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Circulating Pin1 Levels in Patients With Chronic Thromboembolic Pulmonary Hypertension: A Case-Control Study

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Keywords: chronic thromboembolic pulmonary hypertension | Pin1 | pulmonary embolism | sex-specificity

ABSTRACT

Chronic thromboembolic pulmonary hypertension (CTEPH) is a rare but severe complication of pulmonary embolism (PE), yet its underlying mechanisms remain poorly understood. Peptidyl-prolyl cis/trans isomerase (Pin1), a regulatory enzyme involved in thrombosis, inflammation, and vascular remodeling, may contribute to pulmonary vascular disease. We investigated whether circulating Pin1 levels are associated with the risk of CTEPH and its severity. In this case-control study, we measured circulating Pin1 levels by ELISA and compared them across 329 patients with CTEPH, 350 patients after acute PE, and 350 healthy individuals. Associations between Pin1 levels and clinical parameters were assessed with multivariable regression, stratified by sex. Overall, circulating Pin1 levels did not differ between patients with CTEPH, patients after acute PE, and healthy individuals. However, male patients with CTEPH had higher Pin1 levels than the two control groups, while female patients with CTEPH had slightly lower levels than female healthy individuals. Elevated Pin1 levels were associated with improved hemodynamics and exercise capacity, lower N-terminal prohormone of brain natriuretic peptide, and increased probability of CTEPH in men. Among healthy individuals, circulating Pin1 levels varied based on age, sex, and history of cancer. In conclusion, circulating Pin1 did not distinguish between patients with CTEPH,

Lynn Willems and Eleonora Camilleri contributed equally to this study.

acute PE or healthy individuals. Nonetheless, our results suggest a possible sex-specific association between circulating Pin1 levels and CTEPH. Although Pin1 is unlikely to serve as a standalone biomarker, it may reflect underlying sex-specific pathological mechanisms, and further investigation on its utility within multimodal strategies for early risk stratification of CTEPH is warranted.

1 | Introduction

Chronic thromboembolic pulmonary hypertension (CTEPH) is a rare and severe complication of pulmonary embolism (PE), affecting approximately 3% of patients [1–3]. Persistent fibro-thrombotic material in the pulmonary arteries is responsible for an increased pulmonary vascular resistance (PVR), leading to progressive pulmonary hypertension (PH) and ultimately right ventricular failure. Early stages of the disease are often asymptomatic, or patients present with nonspecific symptoms, making timely diagnosis challenging. This frequently results in significant delays in diagnosis, even in patients with a known history of PE [4].

There is thus an urgent need to understand why some patients develop CTEPH following acute PE, to identify individuals at risk and to treat affected individuals at an early stage. Proposed mechanisms underlying the pathogenesis of CTEPH include inflammatory thrombosis [2, 4], dysregulated clot resolution [2], abnormal pulmonary vascular and progenitor cell activity [2, 5–8], and deficient angiogenesis [2, 4, 9]. Emerging evidence points to peptidyl-prolyl cis/trans isomerase NIMA-interacting protein 1 (Pin1) as a potential contributor to CTEPH pathogenesis. Pin1 is an enzyme that catalyzes conformational changes in phosphorylated serine/threonine-proline motifs, thereby regulating proteins involved in cell proliferation and survival [10–12]. Notably, Pin1 promotes tissue factor expression [13], a key initiator of coagulation [14], and reduces angiogenesis and vascular remodeling in an experimental rat model of PH [15]. These findings suggest that Pin1 may contribute to the development of CTEPH by modulating clot resolution, angiogenesis, and vascular remodeling. Noteworthy, preliminary data from our group have shown that Pin1 levels are elevated in pulmonary arterial endothelial cells and plasma from patients with CTEPH, compared with healthy individuals [16].

Currently, there are no established biomarkers to diagnose or predict CTEPH. Given that elevated Pin1 expression has been associated with poor outcomes in proliferative and inflammatory disorders [17, 18], along with promising preclinical findings on Pin1 inhibition in PH [15], we hypothesized that Pin1 could serve as a diagnostic or prognostic biomarker for CTEPH after acute PE. As a first step to address this, we investigated whether Pin1 levels were associated with CTEPH and with clinical and hemodynamic markers of its severity in a case-control study. Second, we aimed to identify clinical and biochemical factors that are associated with circulating Pin1 levels, including procoagulant, antifibrinolytic, and inflammatory markers.

2 | Methods

2.1 | Study Design

We established a case-control study, including as cases patients diagnosed with CTEPH at the University Hospitals of Leuven, Belgium (Figure 1). We included two control groups, consisting

