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Plasma von Willebrand factor as a predictor of survival in pulmonary arterial hypertension associated with congenital heart disease

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Abstract

Biomarkers have been identified for pulmonary arterial hypertension, but are less well defined for specific etiologies such as congenital heart disease-associated pulmonary arterial hypertension (CHDPAH). We measured plasma levels of eight microvascular dysfunction markers in CHDPAH, and tested for associations with survival. A cohort of 46 inoperable CHDPAH patients (age 15.0 to 60.2 years, median 33.5 years, female:male 29:17) was prospectively followed for 0.7 to 4.0 years (median 3.6 years). Plasma levels of von Willebrand factor antigen (VWF:Ag), tissue plasminogen activator (t-PA) and its inhibitor (PAI-1), P-selectin, reactive C-protein, tumor necrosis factor alpha, and interleukin-6 and -10 were measured at baseline, and at 30, 90, and 180 days in all subjects. Levels of six of the eight proteins were significantly increased in patients versus controls (13 to 106% increase, $P < 0.003$). Interleukin-10 level was 2.06 times normal ($P = 0.0003$; Th2 cytokine response). Increased levels of four proteins (t-PA, PAI-1, P-selectin, and interleukin-6) correlated with disease severity indices ($P < 0.05$). Seven patients died during follow-up. An average VWF:Ag (mean of four determinations) above the level corresponding to the 95th percentile of controls (139 U/dL) was independently associated with a high risk of death (hazard ratio = 6.56, 95%CI = 1.46 to 29.4, $P = 0.014$). Thus, in CHDPAH, microvascular dysfunction appears to involve Th2 inflammatory response. Of the biomarkers studied, plasma vWF:Ag was independently associated with survival.

Key words: Pulmonary hypertension; Congenital heart disease; Eisenmenger syndrome; Endothelial dysfunction; von Willebrand factor; Th2 cytokine response

Introduction

There has been growing interest in the identification of biomarkers in pulmonary arterial hypertension (PAH). The most extensively analyzed marker is the cardiac hormone brain natriuretic peptide (BNP), either the whole molecule or its intact N-terminal-pro-fragment (NT-proBNP). Increased circulating levels of these peptides have been associated with a poor prognosis in pulmonary hypertension (1), as they are believed to reflect right ventricular dysfunction. In addition, serum uric acid levels correlate with the severity of idiopathic PAH and are associated with mortality (2), as is also the case for PAH due to the Eisenmenger syndrome (3). The circulating levels of endothelin-1 (4) and D-dimer (5) have also been proposed as biomarkers in pulmonary hypertension.

We have shown (6) that increased circulating levels of von Willebrand factor antigen (VWF:Ag), a known marker of endothelial dysfunction, were associated with decreased short-term survival in “primary” and “secondary” (Eisenmenger syndrome) pulmonary hypertension. However, the small patient populations and the short follow-up time prevented us from performing a detailed survival analysis. Others, however, have recently confirmed that VWF:Ag is

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an independent predictor of survival (7) in PAH (idiopathic, familial or associated with anorexigen use). In the present study, we looked at eight circulating plasma proteins to characterize microvascular dysfunction in the specific setting of PAH associated with congenital heart disease and to determine if they reflected the severity of the disease. We measured plasma levels of VWF:Ag, tissue-type plasminogen activator (t-PA), plasminogen activator inhibitor 1 (PAI-1) and P-selectin to monitor endothelial dysfunction. We also measured plasma levels of reactive C-protein and interleukin-6 (IL-6) as nonspecific markers of inflammation, and tumor necrosis factor alpha (TNF-α) and IL-10 as markers of Th1 and Th2 cytokine response, respectively. Additionally, we prospectively investigated possible associations between these biomarkers and survival over a period of 4 years. Demographic, functional and treatment-related variables were included in the analysis.

Patients and Methods

Study population and design

The patient population consisted of 46 patients from a previous study of 60 consecutive individuals with PAH who were randomly assigned to 6-month rosuvastatin or placebo treatment in order to investigate the effects of the statin on endothelial dysfunction markers (8). In the initial study, the first patient was enrolled in July 2005; in the present study, the follow-up was terminated in February 2010. In 46 patients (statin:placebo, 23:23), PAH was associated with uncorrected, inoperable congenital cardiac defects (Table 1). Of these, 18 were acyanotic, while 28 individuals had typical features of the Eisenmenger syndrome, with systemic oxygen saturation <90%. All patients were on chronic anticoagulant therapy with warfarin, and some of them were treated with oral vasodilator therapy for PAH. Vasodilators were given to all functional class III patients, and some who were class II based on the life style and the degree of exercise-induced hypoxemia.

