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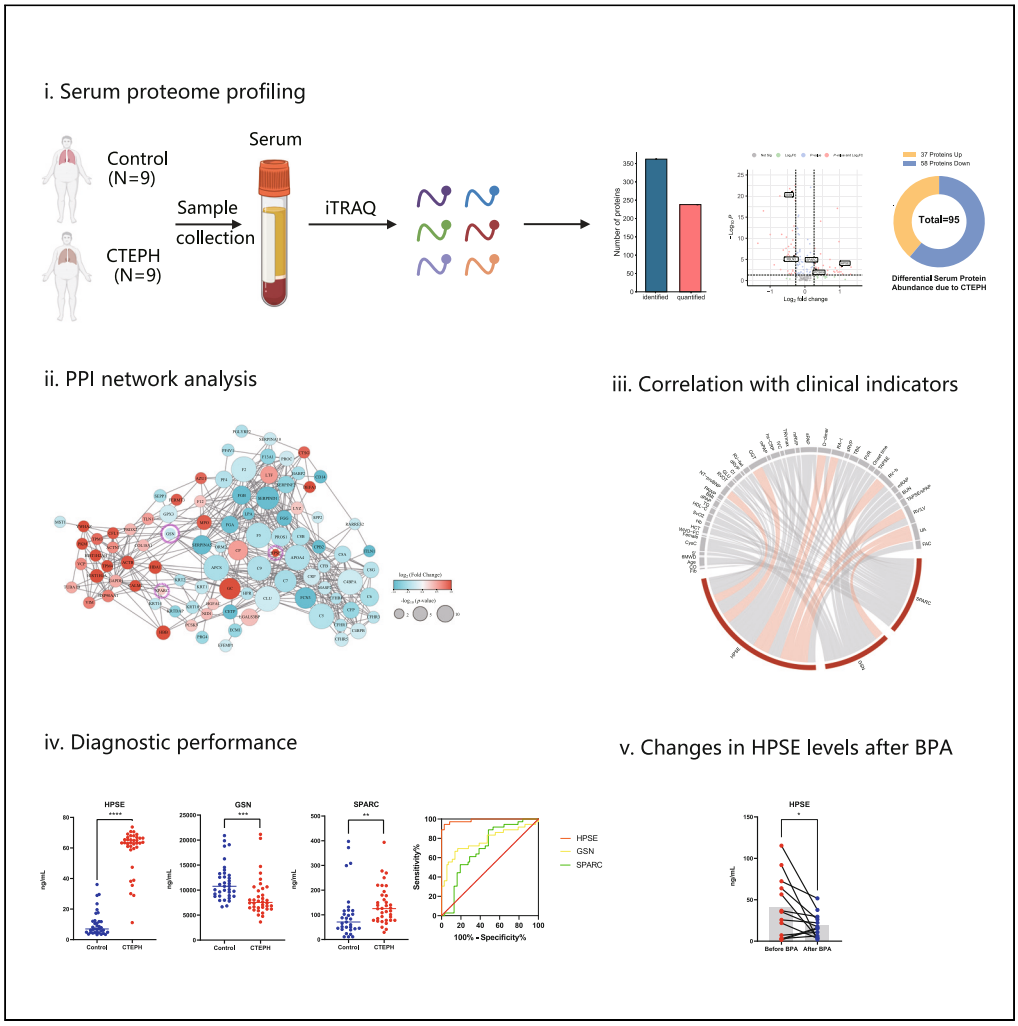

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Highlights

HPSE had significant changes and diagnostic values in CTEPH

HPSE was correlated with parameters of right ventricular function in CTEPH

Treated CTEPH patients had significantly lower HPSE levels after BPA

Zhang et al., iScience 27, 108930
February 16, 2024
https://doi.org/10.1016/j.isci.2024.108930

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Serum proteome profiling reveals heparanase as a candidate biomarker for chronic thromboembolic pulmonary hypertension

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SUMMARY

Determining novel biomarkers for early identification of chronic thromboembolic pulmonary hypertension (CTEPH) could improve patient outcomes. We used the isobaric tag for relative and absolute quantitation approach to compare the serum protein profiles between CTEPH patients and the controls. Bioinformatics analyses and ELISA were also performed. We identified three proteins including heparanase (HPSE), gelsolin (GSN), and secreted protein acidic and rich in cysteine (SPARC) had significant changes in CTEPH. The receiver operating characteristic curve analysis showed that the areas under the curve of HPSE in CTEPH diagnosis were 0.988. Furthermore, HPSE was correlated with multiple parameters of right ventricular function. HPSE concentrations were significantly higher in patients with a low TAPSE/sPAP ratio ($\leq$0.31 mm/mmHg) (65.4 [60.5,68.0] vs. 59.9 [35.9,63.2] ng/mL, $p < 0.05$). The CTEPH patients treated by balloon pulmonary angioplasty had significantly lower HPSE levels. The study demonstrates that HPSE may be a promising biomarker for noninvasive detection of CTEPH.

INTRODUCTION

Chronic thromboembolic pulmonary hypertension (CTEPH) is a devastating disease characterized by the combination of large vessel obstruction and small vessel disease leading to elevated pulmonary artery pressure and increased workload on the right ventricle, ultimately resulting in right heart failure.[1] The prevalence of CTEPH varies by country, ranging from 19 per million in Japan to 30–50 per million in the United States and Europe.[2] However, as the disease often remains undiagnosed or misdiagnosed, the data may be underestimated.[3] A multimodal approach to the treatment of CTEPH is recommended including pulmonary endarterectomy (PEA), balloon pulmonary angioplasty (BPA), and medical therapy.[4] PEA and BPA have greatly improved the survival rate of patients with CTEPH, while the prognosis with untreated CTEPH was still very poor.[5,6] Therefore, timely recognition is critical for CTEPH, as it may be curable.

Echocardiography is integral for screening and is usually the first imaging tool used in patients with suspected pulmonary hypertension (PH). However, the accuracy of echocardiography depends on the experts' experience. Right heart catheterization is the gold-standard method to diagnose PH but cannot be broadly applied owing to its invasive nature.[7] Various biomarkers have been looked into, to develop an inexpensive and noninvasive screening tool for CTEPH. BNP and N-terminal pro-brain natriuretic peptide (NT-proBNP) are currently the

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https://doi.org/10.1016/j.isci.2024.108930

Table 1. Clinical characteristics of the patients enrolled in this study

	iTRAQ			Validation cohort1			Validation cohort2		
	Control	CTEPH	p	Control	CTEPH	p	Control	CTEPH	p
N	9	9		36	36		30	30	
Age, years	39.1 ± 10.5	39.1 ± 13.6	1.00	56.1 ± 12.1	62.0(52.3,68.5)	0.18	56.4 ± 6.6	56.2 ± 11.6	0.92
Female	6(66.7%)	6(66.7%)	1.00	11(30.6%)	11(30.6%)	1.00	15(50.0%)	15(50.0%)	1.00
BMI, kg · m^{-2}	–	26.2 ± 4.6		–	23.9 ± 2.9				
Comorbidity									
CHD	0	1		0	4		0	2	
Hypertension	0	1		0	10		0	4	
Lung Disease	0	0		0	3		0	24	
Sleep Disorder	0	0		0	0		0	0	
VTE	0	0		0	31		0	8	
Diabetes mellitus	0	1		0	3		0	1	
Renal Insufficiency	0	0		0	0		0	3	
Malignant Tumor	0	0		0	2		0	0	
Any comorbidities	0	8		0	35		0	29	
Mean time from baseline RHC to Sample collection, days		0.0 (0.0,132.0)			109.5 (0.0,283.3)			88.5 (9.8,590.3)	
6MWD, m	–	397.3 ± 94.0			435.9 ± 119.3			421.2 ± 126.9	
WHO functional class									
II	–	8(88.9%)			21(60.0%)			16(53.3%)	
III	–	1(11.1%)			9(25.7%)			8(26.7%)	
IV	–	0(0%)			4(11.4%)			2(6.7%)	
Hemodynamics									
mPAP, mmHg	–	46.8 ± 4.8			34.3 ± 10.9			37.8 ± 10.9	
PVR, dyn·s·cm^{-5}	–	779.6 ± 332.7			625.2 ± 296.1			677.5 ± 388.1	
CO, L/min	–	4.7 ± 1.7			3.4 ± 0.8			2.1 ± 0.5	
SvO_2, %		55.9 ± 11.3			66.8 ± 6.4			67.8 ± 7.0	
Laboratory									
NT-proBNP, ng/mL		507.7 (199.0, 943.6)			540.0 (175.0,1841.5)			496.5 (137.8, 1996.3)	
Echocardiography									
sPAP, mmHg		78.4 ± 15.6			73.7 ± 24.3				
TAPSE, mm		14.7 ± 3.6			16.5 ± 3.8				
TAPSE/sPAP		0.20 ± 0.09			0.19(0.17,0.36)				
S', cm/s		–			10.1 ± 2.2				
LVEF, %		68.4 ± 4.8			67.7 ± 5.6			68.0 ± 5.2	
Targeted therapy									
sGCs		0			25(69.4%)			25(83.3%)	
ERA		0			8(22.2%)			1(3.3%)	
PDE5i		3(33.3%)			0			2(6.7%)	
Monotherapy[a]		3(33.3%)			23(63.9%)			27(9.0%)	