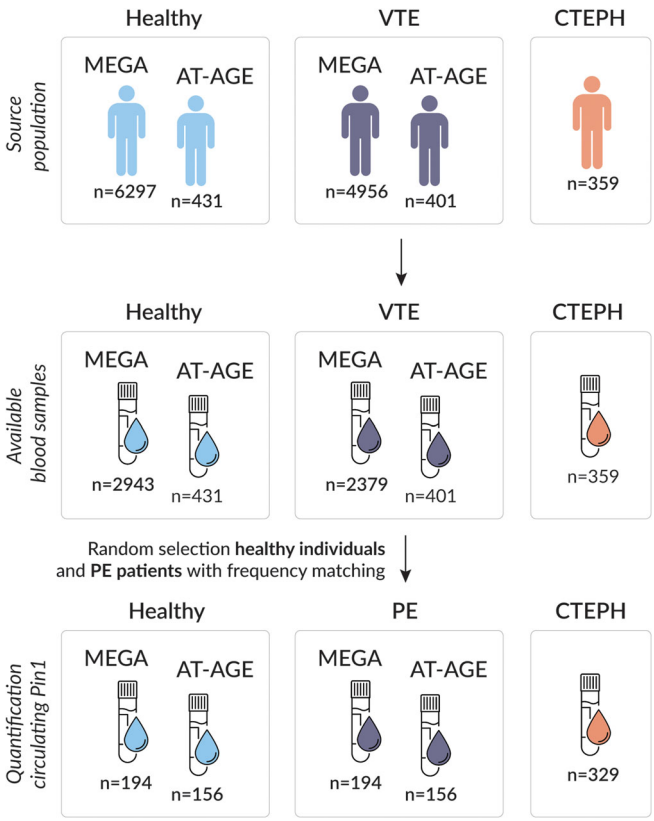

FIGURE 1 | Overview of the source population, available blood samples, and those used for quantification of circulating Pin1 levels. This case-control study selected controls participants originally included in two studies: healthy individuals (MEGA: $n = 6297$, AT-AGE: $n = 431$) and patients after VTE (MEGA: $n = 4956$, AT-AGE: $n = 401$) from the MEGA and AT-AGE study. In addition, cases with CTEPH ($n = 359$) were recruited at University Hospitals of Leuven. Blood samples were collected from a subset of participants (Healthy MEGA: $n = 943$, VTE MEGA: $n = 2379$, AT-AGE: healthy $n = 431$ and VTE 401, CTEPH: $n = 359$). After random selection with frequency matching of the healthy individuals and of patients after PE (among the VTE cases) in the MEGA and AT-AGE study, we quantified circulating Pin1 levels in further selected subgroups (Healthy MEGA and PE MEGA, $n = 194$; AT-AGE Healthy and PE, $n = 156$; CTEPH, $n = 329$). CTEPH, patients with chronic thromboembolic pulmonary hypertension; Healthy, healthy individuals; PE, patients after pulmonary embolism; VTE, venous thromboembolism.

of healthy individuals and patients recently diagnosed with acute PE, recruited from the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA) study and the Age and Thrombosis, Acquired and Genetic risk factors in the Elderly (AT-AGE) study, which were conducted in Leiden, the Netherlands [19, 20].

2.2 | Study Population

The cases consisted of 359 patients undergoing right heart catheterization for suspected CTEPH between April 2004 and February 2024. All patients were diagnosed with CTEPH based on hemodynamic and imaging criteria according to the most recent guidelines: the presence of a mean pulmonary arterial pressure (mPAP) > 20 mmHg, pulmonary arterial wedge pressure (PAWP) ≤ 15 mmHg, PVR > 160 dyn·s·cm^{-5}, perfusion defects at ventilation perfusion scan and signs of occlusion on a computed tomography scan, after at least 3 months of anticoagulation [1, 21]. Clinical data, including comorbidities, hemodynamic parameters, exercise capacities, and biochemical markers, were extracted from electronic health records. Written informed consent was obtained from all participants, and the study was approved by the Medical Ethical Committee of the UZ Leuven/KU Leuven (S57114). For this study, we included all cases of CTEPH from whom plasma samples were available in citrate or EDTA (329 patients out of 359) from the KU Leuven (Figure 1).

The MEGA and AT-AGE studies, which provided control participants, have been described previously [19, 20]. The MEGA study included 4956 patients aged 18–70 years with a first deep vein thrombosis (DVT) or PE (i.e., venous thromboembolism [VTE]) and 6297 age- and sex-matched controls in Leiden (the Netherlands) between March 1999 and September 2004. Controls were either partners of participants ($n = 3297$) or identified through random digit dialing (RDD, $n = 3000$). Participants completed detailed questionnaires on risk factors and comorbidities. Venous blood samples were collected from 2379 patients and 2943 controls. The AT-AGE study enrolled 401 elderly VTE patients (≥70 years) and 431 matched controls from primary care practices in Leiden (The Netherlands) and Burlington (Vermont, United States of America [USA]) from 2008 to 2011. Participants with active cancer, severe psychiatric or cognitive disorders, or a history of VTE were excluded. Structured home visits were conducted to collect blood samples and clinical information. In both studies, the index date was defined as the date of thrombosis for VTE cases and the date of filling the questionnaire or interview for controls. Written informed consent was obtained from all participants in the MEGA and the AT-AGE study, and both studies were approved by the Medical Ethical Committee of Leiden University Medical Center. For this study, we included two control groups of 350 patients after PE (with or without a concomitant DVT) selected from the cases and 350 age and sex-matched healthy individuals selected from the RRD controls of the MEGA study and AT-AGE study (Figure 1). From the AT-AGE study, we excluded cases and controls from the USA. Patients after PE and healthy individuals were randomly sampled with frequency matching from all the VTE cases with PE and controls of the MEGA study and AT-AGE study, respectively. Given that the MEGA study included patients up to 70 years of age and the AT-AGE included patients older than 70 years, the two control groups were selected from these two studies to reflect the age distribution of patients with CTEPH. Consequently, 194 PE patients and 194 age- and sex-matched healthy individuals under the age of 70 were selected from the MEGA study. In addition, 156 PE patients and 156 age- and sex-matched healthy individuals aged 70 and above were selected from the AT-AGE study.

