Clinical phenotyping of plasma thrombospondin-2 reveals relationship to right ventricular structure and function in pulmonary hypertension

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Converging evidence promotes thrombospondin-2 as a biomarker in pulmonary hypertension, that informs on the presence and progression of the disease, and helps to identify right ventricular responses to chronically increased afterload https://bit.ly/3XoVJIf

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Abstract

Background Converging evidence from proteogenomic analyses prioritises thrombospondin-2 (TSP2) as a potential biomarker for idiopathic or heritable pulmonary arterial hypertension (PAH). We aimed to assess TSP2 levels in different forms of pulmonary hypertension (PH) and to define its clinical phenotype.

Methods Absolute concentrations of TSP2 were quantified in plasma samples from a prospective single-centre cohort study including 196 patients with different forms of PH and 16 disease controls (suspected PH, but normal resting pulmonary haemodynamics). In an unbiased approach, TSP2 levels were related to 152 clinical variables.

Results Concentrations of TSP2 were increased in patients with PH *versus* disease controls ($p<0.001$ for group comparison). The discriminatory ability of TSP2 levels to distinguish between patients and controls was superior to that of N-terminal pro-brain natriuretic peptide ($p=0.0023$ for comparison of areas under the curve). Elevation of TSP2 levels was consistently found in subcategories of PAH, in PH due to lung disease and due to left heart disease. Phenotypically, TSP2 levels were robustly related to echocardiographic markers that indicate the right ventricular (RV) response to chronically increased afterload with increased levels in patients with impaired systolic function and ventriculoarterial uncoupling. Focusing on PAH, increased TSP2 levels were able to distinguish between adaptive and maladaptive RV phenotypes (area under the curve 0.87, 95% CI 0.76–0.98).

Interpretation The study indicates that plasma TSP2 levels inform on the presence of PH and associate with clinically relevant RV phenotypes in the setting of increased afterload, which may provide insight into processes of RV adaptability.

Introduction

Pulmonary vascular disease comprises several forms of pulmonary hypertension (PH) ranging from rare vasculopathies such as idiopathic or heritable pulmonary arterial hypertension (PAH) to common forms of PH underlying chronic lung or heart conditions [1]. Despite differences in aetiology, alterations of small pre-capillary pulmonary arteries (PA) can be detected in all forms of PH, through processes of loss and obliteration, abnormal muscularisation and perivascular inflammation [2–5]. High mortality and morbidity in these patients result from maladaptive pathological remodelling and failure of the right ventricle (RV) if PH progresses and is left untreated [6]. A circulating marker directly involved in the pulmonary vascular and/or RV pathology across the spectrum of PH may be useful to inform on (early) pathology, selection for targeted therapy, assessment of treatment response, drug development and innovative clinical trial design [7, 8].

Unbiased screening analyses of the plasma proteome in patients with idiopathic or heritable PAH have recently identified thrombospondin-2 (TSP2), a secreted matricellular protein, as a likely disease-related plasma marker, which was the only protein that emerged from each analytical approach: circulating TSP2 levels were increased in patients with PAH compared to healthy subjects, associated with survival, provided independent prognostic information in a combinatory protein panel score and tracked disease onset in genetically susceptible individuals [9, 10]. Integrating protein data with common genetic variation has additionally revealed that TSP2 probably protects against PAH, where an allele that confers lifelong higher TSP2 levels reduced risk of having PAH in a case–control comparison [9]. To translate these findings to potential clinical applicability and therapeutic strategies, in-depth clinical phenotyping of plasma TSP2 levels is needed within the full spectrum of PH.

Therefore, we aimed to investigate plasma TSP2 levels in patients with different forms of PH and to determine the clinical phenotype that links to TSP2 focusing on cardiopulmonary parameters.

Methods

Patient selection

Plasma samples were obtained from a prospectively enrolled cohort of consecutive patients with PH attending the University Medical Centre Hamburg-Eppendorf, Germany, between January 2021 and January 2022 (ClinicalTrials.gov identifier NCT04654650). Haemodynamic diagnosis, a mean pulmonary artery pressure (mPAP) >20 mmHg during right heart catheterisation at rest and classification of PH followed recent international recommendations [1]. Pulmonary vascular resistance (PVR) >3 Wood units (WU) and mean pulmonary arterial wedge pressure (PAWP) ⩽15 mmHg were used to differentiate pre-capillary from post-capillary PH. Cardiac output was measured by thermodilution. Symptomatic patients referred with suspected PH were included as disease controls if PH was ruled out by right heart catheterisation (mPAP ⩽20 mmHg and/or PVR ⩽3 WU with PAWP ⩽15 mmHg). The study protocol was approved by the local ethics committee and written informed consent was obtained from each participant (ethics reference identifier 2020-10122-BO-ff). The investigation conformed to the principles outlined in the Declaration of Helsinki.

