DOI: 10.1002/pul2.12041

RESEARCH ARTICLE

Pulmonary Circulation Open Access | PVRI Pulmonary Vascular Research Institute | WILEY

Plasma matrix metalloproteinase 2 is associated with severity and mortality in pulmonary arterial hypertension

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Funding information
Swedish Society of Pulmonary Hypertension; "Avtal om läkarutbildning och forskning"; Janssen-Cilag AB

Abstract

Pulmonary arterial hypertension (PAH) is a life-threatening disease characterized by vasoconstriction and remodeling of the pulmonary vessels. Risk stratification in PAH could potentially be improved by including novel biomarkers related to PAH pathobiology. We aimed to investigate the relationship between extracellular matrix (ECM)-related proteins, survival, and European Society of Cardiology and European Respiratory Society (ESC/ERS) risk stratification scores in patients with PAH. Plasma samples and hemodynamics were collected from PAH patients during right heart catheterizations at diagnosis ($n = 48$) and early follow-up, after treatment initiation ($n = 33$). Plasma levels of 14 ECM-related proteins, with altered levels in PAH compared to healthy controls, were analyzed with proximity extension assays, and related to hemodynamics, transplant-free survival time, and ESC/ERS risk score. Glypican-1 levels were higher before versus after treatment initiation ($p = 0.048$). PAH patients with high plasma levels of matrix metalloproteinase (MMP) -2, MMP-7, MMP-9, MMP-12, perlecan, and tissue inhibitor of metalloproteinase 4 (TIMP-4) at baseline, had worse transplant-free survival ($p < 0.03$) than patients with low levels. Hazard ratio (95% confidence interval) was for MMP-2 1.126 (1.011–1.255), perlecan 1.0099 (1.0004–1.0196), and TIMP-4 1.037 (1.003–1.071) in age and sex-adjusted Cox-regression model. MMP-2 correlated with ESC/ERS risk scores ($r_s = 0.34$, $p = 0.019$), mean right atrial pressure ($r_s = 0.44$, $p = 0.002$), NT-proBNP ($r_s = 0.49$, $p \leq 0.001$), and six-minute walking distance ($r_s = -0.34$, $p = 0.02$). The present study indicates that high levels of MMP-2, perlecan, and TIMP-4 are associated with poor survival in PAH. High plasma MMP-2, correlated with poor prognosis in PAH. Further validation in larger studies is needed to better determine this association.

KEYWORDS
extracellular matrix, matrix metalloproteinase-2, prognosis, pulmonary arterial hypertension, risk assessment

INTRODUCTION

Pulmonary arterial hypertension (PAH) develops due to vasoconstriction and remodeling of pulmonary small muscular arterial vessels, including hypertrophy of the media, as well as intimal and adventitial fibrosis, leading to increased mean pulmonary arterial pressure (MPAP) and pulmonary vascular resistance (PVR), resulting in progressive right heart failure and ultimately death.[1] Despite the development of PAH-specific therapies during the last three decades, prognosis in patients with PAH remains poor.[2,3] Risk stratification is an important part of PAH management and in the European Society of Cardiology and European Respiratory Society (ESC/ERS) 2015 PH guidelines, a risk assessment tool for 1-year mortality was introduced to estimate prognosis and guide treatment decisions.[4] The ESC/ERS risk stratification model has since 2017 been validated in three independent cohorts, but its discriminatory ability for the wide span of intermediate-risk patients is a limitation.[5–7]

Utilizing additional prognostic bloodborne biomarkers related to PAH vascular pathobiology, as opposed to N-terminal pro-brain natriuretic peptide (NT-proBNP), which reflects a currently strained myocardium, in combination with the risk stratification tools, may potentially allow for a more precise and individualized prediction of risk of mortality. For instance, a patient with otherwise low-risk or intermediate-risk characteristics, that would display a biomarker profile of poor prognosis could benefit from an earlier and more aggressive escalation of PAH-specific treatment.

Expansion of extracellular matrix (ECM) across the pulmonary vessel walls and the associated vascular fibrosis cause stiffening and reduced compliance of the pulmonary arteries.[8] Matrix metalloproteinases (MMPs) are important regulators of ECM and have been implicated in the development of pulmonary vascular remodeling in PAH.[9,10] For instance, MMP-2 is increased in cultured idiopathic PAH (IPAH) pulmonary artery smooth muscle cells.[10] Likewise, proteoglycans are components of the ECM that can influence ECM remodeling. One proteoglycan, decorin, inhibits the fibrotic effect of transforming growth factor-beta.[11,12] Previous studies have investigated MMPs and proteoglycans as diagnostic markers for PAH differentiation and found that several ECM-related proteins were altered in PAH including MMP-7 and prolargin.[13,14]

We hypothesized that ECM-related proteins with known abnormal expression in PAH patients may serve as biomarkers with prognostic value in PAH. Our aim was to study the relationship between ECM-related plasma protein levels in PAH patients in relation to survival and ERS/ESC risk stratification score.

MATERIALS AND METHODS

Study population

Patients aged ≥18 years diagnosed with PAH and included in the Lund Cardio Pulmonary Register (LCPR) cohort in the Region Skåne biobank between September 2011 and September 2016 were included. Patients lacking data from the hemodynamic assessment at diagnosis were excluded.

PAH diagnosis required hemodynamic assessment with right heart catheterization (RHC) at rest, exhibiting an MPAP ≥ 25 mmHg, a pulmonary arterial wedge pressure (PAWP) ≤ 15 mmHg, and a PVR > 3 wood units (WU), corresponding to the 2015 ESC/ERS guidelines.[4] Spirometry with diffusion capacity and/or high-resolution computed tomography was used to exclude PH due to lung disease and/or hypoxia. Chronic thromboembolic PH was excluded with pulmonary scintigraphy. Echocardiography and/or MRI were/was additionally used to exclude left heart disease.

A total of 48 PAH patients were identified at diagnosis and were included in the present study. Thirty-three of these patients had RHC assessment and plasma samples from an early follow-up (median: 116, range: 18–289 days) For categorization, IPAH and familial PAH were considered as one entity called IPAH/FPAH, and PAH associated with systemic sclerosis or other connective tissue diseases were considered as one entity called connective tissue disease-associated PAH (CTD-PAH).