2.3 | Sample Collection and Measurement of Circulating Pin1 Levels

Venous blood samples from patients with CTEPH were collected either on citrate or EDTA at the time of diagnostic right heart catheterization and stored in a biobank. At the time of blood sampling, all patients were on anticoagulant therapy (low molecular weight heparin, vitamin K inhibitors, thrombin inhibitors, or factor Xa inhibitors) for at least 3 months. Citrated plasma collection for patients with CTEPH began in March 2010. For patients diagnosed before that date only EDTA plasma samples were collected. In total, 186 citrated and 143 EDTA plasma samples from patients with CTEPH were analyzed.

In patients after PE from the MEGA study, citrated venous blood samples were obtained at least 3 months after discontinuation of anticoagulant treatment or after 1 year when treatment was still ongoing. In patients after PE from the AT-AGE study, venous blood samples were collected on citrate either immediately following VTE or 1 year later. Venous blood samples from healthy individuals were collected on citrate at the time of study inclusion in both AT-AGE and MEGA studies. Only citrated plasma samples were included, as EDTA samples were not collected in these studies.

From these venous blood samples on citrate or EDTA, plasma was prepared by centrifugation at 2500 g for 10 min at 4°C, and plasma samples were stored at −80°C until analysis. All plasma samples were diluted 1:10 in the assay diluent provided with the ELISA kit (CSB-E11308h, Cusabio, USA), and circulating Pin1 levels were measured, according to the manufacturer's instructions. To ensure that the type of anticoagulant used for plasma collection (i.e. citrate or EDTA) did not influence the measurement of circulating Pin1 levels, the assay was first optimized for citrate samples. Matched plasma samples collected in both citrate and EDTA from patients with CTEPH were analyzed, and no significant difference in Pin1 levels was observed (data not shown).

2.4 | Statistical Analysis

Circulating Pin1 levels were visually assessed for normality and found to be non-normally distributed (Figure S1) and were therefore log-transformed. To describe circulating Pin1 levels across patients with CTEPH, patients after PE and healthy individuals, linear regression was used, adjusted for age (continuous) and sex. Mean differences in log-transformed Pin1 levels between patients with CTEPH and the two control

groups, obtained from the linear regression, were back transformed to obtain mean ratios (i.e., relative difference in circulating Pin1 levels compared with the control group), since the difference between two natural logarithm corresponds to the logarithm of their ratio. Additionally, Pin1 levels were stratified by sex to explore differences between men and women. To assess whether Pin1 levels were associated with CTEPH, multinomial logistic regression was used to estimate odds ratios (ORs) and 95% confidence intervals (95% CI) for CTEPH per increase in 10 unit of Pin1 levels, adjusted for age (continuous) and sex. Pin1 was also analyzed as a categorical variable (by quartiles, based on the distribution in healthy individuals), and using restricted cubic splines, to assess potential nonlinear association with the odds of CTEPH. We examined trends in Pin1 levels in patients with CTEPH and in patients after acute PE based on the timing of blood sampling after VTE. For patients with CTEPH, the VTE event was defined as the most recent episode before CTEPH diagnosis. For patients after acute PE, it was defined as the first VTE (as these patients were included after their first VTE). Associations of time since VTE with Pin1 levels were assessed through linear regression, in which days after VTE were modeled using restricted cubic splines to allow for nonlinear associations. To assess whether Pin1 circulating levels were associated with symptoms of dyspnea, patients with CTEPH were stratified according to the New York Heart Association Functional Class (NYHA-FC) evaluated at the time of diagnosis [21]. Similarly, we stratified patients with CTEPH according to the COMPERA risk assessment model, which utilizes NYHA-FC, circulating levels of N-terminal prohormone of brain natriuretic peptide (NT-proBNP) and 6-min walking distance (6MWD) for risk stratification of long-term survival of patients with CTEPH [22, 23].

The association between Pin1 levels (continuous and categorical) and NYHA-FC and COMPERA score was assessed using logistic regression in patients with CTEPH, adjusted for age and sex, and using NYHA-FC I-II and COMPERA class 1–2 as reference category, respectively. Furthermore, the association between Pin1 levels and clinical parameters, including hemodynamic parameters evaluated by right heart catheterization at diagnosis, such as mPAP, right atrial pressure (RAP), cardiac index (CI), PVR, NT-proBNP, and 6MWD, was also assessed through linear regression, adjusted for age and sex. All aforementioned models were additionally stratified by sex. Lastly, possible clinical and biochemical determinants of Pin1 levels were studied in healthy individuals to avoid the influence of CTEPH and PE occurrence on Pin1 circulating levels. We assessed the association of patient characteristics (age, sex, body mass index [BMI]), comorbidities (cardiovascular disease, cancer, diabetes, hormone use) and biochemical markers (fibrinogen activity, factor VIII activity, Von Willebrand Factor levels, plasminogen activator inhibitor 1 levels, thrombin-activatable fibrinolysis inhibitor activity, C-reactive protein [CRP] levels) with Pin1 levels (log-transformed) by univariable linear regression.

For all analyses, samples with Pin1 values outside the assay detection range were remeasured. If the out-of-range values were not confirmed, they were considered missing and excluded. All analyses were conducted excluding missing values in RStudio (R version 4.3.1, R Foundation for Statistical Computing, Vienna, Austria, 2023: http://www.R-project.org/).