(*Continued on next page*)

Table 1. *Continued*

	iTRAQ			Validation cohort1			Validation cohort2		
	Control	CTEPH	p	Control	CTEPH	p	Control	CTEPH	p
Combination therapy[a]		0			5(13.9%)			1(3.3%)	
Anticoagulation		9(100.0%)			36(100.0%)			30(100.0%)	

Abbreviations: BMI, body mass index; CHD, coronary heart disease; VTE, venous thromboembolism; RHC, right heart catheterization; 6MWD, 6-min walking distance; WHO FC, World Health Organization Functional Class; mPAP, mean pulmonary artery pressure; PVR, pulmonary vascular resistance; CO, cardiac output; SvO_2, mixed venous oxygen saturation; NT-proBNP, N-terminal pro-brain natriuretic peptide; sPAP, pulmonary artery systolic pressure; TAPSE, tricuspid annular plane systolic excursion; S', tricuspid lateral annular longitudinal systolic velocity; LVEF, Left ventricular ejection fraction; sGCs, soluble guanylate cyclase stimulator; ERA, endothelin receptor antagonist; PDE5i, Phosphodiesterase type 5 (PDE5) inhibitor.

[a]Patients were categorized as Monotherapy if they received only one PH-specific medical therapy between diagnosis and sample collection, although they could switch between different monotherapies; whereas they were categorized as Combination therapy if they received two or more PH-specific medical therapies simultaneously.

only plasma markers widely used in clinical practice.[8] However, there still exist many disadvantages including poor sensitivity to early vascular pathology.[9] The ideal CTEPH biomarker should be easy to detect and have high sensitivity and specificity for early identification. The search for a marker that fulfills the aforementioned criteria is still ongoing.

Proteomics has been increasingly applied to the discovery of disease biomarkers. The isobaric tags for relative and absolute quantitation (ITRAQ)-based proteomic analysis are a highly efficient technique developed by AB SCIEX to examine biomarkers for various diseases.[10,11] In the past, few proteomic studies on the biomarker for CTEPH have been performed.[12,13] Thus, we launched a study on the plasma proteome features to identify novel CTEPH-specific biomarkers.

RESULTS

Schematic workflow of screening CTEPH proteins

The clinical characteristics of CTEPH patients and controls are summarized in Table 1. We collected 9 serum samples from each group, and protein digestion was performed on samples depleted of highly abundant proteins. After protein extraction and trypsin digestion, an iTRAQ labeling experiment was performed. For iTRAQ experiments, the labeled peptides were pooled together for high performance liquid chromatography (HPLC) fractionation and subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Database searching and intensive bioinformatics analyses were performed to identify potential CTEPH biomarkers.

This assay identified 362 proteins, and 238 of them contained quantitative information (Figure 1A). 37 proteins were upregulated and 58 proteins were downregulated in the serum from CTEPH patients compared with healthy controls (Figures 1B and 1C).

Functional enrichment analyses of differentially expressed proteins

To exploit the potential functions of differentially expressed proteins (DEPs), we analyzed Gene Ontology (GO) function and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment. The top 6 most enriched KEGG pathways of DEPs were involved in complement and coagulation cascades, coronavirus disease-COVID-19, systemic lupus erythematosus (SLE), prion disease, neutrophil extracellular trap (NET) formation, and platelet activation (Figure 2D).

Moreover, we were able to obtain a global perspective of the changes in protein expression patterns in GO enrichment analysis. In biological process (BP) term, DEPs were centered on "humoral immune response", "complement activation", "coagulation", "hemostasis", and "platelet degranulation" (Figure 2A). As for the cellular component (CC) category, the core DEPs were significantly enriched related to "blood microparticle", "secretory granule lumen", "cytoplasmic vesicle lumen", "vesicle lumen", "collagen-containing extracellular matrix", and "platelet alpha granule" (Figure 2B). In addition, DEPs were enriched in the molecular function (MF) category focused on "serine-type endopeptidase activity", "serine-type peptidase activity", "serine hydrolase activity", "glycosaminoglycan binding", "complement binding", and "heparin binding" (Figure 2C).

Protein-protein interaction network analysis of differentially expressed proteins

To search hub proteins that might be useful as biomarkers and therapeutic targets for CTEPH, a protein-protein co-expression network was constructed for the DEPs using the STRING database and Cytoscape software. The protein-protein interaction (PPI) network contained a total of 94 nodes and 412 edges (Figure 3A). Subsequently, the co-expression network was further analyzed to detect potential critical modules. We have defined the critical module as one target protein that interacts with more than five proteins and is potentially associated with the pathogenesis of CTEPH based on literature searches, and three significant modules heparanase (HPSE), secreted protein acidic and rich in cysteine (SPARC), and gelsolin (GSN) were determined.

HPSE interacted with ACTB, CLU, GAPDH, PF4, SERPINF4, and VIM (Figure 3B). SPARC interacted with ACTB, CLU, GAPDH, COL18A1, NID1, and VIM (Figure 3C). GSN interacted with ACTB, ACTN1, APOA4, APCCS, CLU, CP, CFL1, GC, TLN1, TPM3, TPM4, FGA, LTF, and LYZ (Figure 3D).

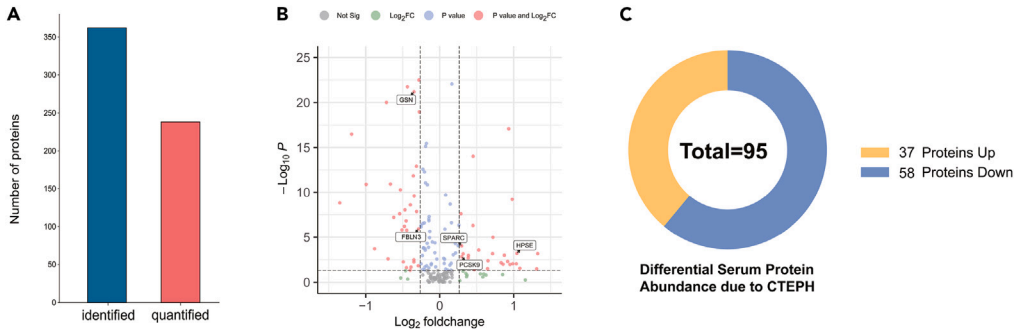

Figure 1. Proteomics analysis reveals differential protein expression in the serum of CTEPH patients

(A) Total number of identified (blue bar) and quantified (red bar) proteins in the iTRAQ experiment.

(B) Volcano plots for expression of differentially expressed proteins, red represent differentially expressed proteins.

(C) A total of 37 upregulated proteins and 58 downregulated proteins were identified.