Hemodynamics and clinical data

Patients underwent RHC as part of clinical diagnosis and follow-up. The RHC procedure was performed by experienced cardiologists at the regional PH center at the Hemodynamic Lab at Skåne University Hospital in Lund, using Swan Ganz catheters (Baxter Health Care Corp.). Mean arterial pressure (MAP), MPAP, mean right atrial pressure (MRAP), and PAWP were registered during RHC. Cardiac output (CO) was measured by thermodilution and heart rate by electrocardiogram (ECG). Body surface area (BSA), cardiac index (CI), PVR, and transpulmonary pressure gradient (TPG) were calculated by the following formulae: $BSA = weight^{0.425} \times height^{0.725} \times 0.007184$; $CI = CO/BSA$; $PVR = TPG/CO$.

World health organization functional class (WHO-FC), 6-min walking distance (6MWD), and NT-proBNP were retrieved from LCPR and had previously been obtained from medical records.

Plasma sampling and protein analysis

Mixed venous blood samples were collected from the introducer in the right internal jugular vein during RHC, which the patients underwent as part of clinical diagnosis and early follow-up. Plasma was extracted using centrifugation and stored in the LCPR cohort of the Region Skåne biobank at −80°C in accordance with the biobank's standardized procedures. Plasma samples were stored with edetic acid (EDTA). Median storage time was 3.7 years, total range 1.2–5.7 years, until analysis in May 2017.

Protein analysis of plasma samples was performed using Proseek Multiplex cardiovascular I, cardiovascular II, and oncology II 96-plex immunoassay panels (Olink Proteomics) which use proximity extension assays (PEA) and quantitative real-time polymerase chain reaction (qPCR). Briefly stated, PEA use antibody pairs labeled with complementary oligonucleotide strands. As antibodies bind the target protein corresponding tails in the proximity of each other join and create a DNA sequence which is extended by DNA polymerase. Resulting DNA sequences are amplified and read out with qPCR.[15] Relative protein levels were reported in normalized protein expression (NPX) values, which is an arbitrary unit on a log2-scale, and then transformed into linear values. Protein analyses were due to logistics done in two different runs. One sample before treatment initiation and one sample after treatment initiation were analyzed for 33 patients in the first run. For 15 patients, one sample before treatment initiation was analyzed in the second run. Four internal controls, added to each sample, and 10 separate external samples per analysis plate, were used to adjust for intra- and interanalysis variability. Ten overlapping samples were in addition used to normalize protein levels between the analysis runs.

Fourteen ECM-related proteins, which we previously had found to be altered in PAH compared to healthy controls, were selected from the aforementioned Olink panels.[13,14] Proteins included were CYR61 protein (CYR61) (also known as CCN family member 1), decorin, glypican-1, matrix extracellular phosphoglycoprotein (MEPE), matrix metalloproteinase (MMP)-2, MMP-7, MMP-9, MMP-12, perlecan, prolargin (also known as proline-arginine-rich end leucine-rich repeat protein), syndecan-1, thrombospondin-2, tissue inhibitor of metalloproteinases 4 (TIMP-4), and WNT1 inducible signaling pathway protein 1 (WISP-1). NT-proBNP was also analyzed with PEA for consistency. All included proteins except MEPE and glypican-1 had previously been found to have higher levels in PAH compared to healthy controls, whereas MEPE and glypican-1 had been found to have lower levels in PAH compared to healthy controls.[13,14]

Risk scores

Patients' 1-year mortality risk scores were calculated using ERS/ESC 2015 guidelines risk assessment tool.[4] WHO-FC, 6MWD, NT-proBNP, MRAP, CI, and SvO_2 were graded as 1 "low risk," 2 "intermediate risk," and 3 "high risk" according to the guidelines cut-offs.[4] The scores of all parameters were combined, and a mean risk score was calculated and rounded to the nearest integer as outlined by Kylhammar et al.[5] For correlations with the proteins' levels, the rounded-off scores were used.

Statistical analyses

Histograms, Wilcoxon matched-pairs signed-ranks test, Mann–Whitney test and Spearman correlations were performed in GraphPad Prism version 9.0.0 for Windows, GraphPad Software https://www.graphpad.com/.

Receiver operating characteristic (ROC) analysis, Kaplan–Meier with log-rank tests, univariable, and multivariable Cox-regression models were performed in R 4.0.2: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria https://www.R-project.org/.

Histograms were used to check for normality. Wilcoxon matched-pairs signed-ranks test was used to compare protein levels at baseline and follow-up, and Mann–Whitney test between PAH etiologies. ROC analysis was used to measure the proteins' discriminative ability for the outcome of death or lung transplantation and Youden's index was used to identify protein level cut-off points. Kaplan–Meier plots with log-rank tests were done on proteins with an area under the ROC curve (AUC) CI not overlapping 0.5. Proteins' levels were dichotomized with the identified cut-off points. Survival data for the follow-up time were censored at April 9, 2021. Death or lung transplantation were defined as events. The median follow-up time was 3.33 (interquartile range: 1.54–4.64) years.

Univariable Cox proportional-hazards regression models were used to evaluate the prognostic value of the continuous values of the proteins analyzed with Kaplan–Meier, as well as age and sex. Proteins with a significant crude model were further analyzed with multivariable Cox proportional-hazards regression models, adjusted for age and sex.

Spearman correlations were used to investigate the association between proteins' levels and the ESC/ERS risk scores as well as parameters included in the risk score model. $p < 0.05$ were considered statistically significant.

The study was conducted in accordance with the declaration of Helsinki. It was approved by the local ethics committee in Lund (Dnr 2010/114, 2010/248,

2010/442, 2011/368, 2015/270), and all participants had given their informed and written consent.