All patients had demographic data and functional parameters (functional class, distance walked in 6 min, systemic oxygen saturation, and noninvasive hemodynamics) recorded at baseline. Plasma levels of 4 endothelial dysfunction markers were determined at baseline, and 30, 90, and 180 days later during treatment (rosuvastatin versus placebo). Using additional plasma samples that were stored in parallel, we subsequently extended the biochemical analyses to include three cytokines, and C-reactive protein. Demographic and functional data obtained at patient entry in the first study, as well as results of all biochemical analyses, were used in the present study to define a general endothelial and inflammatory response for this group, and to identify factors that might be associated with an increased risk of death.

The study protocol was approved by the Scientific Committee of Instituto do Coração (SDC2613/05/033), and the Ethics Committee of Hospital das Clínicas (CAPPesq 380/05), Faculdade de Medicina, Universidade de São Paulo, São Paulo, SP, Brazil. Written informed consent was obtained from all patients (or their parents in case of adolescents) before inclusion in the study.

Demographic, functional and treatment-related variables

Patient age and gender were recorded at baseline. The functional class was graded according to the classification modified for pulmonary hypertension by the World Health Organization. The six-minute walk distance was determined according to a standardized protocol (9). Systemic oxygen saturation was measured by pulse oximetry, at rest and at the end of the six-minute walk. Pulmonary artery systolic and mean pressures were estimated noninvasively by Doppler echocardiography (10,11). Catheterization data that confirmed the diagnosis of PAH and the congenital heart defect were obtained for all patients at different times prior to patient inclusion in the trial, and for this reason were not used in the analyses that were carried out in the study. The use of rosuvastatin during the first 6 months and the duration of vasodilator therapy (bosentan, sildenafil or a combination of both) were considered in the survival analysis.

Biochemical analyses

Proteins were measured by high-sensitivity enzyme-linked immunosorbent assays. Commercial kits were purchased from R&D Systems Inc., USA (P-selectin, TNF-α, IL-6, and IL-10), Diagnostica Stago, France (VWF:Ag, t-PA and PAI-1), or American Diagnostica Inc., USA (C-reactive protein). The following results were obtained for each patient (and each biomarker): 1) baseline plasma level;

Table 1. Congenital cardiac anomalies of the patients studied.

Anomaly	No. of patients
Ventricular septal defect	19
Atrial septal defect	10
Atrioventricular septal defect	7
Patent ductus arteriosus	4
Atrial and ventricular septal defects	2
Ventricular septal defect and patent ductus arteriosus	1
Double outlet right ventricle	1
Transposition of the great arteries with a ventricular septal defect	1
Single ventricle	1
Total	46

2) average level (mean of determinations performed at baseline and at 30, 90, and 180 days); 3) highest level of four determinations. Control plasmas were obtained from 30 healthy volunteers (consecutively selected blood donors or adolescents with innocent cardiac murmur) in the same age range as the patients. Blood donors were non-smokers, had no clinical history of systemic hypertension, diabetes, vascular diseases or stroke, and had no evidence of ongoing infections or disorders. In adolescents, the diagnosis of innocent cardiac murmur was established after screening echocardiography. For each protein, upper levels corresponding to the 90th and 95th percentiles of controls were determined and tested as cutoff values in the prediction of increased risk of death.

Statistical analysis

Comparisons between patients and controls or patient groups were performed by the non-parametric Mann-Whitney test. Correlations of protein levels with demographic and functional parameters were analyzed by calculating the Spearman correlation coefficient. Univariate and multivariable Cox regression models were used to investigate possible associations of the specified proteins with survival time. Plasma levels were analyzed both as continuous and as categorical variables (i.e., above versus below the level corresponding to the 90th and 95th percentiles of controls). Once a potential risk factor was identified, it was analyzed for the possible existence of confounders. When each potential confounder was added to the model, a >20% change (decrease) in the hazard ratio associated with the risk factor was considered to indicate confounding. Kaplan-Meier survival curves were constructed and compared using the log-rank (Cox-Mantel) test. In all procedures, the level of significance was set at 0.05.