Verification of significantly dysregulated proteins in the CTEPH and reference groups

According to the results of PPI analysis and the clinical relevance, we selected some candidate proteins for in-depth research, including HPSE, GSN, SPARC, Fibulin-3, and proprotein convertase subtilisin/kexin-9 (PCSK9). To validate the expression level of these candidate proteins, serum derived from another 36 CTEPH patients and 36 healthy individuals were determined by ELISA. The levels of HPSE (CTEPH: 63.4 [59.8, 66.8] vs. control: 7.0 [4.7, 12.0] ng/mL, $p < 0.0001$) and SPARC (CTEPH: 125.3 [77.9,179.3] vs. control: 70.9 [43.4,117.3] ng/mL, $p < 0.01$) were higher in CTEPH patients compared to normal controls according to ELISA, whereas GSN was reduced (CTEPH: 7535.8 [6459.0, 10191.6] vs. control: 10763.0 [8829.0, 13206.7] ng/mL, $p < 0.001$) (Figures 4A–4C). No differences were observed for Fibulin-3 (CTEPH: 1535.3 [1175.4, 1645.9] vs. control: 1512.4 [1367.5, 1575.0] ng/mL; $p = 0.719$) and PCSK9 (CTEPH: 215.8 [170.7, 293.7] vs. control: 187.2 [153.5, 250.4] ng/mL; $p = 0.203$) (Figures 4D and 4E). Further analysis based on the independent cohort found that the levels of HPSE (CTEPH: 33.1 [22.9, 55.9] vs. control: 7.5 [4.2, 9.4] ng/mL, $p < 0.0001$) were also higher in CTEPH patients compared to normal controls (Figure S1). The results were consistent with iTRAQ results.

According to the heat maps for five groups (idiopathic pulmonary arterial hypertension (IPAH), connective tissue disease-associated pulmonary hypertension (CTD-PH), PTE, CTEPH, and healthy control), HPSE was higher expressed in CTEPH patients than any other groups, SPARC was higher expressed in CTEPH patients than IPAH, PTE, and control groups. GSN was lower expressed in CTEPH than in healthy controls (Figure 5).

Diagnosis performance of candidate plasma biomarkers

Since we discovered the difference of plasma HPSE, GSN, and SPARC concentrations between CTEPH and healthy controls, we intended to find the discriminate point to help diagnose CTEPH. In Figure 4F, the area under the receiver operating characteristic (ROC) curve (AUC) of HPSE was 0.988 and those of other proteins were in the range of 0.6–0.8 in the CTEPH diagnosis. The sensitivity and the specificity of HPSE were 94.4% and 97.2%, respectively, at the cutoff value of 29.8 ng/mL; the sensitivity and the specificity of GSN were 86.1% and 66.7%, respectively, at the cutoff value of 8337.1 ng/mL; the sensitivity and the specificity of SPARC were 88.9% and 51.6%, respectively, at the cutoff value of 71.8 ng/mL. In Figure S1, the area under the ROC curve (AUC) of HPSE was 0.948 in the CTEPH diagnosis of validation cohort2, while the sensitivity and specificity were 96.7% and 90.0%, respectively.

Correlation analysis between clinical data and biomarkers

We then investigated the correlation of serum HPSE, GSN, and SPARC levels with a cluster of clinical parameters including hemodynamic parameters, 6-min walking distance (6MWD), echocardiographic parameters, WHO functional class, and laboratory test. In Figure 6, serum HPSE concentrations inversely correlated with tricuspid lateral annular longitudinal systolic velocity. Furthermore, serum HPSE concentrations positively correlated with right atrial transverse diameter (RA-t, $r = 0.371$, $p = 0.034$), right ventricular basal transverse diameter (RV-b, $r = 0.489$, $p = 0.004$), right ventricular transverse diameter/left ventricular transverse diameter (RV/LV, $r = 0.467$, $p = 0.012$), glutamyl transpeptidase (GGT, $r = 0.489$, $p = 0.008$), D-dimer ($r = 0.399$, $p = 0.016$), uric acid (UA, $r = 0.344$, $p = 0.04$), NT-proBNP ($r = 0.378$, $p = 0.023$). Serum GSN concentrations positively correlated with UA ($r = 0.384$, $p = 0.021$)[14] and negatively correlated with direct bilirubin (DBIL, $r = -0.378$, $p = 0.047$), NT-proBNP ($r = -0.379$, $p = 0.022$), erythrocyte sedimentation rate (ESR, $r = -0.651$, $p = 0.003$). SPARC inversely correlated with urea nitrogen (BUN, $r = -0.367$, $p = 0.028$). While all three biomarkers are not associated with 6MWD and hemodynamics parameters (Table S1).

We further verified HPSE expression levels in different groups based on echocardiography indicators due to the correlation between HPSE levels and multiple echocardiography indicators. Higher expression levels of HPSE were observed in the group with higher RV-b levels ($p = 0.007$) as well as lower S′ ($p = 0.02$) (Table 2).

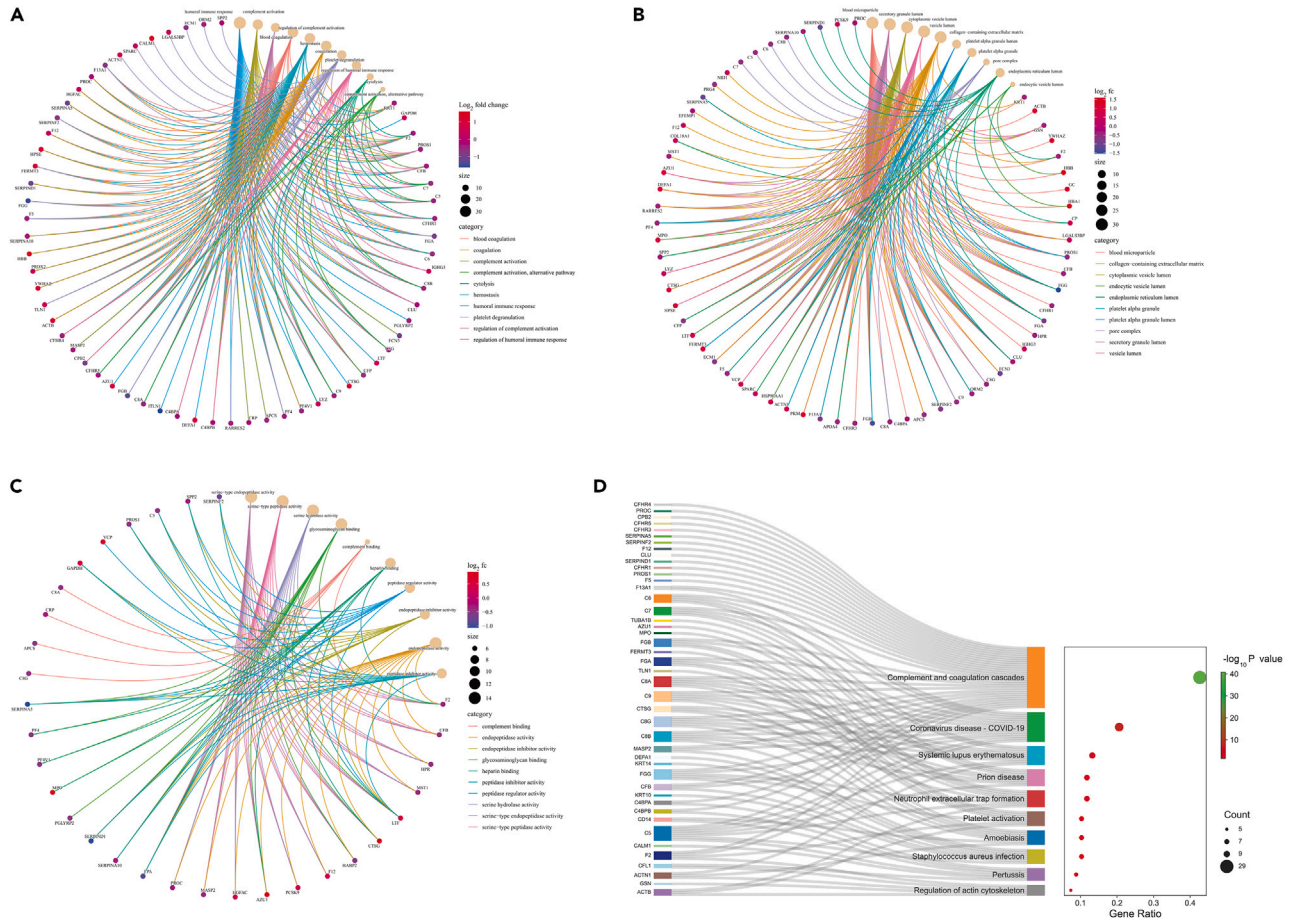

Figure 2. Functional enrichment analyses of differentially expressed proteins

(A) The top 10 enrichment GO Biological Process (BP) pathways ranked by enrichment score.