RESULTS

Patient characteristics and PAH-specific treatment at early follow-up

Twenty-four (72.7%) patients received initial monotherapy, and nine (27.3%) received initial combination therapy. Sixteen patients were treated with endothelin receptor antagonist (ERA), six with phosphodiesterase Type 5 inhibitor (PDE5i), and eight with triple combination therapy. Two (6.1%) of the patients with follow-up were acute vaso-responders. One of these was treated with calcium channel blockers (CCB) and the other with CCB and ERA. The patients' characteristics are included for descriptive purposes (Table 1) and have previously been characterized in a related manuscript on the same population focusing on other bloodborne biomarkers.[16]

Comparison of PAH groups

At follow-up, gypican-1 levels were higher than baseline ($p = 0.048$) (Figure 1). None of the other proteins had a significant difference in their plasma levels between baseline and follow-up. *p* values are presented in Table 2. Glypican-1 was also significantly higher in the CTD-PAH

TABLE 1 Population characteristics

Patient characteristics	All PAH patients[a]	IPAH/FPAH[a]	CTD-PAH[a]	PAH before treatment	PAH after treatment
Sample size, *n* (% females)	48 (88.3)	23 (73.9)	25 (92)	33 (87.9)	33 (87.9)
Age, years	71.5 (64–76)	73 (57–77)	71 (64.5–76)	71 (60.5–76.5)	NA
BSA, m^2	1.75 (1.59–1.97)	1.77 (1.59–1.98)	1.70 (1.60–1.80)	1.73 (1.58–1.79)	1.73 (1.58–1.79)
Hemodynamics					
MPAP, mmHg	43 (37–54.75)	51 (42–56)	39 (30–43.5)	43 (37–55)	36 (32–48)
PAWP, mmHg	8 (6–11)	9 (6–12)	8 (5–10)	6 (5–9.5)	8 (5–11)
PVR, WU	9.5 (6.23–11.83)	11.47 (8.86–14.52)	6.88 (4.73–9.92)	9.56 (6.95–12.06)	5.79 (4.3–8.69)
CI, $L/min/m^2$	2.19 (1.75–2.82)	1.9 (1.69-2.24)	2.62 (1.92–3.06)	2.25 (1.8–2.85)	2.7 (2.14–3.45)
MRAP, mmHg	7 (4–11)	9 (6–11)	6 (2.5–9)	6 (3–9.5)	6 (3–9.5)
Clinical parameters					
6MWD, m	242 (172.5–349)[c]	225 (150–280)[c]	267 (180–352)	242 (183.75–345.5)[b]	270 (222.25–337.5)[d]
SvO_2, %	59.25 (51.05–66.18)	55.2 (49–61)	64.9 (54.5–71.25)	62.3 (54.45–66.15)	63.4 (58.4–72.2)
NT-proBNP	2149 (864.5–3631)[c]	2213 (1678–4747)[b]	1169 (411.3–3370)[b]	2104 (767–3139)[b]	695 (242.5–1796.75)[d]
WHO-FC, I/II/III/IV/NA, *n*	1/9/28/2/8	1/3/16/0/3	0/6/12/2/5	1/6/22/2/2	2/10/15/0/6
Comorbidities					
Thyroid disease, *n*	11 (22.9)	5 (21.7)	6 (24)	10 (30.3)	NA
Ischemic heart disease, *n*	7 (14.6)	4 (17.4)	3 (12)	5 (15.2)	NA
Stroke, *n*	2 (4.2)	2 (8.7)	0 (0)	2 (6.1)	NA
Atrial fibrillation, *n*	4 (8.3)	2 (8.7)	2 (8)	3 (6.1)	NA
Diabetes mellitus, *n*	12 (25)	10 (43.5)	2 (8)	8 (24.2)	NA
Systemic hypertension, *n*	17 (35.4)	12 (52.2)	5 (20)	9 (24.2)	NA

Note: The study population have previously been characterized.[16] Continuous variables are presented as median (interquartile range: 25–75 percentile). Categorical variables are presented as numbers and percentage, *n* (%).

Abbreviations: 6MWD, 6-min walk distance; BSA, body surface area; CI, cardiac index; CTD-PAH, connective tissue disease-associated PAH; MPAP, mean pulmonary artery pressure; MRAP, mean right atrial pressure; NA, not available; PAH, pulmonary arterial hypertension; PAWP, pulmonary arterial wedge pressure; PVR, pulmonary vascular resistance; SvO2, venous oxygen saturation; WHO-FC, World health organization functional class; WU, wood units.

[a]At baseline before PAH-specific treatment. Missing values [b] = 1, [c] = 2, [d] = 3.

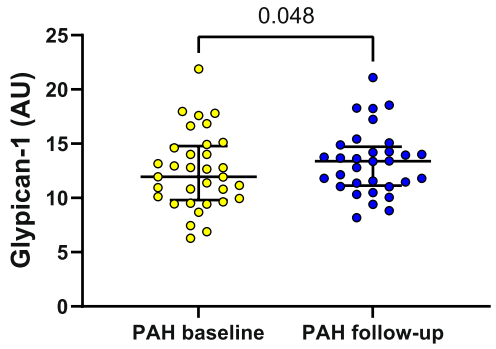

FIGURE 1 Glypican-1 levels at PAH baseline and follow-up. Glypican-1 levels are significantly higher in PAH patients at follow-up compared to baseline values. AU, arbitrary units; PAH, pulmonary arterial hypertension

group compared to the IPAH/FPAH group ($p = 0.029$) as the only protein with different levels in the CTD-PAH compared to the IPAH/FPAH group (Table 2).

ROC analyses

MMP-2, -7, -9, -12, perlecan, and TIMP-4 had an AUC with a CI that did not overlap 0.5 and were consequently selected for further analyses. Cyr61, decorin, glypican-1, MEPE, prolargin, syndecan-1, thrombospondin-2, and WISP-1 did not generate AUCs that differed from 0.5. Generated AUCs with 95% CI, thresholds, sensitivity, and specificity are found in Table 3.

Kaplan–Meier and log-rank test

During the observation period, 30 (62.5%) patients died, and 3 (6.3%) patients underwent lung transplantation. Patients' transplant-free survival based on proteins' levels above the threshold attained from the ROC analyses for prediction of death, were significantly lower for MMP-2 ($p < 0.001$), MMP-7 ($p < 0.001$), MMP-9 ($p = 0.007$), MMP-12 ($p = 0.028$), perlecan ($p = 0.025$), and TIMP-4 ($p < 0.001$) (Figure 2).