3 | Results

3.1 | Baseline Characteristics

Patients with CTEPH had a median age of 68 years (interquartile range [IQR] 56–76) at the time of diagnosis, and the majority were women ($n = 188$, 57%). Healthy individuals and patients after PE, after frequency matching, had a similar median age as patients with CTEPH (65 and 66 years, IQR 46–76 and IQR 48–77, respectively), although both control groups included a lower proportion of women ($n = 172$, 49%, in both groups). At the time of diagnosis, most patients with CTEPH were classified as NYHA-FC class III ($n = 163$, 50%) (Table 1). Circulating Pin1 levels were not measurable in 3 (0.8%) healthy individuals, 9 (2.5%) patients after PE, and 9 (2.7%) patients with CTEPH. In addition, six patients after PE had Pin1 levels exceeding the assay upper detection limit of 1000 pg/mL. After repeated measurements failed to confirm these high levels, these samples were excluded from further analysis.

3.2 | Association of Circulating Pin1 Levels With CTEPH

Overall, median circulating Pin1 levels did not differ between patients with CTEPH, patients after PE or healthy individuals (Figure 2A). When stratified by sex, female patients with CTEPH had 9% lower Pin1 levels than healthy individuals (mean ratio of 0.91, 95% confidence intervals [CI] 0.83–1.01), while Pin1 levels were similar between female patients with CTEPH and patients after PE (mean ratio of 0.98, 95% CI 0.89–1.09, Figure 2B). By contrast, in men, Pin1 levels were 27% higher in patients with CTEPH than in healthy individuals and 18% higher than in patients after PE, respectively (mean ratio of 1.27, 95% CI 1.14–1.42, and mean ratio of 1.18, CI 95% 1.31–1.06, respectively; Figure 2B). After adjustment for age and sex, circulating Pin1 levels remained elevated in patients with CTEPH compared with both healthy individuals and patients after PE, by 10% (mean ratio of 1.10, 95% CI 1.02–1.18) and 9% (mean ratio of 1.09, 95% CI 1.02–1.17), respectively. An increase of 10 pg/mL in Pin1 levels was associated with 3% higher odds of CTEPH compared with both healthy individuals and patients after PE (OR 1.03, 95% CI 1.01–1.04 and OR 1.03, 95% CI 1.01–1.05 respectively; Table 2). However, this association was nonlinear, as only very high levels of Pin1 (in the highest quartile > 219.0 pg/mL) were associated with increased odds of CTEPH (Table 2 and Figure S2). In addition, this association differed by sex (Table 2 and Figure S3). In men, patients with Pin1 levels in the highest quartile had higher odds of CTEPH than patients in the lowest quartile (OR 2.78, 95% CI 1.42–5.43 compared with healthy individuals and OR 1.92, 95% CI 0.99–3.73 compared with patients after PE). In contrast, among women, higher Pin1 levels were associated with reduced odds of CTEPH. Specifically, Pin1 levels in the highest quartile were associated with lower odds of CTEPH compared with the

TABLE 1 | Baseline characteristics of patients with CTEPH and the two control groups (healthy individuals and patients after PE).

	Healthy, $n = 350$	PE, $n = 350$	CTEPH, $n = 329$
Age, years	65 (46–76)	66 (48–77)	68 (56–76)
Women, *n* (%)	172 (49)	172 (49)	188 (57)
BMI, kg/m^2	25.0 (22.5–28.0)	26.1 (23.5–29.2)	27.1 (23.6–30.5)
Type of PE			
Isolated PE, *n* (%)	—	302 (86)	219 (78)
PE + DVT, *n* (%)	—	48 (14)	63 (22)
Comorbidities at index			
Diabetes, *n* (%)	29 (8)	35 (11)	34 (10)
History of cardiovascular disease, *n* (%)	45 (13)	71 (21)	—
History of cancer, *n* (%)	25 (7)	30 (9)	—
CTEPH and clinical parameters			
NYHA—FC			
I, *n* (%)	—	—	14 (4)
II, *n* (%)	—	—	136 (41)
III, *n* (%)	—	—	163 (50)
IV, *n* (%)	—	—	15 (5)
mPAP, mmHg	—	—	40 (31–49)
RAP, mmHg	—	—	6.0 (4.0–9.0)
CI, L.min^{-1}.m^2	—	—	2.3 (1.9–2.6)
PVR, dyn.s.cm^{-5}	—	—	523 (299–796)
6MWD, *m*	—	—	349 (240–437)
NT-proBNP, pg/mL	—	—	510 (209–1635)

Note: Continuous variables were expressed as median (interquartile range). Information on the history of cardiovascular disease and of cancer was not available for patients with CTEPH. Measurements of RAP (right atrial pressure) were missing in two patients, PVR (pulmonary vascular resistance) in one patient, 6MWD (6-min walking distance) in 24 patients and NT-proBNP (N-terminal prohormone of brain natriuretic peptide) in 49 patients.
Abbreviations: BMI, body mass index; CI, cardiac index; CTEPH, chronic thromboembolic pulmonary hypertension; DVT, deep vein thrombosis; IQR, interquartile range; mPAP, mean pulmonary arterial pressure; NYHA-FC, New York Heart Association functional class; PE, pulmonary embolism; RHC, right heart catheterization.

lowest quartile in both controls (OR 0.56, 95% CI 0.30–1.04 compared with healthy individuals and OR 0.86, 95% CI 0.45–1.64 compared with patients after PE). No trends in circulating Pin1 levels were observed based on the timing of blood sampling after the initial VTE in patients with CTEPH and patients after PE (Figure S4).