(B) The top 10 enrichment GO Cellular Component (CC) pathways ranked by enrichment score.

(C) The top 10 enrichment GO Molecular Function (MF) pathways ranked by enrichment score.

(D) The top 10 enrichment KEGG pathways ranked by enrichment score.

HPSE levels and right ventricular-pulmonary artery coupling

The tricuspid annular plane systolic excursion (TAPSE)/sPAP ratio is a non-invasive echocardiographic measure of the right ventricular-pulmonary artery (RV-PA) coupling, with a validated prognostic role in different types of PH including CTEPH.[15] A recent study revealed that patients with PH and TAPSE/PASP<0.31 mm/mmHg had a significantly worse prognosis. TAPSE/sPASP also could predict the overall mortality of PH.[16] Patients with CTEPH were dichotomized into high or low TAPSE/sPAP ratio assuming the value of 0.31 mm/mmHg as the threshold. As shown in Table 3, serum HPSE concentrations (65.4 [60.5, 68.0] vs. 59.9 [35.9, 63.2] ng/mL, p = 0.036) and Fibulin-3 concentrations ([1499.0 ± 239.9] vs. [1080.5 ± 575.5] ng/mL, p = 0.026) were significantly higher in patients with a low TAPSE/sPAP ratio (≤0.31 mm/mmHg) compared to patients with a high TASPE/sPAP ratio (>0.31 mm/mmHg).

Changes in HPSE levels after treatment

Changes to circulating serum proteins following BPA and PEA were assessed. Serum protein levels were measured in 13 CTEPH patients pre- and post-BPA. Significant reductions in HPSE were observed ($p < 0.05$; Figure 7). Serum protein levels were measured in 3 CTEPH patients pre- and post-PEA. Due to the small sample size, there was no significant change in HPSE ($p > 0.05$; Figure 7), but it shows a downward trend. When combined BPA and PEA, the treated CTEPH patient group had significantly lower HPSE levels ($p < 0.05$; Figure 7).

DISCUSSION

The present study profiled the serum proteome of CTEPH patients and detected 37 upregulated proteins and 58 downregulated proteins, which were mainly associated with the top 6 most enriched KEGG pathways. Subsequently, we identified three candidate biomarkers

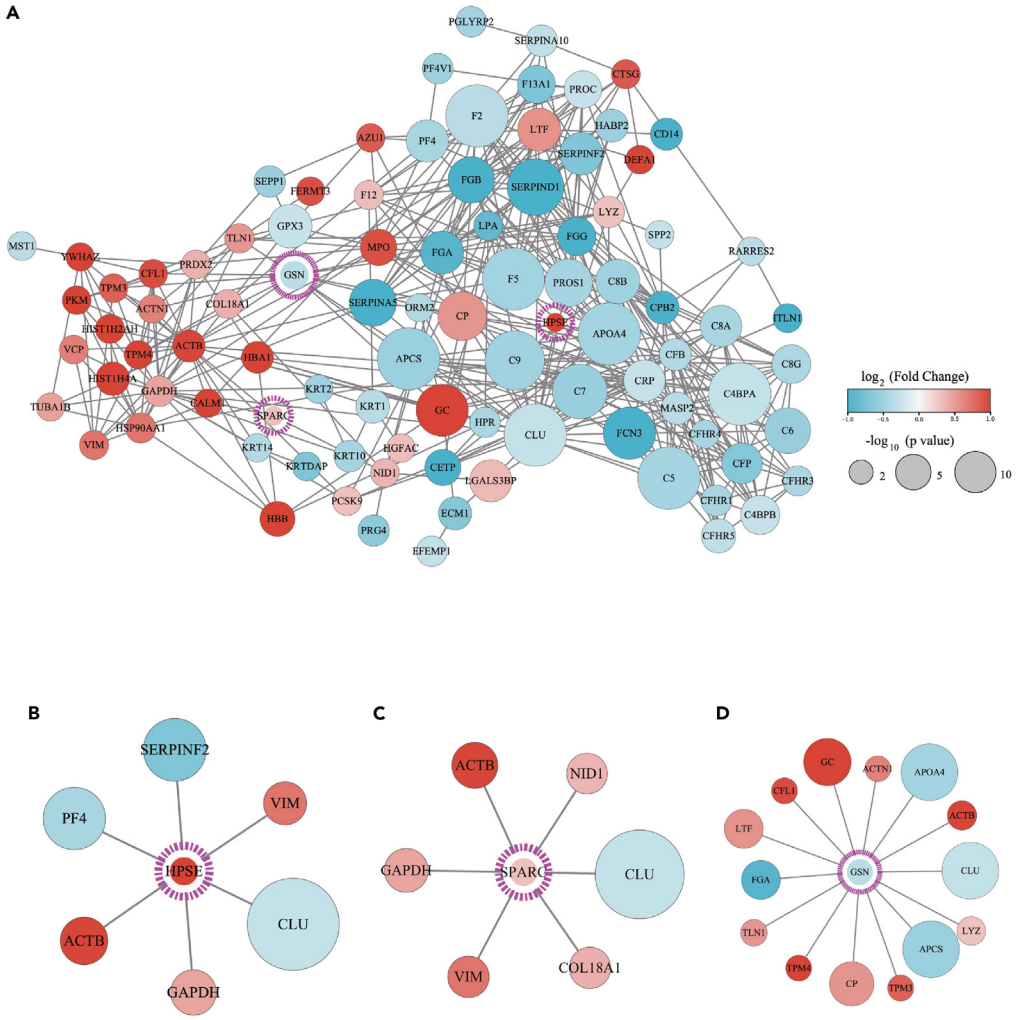

Figure 3. PPI network and module analysis

(A) PPI network for DEPs.

(B) The key module containing HPSE in the PPI network.

(C) The key module containing SPARC in the PPI network.

(D) The key module containing GSN in the PPI network. Different colored nodes represent differentially expressed proteins.

according to the results of PPI analysis and the clinical relevance, including 2 upregulated proteins (HPSE and SPARC) and 1 downregulated protein (GSN), among which HSPE performed best in CTEPH early identification from healthy control. What's more, serum HPSE concentration was correlated with parameters of RV function and RV-PA coupling. The serum levels of HPSE showed an obvious decrease after BPA treatment of CTEPH group.

The underlying pathophysiological mechanisms of CTEPH have not been fully elucidated. Here, we report that the top 6 most enriched KEGG pathways of CTEPH DEPs include complement and coagulation cascades, coronavirus disease-COVID-19, SLE, prion disease, NET formation, and platelet activation. The coagulation system is a physiological process involving platelets and multiple coagulation factors, which are involved in hemostasis and vascular repair processes. Coagulation abnormalities play an important role in the formation of CTEPH. Elevated levels of coagulation factor VIII may be a possible cause of disease progression in CTEPH patients.[17] The complement system has also been studied in the pathogenesis of CTEPH, complement c5a receptor 1 may be an important gene for the pathogenesis of CTEPH,[18] which is consistent with our findings. The previous study uncovered that NET formation increased in CTEPH patients, which mediated fibrotic remodeling of thrombi.[19] However, the relationship between NET and CTEPH needs further studies. Platelets play a key role in the coagulation and hemostasis process and are also closely associated with inflammation in the pathophysiological mechanisms of CTEPH. Histopathological specimens and blood samples from CTEPH patients often presented with highly activated platelets signs, contributing to the pro-thrombotic state of CTEPH.[20] To summarize, our functional enrichment analysis of DEPs could provide deep insights into the pathophysiology of CTEPH, which provide potential targets for intervention.