Clinical phenotyping of patients

Clinical data were collected during routine appointments (prevalent patients) or diagnostic work-up for suspected PH (incident patients or disease controls) and included demographic characteristics, anthropomorphic measures, medication (grouped by the World Health Organization (WHO) Anatomic Therapeutic Chemical/Defined Daily Dose Index 2022), right heart catheterisation data, transthoracic echocardiography data, biochemical data from routine laboratory testing including N-terminal pro-brain natriuretic peptide (NT-proBNP), pulmonary function data, capillary blood gas analysis data, diffusing capacity of the lung for carbon monoxide, 6-min walk distance (6MWD) and WHO functional class (FC). The majority of data were obtained within 3 months of sampling (distribution of time periods are provided in supplementary table S1). RV dimensions and areas were not available due to suboptimal echocardiographic images in 22 (11%) subjects.

Risk groups in patients diagnosed with PAH were calculated based on four-stratum cut-off criteria as presented recently for WHO-FC, 6MWD and NT-proBNP [11, 12]. Each variable was graded from 1 to 4; the mean was calculated and rounded to the next integer [11, 12].

Maladaptation of the RV in patients diagnosed with PAH was defined by reduced tricuspid annular plane systolic excursion (TAPSE) and reduced cardiac index in the setting of increased RV end-diastolic area (RVEDA). The median of each parameter was selected as cut-off criteria: TAPSE ⩽17 mm, cardiac index ⩽2.5 $L \cdot min^{-1} \cdot m^{-2}$ and RVEDA ⩾27 cm^2.

Sample collection and measurement of plasma TSP2

Plasma samples were collected at study enrolment and subjects were nonfasting. Peripheral venous blood was collected using EDTA tubes, centrifuged immediately and plasma aliquots were stored at −80°C until required without additional freeze–thaw cycles. Absolute concentration of plasma TSP2 levels were measured using a quantitative enzyme linked immunosorbent assay (DTSP20; R&D Systems, MN, United States). Based on duplicative measurements, mean coefficient of variation was 2% for intra-assay and 7.2% for inter-assay variability.

TSP2 gene expression in RV tissue

TSP2 gene (*THBS2*) expression was re-examined in publicly available transcriptome profiles from human RV tissue obtained by RNA-sequencing (Gene Expression Omnibus accession number GSE198618) [13].

In brief, RV samples were collected either through biopsy or during early autopsy from 1) controls with normal RV function; 2) patients with adaptive/compensated RV function (RV hypertrophy, TAPSE ⩾17 mm and/or cardiac index >2.2 $L \cdot min^{-1} \cdot m^{-2}$); and 3) patients with maladaptive/decompensated RV and end-stage PAH [13].

Statistical analyses

Distribution of data were assessed on histograms and using Shapiro–Wilk test. Given the skewed distributions of many clinical parameters and TSP2 measurements, group comparisons were performed using the Mann–Whitney U-test or Kruskal–Wallis one-way ANOVA. Categorical variables were compared using the Chi-squared test. Generalised linear models with γ-distribution and log link were used to test for an association between TSP2 levels and clinical variables, as the data did not span negative values. In these models TSP2 was the dependent variable and possible confounders were included as covariates. Missing data were handled by pairwise removal without imputation. Receiver operating characteristic (ROC) curve analyses were performed to assess the discriminatory ability for TSP2 and NT-proBNP. The DeLong test was applied to compare area under the curve (AUC) from ROC using the pROC R-package (version 1.18.0). To account for multiple testing, we applied the Benjamini–Hochberg false discovery rate (FDR) or the more conservative Bonferroni procedure. Data are presented as numbers with percentages, 95% confidence intervals or median (interquartile range (IQR)). Analyses were performed in R (version 4.0.4) and visualisation in GraphPad Prism (version 9.0.1).

Results

Details of the PH patients (n=196) and disease control subjects (n=16) who provided plasma samples are presented in table 1. The median age was 67 (IQR 52–77) years; 66% were female; and 60% of PH patients had PAH. Patients with PAH were mostly prevalent (88%) and on combination treatment with targeted drugs at time of sampling (74%).

TSP2 plasma levels in PH and controls

Median TSP2 levels were 29.59 (IQR 20.27–38.9) $ng \cdot mL^{-1}$ in PH patients and 19.45 (IQR 16.26–22.65) $ng \cdot mL^{-1}$ in controls ($p<0.001$ for group comparison). Elevated TSP2 plasma levels distinguished between PH patients and controls in ROC analysis (AUC 0.75, 95% CI 0.63–0.88; $p=6.04\times10^{-5}$). Elevated NT-proBNP levels also distinguished between PH patients and controls (AUC 0.63, 95% CI 0.53–0.72; p=0.0068), but the discriminatory ability of TSP2 was superior to that of NT-proBNP in this cohort (p=0.0023 for differences in AUC; supplementary figure S1).

Next, we classified patients according to PH aetiology (table 1). Levels of TSP2 were increased in patients with PAH, both idiopathic/heritable and associated PAH, in PH due to lung diseases and in PH due to left heart disease, as compared to disease controls (FDR $q<0.05$ for comparison with controls; figure 1). In PAH, the elevation of TSP2 levels was pronounced in incident cases (supplementary figure S2). We further classified patients with associated PAH due to underlying conditions, connective tissue diseases or congenital heart disease, and similarly observed increased TSP2 levels compared to controls (supplementary figure S2). Likewise, TSP2 levels were increased in both PH due to COPD and interstitial lung diseases (ILDs) (supplementary figure S2). Levels of TSP2 were not significantly increased in patients with chronic thromboembolic PH compared to controls in our cohort (figure 1).