3.3 | Association of Circulating Pin1 Levels With Clinical and Hemodynamic Markers of CTEPH Severity

The next step was to investigate whether circulating Pin1 levels were associated with clinical and hemodynamic markers of CTEPH severity. Higher circulating Pin1 levels were associated with lower odds of having a NYHA-FC III or IV than with I or II in patients with CTEPH, overall and in both sexes, although the confidence intervals were wide (Table 3). Similarly, higher circulating Pin1 levels were associated with lower odds of a COMPERA risk score of 3 or 4 compared with a score of 1 or 2. Each 10 pg/mL increase in circulating Pin1 levels was also associated with a decrease in mPAP (−0.01 mmHg, 95% CI −0.18 to 0.00), in PVR (−2.49 dyn.s.cm^{-5}, 95% CI −4.89 to −0.01) and in NT-proBNP levels (−30.37 pg/mL, 95% CI −58.96 to −1.77) (Figure 3 and Figure S5). There was no association of circulating Pin1 levels with other hemodynamic parameters including RAP and CI, neither with exercise capacity (6MWD), both in the total population or in sex-stratified analyses (Figure 3 and Figure S5).

3.4 | Clinical and Biochemical Determinants of Circulating Pin1 Levels

In healthy individuals, age was associated with slightly lower circulating Pin1 levels, indicating a decline with age (mean ratio = 0.99, 95% CI 0.99–1.00, Table 4). Similarly, men had lower Pin1 levels than women (mean ratio = 0.83, 95% CI 0.76–0.92). Healthy individuals with a history of cancer also showed reduced Pin1 levels (mean ratio = 0.77, 95% CI 0.64–0.93). No other clinical or biochemical determinants were associated with Pin1 levels in healthy individuals.

4 | Discussion

Overall, we found that circulating Pin1 levels did not significantly differ among patients with CTEPH, patients after recent

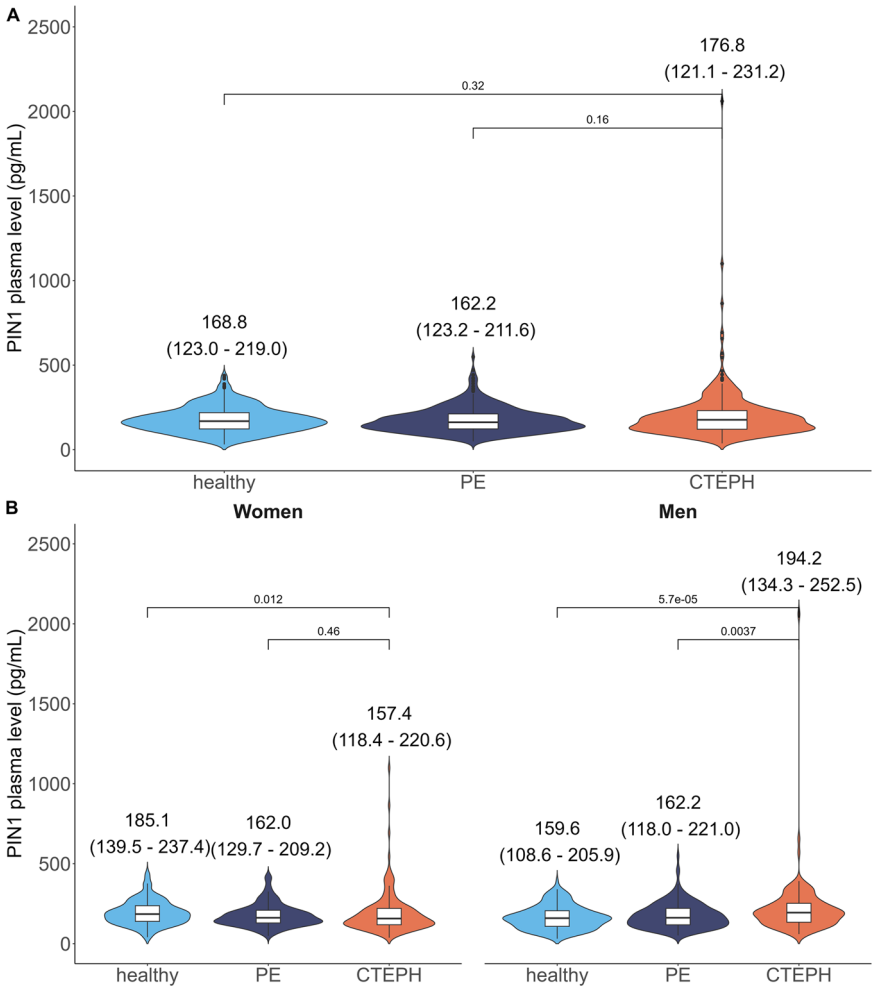

FIGURE 2 | Circulating Pin1 levels in healthy individuals, patients after PE, and patients with CTEPH. (A) Violin plots of Pin1 levels in the overall population. The white box plots within the violin plots, and the text above represents the median levels and the interquartile range. (B) Violin plots of Pin1 levels stratified by sex. CTEPH, patients with chronic thromboembolic pulmonary hypertension; Healthy, healthy individuals; PE, patients after pulmonary embolism.

acute PE, and healthy individuals. However, our results suggest a sex-specific association between circulating Pin1 levels and CTEPH. Male patients with CTEPH exhibited higher circulating Pin1 levels than male healthy individuals and patients after PE, while in women, patients with CTEPH presented slightly lower Pin1 levels than healthy individuals. Interestingly, healthy men had lower circulating Pin1 levels than healthy women, which was the opposite in patients with CTEPH. This suggests that the sex-specific differences in circulating Pin1 levels might depend on disease status. Furthermore, high circulating Pin1 levels were associated with increased odds of CTEPH in men. In contrast, elevated circulating Pin1 levels correlated with more favorable hemodynamic and clinical markers of CTEPH severity.

