ORIGINAL RESEARCH

Prognostic Significance of Biomarkers in Pulmonary Arterial Hypertension

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Abstract

Rationale: Pulmonary arterial hypertension (PAH) is a rare progressive disease of the pulmonary vasculature that is characterized by endothelial dysfunction, inflammation, and right ventricular dysfunction.

Objectives: The main objective was to determine whether endothelial, inflammatory, and cardiac biomarkers would be associated with the World Health Organization functional assessment and survival in patients with PAH.

Methods: We performed a retrospective cohort study of patients with PAH enrolled in the Randomized Clinical Trial of Aspirin and Simvastatin for Pulmonary Arterial Hypertension (ASA-STAT). Biomarkers (N-terminal fragment of pro-BNP [NT-pro-BNP], von Willebrand factor [vWF], soluble P selectin, C-reactive protein, total and high-density lipoprotein cholesterol, triglycerides, tumor necrosis factor, IL-6, β-thromboglobulin, and thromboxane B_2) were measured at baseline. Patients from the study were followed until lung transplantation, death, or August 1, 2013. Ordinal logistic regression and Cox regression analyses were performed.

Measurements and Main Results: Sixty-five patients with PAH were enrolled. The mean age was 51 years, and 86% were women. Higher vWF activity, lower high-density lipoprotein cholesterol, and higher thromboxane B_2 levels were associated with worse World Health Organization functional class after adjustment for age, sex, and etiology of PAH. Higher NT-pro-BNP levels, lower vWF activity, and lower total cholesterol were associated with an increased risk of death or lung transplant after adjustment for age, sex, etiology of PAH, and 6-minute-walk distance.

Conclusions: In patients with PAH, lower vWF activity and cholesterol levels and higher NT-pro-BNP levels at baseline were associated with an increased risk of death or transplantation.

Clinical trial registered with www.clinicaltrials.gov (NCT00384865).

Keywords: biomarkers; pulmonary arterial hypertension; World Health Organization functional class; survival

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Pulmonary arterial hypertension (PAH) is a rare and progressive disease of the pulmonary vasculature that leads to exercise limitation and eventually right ventricular (RV) failure and death. Endothelial dysfunction of the pulmonary vasculature is characterized by inflammation, impaired vasoactive balance, platelet activation, and *in situ* thrombosis (1–3). Increased afterload leads to RV strain and dysfunction, with resultant release of molecular mediators from the heart.

Biomarkers that reflect underlying pathophysiologic processes as well as the patient's functional status would be helpful in diagnosis, evaluation, and follow up of patients with PAH. Several potential biomarkers have been evaluated in PAH; however, their usefulness in predicting functional status and survival in PAH has not been definitively established. Current PAH guidelines only recommend the use of brain natriuretic peptide (BNP) or the N-terminal fragment of pro-BNP (NT-pro-BNP) at diagnosis and for longitudinal follow up of patients (4). Markers of inflammation (IL-6, tumor necrosis factor [TNF], and C-reactive protein [CRP]) are increased in patients with PAH (5–7). Markers of platelet and endothelial dysfunction, such as von Willebrand factor (vWF), soluble P selectin, β-thromboglobulin (BTG), and thromboxane B_2 (TX), are also elevated in patients with PAH (2, 8–10). Higher levels of BNP and NT-pro-BNP, indicators of ventricular stretch, are associated with an increased risk of death in patients with PAH (11, 12). Brachial artery flow-mediated dilation (FMD) is a measure of nitric oxide (NO)-dependent vasodilation in response to shear stress from transient flow restriction and is associated with disease severity in children with idiopathic PAH (13).

The Randomized Clinical Trial of Aspirin and Simvastatin for Pulmonary Arterial Hypertension (ASA-STAT) was a multicenter randomized clinical trial of aspirin and simvastatin in patients with PAH with a primary end point of 6-minute-walk distance (6MWD) at 6 months. We used the ASA-STAT cohort to evaluate the relationship between biomarkers of inflammation, endothelial dysfunction, thrombosis, and RV function with World Health Organization (WHO) functional class and long-term transplant-free survival in patients with prevalent PAH. Some of the results of these studies have been previously reported in the form of abstracts (14, 15).

Methods

Study Design and Participants

We performed a retrospective cohort study of patients enrolled in ASA-STAT (16). ASA-STAT was a multicenter phase II randomized, double-blind, placebo-controlled 2×2 factorial study to determine the efficacy and safety of aspirin and simvastatin in patients with PAH receiving background therapy. Patients with PAH from four academic medical centers were recruited into the trial: Columbia University, Johns Hopkins University, University of Pennsylvania, and Tufts Medical Center. Patients were eligible if they were older than 18 years of age and without an indication (or contraindication) for aspirin or simvastatin. Sixty-five patients were randomized between January 2007 and September 2009, when the study was terminated for futility in reaching the primary end point. The trial had four study visits over a 6-month treatment period and three telephone follow ups. For this analysis, patients from the study were followed until lung transplantation, death, or August 1, 2013. Details of the methods have been published elsewhere (16, 17).