Clinical phenotypes of TSP2 levels in PH

To elucidate the phenotypic profile that links to increased TSP2 levels in PH, we tested 152 clinical variables for association with TSP2 using regression models, adjusting for age, body mass index and self-reported gender. This identified 12 clinical measures at a conservative Bonferroni-corrected level of significance ($q<0.05$; table 2). The majority (nine out of 12) of these measures were directly related to the right heart structure and function (table 2); namely, right atrial pressure (RAP), right atrial area, TAPSE, TAPSE adjusted for systolic pulmonary artery pressure (sPAP), RV fractional area change (RVFAC), RV end-diastolic pressure, stroke volume, stroke volume indexed for body surface area and myocardial biomarker NT-proBNP. Measures that reflect pulmonary vascular disease reached nominal significance, such as mPAP (p=0.0009), PA compliance (p=0.0008) and PVR (p=0.001). Results for all 152 clinical variables are presented in supplementary table S2.

Increased RAP with elevated central venous pressure and decreased stroke volume with low perfusion pressure can cause hepatopathy as well as nephropathy in PH patients [14]. As both γ-glutamyl transferase and estimated glomerular filtration rate were associated with TSP2 levels (table 2), we additionally adjusted models for these variables in sensitivity analyses and revealed that TSP2 remained independently related to RV measurements (supplementary table S3).

TABLE 1 Characteristics of patients with pulmonary hypertension (PH) and disease controls who provided plasma samples

	All PH	IPAH/HPAH	APAH	PH due to heart disease	PH due to lung disease	CTEPH	Disease controls
Subjects, n	196	70	47	14	28	37	16
Age, years	67 (52–77)	69 (54–78)	58 (39–73)	74 (68–80)	67 (56–74)	77 (65–80)	67 (56–76)
Female	71	67	68	79	46	65	75
BMI, kg·m^{-2}	25 (22–30)	26 (22–31)	24 (21–28)	25 (22–26)	26 (23–30)	25 (23–31)	30 (25–32)
Exercise							
WHO-FC ⩾3	50	50	38	71	57	38	31
6MWD, m	390 (278–466)	377 (267–458)	405 (330–468)	240 (189–289)	303 (246–394)	413 (340–458)	396 (305–462)
Right heart catheterisation							
sPAP, mmHg	62 (46–76)	71 (59–86)	61 (43–74)	68 (52–79)	60 (48–72)	58 (46–76)	33 (25–36)
mPAP, mmHg	38 (29–48)	43 (33–52)	36 (28–45)	43 (36–51)	38 (31–42)	35 (28–42)	21 (17–23)
dPAP, mmHg	22 (17–29)	26 (19–34)	25 (17–28)	24 (22–29)	24 (21–27)	20 (16–23)	12 (10–15)
mPAWP, mmHg	11 (7–14)	10 (6–13)	10 (6–15)	20 (19–22)	11 (9–14)	10 (7–13)	10 (8–13)
RAP, mmHg	6 (4–9)	6 (4–9)	7 (4–10)	11 (8–14)	6 (5–8)	5 (4–8)	6 (3–7)
Cardiac index, L·min^{-1}·m^{-2}	2.5 (2–2.9)	2.6 (2–2.9)	2.7 (2.2–3)	2.1 (1.8–2.3)	2.5 (2–3.1)	2.3 (1.9–2.9)	2.6 (2.4–2.9)
PVR, WU	5.5 (3.9–8.6)	6.7 (4.8–10.4)	5.7 (4–8.1)	4.2 (3.3–6.1)	5.6 (4.5–7.5)	4.8 (3.9–7.8)	2.1 (1.5–2.3)
Echocardiography							
RAA, cm^2	19 (15–26)	20 (16–27)	20 (15–25)	27 (20–29)	16 (13–25)	18 (13–22)	15 (13–18)
TAPSE, mm	19 (17–22)	19 (17–23)	19 (17–22)	17 (14–22)	19 (16–21)	19 (17–22)	21 (20–30)
TAPSE/sPAP, mm·mmHg^{-1}	0.32 (0.22–0.4)	0.32 (0.22–0.4)	0.33 (0.26–0.4)	0.25 (0.2–0.37)	0.31 (0.27–0.42)	0.36 (0.25–0.45)	0.80 (0.59–0.87)
RVFAC, %	38 (32–45)	37 (28–42)	37 (31–41)	32 (29–40)	41 (33–45)	42 (38–48)	41 (39–47)
LAA, cm^2	17 (13–23)	17 (13–25)	19 (13–21)	24 (19–29)	14 (13–23)	17 (13–20)	16 (14–22)
LVEF, %	58 (55–62)	60 (56–62)	59 (56–63)	58 (55–63)	58 (55–60)	58 (55–60)	58 (57–62)
Pulmonary function test							
FEV_1/FVC, %	75 (68–80)	75 (67–79)	75 (70–81)	76 (73–79)	67 (45–79)	76 (71–81)	77 (73–79)
TLC, % pred	90 (80–100)	90 (82–99)	90 (78–98)	89 (84–99)	78 (59–108)	91 (84–99)	94 (80–103)
Biochemistry							
TSP2, ng·mL^{-1}	29 (21–41)	30 (23–46)	29 (22–41)	44 (26–61)	32 (23–36)	24 (19–33)	19 (17–24)
NT-proBNP, ng·L^{-1}	547 (163–1660)	981 (286–2164)	460 (184–1005)	2701 (849–5059)	337 (144–933)	329 (130–1287)	315 (204–514)
Comorbidities							
Atrial fibrillation	22	22	20	77	15	14	33
Diabetes	16	22	13	23	11	11	7
CAD	17	14	7	8	22	14	7