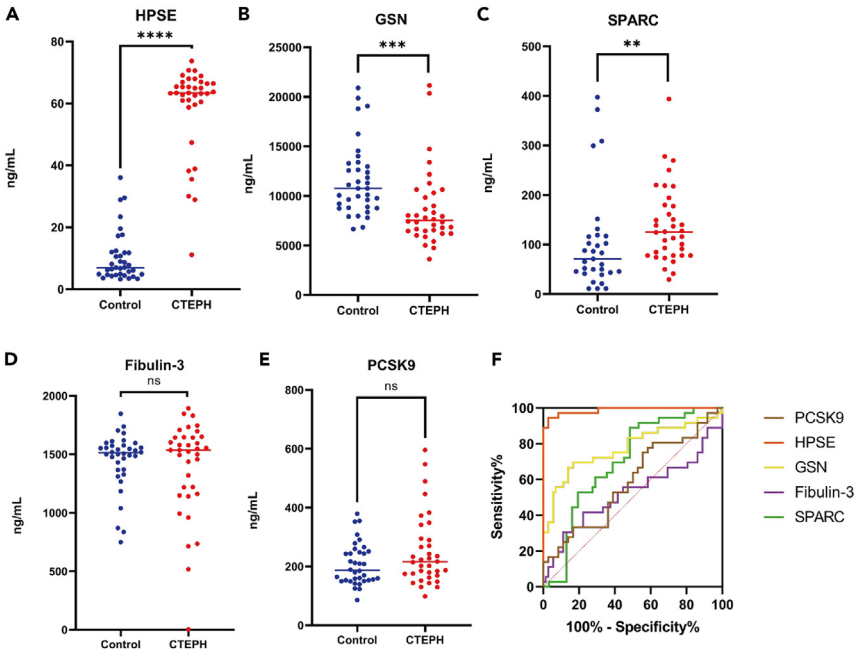

Figure 4. Verification of differentially expressed proteins by ELISA in validation cohort1

(A) HPSE.

(B) GSN.

(C) SPARC.

(D) Fibulin-3.

(E) PCSK9 in CTEPH patients and healthy controls.

(F) Receiver operating characteristic (ROC) results of different proteins between the CTEPHs and healthy controls. As the result of significance test, * means p value <0.05; ** means p value <0.01; *** means p value <0.001; **** means p value <0.0001; ns means p value >0.05.

The concentration difference of 5 serum proteins was verified between CTEPH patients and healthy controls. HPSE and SPARC elevated in CTEPH patients, whereas GSN was reduced. We focused on HPSE due to its significantly higher expression levels in CTEPH than any other groups (IPAH, CTD-PH, PTE patients, and healthy control). HPSE is an endoglycosidase, which is the only mammalian enzyme capable of degrading the carbohydrate moiety of heparan sulphate (HS) proteoglycans, a key component of vascular extracellular matrix (ECM) and basement membrane. Recent evidence suggests a multifactorial role for HPSE in atherosclerosis by promoting underlying inflammatory processes giving rise to plaque formation, as well as regulating lesion stability.[21] HPSE also plays a procoagulant role in several arterial and venous thrombotic diseases.[22] The subsequent study demonstrated that elevated HPSE levels were associated with higher thrombus load and rates

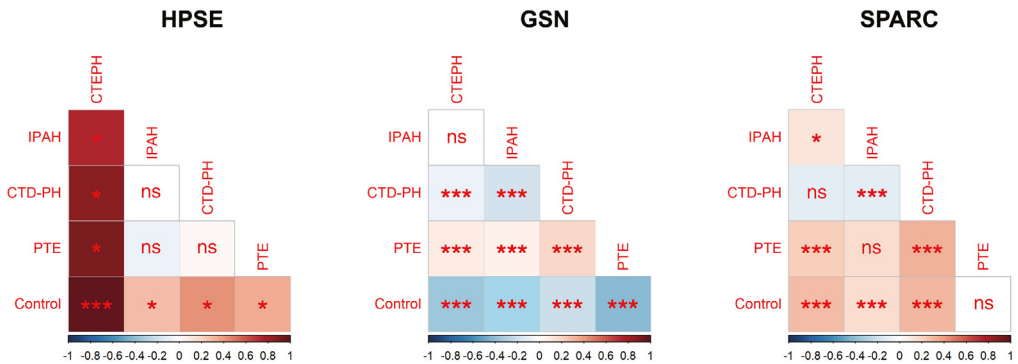

Figure 5. Comparison of protein expression in multiple groups

Depth of color represents log (FC) of DEPs, the FC is calculated by dividing the vertical corresponding group expression level by the horizontal corresponding group expression level in a square matrix. As the result of significance test, * means p value <0.05; ** means p value <0.01; *** means p value <0.001; ns means p value >0.05.

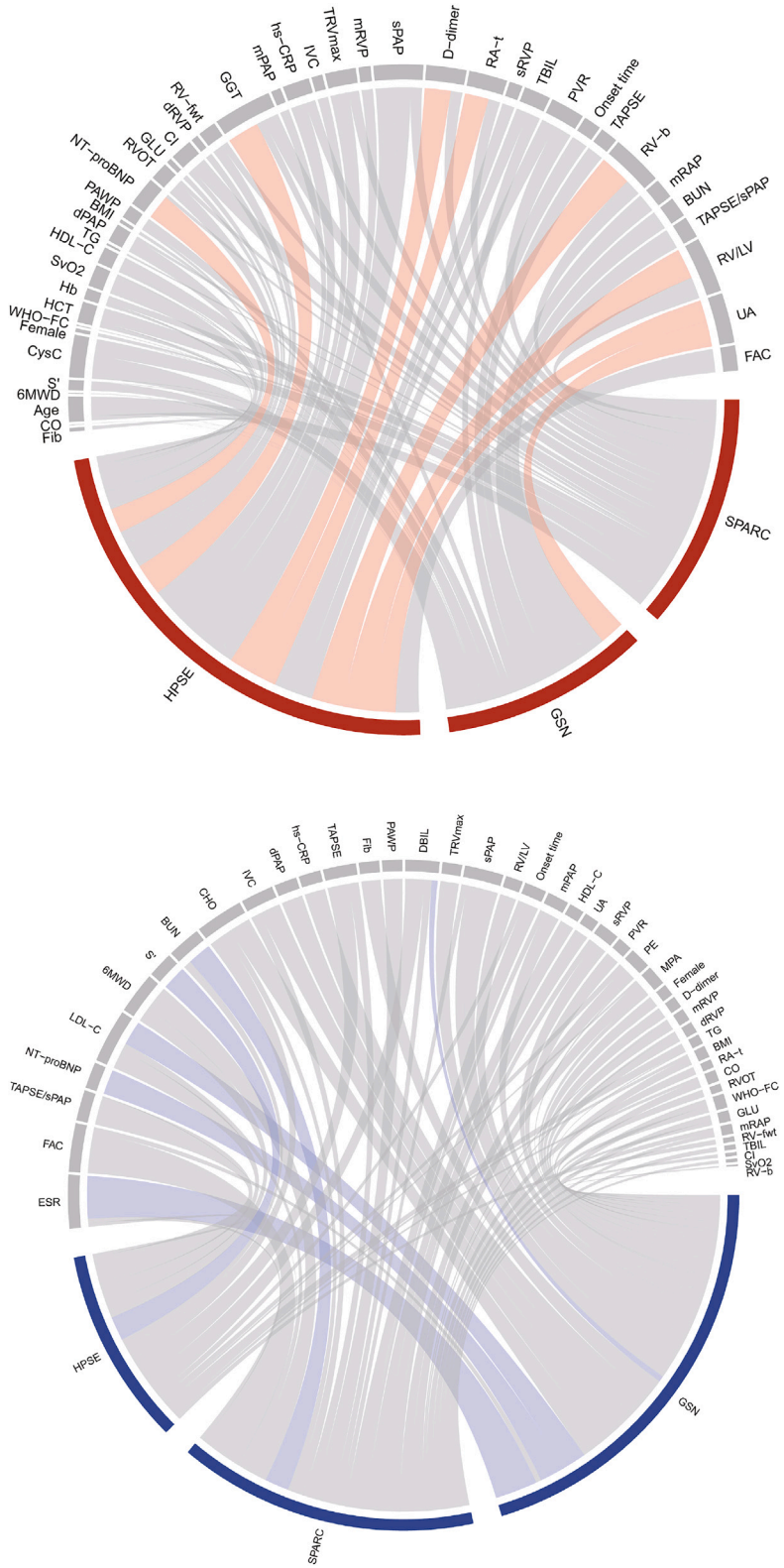

Figure 6. Correlation network of three biomarkers and clinical indicators in CTEPH patients

Correlations with statistical significance (p value <0.05) are indicated in red for positive correlations and in blue for negative correlations. Non-significant correlations (p value ≥0.05) are indicated in gray.