Results

The demographic and functional data of 46 patients included in the study are summarized in Table 2. Twenty-one received vasodilator therapies for PAH (bosentan, sildenafil, or a combination of both, mean treatment duration of 2.25 years). During a follow-up of 0.7 to 4.0 years (median 3.6 years), 7 patients died, 5 of them with initial Eisenmenger syndrome presentation. A fatal outcome was associated with worsening of functional capacity, right ventricular failure and progressive hypoxemia. In 3 patients, progression of pulmonary arterial thrombosis (despite chronic use of warfarin) was considered as an important event associated with death. Two patients died suddenly. There were no deaths during the first 6 months of follow-up.

Baseline and average plasma levels of endothelial dysfunction markers and cytokines are shown in Table 3. Levels of six plasma proteins were significantly elevated in patients compared to controls. The predominant cytokine response was of the Th2 subtype, as suggested by the increased levels of IL-10 relative to TNF-α. As shown in Table 4, altered plasma levels of four proteins correlated significantly with disease severity indices. Plasma VWF:Ag, reactive C-protein, TNF-α, and IL-10 did not correlate significantly with any of the variables listed in Table 4. Furthermore, there was no correlations between protein levels and pulmonary artery systolic pressure or functional class.

A significant association was observed between the average plasma VWF:Ag level and survival. An average VWF:Ag above the 90th percentile of normal subjects (135 U/dL) was associated with increased risk of death (P = 0.0404), as was the level corresponding to the 95th percentile of controls (139 U/dL; P = 0.0140). Four of the 9 patients with an average VWF:Ag level higher than 139 U/dL died within 2.75 years of follow-up. Neither the baseline nor the highest level of VWF:Ag correlated with survival. The highest value of 4 determinations seemed to be less specific, since it was >139 and >135 U/dL in 19 and 24 patients, respectively (with the same 4 deaths in each situation). Survival curves for the different levels of VWF:Ag are shown in Figure 1. Plasma levels of other proteins did not correlate significantly with survival. No association with survival could be established when protein levels were analyzed as continuous variables.

The risk of death associated with an average VWF:Ag above the level corresponding to the 90th or 95th percentile of controls was high (respective hazard ratios of 4.79, 95%CI = 1.07-21.44, and of 6.56, 95%CI = 1.46-29.40). As shown in Table 5, the hazard ratio related to increased VWF:Ag was not significantly reduced by the inclusion of any potential confounder in the model. None of the demographic, functional or treatment-related variables listed in Table 5 had a significant association with survival during the period of the study.

Table 2. Demographic and functional data of the patients studied (N = 46).

Age (years)	33.5 (15.0-60.2)
Female:male	29:17
Acyanotic:Eisenmenger syndrome	18:28
Functional class (II:III)	38:8
$PAP_{systolic}$ (mmHg)	97 ± 25
PAP_{mean} (mmHg)[a]	52 ± 17
Resting SpO_2 (%)	89 (79-96)
6-min walk SpO_2 (%)	71 (31-94)
6-min walk distance (m)	402 ± 112
Hematocrit (%)	52 (37-79)

Data are reported as means ± SD, or median (range). PAP = pulmonary artery pressure estimated by Doppler-echocardiography; SpO_2 = peripheral oxygen saturation. [a]Available for 31 patients.

Table 3. Endothelial dysfunction markers and cytokines of patients (N = 46) and controls (N = 30).