Data are presented as median (interquartile range) or %, unless otherwise stated. IPAH: idiopathic pulmonary arterial hypertension; HPAH: heritable pulmonary arterial hypertension; APAH: associated pulmonary arterial hypertension; CTEPH: chronic thromboembolic PH; BMI: body mass index; WHO-FC: World Health Organization functional class; 6MWD: 6-min walk distance; sPAP: systolic pulmonary arterial pressure; mPAP mean pulmonary arterial pressure; dPAP: diastolic pulmonary arterial pressure: mPAWP: mean pulmonary arterial wedge pressure; RAP: right atrial pressure; PVR: pulmonary vascular resistance; WU: Wood unit; RAA: right atrial area; TAPSE: tricuspid annular plane systolic excursion; RVFAC: right ventricle fractional area change; LAA: left atrial area; LVEF: left ventricle ejection fraction; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; TLC: total lung capacity; TSP2: thrombospondin-2; NT-proBNP: N-terminal pro-brain natriuretic peptide; CAD: coronary artery disease.

TSP2 levels and RV structure and function in PH

To further explore the relationship between TSP2 and RV function, we divided RVFAC and TAPSE, both estimates for RV systolic function, into tertiles. Concentrations of TSP2 were significantly higher in patients with low RVFAC (⩽33%) or low TAPSE (⩽17 mm) compared to corresponding high tertiles (FDR q<0.05; figure 2). To assess whether the association between TSP2 levels and estimates of RV systolic function were independent of PA pressure (*i.e.* afterload), we used the TAPSE/sPAP ratio as surrogate marker for RV–PA coupling [15]. Dividing TAPSE/sPAP into tertiles depicted that concentrations of TSP2 were significantly increased in patients with low TAPSE/sPAP (⩽0.24 mm·mmHg^{-1}) compared to the medium and high tertiles (FDR q<0.05; figure 2).

TSP2 levels and maladaptive RV in PAH

The RV profile linked to TSP2 levels in PH was consistently detected in the subset of patients with PAH (table 2). In PAH, maladaptive RV remodelling in response to persistently increased afterload causes ventriculoarterial uncoupling resulting in reduced cardiac output [6]. We defined maladaptation of the RV based on functional (cardiac index, TAPSE) and structural (RVEDA) criteria. A maladaptive RV