Table 2. Relationship between HPSE and echocardiographic indices of right heart

	HPSE (ng/mL)	p value
RV/LV		0.055
>1.0	64.5(61.0, 66.8)	
≤1.0	61.0(35.6, 65.0)	
RV-b		0.007**
>42 mm	66.0 ± 4.0	
≤42 mm	62.2(36.2, 65.0)	
TAPSE		0.113
<16 mm	65.4(61.0, 68.0)	
≥16 mm	63.2(59.0, 65.3)	
IVC-CI		0.141
<50%	66.0 ± 5.3	
≥50%	63.3(60.3, 65.1)	
S′		0.020*
<9.5 cm/s	65.4(65.0, 70.6)	
≥9.5 cm/s	62.9(43.4, 65.0)	
Pericardial effusion		0.633
Yes	55.9 ± 14.8	
No	65.0 (60.5, 66.9)	

Abbreviations: HPSE, heparanase; RV/LV, right ventricular transverse diameter/left ventricular transverse diameter; RV-b, right ventricular basal transverse diameter; TAPSE, tricuspid annular plane systolic excursion; IVC-CI, inferior vena cava collapsibility index; S′, tricuspid lateral annular longitudinal systolic velocity; **:p < 0.01; *:p < 0.05.

of thromboembolic complications.[23] HPSE neutralizes the anticoagulation properties of heparin and low-molecular-weight heparin (LMWH).[24] Most patients with acute pulmonary emboli are treated with either unfractionated heparin or LMWH before longer-term oral anticoagulants. Some remain on LMWH throughout their treatment period. It is known that heparin can contribute to the fibrinolytic process.[25] It is conceivable that patients with acute pulmonary emboli and high endogenous HPSE levels might clear their clots less effectively and end up with CTEPH. The expression and the role of HPSE in PH, particularly CTEPH, remained unclear. Our study is the first to experimentally demonstrate an increase of HPSE in the CTEPH patients' serum.

To further investigate the role of HPSE in CTEPH, we evaluated its diagnostic performance. HPSE showed good performance for the CTEPH diagnosis with 97.3% specificity and 94.6% sensitivity. To further demonstrate the role of HPSE in clinical practice, we analyzed the relationship between HPSE and a cluster of clinical parameters. HPSE was found to be associated with several indicators of right heart function such as RA-t, RV-b, RV/LV, S′, and NT-proBNP. The diagnostic accuracy of HSPE and its correlation with right heart function parameters confirmed that HPSE has the potential to be a stable and independent serum biomarker for CTEPH diagnosis, and may be a crucial target for mediating the disease progression of CTEPH.

As we know, CTEPH leads to right heart failure, which is strongly associated with adverse outcomes, whereas PEA or BPA restores pulmonary hemodynamics and allows cardiac recovery. The severity of CTEPH is correlated inversely with RV-PA coupling,[26] which could

Table 3. Differences in proteomic indicators between groups based on TAPSE/sPAP

	TAPSE/sPAP		
	>0.31	≤0.31	p value
N	6	15	
Fibulin-3 (ng/mL)	1080.5 ± 575.5	1499.0 ± 239.9	0.026*
GSN (ng/mL)	8852.3 ± 3212.7	6857.7 (6024.4, 8021.1)	0.267
HPSE (ng/mL)	59.9(35.9, 63.2)	65.4 (60.5, 68.0)	0.036*
PCSK9 (ng/mL)	252.1 ± 103.6	265.8 ± 130.3	0.822
SPARC (ng/mL)	115.0(66.1,203.3)	151.8 ± 64.6	0.340

TAPSE, tricuspid annular plane systolic excursion; sPAP, pulmonary artery systolic pressure; GSN, gelsolin; HPSE, heparanase; SPARC, secreted protein acidic and rich in cysteine; PCSK9, proprotein convertase subtilisin/kexin-9.
*p < 0.05.

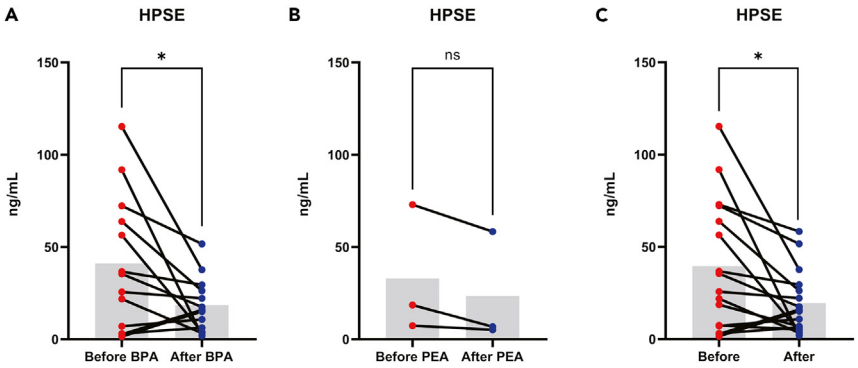

Figure 7. Changes in HPSE levels after treatment

(A) BPA.

(B) PEA.

(C) Combine BPA and PEA. As the result of significance test, * means p value <0.05; ns means p value >0.05.

be assessed by TAPSE/sPAP.[27] Furthermore, recent studies showed that RV-PA coupling is a strong prognostic indicator.[28] In our study, serum HPSE concentrations were associated with several indicators of right ventricular enlargement such as RA-t, RV-b, RV/LV, and TASPE/sPAP. Serum HPSE concentrations were significantly higher in patients with a low TAPSE/sPAP ratio ($\leq$0.31 mm/mmHg) compared to patients with a high TASPE/sPAP ratio (>0.31 mm/mmHg). The results suggest that HPSE may be useful in assessing PV-PA coupling and predicting progression in CTEPH patients. Our finding further confirms the validity and importance of BPA, and also shows the potential value of HPSE in the individual assessment of disease severity, risk stratification, and therapeutic monitoring of CTEPH patients. To fully understand the significance of HPSE in CTEPH, prognosis such as mortality and disease recurrence needs to be investigated in future studies.

Limitations of the study

Our study has certain limitations. Firstly, the sample size of plasma specimens was limited, and further validation of the results is necessary in a larger population. Secondly, our study chose a control group to match the CTEPH group, it will be better to compare patients with chronic thromboembolic pulmonary disease (CTEPD) without PH and patients with CTEPH and other types of PH. Thirdly, it is necessary to elaborate on potential confounders and biases in the study. Finally, the results have not been validated at the cellular level, which is an ongoing study currently.

METHOD DETAILS

Blood sample processing and proteomics profiling

Blood samples were collected at baseline and centrifuged shortly. All samples were stored at −80°C in aliquots and thawed only before the test. To identify specific proteins of CTEPH, serum samples from CTEPHs and control subjects were pooled for iTRAQ analysis. The ProteoMiner was used to remove the high abundance proteins from the samples. Peptide mixtures from each group were labeled using the iTRAQ Reagent-8 Plex Multiplex Kit (Applied Biosystem). The digested peptides were labeled with iTRAQ tags (reagent 116: CTEPH; reagent 118: Control). The labeled peptides of each group were separated by HPLC system. Then liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analysis was performed on Q–Exactive MS (Thermo Fisher Scientific, Waltham, MA, USA).

Database searching and bioinformatics analysis

The original data of iTRAQ assay were collected by mass spectrometry, and the search of the database was conducted by the Proteome Discoverer software (version 1.3). The primary quality deviation of 15 ppm, and the secondary quality deviation of 20mmu. The database is UniProt-Swiss human database. Log_2 transformation of the original quantitative value is carried out make it to meet the normal distribution.