	VWF:Ag (U/dL)	t-PA (ng/mL)	PAI-1 (ng/mL)	P-selectin (ng/mL)	CRP (µg/mL)	TNF-α (pg/mL)	IL-6 (pg/mL)	IL-10 (pg/mL)	IL-10/ TNF-α
Controls									
Median	109	5.58	16.46	24.79	4.14	0.60	0.90	0.94	2.02
Lower	62	2.87	5.23	8.82	0.67	0.08	0.29	0.003	0.04
Upper	147	10.25	42.88	53.96	9.91	2.86	8.48	3.32	27.26
90th percentile	135	8.50	35.28	39.66	9.45	2.25	6.57	2.37	4.84
95th percentile	139	9.70	41.15	49.84	9.76	2.86	8.02	2.58	27.26
Patients (baseline)									
Median	123	7.82	24.53	43.12	6.50	0.70	1.44	1.94	3.18
Lower	73	3.01	9.69	10.71	0.18	0.11	0.37	0.04	0.03
Upper	177	23.93	134.71	136.94	11.80	5.61	10.58	11.88	20.13
Patients (average)[a]									
Median	125	7.72	25.25	42.70	6.04	0.84	1.80	1.89	2.92
Lower	60	4.11	10.54	11.51	0.23	0.13	0.34	0.42	0.23
Upper	197	21.67	127.55	138.12	13.00	5.93	8.67	6.24	31.73
P value (baseline *vs* controls)	0.0025	<0.0001	<0.0001	<0.0001	0.4961	0.3492	0.0006	0.0003	0.0414
P value (average *vs* controls)	0.0015	<0.0001	<0.0001	<0.0001	0.5355	0.2244	<0.0001	0.0002	0.0727

VWF:Ag = plasma von Willebrand factor antigen; t-PA = plasma tissue-type plasminogen activator; PAI-1 = plasma plasminogen activator inhibitor 1; CRP = plasma C-reactive protein; TNF-α = plasma tumor necrosis factor alpha; IL-6 and IL-10 = plasma levels of interleukin-6 and -10, respectively; IL-10/TNF-α = plasma IL-10 relative to TNF-α concentration. [a]Mean value of determinations performed at baseline, 30, 90, and 180 days. P values obtained with the Mann-Whitney test.

Table 4. Endothelial dysfunction markers and cytokines versus demographic and functional parameters at baseline (N = 46).

	Age	Gender[a]	Clinical presentation[b]	Resting SpO_2	6-min walk SpO_2	6-min walk distance	Hematocrit
t-PA	r_s = 0.35 P = 0.0172	NS	P = 0.0046	r_s = -0.29 P = 0.049	NS	NS	r_s = 0.49 P = 0.0005
PAI-1	NS	NS	P = 0.0133	r_s = -0.31 P = 0.0345	NS	r_s = -0.39 P = 0.0073	NS
P-selectin	NS	P = 0.004	P = 0.0069	NS	r_s = -0.31 P = 0.0379	NS	r_s = 0.61 P < 0.0001
IL-6	NS	P = 0.0373	NS	NS	NS	NS	r_s = 0.35 P = 0.0186

[a]Male relative to female: P-selectin and IL-6 levels were 71 and 59% higher in the former, respectively. [b]Eisenmenger syndrome (with resting SpO_2 <90%), relative to acyanotic patients: t-PA, PAI-1, and P-selectin levels were 44, 60, and 64% higher in the former, respectively. SpO_2 = peripheral oxygen saturation; t-PA = plasma tissue-type plasminogen activator; PAI-1 = plasma plasminogen activator inhibitor 1; IL-6 = interleukin-6. Correlations were tested by the Spearman correlation coefficient (r_s). Comparisons between groups were performed by the Mann-Whitney test. NS = not significant.

Discussion

Microvascular dysfunction has been extensively studied in pulmonary hypertension (12). It includes decreased expression of prostacyclin and nitric oxide synthase and increased expression and secretion of vasoconstrictors (13-16). There is a shift toward a hypercoagulable state, with decreased expression of thrombomodulin (17) and increased production of tissue factor (18). There are increased plasma levels of proteins that are normally stored within endothelial Weibel-Palade bodies (von Willebrand factor, P-selectin and t-PA) (6,17). Circulating endothelial cells and endothelial progenitor cells have been described in PAH (19), particularly in the congenital heart disease-associated form (20). Some of these markers seem to be related to the severity of the disease or change favorably in response to treatments (17,20,21).