Univariable Cox-regression models

Proteins with a significant log-rank test were further analyzed in a univariable Cox-regression analysis. Age ($p = 0.032$), female sex ($p = 0.019$), MMP-2 ($p = 0.009$), MMP-7 ($p = 0.023$), perlecan ($p = 0.02$), and TIMP-4 ($p = 0.015$) were significant in the univariable Cox-regression model (Table 4). MMP-2 had the largest hazard ratio (HR) of the proteins per increase in protein level (AU unit), followed by TIMP-4, perlecan, and MMP-7. Female sex was a strong predictor of transplant-free survival with a HR of 0.355 (95% CI: 0.15–0.841). Age increased the risk by 3.8% per year (Table 4), whereas MMP-9 ($p = 0.169$) and MMP-12 ($p = 0.263$) did not have a significant effect on prognosis (Table 4).

Age and sex-adjusted multivariable Cox-regression models

Proteins with a significant crude Cox-regression were further investigated in a multivariable model adjusted for age and sex. MMP-2, perlecan, and TIMP-4, but not MMP-7, remained prognostic in the multivariable Cox-regression model (Table 4). Female sex was a strong predictor of transplant-free survival with an HR of around 0.2 in all four multivariable models. An increase in age by 1 year increased the hazard of death by around 4% but was not significant ($p = 0.057$) in the adjusted MMP-2 model (Table 4).

Correlation with the ESC/ERS risk score

Proteins with a prognostic value in the multivariable Cox-regression model were subsequently analyzed for correlation with the ESC/ERS risk score. MMP-2 levels correlated with ESC/ERS risk score ($r_s = 0.34$, $p = 0.019$) (Figure 3a). Perlecan ($p = 0.45$) and TIMP-4 ($p = 0.57$) did not display a significant correlation with the ESC/ERS risk score.

Correlation with hemodynamic parameters

MMP-2 levels were further investigated for correlations with the parameters included in the ESC/ERS risk score. MMP-2 correlated significantly with MRAP ($r_s = 0.44$, $p = 0.002$), NT-proBNP (AU) ($r_s = 0.49$, $p < 0.001$), and 6MWD ($r_s = -0.34$, $p = 0.02$) (Figure 3b–d). They did, however, not correlate with WHO-FC ($r_s = 0.18$, $p = 0.26$), CI ($r_s = -0.19$, $p = 0.19$), SvO_2 ($r_s = -0.27$, $p = 0.06$), PVR ($r_s = -0.033$, $p = 0.82$), or MPAP ($r_s = 0.082$, $p = 0.58$).

DISCUSSION

PAH is a severe disease characterized by pulmonary vasoconstriction, remodeling, and endothelial dysfunction.[1] Despite the development of potent vasoactive therapies for PAH, which have improved prognosis, mortality is still high.[2,3] Remodeling of pulmonary arterial ECM has

TABLE 2 Proteins' levels in PAH subgroups

Proteins (AU)	All PAH patients[a] ($n = 48$)	IPAH/FPAH[a] ($n = 23$)	CTD-PAH[a] ($n = 25$)	PAH before treatment ($n = 33$)	PAH after treatment ($n = 33$)	p values after versus before treatment	p values IPAH/FPAH versus CTD-PAH
CYR61	32.3 (24.72–38.92)	29.92 (22.04–37.36)	32.98 (29.2–41.44)	32.2 (25.06–39.5)	30.21 (24.27–36.98)	0.13	0.28
Decorin	25.81 (21.6–31.5)	26.96 (22.4–34.1)	24.35 (19.86–30.53)	24.72 (21.47–29.38)	26.19 (22.55–30.7)	0.12	0.38
Glypican-1	12.27 (9.5–14.66)	10.53 (8.68–13.15)	13.02 (10.89–15.96)	11.94 (9.79–14.78)	13.38 (11.13–14.72)	**0.048***	**0.029***
MEPE	4.75 (3.5–6.16)	4.58 (3.53–6.4)	4.84 (3–5.65)	4.97 (3.48–6.3)	4.61 (3.84–6.09)	0.9	0.44
MMP-2	10.15 (7.21–12.29)	10.53 (8.05–12.74)	9.68 (6.26–11.68)	9.68 (7.31–11.67)	10.3 (7.96–12.08)	0.87	0.45
MMP-7	600.35 (479.52–724)	557.58 (376.06–725.88)	618.34 (521.79–731.27)	578.89 (443.98–707.73)	618.38 (429.86–693.24)	0.41	0.33
MMP-9	16 (9.99–25.9)	16.34 (10.89–26.54)	14.59 (9.64–25.14)	12.17 (8.57–23.85)	11.26 (7.92–15.83)	0.058	0.61
MMP-12	174.06 (117.74–254.65)	203.1 (120.23–285.21)	162.67 (103.41–248.09)	191.68 (120.05–285.21)	201.42 (131.09–307.69)	0.076	0.34
Perlecan	91.68 (71.7–121.15)	91.07 (77.05–107.97)	97.72 (70.92–130.42)	92.7 (71.1–118.92)	104.06 (78.05–137.16)	0.3	0.67
Prolargin	83.12 (71.7–95.16)	84.03 (76.34–98.45)	80.87 (68.79–92.63)	79.15 (69.28–91.95)	75.8 (68.41–87.57)	0.52	0.14
Syndecan-1	87.52 (71.38–120.1)	78.62 (70.11–118.31)	92.68 (74.63–122.92)	84.39 (70.06–102.71)	83.81 (68.62–104.62)	0.89	0.23
Thrombospondin-2	47.67 (39.8–53.26)	49.22 (41.13–53.68)	44.17 (38.03–52.65)	45.42 (38.47–51.32)	43.84 (36.68–48.83)	0.22	0.22
TIMP-4	19.79 (15.92–29.34)	19.15 (14.69–32.18)	20.56 (16.07–26.57)	21.53 (14.17–28.7)	19.12 (14.74–32.54)	0.97	0.79
WISP-1	19.55 (16.51–27.08)	19.28 (16.73–30.56)	21.67 (16.44–27.07)	18.23 (16.44–26.58)	18.76 (15.95–26.68)	0.61	0.87
NT-proBNP	8.79 (4.21–14.25)	9.73 (7.67–14.38)	5.83 (2.41–13.92)	7.67 (3.67–10.65)	4.22 (2.38–8.07)		

Note: Protein levels presented in median (interquartile range).