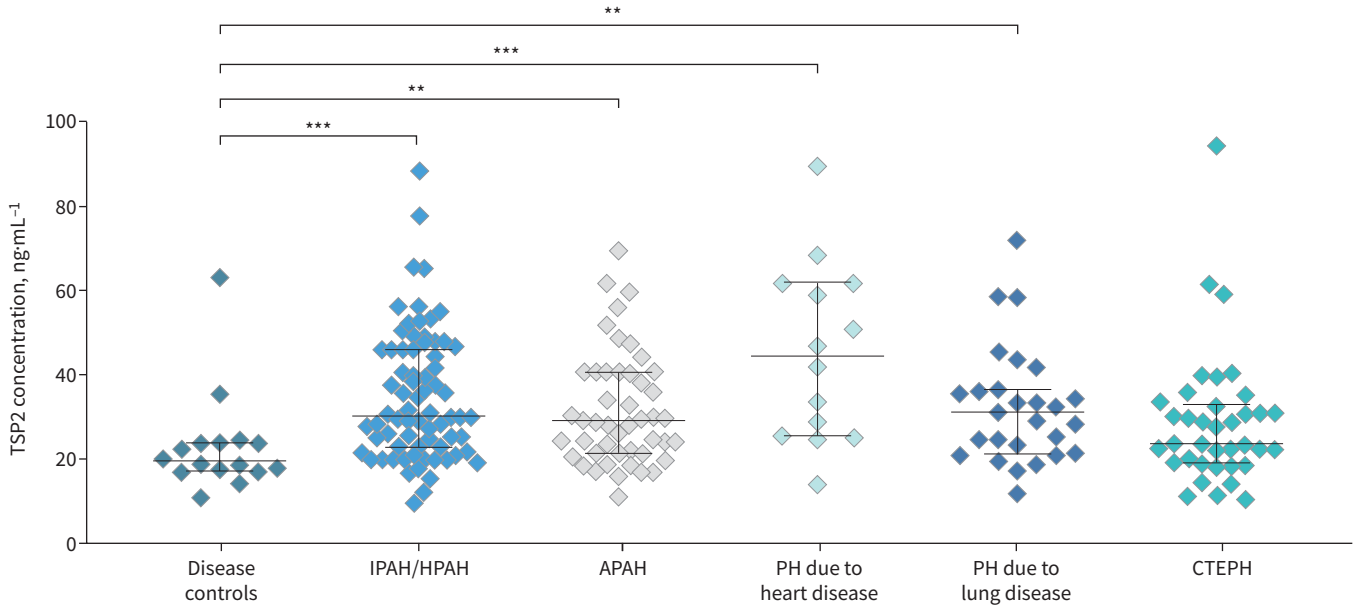

FIGURE 1 Comparison of plasma thrombospondin-2 (TSP2) concentrations in patients with different forms of pulmonary hypertension (PH) and disease controls presenting with suspected PH, but normal pulmonary artery pressure at cardiac catheterisation. Data are presented along with median and interquartile range. Nonsignificant comparisons are not shown. False discovery rate procedure was applied to obtain q-value. IPAH: idiopathic pulmonary arterial hypertension; HPAH: heritable pulmonary arterial hypertension; APAH: associated pulmonary arterial hypertension; CTEPH: chronic thromboembolic PH. **: q<0.01, ***: q<0.001.

phenotype was found in 10% of PAH patients with completely available data (n=100) and concentrations of TSP2 were significantly increased in this group of patients (p<0.0001 for group comparison; figure 3, supplementary table S4). ROC curve analysis showed that both TSP2 and NT-proBNP levels distinguished between patients with maladaptive and adaptive RV phenotypes (both p<0.0001; figure 3). There was no

TABLE 2 Results from linear regression models associating clinical measures with thrombospondin-2 (TSP2) levels in patients with pulmonary hypertension (PH) and pulmonary arterial hypertension (PAH), independent of age at sampling, body mass index and gender

	In patients with PH (primary analysis)		In patients with PAH (secondary analysis)	
	HR±SE	q-value	HR±SE	p-value
GGT, IU·L^{-1}	1.13±0.02	3.38×10^{-6}	1.21±0.04	6.63×10^{-6}
RAP, mmHg	4.10±0.25	1.00×10^{-5}	2.57±0.29	1.44×10^{-3}
NT-proBNP, 100 ng·L^{-1}	1.14±0.03	6.40×10^{-5}	1.11±0.03	1.44×10^{-3}
RAA, cm^2	1.84±0.14	2.02×10^{-3}	1.83±0.15	1.16×10^{-4}
RVFAC, %	0.58±0.12	2.88×10^{-3}	0.50±0.15	1.77×10^{-5}
TAPSE, mm	0.35±0.26	8.67×10^{-3}	0.40±0.32	4.55×10^{-3}
eGFR, mL·min^{-1}	0.81±0.05	1.02×10^{-2}	0.77±0.06	1.30×10^{-4}
RVEDP, mmHg	2.44±0.22	1.0 x10^{-2}	1.81±0.24	1.69×10^{-2}
TAPSE/sPAP, 0.01 mm·mmHg^{-1}	0.68±0.09	1.38×10^{-2}	0.74±0.12	1.74×10^{-2}
SVI, mL·m^{-2}	0.66±0.11	2.47×10^{-2}	0.72±0.12	7.58×10^{-3}
S_{vO_2}, %	0.59±0.14	2.80×10^{-2}	0.56±0.17	6.95×10^{-4}
Stroke volume, mL	0.81±0.05	3.08×10^{-2}	0.86±0.06	1.24×10^{-2}

Hazard ratio (HR) and standard error (SE) refer to increase by one unit of clinical variable except N-terminal pro-brain natriuretic peptide (NT-proBNP: increase by 100 ng·L^{-1}) and tricuspid annular plane systolic excursion (TAPSE) to systolic pulmonary artery pressure (sPAP) ratio (increase by 0.01 mm·mmHg^{-1}). Bonferroni procedure was applied to obtain q-values in the primary analysis. Unadjusted p-values are presented for the secondary analysis. GGT: γ-glutamyl transferase; RAP: right atrial pressure; RAA: right atrial area; RVFAC: right ventricle fractional area change; eGFR: estimated glomerular filtration rate; RVEDP: right ventricle end-diastolic pressure; SVI: stroke volume indexed for body surface area; S_{vO_2}: mixed venous oxygen saturation.