Our study aimed to assess whether circulating Pin1 levels could distinguish between healthy individuals, patients after acute PE, and those with established CTEPH. However, we did not find any overall differences in circulating Pin1 levels across these groups, which suggests that Pin1 alone is unlikely to serve as a reliable diagnostic or risk stratification biomarker for CTEPH. Currently, there are no validated diagnostic and prognostic tools to diagnose CTEPH timely and to identify PE survivors at risk of developing CTEPH. Clinicians must rely on symptom monitoring, imaging, and functional testing during follow-up, approaches that are often delayed, nonspecific, and resource-intensive [24, 25]. Several circulating markers have been explored in this context. While NT-proBNP levels correlate with preoperative hemodynamic severity and postoperative risk after pulmonary endarterectomy (PEA) in CTEPH, it lacked sensitivity for early detection [26, 27]. Similarly, CRP levels are increased in patients with CTEPH but also in numerous other inflammatory and thrombotic conditions [28, 29]. Moreover, these biomarker studies have relied on healthy controls rather than clinically appropriate comparators, such as PE survivors who do not develop CTEPH, leading to overestimated biomarker performance and limited clinical applicability. Among more promising candidates, circulating angiopoietin-2 has been shown to be elevated in patients with CTEPH and is strongly associated with an increased risk of developing CTEPH during follow-up in patients with acute PE [29–31]. Other emerging biomarkers, including miR-let7a [32], heparanase [33], soluble ST2 [34], and a set of five hub genes [35], also show potential for predicting or diagnosing CTEPH after PE. So far, none have progressed beyond early discovery stages, due to poor specificity, lack of large-scale validation, and unclear mechanistic

TABLE 2 | Association of circulating Pin1 levels with the odds of developing CTEPH in the total population and stratified by sex.

Model	CTEPH versus healthy individuals, OR (95% CI)			CTEPH versus PE, OR (95% CI)		
	Overall	Men	Women	Overall	Men	Women
Pin1, continuous (10 pg/mL)	1.03 (1.01–1.04)	1.06 (1.03–1.09)	1.00 (0.99–1.01)	1.03 (1.01–1.05)	1.03 (1.00–1.06)	1.00 (0.99–1.01)
Pin1, quartiles						
Q1 (31.2–123.0 pg/mL)	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.
Q2 (123.0–168.8 pg/mL)	0.84 (0.54–1.31)	0.96 (0.48–1.91)	0.70 (0.37–1.32)	0.75 (0.48–1.18)	1.04 (0.51–2.10)	0.56 (0.30–1.03)
Q3 (168.8–219.0 pg/mL)	1.05 (0.67–1.64)	2.31 (1.19–4.47)	0.46 (0.24–0.89)	1.04 (0.66–1.63)	1.77 (0.91–3.42)	0.57 (0.30–1.08)
Q4 (> 219.0 pg/mL)	1.26 (0.81–1.96)	2.78 (1.42–5.43)	0.56 (0.30–1.04)	1.34 (0.86–2.09)	1.92 (0.99–3.73)	0.86 (0.45–1.64)

Note: All odds ratios are adjusted for age and sex, except for the models in men and women, which are only adjusted for age. Quartiles of Pin1 levels are based on the distribution of Pin1 in healthy individuals.
Abbreviations: 95% CI, 95% confidence intervals; CTEPH, chronic thromboembolic pulmonary hypertension; PE, pulmonary embolism; OR, odds ratio; Q, quartiles.

relevance. These challenges underscore the limitations of relying on single biomarkers and have spurred growing interest in multimodal diagnostic approaches that integrate biochemical, imaging, and clinical parameters to improve risk stratification and early diagnosis. For example, Klok et al. demonstrated that combining electrocardiographic features of right ventricular hypertrophy with normal circulating NT-proBNP levels help distinguishing between CTEPH and PE survivors [36]. Although our findings indicate limited utility for Pin1 as a standalone biomarker in CTEPH, our study suggests sex-specific associations in circulating Pin1 levels, which may reflect underlying pathophysiological differences between men and women in the development of CTEPH. These observations highlight the potential for Pin1 to possibly contribute to a more nuanced, sex-informed risk stratification framework, especially when used in combination with other clinical or biochemical parameters (i.e. NT-proBNP).