Abbreviations: AU, arbitrary units; CTD-PAH, connective tissue disease-associated PAH; IPAH/FPAH: Idiopathic or familial PAH; MEPE, matrix extracellular phosphoglycoprotein; MMP, matrix metalloproteinase; NT-proBNP: N-terminal pro-brain natriuretic peptide; PAH, pulmonary arterial hypertension; TIMP-4, tissue inhibitor of metalloproteinases 4; WISP-1, WNT1 inducible signaling pathway protein 1.

*Statistically significant $p < 0.05$ are marked as bold.

[a]At baseline before PAH-specific treatment.

TABLE 3 ROC analyses

Protein (AU)	AUC (95% CI)	Threshold (AU)	Sensitivity	Specificity
CYR61	0.54 (0.36–0.72)	44.73	0.24	0.93
Decorin	0.58 (0.4–0.76)	24.9	0.64	0.67
Glypican-1	0.48 (0.28–0.68)	14.13	0.76	0.47
MEPE	0.53 (0.33–0.72)	4.57	0.61	0.60
MMP-2	**0.76 (0.61–0.91)**	10.73	0.52	0.93
MMP-7	**0.74 (0.56–0.91)**	473.49	0.94	0.6
MMP-9	**0.7 (0.52–0.89)**	9.46	0.94	0.47
MMP-12	**0.68 (0.505–0.85)**	159.48	0.70	0.67
Perlecan	**0.71 (0.54–0.87)**	83.83	0.70	0.67
Prolargin	0.49 (0.31–0.66)	75.01	0.36	0.80
Syndecan-1	0.61 (0.43–0.79)	102.92	0.45	0.87
Thrombospondin-2	0.6 (0.43–0.76)	41.55	0.39	0.93
TIMP-4	**0.76 (0.59–0.93)**	17.47	0.85	0.73
WISP-1	0.52 (0.33–0.71)	29.67	0.85	0.27

Note: MMP-2, -7, -9, -12, perlecan, and TIMP-4 had a significant (bold) AUC not overlapping 0.5.

Abbreviations: AU, arbitrary units; AUC, area under the curve; CI, confidence interval; MEPE, matrix extracellular phosphoglycoprotein; MMP, matrix metalloproteinases; ROC, receiver operating characteristic; TIMP-4, tissue inhibitor of metalloproteinases 4; WISP-1, WNT1 inducible signaling pathway protein 1.

been suggested to occur early in PAH.[8] Consequently, ECM proteins could be of interest as prognostic markers in PAH. The present study, therefore, investigated ECM-related proteins that we previously had found to be altered in the plasma of patients with PAH compared to healthy controls.[13,14] Among the ECM proteins evaluated in the present study, MMP-2 was the most promising prognostic marker of outcome in PAH.

MMP-2 is a gelatinase that degrades collagen and gelatins.[17] It has functions in angiogenesis and vascular smooth muscle cell mitogenesis and migration.[18] MMP-2 has been found to be increased in pulmonary artery endothelial cells (PAEC), in a mouse hypoxia model of PH, contributing to PAEC proliferation, migration, and angiogenesis.[19] Furthermore, in mesenteric arteries, endothelial dysfunction, vascular injury, and remodeling induced by angiotensin 2 have been demonstrated to be dependent on MMP-2 expression.[20] Some studies furthermore indicate that PAH-specific therapies influence MMP-2.[21–23] In a study by Schermuly et al.,[21] MMP-2 levels in lung tissue were induced by monocrotaline (MCT), in a rat model of PAH, and were reduced by the prostacyclin analogue iloprost, indicating a treatment response on MMP-2 levels. In addition, Sun et al.[22] reported that the phosphodiesterase 5 inhibitor sildenafil inhibits endothelin-1 (ET-1)-induced increase of MMP-2 levels in pulmonary artery smooth muscle cells. Furthermore, in an MCT PH model in rats, gene expression and serum levels of MMP-2 were found to be increased, and gene expression but not serum MMP-2 levels were reduced as an effect of bosentan.[23] This is in line with the present study where there was not a significant difference in plasma MMP-2 levels at follow-up with PAH-specific treatment compared to at PAH baseline diagnosis.

Emerging evidence suggests that MMP-2 also has intracellular activity, for instance, cleaving troponin I in cardiomyocytes in ischemia-reperfusion injuries.[24] Thus, that gene expression and serum levels of MMP-2 can differ is not surprising as some of the protein may be acting intracellularly. This underscores that MMP-2 levels derived from peripheral blood may not always reflect protein levels locally in the lung vessels.

In addition to the ET-1 stimulation of MMP-2, the other way around, vascular MMP-2 cleaves the precursor big ET-1 into the potent vasoconstrictor ET-1.[22,25] Bosentan is a dual endothelin receptor types A and B antagonist and part of the ERAs that targets the endothelin pathway, which is one of the main pathological pathways in PAH.[4] Increased plasma levels of MMP-2 may thus potentially reflect an increased activation of ET-1.