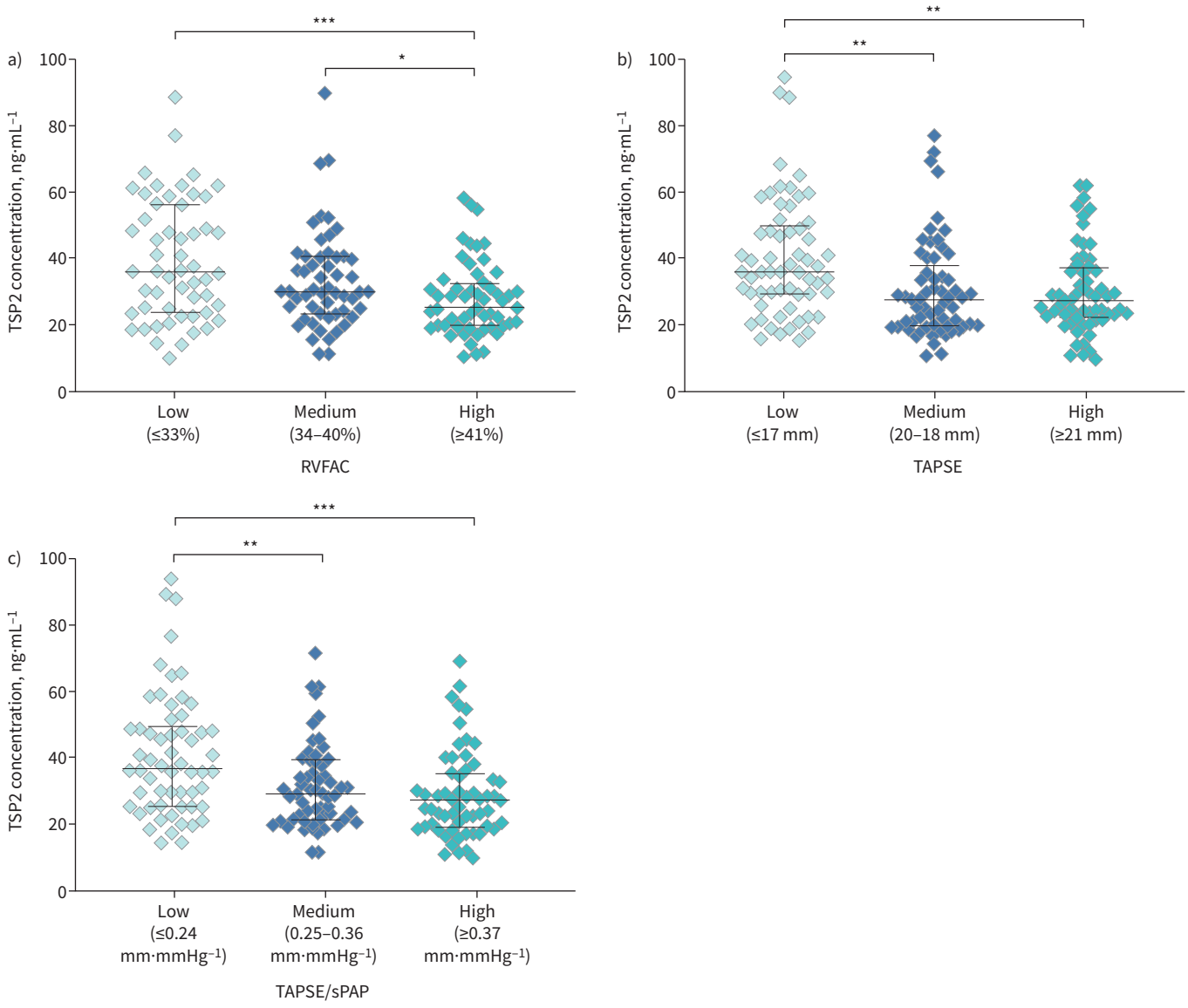

FIGURE 2 Comparison of plasma thrombospondin-2 (TSP2) concentrations in patients with pulmonary hypertension (PH) with respect to tertiles of a) right ventricle fractional area change (RVFAC), b) tricuspid annular plane systolic excursion (TAPSE) and c) TAPSE adjusted for systolic pulmonary artery pressure (sPAP). Data are presented along with median and interquartile range. Nonsignificant comparisons are not shown. False discovery rate procedure was applied to obtain q-values. *: q<0.05, **: q<0.01, ***: q<0.001.

significant difference between the two AUC curves (p=0.54). Exercise intolerance is a consequence of RV maladaptation and recent risk assessment strategies in PAH adopted from international guidelines are primarily based on measurements of exercise capacity (6MWD and WHO-FC) in combination with myocardial marker NT-proBNP [11, 12]. Higher risk groups were significantly enriched in PAH patients with a maladaptive RV phenotype (p=0.024 for group comparison) and concentrations of TSP2 increased consecutively from low- to high-risk group (FDR q<0.05; figure 3).

In line with elevated plasma levels in PAH patients with RV maladaptation, expression levels of TSP2 were increased in RV tissue obtained from PAH patients with maladaptive/decompensated RV compared to tissue from patients with adaptive/compensated RV or from controls (FDR q<0.05; supplementary figure S3) [13].

Discussion

We performed a comprehensive investigation of plasma TSP2 levels in patients with PH. TSP2 levels were consistently increased in different forms of PH as compared to subjects presenting with suspected PH, but