In the present study, specifically focused on PAH associated with congenital heart disease, circulating levels of four endothelial dysfunction markers and two cytokines were

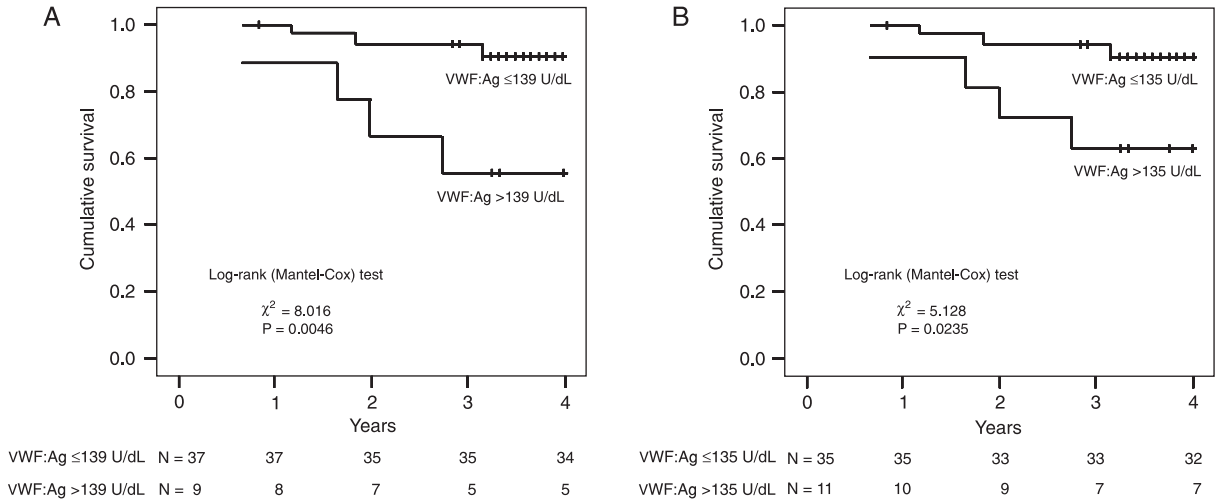

Figure 1. Kaplan-Meier survival curves for patients with pulmonary arterial hypertension associated with congenital heart disease according to plasma von Willebrand factor antigen (VWF:Ag). *A*, Patients with an average VWF:Ag (mean of determinations performed at baseline, 30, 90, and 180 days) above the 95th percentile of controls (in this case, 139 U/dL) had a decreased life expectancy (mean survival of 3.01 years) compared with those at or below this limit (mean survival of 3.85 years). *B*, Patients with an average VWF:Ag above the 90th percentile of controls (in this case, 135 U/dL) also had a shortened survival time (mean 3.19 years) compared with those at or below this level (mean survival 3.82 years). (+) represents censored cases.

elevated compared to normal subjects. The abnormalities in the plasma levels of four proteins correlated with clinical parameters of severity of the disease such as the magnitude of hypoxemia, erythrocytosis and decreased physical capacity. Of notice, hypoxia has been shown to induce endothelial PAI-1 gene expression and secretion of P-selectin and von Willebrand factor protein (22,23). In this patient population, plasma VWF:Ag did not correlate with any of the functional parameters, but was identified as an independent predictor of survival. We also observed a mild but significant inflammatory response, which seemed to be predominantly Th2-cytokine related, as judged by the increased levels of IL-10 relative to TNF-α. Taken together with previous reports in humans (24), this observation points toward a pathophysiological role of inflammation in PAH associated with congenital heart disease, similar to its role in idiopathic PAH. Interestingly, in a recent report (25), a Th2 immune response was shown to induce pulmonary artery remodeling in an animal model.

Quantitative and qualitative abnormalities of circulating von Willebrand factor have been reported in PAH asso-

Table 5. Multivariate analysis of the association of plasma VWF:Ag with the risk of death (N = 46) before and after addition of possible confounders.

	HR (95%CI)[a]	P	HR (95%CI)[b]	P
Unadjusted	4.79 (1.07-21.44)	0.040	6.56 (1.46-29.40)	0.014
Adjusted for:				
Age	4.23 (0.86-20.70)	0.075	5.94 (1.23-28.75)	0.027
Gender	4.71 (1.03-21.57)	0.046	6.73 (1.41-32.17)	0.017
Clinical presentation[c]	4.67 (1.01-21.68)	0.049	6.39 (1.41-28.95)	0.016
Functional class	5.06 (1.12-22.91)	0.035	6.61 (1.47-29.79)	0.014
Resting SpO_2	4.51 (1.00-20.37)	0.050	5.75 (1.26-26.34)	0.024
6-min walk SpO_2	4.83 (1.07-21.78)	0.040	6.41 (1.42-28.83)	0.015
6-min walk distance	4.98 (1.09-22.86)	0.039	6.86 (1.49-31.51)	0.013
Hematocrit	4.87 (1.08-21.89)	0.039	6.58 (1.47-29.52)	0.014
PAH advanced therapy[d]	4.70 (1.04-21.13)	0.044	6.88 (1.52-31.16)	0.012
Duration of treatment[d]	4.85 (1.08-21.75)	0.039	5.75 (1.27-26.05)	0.023
$PAP_{systolic}$	4.88 (1.09-21.89)	0.038	8.45 (1.72-41.43)	0.009
Statin[e]	5.30 (1.16-24.20)	0.031	7.71 (1.65-35.95)	0.009