Of interest, the present study did not find a difference in MMP-2 levels at PAH follow-up compared to PAH baseline, despite that the patients were treated with

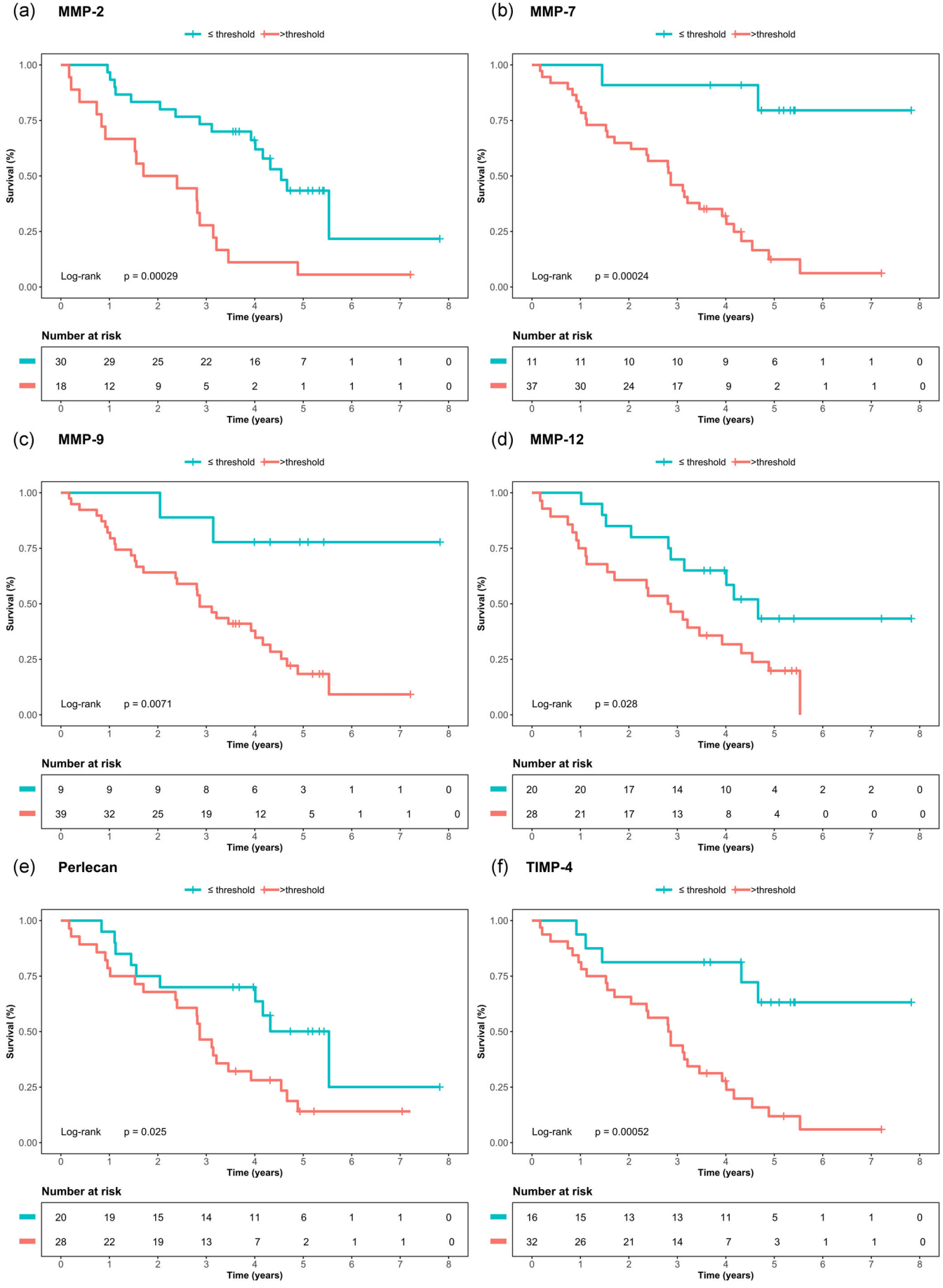

FIGURE 2 (See caption on next page)

PAH-specific treatment at PAH follow-up. Thus, despite the patients being treated with ERAs, PDE5i, or both, one could expect a reduction of the MMP-2 levels, which did not occur. This may be attributable to an interplay of the therapies or that the change of MMP-2 levels predominantly occurs in the local lung tissue. The present study was, however, not designed to determine whether circulating protein levels represent local levels in the pulmonary vasculature, but instead to evaluate whether local plasma levels are related to hemodynamics, prognosis, and risk stratification in PAH.

TABLE 4 Univariable and multivariable Cox-regression analysis

Explanatory variable	HR (95% CI)	*p* value
Univariable Cox-regression		
Age, years	1.038 (1.003–1.074)	**0.032**
Female	0.355 (0.15–0.841)	**0.019**
MMP-2 (AU)	1.141 (1.033–1.259)	**0.009**
MMP-7 (AU)	1.002 (1.0003–1.004)	**0.023**
MMP-9 (AU)	1.018 (0.992–1.044)	0.169
MMP-12 (AU)	1.002 (0.999–1.005)	0.263
Perlecan (AU)	1.010 (1.002–1.019)	**0.02**
TIMP-4 (AU)	1.038 (1.007–1.069)	**0.015**
Multivariable Cox-regression		
MMP-2 (AU)	1.126 (1.011–1.255)	**0.031**
Age, years	1.038 (0.999–1.078)	0.057
Female	0.213 (0.084–0.541)	**0.001**
MMP-7 (AU)	1.002 (0.9997–1.004)	0.098
Age, years	1.041 (1.001–1.084)	**0.046**
Female	0.197 (0.075–0.52)	**0.001**
Perlecan (AU)	1.0099 (1.0004–1.0196)	**0.041**
Age, years	1.045 (1.006–1.086)	**0.023**
Female	0.226 (0.09–0.566)	**0.001**
TIMP-4 (AU)	1.037 (1.003–1.071)	**0.031**
Age, years	1.044 (1.004–1.086)	**0.032**
Female	0.201 (0.079–0.513)	**<0.001**

Note: Age, female sex, MMP-2, MMP-7, perlecan, and TIMP-4 were predictors of transplant-free survival. Bold indicates statistical significance at $p < 0.05$.

Abbreviations: AU, arbitrary unit; CI, confidence interval; HR, hazard ratio; MMP, matrix metalloproteinase; TIMP-4, tissue inhibitor of metalloproteinases 4.