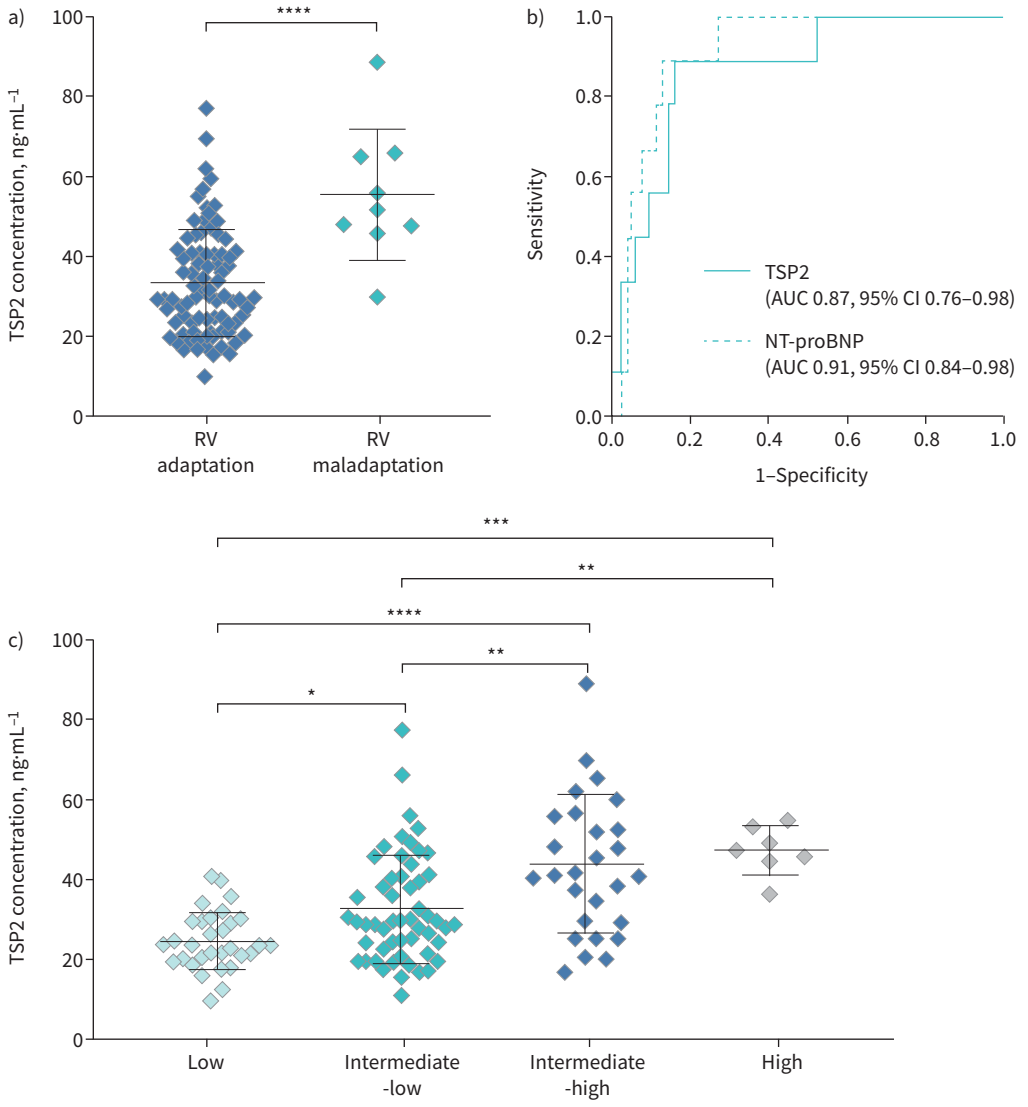

FIGURE 3 a) Comparison of plasma thrombospondin-2 (TSP2) concentrations in patients with pulmonary arterial hypertension (PAH) with respect to right ventricle (RV) adaption and maladaptation. b) Receiver operating characteristic curve showing the discriminatory abilities of TSP2 and N-terminal pro-brain natriuretic peptide (NT-proBNP) to distinguish maladaptive from adaptive RV in PAH (both p<0.0001). c) Comparison of plasma TSP2 concentrations in patients with PAH with respect to risk stratification groups. Data are presented along with median and interquartile range. Nonsignificant comparisons are not shown. False discovery rate procedure was applied to obtain q-value. AUC: area under the curve. *: q<0.05, **: q<0.01, ***: q<0.001, ****: q<0.0001.

normal right heart catheterisation. Increased TSP2 levels were particularly evident in PH patients with reduced RV systolic function and RV–PA uncoupling. Focusing on PAH, increased TSP2 levels were identified in patients with a maladaptive RV phenotype and TSP2 successively increased with exercise intolerance as reflected by risk stratification groups. This was supported by increased expression levels of TSP2 in RV tissue obtained from PAH patients with a maladaptive phenotype. To the best of our knowledge, this is the first study showing that TSP2 has the potential to inform on RV structure and function in the setting of PH.

Phenotyping of *THBS2*-null mice have established that TSP2 is involved in the regulation of the extent of angiogenesis [16]. TSP2 is secreted by microvascular endothelial cells and pericytes, particularly if nitric oxide concentration is low, and induces endothelial cell apoptosis, probably through activation of CD36 [17–19]. Evidence for an involvement in pulmonary vascular disease has emerged from studies in patients.