The sex-specific nature of our findings is notable given that CTEPH affects men and women in roughly equal proportions after acute PE and is diagnosed at a similar age [37, 38]. Despite similar baseline hemodynamic parameters and functional class, women are significantly less likely to undergo surgical removal of thrombotic material by PEA [37, 39–42]. We showed that lower circulating Pin1 levels were associated with higher mPAP, PVR, and NT-proBNP. It has been shown that microvascular disease occurs more frequently in elderly and women, who often display higher PVR values, contributing to right ventricular pressure overload and increased NT-proBNP [26, 43–46]. This suggests that sex-specific differences in circulating Pin1 levels may be driven by differences in the anatomical distribution of lesions in men and women, as men with CTEPH more often present with large-vessel thromboembolic disease, while women with (sub)segmental or microvascular lesions [37, 39–42]. In addition, it has been previously shown that Pin1 is highly expressed in platelets and megakaryocytes, where it regulates cytoskeletal remodeling critical for platelet formation and activation [15, 42]. Consequently, elevated Pin1 in male CTEPH patients may reflect a platelet-driven thrombotic phenotype, associated with large-vessel thromboembolic disease. By contrast, (sub-)segmental and/or microvascular lesions more commonly observed in females may involve less platelet-mediated thrombosis and more chronic vascular remodeling. This pathophysiological distinction supports the notion that circulating Pin1 may act as a marker of in situ thrombosis in CTEPH. Another potential explanation is that Pin1 could modulate sex hormones, since Pin1 directly binds estrogen receptor alpha (ERα) enhancing ERα nuclear localization and stability, its transcriptional activity and thereby amplifying estrogen-mediated gene expression [47, 48]. This in turn can have an effect on platelet activation [49]. In the present study, circulating Pin1 levels were lower in healthy men than women, while no sex differences were observed in patients after PE. This may suggest that although sex-dependent differences in Pin1 levels exist under physiological conditions, they could be masked in pathological states due to increased release of Pin1 from platelets and megakaryocytes.

Several limitations should be mentioned. First, due to the case-control design, we cannot distinguish whether the elevation of circulating Pin1 levels in men might be a cause or a

TABLE 3 | Association of Pin1 levels with the NYHA-FC class in patients with CTEPH, stratified by sex.

Model	Overall	Men	Women
NYHA-FC I-II vs III-IV, OR (95% CI)			
Pin1, continuous (10 pg/mL)	1.00 (0.99–1.00)	1.00 (0.99–1.00)	1.00 (0.99–1.00)
Pin1, quartiles			
Q1 (31.2–123.0 pg/mL)	Ref.	Ref.	Ref.
Q2 (123.0–168.8 pg/mL)	0.69 (0.35–1.35)	0.59 (0.18–1.85)	0.80 (0.34–1.81)
Q3 (168.8–219.0 pg/mL)	0.52 (0.27–1.00)	0.37 (0.13–1.00)	0.69 (0.28–1.70)
Q4 (> 219.0 pg/mL)	0.74 (0.39–1.38)	0.58 (0.21–1.55)	0.82 (0.36–1.89)
COMPERA risk score 1-2 versus 3-4, OR (95% CI)			
Pin1, continuous (10 pg/mL)	0.99 (0.99–0.99)	0.99 (0.99–1.00)	0.99 (0.99–1.00)
Pin1, quartiles			
Q1 (31.2–123.0 pg/mL)	Ref.	Ref.	Ref.
Q2 (123.0–168.8 pg/mL)	0.44 (0.21–0.91)	0.43 (0.11–1.53)	0.42 (0.16–1.051)
Q3 (168.8–219.0 pg/mL)	0.35 (0.16–0.75)	0.46 (0.14–1.42)	0.28 (0.10–0.77)
Q4 (> 219.0 pg/mL)	0.36 (0.17–0.74)	0.33 (0.10–1.02)	0.40 (0.15–1.03)

Note: Measurements of 6MWD (6-min walking distance) were missing in 24 patients and NT-proBNP (N-terminal prohormone of brain natriuretic peptide) in 49 patients. All odds ratios are adjusted for age and sex (except for the models in men and women, which are only adjusted for age). Quartiles of Pin1 levels are based on the distribution of Pin1 in healthy individuals.
Abbreviations: 95% CI, 95% confidence intervals; CTEPH, chronic thromboembolic pulmonary hypertension; NYHA-FC, New York Heart Association functional class; OR, odds ratio; PE, pulmonary embolism; Q, quartiles.

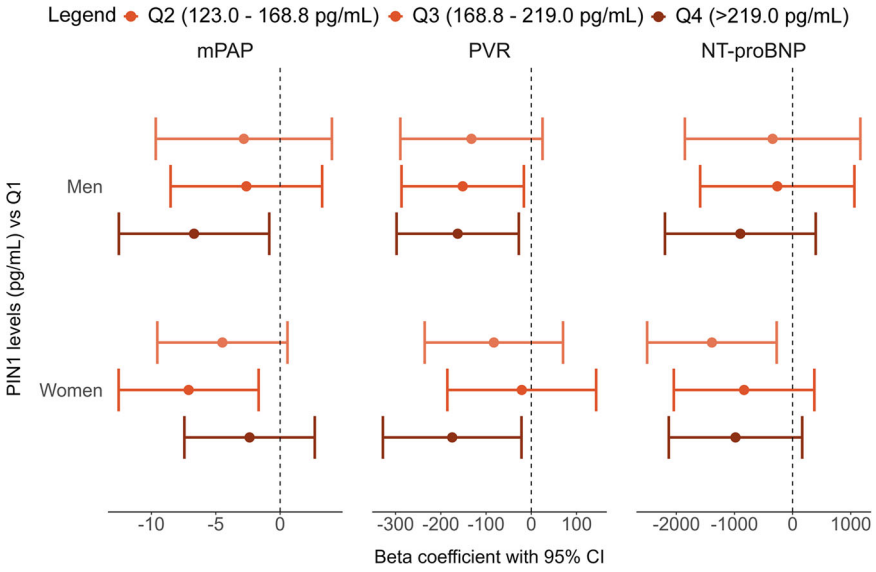

FIGURE 3 | Association of Pin1 levels with hemodynamic parameters and NT-proBNP stratified by sex. mPAP (mean pulmonary arterial pressure), PVR (pulmonary vascular resistance) and NT-proBNP (N-terminal prohormone of brain natriuretic peptide) were measured at the time of right heart catheterization. CI, confidence intervals; Q, quartile of Pin1 levels according to the distribution in the healthy individuals.