The prognostic value of MMP-2 has previously been investigated in PH by Tiede et al.[26] who showed an increased mortality risk in patients with PH, having MMP-2 levels above median values, with an HR of 2.69 in a multivariable Cox-regression model. This is larger than the HR of 1.13 in the present study There are, some differences between the studies. Whereas Tiede et al.[26] investigated the prognostic value of MMP-2 in PH, the present study investigated PAH patients only. Furthermore, the present study displays an HR for continuous MMP-2 levels instead of a dichotomized variable with an HR for supra-median versus inframedian protein levels. This could partly explain the size difference of the HR of the respective studies. Regardless, both studies indicate an increased risk of mortality with higher MMP-2 levels, stressing the potential importance of MMP-2 as a prognostic marker.

In another study, Wetzl et al.[27] investigated the MMP-2/TIMP-4 ratio as a predictor of survival in IPAH. They demonstrated correlations with mPAP, PVR, estimated glomerular filtration rate and tricuspid annular plane systolic excursion. Additionally, they found that patients with a lower MMP-2/TIMP-4 ratio had better survival. Interestingly, the present study found that increasing levels of MMP-2, as well as TIMP-4, were associated with worse prognosis in the multivariable Cox-regression model, whereas an increase in TIMP-4 levels was expected to have a protective effect. On the contrary to Wetzl et al.,[27] the present study included patients with CTD-PAH in addition to patients with IPAH/FPAH, which could have influenced the results as TIMP-4 levels may differ between those two diverse etiologies of PAH. Elias et al.[28] reported increased circulating levels of TIMP-4 in systemic sclerosis patients with high pulmonary artery systolic pressures. The present study did not, however, demonstrate a significant difference in MMP-2 levels between the IPAH/FPAH and the CTD-PAH group.

Older patients with IPAH have been found to exhibit worse survival than young patients.[29] Similarly, the present study found age to be a predictor of transplant-free survival in the univariable Cox-regression model. The present study furthermore found female sex to be a

FIGURE 2 Kaplan–Meier plots. Kaplan–Meier plots with Log-rank tests for (a) MMP-2, (b) MMP-7, (c) MMP-9, (d) MMP-12, (e) perlecan, (f) TIMP-4. $p < 0.05$ were considered statistically significant. Protein level threshold set as the cut-off (as described in Table 3) yielding the highest Youden's index of sensitivity and specificity for the outcome death. MMP, matrix metalloproteinase; TIMP-4, tissue inhibitor of metalloproteinases 4

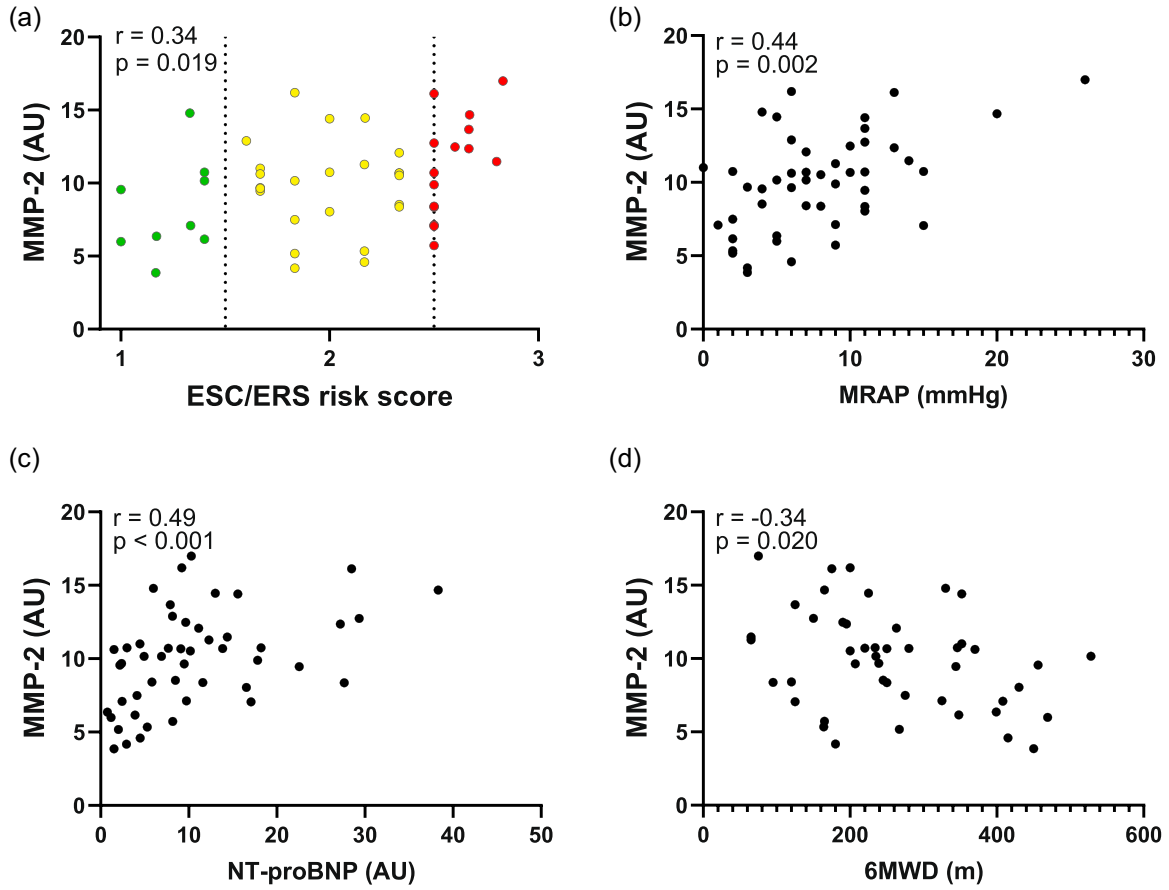

FIGURE 3 MMP-2 correlates with ESC/ERS risk scores and risk parameters. 6MWD, 6-min walk distance; AU, arbitrary unit; NT-proBNP, N-terminal pro-brain natriuretic peptide; MMP-2, matrix metalloproteinase-2; MRAP, mean right atrial pressure; *r*, Spearman's correlation coefficient

predictor of transplant-free survival, which is in line with the findings of Kozu et al.,[30] where males had an increased risk of mortality, but contrary to Hjalmarsson et al.[29] where sex did not predict survival. Furthermore, Kjellström et al.[31] reported that men with incident IPAH had worse crude survival compared to women but not after adjusting for age. A possible explanation could be the large percentage difference in sexes, with 88% females in the present study compared to 56% females in the study of Hjalmarsson et al.[29] However, due to the small number of men included in the present study, the results regarding the predictive value of female sex for transplant-free survival in PAH should be interpreted with caution.