To identify proteins significantly differentially expressed in serum between the CTEPH and control groups, proteins with fold change $\geq$1.20 and p-value <0.05 by t test were defined as upregulated; proteins with fold change $\leq$0.83 and p-value <0.05 by t test were defined as downregulated. Further bioinformatics analysis was performed using R Studio and other necessary websites. Volcano plots were performed by R studio with the R package "ggplot2". GO and KEGG pathway annotation analyses were generated by R studio with the R packages "clusterProfiler".[29] The proteins significantly differentially expressed were submitted to the STRING 11.5 database for PPI network.

Verification of differentially expressed proteins by ELISA

The identification of candidate proteins for further validation was based on (1) differential expression in CTEPH patients and controls; (2) interacted with more than 5 proteins in PPI network; (3) potential functional or pathological significance in CTEPH through literature search.

The candidate biomarkers HPSE, GSN, SPARC, Fibulin-3, and PCSK9 were further validated by using commercially available sandwich ELISA kits (abcam, Waltham, MA, USA or R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions.

Nine IPAH patients, 9 CTD-PH patients, and 9 acute pulmonary thromboembolism (PTE) patients were recruited in another validation stage. Heat maps were generated using log (FC) of DEPs and p value calculated for average expression per group, as indicated in the figure legends, via the ggplot2 package. The FC is calculated by dividing the vertical corresponding group expression level by the horizontal corresponding group expression level in a square matrix. We compared proteins expression with significant differences in ELISA in multiple groups.

Statistical analysis

Sample Size of validation was calculated using a two-sided test with α = 0.05. Based on the results of the discovery stage, the mean difference δ in HPSE expression between the experimental and control groups is 12,500, and the standard deviation σ is 14,000. By using the following formula to calculate the sample size, with a Type I error α of 0.05 and a power (1-β) of 0.95, we obtain a sample size of n = 33.

$$n = 2\left(z_{\alpha}+z_{\beta}\right)^2 \times \sigma^2 \Big/ \delta^2$$

To account for a 10% increase in the sample size, both the experimental and control groups in this study will include 36 participants each.

The Kolmogorov-Smirnov test was used to test the normal distribution of the variables. In case of normal distribution, continuous data were expressed as mean ± standard deviation, while skewed data were expressed as median and corresponding inter-quartile range. We compared continuous variables based on the results of the normality distribution using the Student's *t* test for two independent samples or the nonparametric test. Categorical data were expressed as numbers and percentages and compared using the Chi-Square test. Since the three proteomic indicators for which we performed correlation analysis were skewed, we evaluated the correlation between continuous variables using Spearman's correlation coefficient. The ROC curve and the area under the curve were utilized to assess the diagnostic value of the identified protein for CTEPH. All analyses were performed with the Statistical Package for Social Sciences for Windows (SPSS, Chicago, IL version 22.0).

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.108930.

ACKNOWLEDGMENTS

This study was supported by the Chinese Academy of Medical Sciences Innovation Fund for Medical Sciences (2021-I2M-1-061, 2021-I2M-1-049), National High Level Hospital Clinical Research Funding, Elite Medical Professionals Project of China-Japan Friendship Hospital (ZRJY2023-QM31), Institute of Respiratory Medicine, Chinese Academy of Medical Sciences Foundation for Young Scholars (2023-ZF-22), and National High Level Hospital Clinical Research Funding (2022-NHLHCRF-LX-01-0203).

AUTHOR CONTRIBUTIONS

Study concept and design: Y.Z. and Z. Z.; data acquisition: Y. Z., H.Y., S.M., J.L., Z.F., and Y. L.; drafting of the manuscript: Y.Z., M.Z., H.Y., L.X., and H. L.; statistical analysis: Y.Z., H.Y., H.L., and X.L.; technical support and contributed to the discussion: Z.Z., S.Z., Q.G., Q.H., J.W., W.X., and P.Y. All authors provided final approval of the version to be published.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: August 15, 2023
Revised: November 30, 2023
Accepted: January 12, 2024
Published: January 17, 2024