consequence of CTEPH. The differences we observed in circulating Pin1 levels when stratifying by sex might be due to a chance finding and therefore should be confirmed in further studies. Moreover, we did not have information on whether any of the patients after PE developed CTEPH during the follow-up; thus, we could not assess the value of Pin1 as a prognostic marker for CTEPH. However, given an estimated 3% prevalence of CTEPH after PE, a maximum of approximately 11 patients could have been misclassified. In addition, the timing of blood sampling among patients after PE was not uniform. Samples were obtained at varying intervals after the acute PE event, potentially contributing to variability in Pin1 levels due to differences in disease phase or treatment status at the time of sampling. We did not observe any trends in circulating Pin1 levels according to the timing of blood sampling; however, this remains a potential source of variability. Finally, knowing that Pin1 is an intracellular protein [11], its circulating levels may not directly reflect its intracellular activity or the underlying pathophysiological processes in which Pin1 might be involved. We hypothesize that circulating levels of Pin1 might derive

TABLE 4 | Clinical and biochemical determinants of Pin1 levels in healthy individuals.

	Pin1 levels (mean ratio*, 95% CI)
Clinical determinants	Univariable
Age, years	0.99 (0.99–1.00)
Sex, men versus women	0.83 (0.76–0.92)
BMI, kg/m^2	1.00 (0.99–1.02)
Diabetes	1.08 (0.91–1.29)
History of cardiovascular disease	0.99 (0.86–1.15)
History of cancer	0.77 (0.64–0.93)
Hormone use	1.14 (0.97–1.35)
Biochemical determinants	
Fibrinogen activity, %	1.02 (0.94–1.10)
FVIII activity, %	1.00 (1.00–1.00)
VWF levels, %	1.00 (1.00–1.00)
PAI1 levels, ng/mL	1.00 (1.00–1.00)
TAFI activity, %	1.00 (1.00–1.00)
CRP levels, mg/L	1.00 (1.00–1.00)

Abbreviations: BMI, body mass index; CRP, C-reactive protein; FVIII, factor VIII; PAI1, plasminogen activator inhibitor 1; TAFI, thrombin activatable fibrinolysis inhibitor; VWF, Von Willebrand Factor.
*Mean differences per 1 unit increase of the determinant (if continuous) or compared to the reference category (if categorical) obtained from the linear regression were back transformed to derive a mean ratio, since the difference between two natural logarithm corresponds to the logarithm of their ratio. The mean ratio is to be interpreted as the percentage increase compared to the reference category: e.g., for each year of increase in age, Pin1 levels decrease of 1%.

from platelet activation. However, considering that we still do not have any proof of the origin of circulating Pin1, this remains a speculative hypothesis that could not be investigated within the scope of our study. Nevertheless, the inclusion of a large cohort of CTEPH patients, alongside healthy individuals and patients after acute PE as control groups, and the availability of comprehensive clinical and laboratory data are strong aspects, which allowed enough statistical power to assess for nonlinear associations, and distinction between a CTEPH-specific association and differences related to the initial thrombotic event [11].

In summary, circulating Pin1 did not distinguish between patients with CTEPH, patients after acute PE or healthy individuals. Nevertheless, our results suggest a sex-specific association between circulating Pin1 levels, and CTEPH risk and its severity. Highly elevated Pin1 levels were associated with increased risk of developing CTEPH only in men. Pin1 was inversely associated with hemodynamic markers of disease severity. These findings suggest that Pin1 may reflect distinct sex-dependent mechanisms in CTEPH and merit further research in longitudinal and mechanistic settings to elucidate its role in CTEPH pathogenesis.

Author Contributions

Lynn Willems and Eleonora Camilleri collected the data, performed data analysis and interpretation, drafted and reviewed the manuscript. Elise Bosmans collected the data. Janne Verhaegen collected the data and reviewed the manuscript. Astrid van Hylckama Vlieg and Frits R. Rosendaal collected the data and reviewed the manuscript. Kondababu Kurakula reviewed the manuscript. Marion Delcroix, Suzanne C. Cannegieter, Marie-José T. H. Goumans, Frederikus A. Klok, and Rozenn Quarck conceived and designed the study, contributed to data interpretation, and reviewed the manuscript. All authors approved the final version.

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Ethics Statement

The study protocol was approved by the Institutional Ethics Committee of the University Hospital of Leuven (S57114). The protocol of MEGA and AT-AGE studies was approved by the Medical Ethical Committee of Leiden University Medical Center (P224/98 and P08.066).

Conflicts of Interest

The authors declare no conflicts of interest.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.
Figure S1. Distribution of circulating Pin1 levels in healthy individuals, patients after acute PE and patients with CTEPH. **Figure S2.** Non-linear association of circulating Pin1 levels with CTEPH. **Figure S3.** Sex-differences in the non-linear association of circulating Pin1 levels with CTEPH. **Figure S4.** Trends in circulating Pin levels based on the timing of blood sampling after the initial VTE in patients with CTEPH and after acute PE, by including the whole time span (A), or focusing on the 1st year after VTE (B). **Figure S5.** Association of Pin1 levels with hemodynamic parameters.