In the present PAH cohort, glypican-1 levels at follow-up were increased compared to baseline. This indicates that the change in glypican-1 levels may be related to PAH-specific treatment, as we previously have found glypican-1 levels to be decreased at PAH diagnosis compared to healthy individuals.[14] Glypican-1 is a mediator of flow-induced endothelial nitric oxide synthase activation.[32] Thus, it is plausible that the changes in glypican levels are influenced by PAH therapies, such as ERAs, PDE5is, soluble guanylate cyclase (sGC) stimulators, and prostacyclin analogues as they promote vasodilatation and in the case of PDE5i and sGC stimulators directly influence nitric oxide signaling.[4] None of the other investigated proteins displayed a significant change in plasma levels from PAH baseline to follow-up. This is of interest and may indicate that they are not directly influenced by current PAH-specific therapies.

The strengths of the present study included a study population that encompassed only incident PAH cases, naïve of PAH-specific treatment. This allows for an investigation of plasma biomarker levels in patients with PAH without the interference of treatment at baseline and allows for prognostic estimation at the time of diagnosis. Moreover, a thorough assessment also at an early follow-up allowed for the evaluation of hemodynamics and risk score during disease progression in relation to baseline diagnosis. The PEA technique furthermore allows for analysis of large amounts of proteins simultaneously with low cross-reactivity and only requires small amounts of plasma samples. The

limitations of the present study include that the PEA output is in relative values among the same protein in different samples, and values are not directly comparable for two different proteins. A validation cohort from another PAH center was neither available nor utilized, which will be of great importance in future additional studies. In the present patient cohort, less than a third of the patients with early follow-up assessments had received initial combination therapy. This is attributable to that the majority of the patients were included in LCPR before 2015, that is, before that the beneficial effect of initial combination therapy compared to initial monotherapy was demonstrated by the AMBITION trial.[33] This may have had an impact on prognosis and survival as patients were diagnosed and treated according to the prevailing guidelines available at that time. Data on PAH-specific treatment later than the first initial follow-up were not available for analysis in the present study.

Our analysis did not include the endogenous MMP-2 inhibitor TIMP-2 or α-2 macroglobulin, a general inhibitor of MMPs in plasma, as it was not available for analysis.[34] Thus, our results apply specifically to MMP-2 levels in plasma and do not address the relation between MMP-2 and its endogenous inhibitors.

In conclusion, the present study suggests that high levels of the ECM-related proteins MMP-2, perlecan, and TIMP-4 are associated with poor prognosis in PAH. Moreover, increased MMP-2 levels at PAH diagnosis are associated with worse ESC/ERS risk scores, as well as worsening of right heart function and exercise capacity as defined by MRAP, NT-proBNP, and 6MWD. These findings indicate that high MMP-2 may be useful as a negative prognostic marker, indicating the need for escalation of PAH-specific treatment. While we found that elevated MMP-2 levels correlated with poor prognosis in PAH, further validation in larger cohorts are encouraged to better determine this association.

ACKNOWLEDGMENTS

We acknowledge the support of the staff at the Haemodynamic Lab, The Section for Heart Failure and Valvular Disease, VO Heart and Lung Medicine, Skåne University Hospital, Lund, Sweden, and The Department of Clinical Sciences, Lund, Cardiology, Lund University, Lund, Sweden. Special thanks to Anneli Ahlqvist for support in assembling plasma samples and LCPR registration. In addition, we acknowledge the biobank services and retrieval of blood samples from LCPR performed at Lab medicine Skåne, University, and Regional Laboratories, Region Skåne, Sweden. This study was funded by unrestricted research grants from the Swedish Society of Pulmonary Hypertension, "Avtal om läkarutbildning och forskning" (ALF), and a noninterventional investigator-initiated study research grant from Janssen-Cilag AB. The founders had no role in the literature review selection, analysis, and interpretation of the data or publication of the manuscript.

CONFLICT OF INTERESTS

Mr. Arvidsson reports an unrestricted research grant from The Swedish Society of Pulmonary Hypertension. Mr. Ahmed, Miss Säleby, and Mr. Hesselstrand report no conflicts of interest. Dr. Rådegran reports personal lecture fees from Actelion Pharmaceuticals, Bayer Health Care, GlaxoSmithKline, Janssen-Cilag AB, and Nordic Infucare, outside the submitted work. Dr. Rådegran has received unrestricted research grants from Actelion Pharmaceuticals, GlaxoSmithKline, and a noninterventional investigator-initiated study research grant from Janssen-Cilag AB. Dr. Rådegran is and has been primary-, or co-, investigator in; clinical PAH trials for Acceleron, Actelion Pharmaceuticals, Bayer, GlaxoSmithKline, Janssen, Pfizer, and United Therapeutics, and in clinical heart transplantation immunosuppression trials for Novartis.

ETHICS STATEMENTS

The study was conducted in accordance with the declaration of Helsinki. It was approved by the local ethics committee in Lund (approval numbers 2010/114, 2010/248, 2010/442, 2011/368, 2015/270), and all participants had given their informed and written consent.

AUTHOR CONTRIBUTIONS

Mattias Arvidsson, Abdulla Ahmed, Joanna Säleby, Roger Hesselstrand, and Göran Rådegran designed the study. Mattias Arvidsson drafted the manuscript and analyzed the data. Mattias Arvidsson, Abdulla Ahmed, Joanna Säleby, Roger Hesselstrand, and Göran Rådegran interpreted the results and revised the article critically.

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How to cite this article: Arvidsson M, Ahmed A, Säleby J, Hesselstrand R, Rådegran G. Plasma matrix metalloproteinase 2 is associated with severity and mortality in pulmonary arterial hypertension. Pulmonary Circulation. 2022;12:e12041. https://doi.org/10.1002/pul2.12041