TSP2 expression was found to be differentially upregulated in pulmonary arteries of patients with PH due to ILD and COPD [5]. Consistently, we found that protein concentrations of TSP2 in plasma were elevated in patients with PH due to ILD and COPD as compared to controls. In addition, we were able to reproduce and expand previous findings from studies on plasma samples from patients with idiopathic/heritable PAH and healthy controls, demonstrating increased TSP2 levels in patients [9]. We measured absolute concentrations rather than relative units, included a diseased control group, *i.e.* symptomatic patients with suspected PH, but with normal pulmonary pressure/resistance in catheterisation, and extended observations into associated forms of PAH. Collectively, these results indicate that TSP2 can be robustly detected in the circulation of patients with pre-capillary PH with levels above that found in controls without PH. Patients with left heart disease included in our study demonstrated also a relatively strong pre-capillary component (*i.e.* high PVR) [1] and increased TSP2 concentrations could reflected similar biological processes. The large surface area of the pulmonary vasculature and increased TSP2 expression in remodelled PA tissue probably attribute at least a portion of the circulating TSP2 abundance to PA origin. While mechanistic studies are warranted, genetic inference suggested that elevated levels of TSP2 represent an attempt to compensate and protect from pulmonary vascular disease [9].

In PH, the chronically pressure-overloaded RV adapts by hypertrophy and increased contractility to maintain stroke volume (ventriculoarterial coupling). Persistence or progress in pressure-overload eventually triggers maladaptive mechanisms leading to RV dilation and failure to main cardiac output (uncoupling) [6]. We identified that differences in circulating concentrations of TSP2 in patients with PH were strongly related to markers that indicate the RV response to pressure-overload including systolic function and ventriculoarterial coupling. There is currently no established definition of a maladaptive RV phenotype and we applied, as suggested previously, a combination of structural and functional criteria [20]. Accordingly, TSP2 levels were markedly increased in PAH patients with a maladaptive RV phenotype. This distinct RV phenotype linked to TSP2 levels may help to interpret the prediction of all-cause mortality observed for high TSP2 levels in idiopathic/heritable PAH [10]. TSP2 was among only six out of 4152 plasma proteins that, combined in a single panel score, best classified mortality risk in idiopathic/heritable PAH patients [10]. The link between RV and TSP2 could also help to understand the relatively low TSP2 levels in patients with chronic thromboembolic PH in our study, as RV function was relatively preserved in this group of patients (*i.e.* RVFAC). The utility of TSP2 in routine management as novel marker for RV pathological remodelling that is associated with RV maladaptation and ventriculoarterial uncoupling in the setting of PH will need to be prospectively evaluated.

TSP2 is a plausible candidate for a role in RV remodelling. The absence of TSP2 in rodent models of cardiac diseases disrupts myocardial matrix integrity and associates with dilated cardiomyopathy [21–23]. This was possibly due to TSP2-mediated regulation of matrix metalloproteinases that break down the matrix and tissue inhibitors of metalloproteinases resulting in abnormal collagen fibrillogenesis [22]. The alleviating role of TSP2 on myocardial matrix was particularly evident if the heart coped with stress (*e.g.* after myocardial infarction) [24]. The absence of cardiac TSP2 aggravated inflammation, as mice lacking TSP2 experienced increased myocardial inflammation with a decrease in activation of anti-inflammatory T-regulatory cells [25], while selective overexpression of TSP2 in cardiac tissue did not produce a pathological cardiac phenotype [26]. Collectively, TSP2 appears to play a role in maintenance of cardiac integrity and structure [24]. Accordingly, increased levels of TSP2 in the circulation detected in patients with myocardial haemodynamic stress due to pressure-overloaded RV or injury due to myocardial infarction [27] may represent an ameliorating cardiac remodelling process. To this, it has also been speculated that a certain response of the extracellular matrix is needed to provide a scaffold for the remodelled RV myocardium and that the success of RV adaptation depends on the “quality” of cardiac fibrosis [28]. Therapeutic approaches to cardiac remodelling should consequently consider enhancing TSP2 activity.

The data in our study represent a single-centre observational study based on plasma samples from patients with PH. As cardiac tissue was not obtained from patients with plasma samples, involvement of TSP2 in RV remodelling cannot be directly assessed, but is supported by increased expression found in a publicly available transcriptomic dataset [13]. The source of protein abundance in plasma reflects the influx and efflux of proteins synthesised, leaked or eliminated by tissues and cells across the body [29]. The pulmonary vasculature and RV are plausible sources for plasma TSP2, particularly in a stressed system such as PH. In current clinical management, elevated levels of BNP/NT-proBNP, a marker of cardiac origin that is affected by comorbidities, is used to rule-in diagnosis and to assess disease severity in PH. More data are required to establish whether measurements of TSP2 in patients with PH might affect clinical decision-making above that of BNP/NT-proBNP. The sample size was relatively small for PH due to left heart disease, which could limit the validity of our analyses. Nevertheless, our proof-of-concept

study newly identified that TSP2 levels were increased in this group of PH. Across PH groups, elevated plasma TSP2 levels were linked to a low TAPSE/sPAP ratio, an echocardiographic surrogate marker for RV–PA coupling [15]. The gold-standard method evaluating RV–PA coupling is the ratio of end-systolic to arterial elastances from invasively measured pressure–volume relationships over multiple beats.

In conclusion, we present observational evidence from a contemporary cohort that TSP2 levels as measured in plasma samples are consistently increased across the spectrum of PH and that TSP2 can identify phenotypically distinct and clinical meaningful RV responses to chronically increased afterload. Our data complement previous findings, but further research is needed to establish diagnostic accuracy for TSP2 regarding detection of PH and monitoring of RV functionality, and to define the molecular mechanisms responsible.

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