[a]HR (95%CI) = hazard ratio (95% confidence interval) associated with an average VWF:Ag (mean of determinations performed at baseline, 30, 90, and 180 days) above the level corresponding to the 90th percentile of controls. [b]HR (95%CI) associated with an average VWF:Ag above the level corresponding to the 95th percentile of controls. [c]Eisenmenger syndrome (with resting SpO_2 <90%) present or absent. [d]Treatment for pulmonary arterial hypertension included bosentan, sildenafil or a combination of both. [e]Used in the study from which the present cohort was derived (8). SpO_2 = peripheral oxygen saturation; PAH = pulmonary arterial hypertension; PAP = pulmonary artery pressure estimated by Doppler-echocardiography. The search for possible confounders was carried out using bivariate Cox regression analysis.

ciated with congenital heart defects (26). In our patients, increased plasma VWF:Ag levels (i.e., above the values corresponding to the 90th or 95th percentile in controls) were associated with worse outcome, a relationship that was not observed with the other proteins analyzed. This association was based on analyses that were performed with no missing data, and persisted after adjustments for multiple possible confounding factors. Thus, as observed in patients with PAH of other etiologies (7), our preliminary observations suggest that plasma VWF:Ag may be a predictor of prognosis in the congenital heart disease-associated form of the disease. It is important to note that neither the result of a single VWF:Ag determination (at baseline) nor the highest level obtained over six months of observation correlated significantly with survival. Von Willebrand factor is an acute-phase reactant, and its level may vary and can become transiently elevated in a number of acute inflammatory and infectious conditions (27,28). Therefore, in the specific setting of PAH associated with congenital heart disease, persistently elevated VWF:Ag levels (as indicated by increased mean value of replicate observations) seem to be necessary for a "high-risk" status to be characterized. Thus, at least four determinations over a 6-month period may be required. How levels of biomarkers vary over time in patients is largely unknown and needs to be considered in future studies. In normal subjects, a persistent elevation of plasma VWF:Ag is not expected to occur if there are no ongoing vascular, thrombotic or inflammatory disorders. This was the case for controls in the present study. Although it would be desirable to have sequential determinations in controls as well (their absence being a limitation of the study), in practice, this approach has not been used in longitudinal studies (7).

Plasma VWF:Ag is a predictor of prognosis in several acute and chronic disorders (29-32). One may speculate that thrombosis is the link between increased infectious VWF:Ag and poor outcome in PAH. As a powerful vascular growth factor, thrombin may serve as an important substrate for vascular proliferation (33). In the present study, pulmonary arterial thrombosis was observed in three of the seven patients who subsequently died. Alternatively, it is possible that the amount of circulating von Willebrand factor protein reflects the magnitude of other endothelium-related biological events that are themselves determinants of progression of the disease (23,34-39).

Regarding the impact of treatment on survival, in a recent study on adults with Eisenmenger syndrome (40), both the functional class and the use of advanced therapies for PAH significantly influenced survival. In our study, survival was not influenced by the functional status, the use of specific drugs or the duration of treatments. Differences between the studies may be related to the characteristics of patient populations. The majority of our patients were only mildly symptomatic (38 patients in class II), and 18 subjects did not have the full picture of the Eisenmenger syndrome.

Microvascular dysfunction is present in PAH associated with congenital heart disease, indicating an increased inflammatory mechanisms in which the Th2 cytokine response plays a role. Plasma levels of microvascular dysfunction markers correlate with clinical indices of disease severity in these patients. Our data also suggest that in these patients, a sustained increase in plasma VWF:Ag level is indicative of a poor prognosis. Further studies of larger populations of patients with PAH are warranted. These findings suggest that therapies that target a reversal of the prothrombotic tendency of the pulmonary circulation may be beneficial.

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