reported large-scale multicentre studies (CIPHER and its extensions) exploring circulating microRNA signatures for early detection and subclassification of PH across all World Symposium on Pulmonary Hypertension (WSPH) groups. While these studies demonstrated the potential of miRNA panels to classify PH subtypes, including CTEPH, the diagnostic performance for CTEPH remained modest (AUC ∼0.65–0.71), likely due to cohort heterogeneity resulting from the comparison of patients across different PH subtypes. Notably, none of the eight miRNAs identified in our study overlapped with the miRNAs associated with CTEPH in their signatures, suggesting that distinct molecular mechanisms may underlie the differentiation of CTEPH from PE compared to subclassification within PH. In contrast, our study focused on a specific and clinically relevant comparison of CTEPH *versus* PE, resulting in a transparent eight-miRNA signature with higher diagnostic accuracy (AUC 0.843) and direct applicability to this diagnostic challenge.

This study has several limitations. First, although PE patients were sampled early after the index PE event (within 10 days), the design was not a fully prospective screening study. Control patients were selected based on a 2-year uneventful follow-up without developing CTEPH or other complications. Therefore, while this approach allowed for early-phase biomarker assessment in well-defined phenotypes, future prospective studies enrolling unselected incident PE cohorts and evaluating the predictive performance of miRNA panels over time will be required to determine their utility for early CTEPH risk stratification in clinical practice. Second, no direct comparison was performed between the identified miRNA signature and currently used biomarkers such as N-terminal pro-brain natriuretic peptide or echocardiographic parameters, which represents an important area for future investigation.

In conclusion, this study identifies a novel panel of eight plasma miRNAs with strong diagnostic performance (AUC=0.843) for differentiating CTEPH from PE. These miRNAs are implicated in critical biological pathways, such as vascular remodeling, apoptosis and VEGF signalling, reinforcing their relevance in CTEPH pathogenesis. Target analysis also identified PIK3R1 as a key regulator influenced by multiple miRNAs. Although promising, these findings require validation in larger and more diverse cohorts to confirm clinical utility and support the integration of miRNA profiling into CTEPH diagnostic strategies.

Acknowledgements: We extend our deepest gratitude to the patients who voluntarily and generously participated in this study. We also sincerely thank the medical professionals at the Hospital Clinic de Barcelona and the Hospital Virgen del Rocío in Seville for their dedication and expertise in the clinical care of patients with pulmonary embolism and chronic thromboembolic pulmonary hypertension. Additionally, we acknowledge the invaluable contributions of the research staff at IDIBAPS and IBIS for their efforts in the collection, processing, storage and transportation of biological samples to the centralised laboratory. Their commitment has been essential to the success of this study.

Provenance: Submitted article, peer reviewed.

Ethics statement: The study was conducted in full compliance with the principles outlined in the Declaration of Helsinki. Ethical approval was obtained from the Institutional Ethics Committees of the participating institutions: the Ethics Committee on Human Research at Hospital Clínic of Barcelona (HCB/2016-00347-14) and the Coordinating Committee on Biomedical Research Ethics of Andalusia at Hospital Virgen del Rocío of Seville (Comité Coordinador de Ética de la Investigación Biomédica de Andalucía/10-13).

Author contributions: All individuals listed as authors meet the criteria for authorship. J.A. Barberà, J. Oto, O. Tura-Ceide, P. Medina and R. Otero contributed to the conception and design of the study. D. Hervás, V. Sánchez-López, J. Oto, O. Tura-Ceide, P. Medina, R. Otero, I. Blanco, T. Elias, L. Jara-Palomares and M. Gratacós-Aurich were involved in the acquisition, analysis or interpretation of the data. J. Oto, P. Medina and R. Otero drafted the manuscript, and all authors critically reviewed it for significant intellectual content. All authors provided final approval of the version to be published.

Conflict of interest: The authors have nothing to disclose.

Support statement: This research was supported by research grants from Instituto de Salud Carlos III (PI24/01737, PI23/00449, PI21/01400 and PI15/01085), FEDER una manera de hacer Europa, Sociedad Española de Trombosis y

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Hemostasia and Sociedad Española de Neumología y Cirugía torácica. Funding information for this article has been deposited with the Open Funder Registry.

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