REFERENCES

1. Papamatheakis, D.G., Poch, D.S., Fernandes, T.M., Kerr, K.M., Kim, N.H., and Fedullo, P.F. (2020). Chronic Thromboembolic Pulmonary Hypertension: JACC Focus Seminar. J. Am. Coll. Cardiol. *76*, 2155–2169.
2. Gall, H., Hoeper, M.M., Richter, M.J., Cacheris, W., Hinzmann, B., and Mayer, E. (2017). An epidemiological analysis of the burden of chronic thromboembolic pulmonary hypertension in the USA, Europe and Japan. Eur. Respir. Rev. *26*, 160121.
3. Leber, L., Beaudet, A., and Muller, A. (2021). Epidemiology of pulmonary arterial hypertension and chronic thromboembolic pulmonary hypertension: identification of the most accurate estimates from a systematic literature review. Pulm. Circ. *11*, 2045894020977300.
4. Delcroix, M., Torbicki, A., Gopalan, D., Sitbon, O., Klok, F.A., Lang, I., Jenkins, D., Kim, N.H., Humbert, M., Jais, X., et al. (2021). ERS statement on chronic thromboembolic pulmonary hypertension. Eur. Respir. J. *57*, 2002828.
5. Zhang, L., Bai, Y., Yan, P., He, T., Liu, B., Wu, S., Qian, Z., Li, C., Cao, Y., and Zhang, M. (2021). Balloon pulmonary angioplasty vs. pulmonary endarterectomy in patients with chronic thromboembolic pulmonary hypertension: a systematic review and meta-analysis. Heart Fail. Rev. *26*, 897–917.
6. Delcroix, M., Lang, I., Pepke-Zaba, J., Jansa, P., D'Armini, A.M., Snijder, R., Bresser, P., Torbicki, A., Mellemkjaer, S., Lewczuk, J., et al. (2016). Long-Term Outcome of Patients With Chronic Thromboembolic Pulmonary Hypertension: Results From an International Prospective Registry. Circulation *133*, 859–871.
7. Minkin, R., Sandhu, G., Grosu, H., Tartell, L., Ma, S., Lin, Y.Y., Eden, E., and Turino, G.M. (2017). Desmosine and Isodesmosine as a Novel Biomarker for Pulmonary Arterial Hypertension: A Pilot Study. Am. J. Therapeut. *24*, e399–e404.
8. Zhang, M., Zhang, Y., Pang, W., Zhai, Z., and Wang, C. (2019). Circulating biomarkers in chronic thromboembolic pulmonary hypertension. Pulm. Circ. *9*, 2045894019844480.
9. Rhodes, C.J., Wharton, J., Swietlik, E.M., Harbaum, L., Girerd, B., Coghlan, J.G., Lordan, J., Church, C., Pepke-Zaba, J., Toshner, M., et al. (2022). Using the Plasma Proteome for Risk Stratifying Patients with Pulmonary Arterial Hypertension. Am. J. Respir. Crit. Care Med. *205*, 1102–1111.
10. Latosinska, A., Vougas, K., Makridakis, M., Klein, J., Mullen, W., Abbas, M., Stravodimos, K., Katafigiotis, I., Merseburger, A.S., Zoidakis, J., et al. (2015). Comparative Analysis of Label-Free and 8-Plex iTRAQ Approach for Quantitative Tissue Proteomic Analysis. PLoS One *10*, e0137048.
11. Li, H., Zhang, Z., Qiu, Y., Weng, H., Yuan, S., Zhang, Y., Zhang, Y., Xi, L., Xu, F., Ji, X., et al. (2023). Proteome-wide mendelian randomization identifies causal plasma proteins in venous thromboembolism development. J. Hum. Genet. *10*.
12. Hadinnapola, C.M., Southwood, M., Hernández-Sánchez, J., Bunclark, K., Newnham, M., Swietlik, E.M., Cannon, J., Preston, S.D., Sheares, K., Taboada, D., et al. (2023). Angiopoietin 2 and hsCRP are associated with pulmonary hemodynamics and long-term mortality respectively in CTEPH-Results from a prospective discovery and validation biomarker study. J. Heart Lung Transplant. *42*, 398–405.
13. Xi, Q., Liu, Z., Song, Y., Gan, H., Huang, Z., Luo, Q., and Zhao, Z. (2020). Proteomic Analyses of Endarterectomized Tissues from Patients with Chronic Thromboembolic Pulmonary Hypertension. Cardiology *145*, 48–52.
14. Weng, H., Li, H., Zhang, Z., Zhang, Y., Xi, L., Zhang, D., Deng, C., Wang, D., Chen, R., Chen, G., et al. (2023). Association between uric acid and risk of venous thromboembolism in East Asian populations: a cohort and Mendelian randomization study. Lancet Reg. Health. West. Pac. *39*, 100848.
15. Humbert, M., Kovacs, G., Hoeper, M.M., Badagliacca, R., Berger, R.M.F., Brida, M., Carlsen, J., Coats, A.J.S., Escribano-Subias, P., Ferrari, P., et al. (2023). 2022 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension. Eur. Respir. J. *61*, 2200879.
16. Tello, K., Wan, J., Dalmer, A., Vanderpool, R., Ghofrani, H.A., Naeije, R., Roller, F., Mohajerani, E., Seeger, W., Herberg, U., et al. (2019). Validation of the Tricuspid Annular Plane Systolic Excursion/Systolic Pulmonary Artery Pressure Ratio for the Assessment of Right Ventricular-Arterial Coupling in Severe Pulmonary Hypertension. Circ. Cardiovasc. Imaging *12*, e009047.
17. Bonderman, D., Turecek, P.L., Jakowitsch, J., Weltermann, A., Adlbrecht, C., Schneider, B., Kneussl, M., Rubin, L.J., Kyrle, P.A., Klepetko, W., et al. (2003). High prevalence of elevated clotting factor VIII in chronic thromboembolic pulmonary hypertension. Thromb. Haemostasis *90*, 372–376.
18. Miao, R., Dong, X., Gong, J., Wang, Y., Guo, X., Li, Y., Li, J., Yang, S., Kuang, T., Wan, J., et al. (2021). Possible immune regulation mechanisms for the progression of chronic thromboembolic pulmonary hypertension. Thromb. Res. *198*, 122–131.
19. Sharma, S., Hofbauer, T.M., Ondracek, A.S., Chausheva, S., Alimohammadi, A., Artner, T., Panzenboeck, A., Rinderer, J., Shafran, I., Mangold, A., et al. (2021). Neutrophil extracellular traps promote fibrous vascular occlusions in chronic thrombosis. Blood *137*, 1104–1116.
20. Åberg, M., Björklund, E., Wikström, G., and Christersson, C. (2022). Platelet-leukocyte aggregate formation and inflammation in patients with pulmonary arterial hypertension and CTEPH. Platelets *33*, 1199–1207.
21. Nguyen, T.K., Paone, S., Chan, E., Poon, I.K.H., Baxter, A.A., Thomas, S.R., and Hulett, M.D. (2022). Heparanase: A Novel Therapeutic Target for the Treatment of Atherosclerosis. Cells *11*, 3198.
22. Hu, Y., Yu, Y., Bu, Z., Cun, B., Gong, Y., Li, D., Li, J., Lu, L., Li, G., and Yuan, L. (2020). INCREASED SYSTEMIC HEPARANASE IN RETINAL VEIN OCCLUSION IS ASSOCIATED WITH ACTIVATION OF INFLAMMATION AND THROMBOPHILIA. Retina *40*, 345–349.
23. Bayam, E., Kalçık, M., Gürbüz, A.S., Yesin, M., Güner, A., Gündüz, S., Gürsoy, M.O., Karakoyun, S., Cerşit, S., Kılıçgedik, A., et al. (2018). The relationship between heparanase levels, thrombus burden and thromboembolism in patients receiving unfractionated heparin treatment for prosthetic valve thrombosis. Thromb. Res. *171*, 103–110.
24. Nasser, N.J., Sarig, G., Brenner, B., Nevo, E., Goldshmidt, O., Zcharia, E., Li, J.P., and Vlodavsky, I. (2006). Heparanase neutralizes the anticoagulation properties of heparin and low-molecular-weight heparin. J. Thromb. Haemostasis *4*, 560–565.
25. Upchurch, G.R., Valeri, C.R., Khuri, S.F., Rohrer, M.J., Welch, G.N., MacGregor, H., Ragno, G., Francis, S., Rodino, L.J., Michelson, A.D., and Loscalzo, J. (1996). Effect of heparin on fibrinolytic activity and platelet function in vivo. Am. J. Physiol. *271*, H528–H534.
26. Stam, K., Cai, Z., van der Velde, N., van Duin, R., Lam, E., van der Velden, J., Hirsch, A., Duncker, D.J., and Merkus, D. (2019). Cardiac remodelling in a swine model of chronic thromboembolic pulmonary hypertension: comparison of right vs. left ventricle. J. Physiol. *597*, 4465–4480.
27. Zhou, N., Forton, K., Motoji, Y., Scoubeau, C., Klass, M., Naeije, R., and Faoro, V. (2022). Right ventricular-pulmonary arterial coupling impairment and exercise capacity in obese adults. Front. Cardiovasc. Med. *9*, 946155.
28. Claeys, M., Claessen, G., La Gerche, A., Petit, T., Belge, C., Meyns, B., Bogaert, J., Willems, R., Claus, P., and Delcroix, M. (2019). Impaired Cardiac Reserve and Abnormal Vascular Load Limit Exercise Capacity in Chronic Thromboembolic Disease. JACC. Cardiovasc. Imaging *12*, 1444–1456.
29. Wu, T., Hu, E., Xu, S., Chen, M., Guo, P., Dai, Z., Feng, T., Zhou, L., Tang, W., Zhan, L., et al. (2021). clusterProfiler 4.0: A universal enrichment tool for interpreting omics data. Innovation *2*, 100141.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Blood sample of CTEPH patients and control individuals	China-Japan Friendship Hospital	This paper
Critical commercial assays		
iTRAQ Reagent-8 Plex Multiplex Kit	Applied Biosystem	
Human Gelsolin ELISA Kit	abcam, Waltham, MA, USA	ab270215
Human SPARC Quantikine ELISA Kit	R&D Systems, Minneapolis, MN, USA	DSP00
Human Fibulin-3 ELISA Kit	abcam, Waltham, MA, USA	ab269552
Human Heparanase ELISA Kit	abcam, Waltham, MA, USA	ab256401
Human Proprotein Convertase 9/PCSK9 Quantikine ELISA Kit	R&D Systems, Minneapolis, MN, USA	DPC900
Deposited data		
Proteome profiling	This paper	
Software and algorithms		
R software environment	R Project	version 3.3.2
R package ggplot2	R Project	version 3.4.2
R package clusterProfiler	R Project	version 4.1.1
GraphPad-Prism 8.3.0	GraphPad-Software	https://www.graphpad.com/
SPSS 22.0	IBM	https://www.ibm.com/analytics/spss-statistics-software

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Zhenguo Zhai (zhaizhenguo2011@126.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Study cohort and design

CTEPH patients were retrospectively recruited from respiratory inpatients in China-Japan Friendship Hospital and the healthy controls were recruited from physical examination center during the same period. In the discovery study, 9 CTEPHs and 9 healthy controls were involved in iTRAQ assay in 2020. In the validation cohort1, 36 CTEPHs and 36 healthy controls were tested by ELISA in 2022. In the validation cohort2, 30 CTEPHs, and 30 healthy controls were tested by ELISA in 2023. 13 CTEPH patients underwent BPA and 3 CTEPH patients who were treated with PEA were included to compare the change of biomarkers before and after treatment. The diagnosis of CTEPH is based on the 2022 European guidelines for PH including the following key points: (1) at least 3 months of effective anticoagulation to discriminate this condition from "subacute" pulmonary embolism; (2) mean pulmonary artery pressure (mPAP) >20 mmHg with pulmonary artery wedge pressure (PAWP) $\leq$15 mmHg; (3)mismatched perfusion defects on lung scan and specific diagnostic signs for CTEPH seen by multidetector computed tomography angiography, magnetic resonance imaging, or conventional pulmonary cineangiography, such as ring-like stenoses, webs/slits, and chronic total occlusions (pouch lesions or tapered lesions).[15] The design of this study was based on "three-stage strategy," which divides the study into the discovery stage (proteomics) with a small population for screening and the two-stage verification stage (classical immunoassays) with a larger individual population.