The trial protocol and this study were approved by the institutional review board at each participating center. The trial was registered at www.clinicaltrials.gov before recruitment was initiated (NCT00384865).

Study Procedures

Blood samples were collected at every study visit using standardized methodology (17). Patients were *non per os* before midnight the night before, with only water before the study assessment. The blood samples were analyzed for plasma vWF, soluble P selectin, CRP, total and HDL cholesterol, triglycerides, NT-pro-BNP, TNF, IL-6, BTG, and TX. Blood samples drawn at the baseline visit were used for the analysis. Plasma vWF was measured using an immunoturbidimetric assay (Diagnostica Stago, Inc., Parsippany, NJ). Soluble P selectin was measured by an ELISA (R&D Systems, Minneapolis, MN). Plasma CRP was measured using a nephelometric assay (Siemens BNII; Siemens Healthcare Diagnostics, Plainfield, IN). Serum lipid profile (total cholesterol, HDL, and triglyceride) was analyzed via a colorimetric reaction using the Ortho Vitros Clinical Chemistry System 950IRC (Johnson & Johnson Clinical Diagnostics, Rochester, NY). Plasma NT-pro-BNP was measured using a chemiluminescent immunometric assay (Roche Elecsys 2010; Roche Diagnostics, Indianapolis, IN). TX was measured in serum using ELISA (Cayman Chemical, Ann Arbor, MI). BTG was also measured in serum using ELISA (Asserachrom B-TG; Diagnostica Stago).

Brachial artery ultrasound was performed on subjects at each study visit (ultrasound was not performed at the University of Pennsylvania Field Center). Baseline images of the brachial artery were acquired at rest and after a blood pressure cuff was inflated and deflated according to a standard protocol (17). FMD was calculated as the maximal percent change in brachial artery diameter after forearm cuff release compared with resting brachial artery diameter. The FMD from the baseline visit was used in the analysis.

The WHO functional classification was assessed by one of the study physicians at each visit, with class I defined as no symptoms, class II as symptoms with more than usual activity, class III as symptoms with less than usual activity, and class IV with symptoms at rest (18). All assessments were made while blinded to other variables. The WHO functional class from the baseline visit was used in the analysis.

The 6-minute-walk test was performed at each study visit using a standardized protocol in accordance with the American Thoracic Society statement (19). The total distance walked was recorded and rounded to the nearest meter.

The main outcome for our analysis was transplant-free survival. Subjects were censored at the end of follow up, which was August 1, 2013.

Statistical Analysis

Continuous variables were expressed as mean $\pm$ SD or median and interquartile range. Categorical variables were summarized by frequencies. Pearson or Spearman correlations, as appropriate, were calculated to assess the relationship between baseline biomarker levels and baseline WHO functional class and baseline 6MWD. Multivariate ordinal logistic

regression was performed to assess the relationship between biomarker levels and the WHO functional class at baseline, with adjustment for age, sex, and etiology of PAH. The results are presented as odds ratios (ORs) and 95% confidence intervals (CIs). Cox regression analysis was used to assess the relationship between biomarker levels and transplant-free survival, with adjustment for age, sex, etiology of PAH, and 6MWD. All analyses were performed using available data; no imputation was performed. Sensitivity analyses were performed by additionally adjusting for warfarin use in the regression models. R version 3.1.1 was used for all analyses (20). The Cox regression analysis was performed using the survival package in R (version survival 2.37-7) (21), and the ordinal logistic regression analysis was performed using the MASS package (22) in R.

Results

Sixty-five patients with PAH were enrolled in the ASA-STAT study (Table 1). The mean age of subjects was 51 ± 14 years, and 86% were women. Sixty percent were non-Hispanic white and 20% were black. Fifty-one percent of subjects had idiopathic PAH, and 32% had PAH associated with systemic sclerosis or other connective tissue diseases. Approximately two-thirds received an endothelin receptor antagonist, sildenafil, or a combination. The majority of patients (78%) were receiving anticoagulation. Subjects were mostly classified as WHO functional class II or III (63% and 29%, respectively). The average 6MWD at the baseline visit was 433 ± 116 m.

Follow-Up and Outcomes

The median follow-up time was 4.5 ± 1.2 years, and there were 101,030 patient-days of follow up. There were 17 deaths, two lung transplants, and only one patient lost to follow up. Transplant-free survival at 1, 3, and 5 years was 92%, 86%, and 77%, respectively.

At baseline, higher NT-pro-BNP, TNF, and IL-6 levels were significantly associated with higher WHO functional class, whereas higher HDL was significantly associated with lower WHO functional class (*see* Table E1 in the online supplement). Similarly, higher NT-pro-BNP, vWF, BTG, and IL-6 were significantly associated with lower baseline 6MWD, and lower HDL was strongly associated with lower 6MWD.

Higher plasma vWF at baseline was associated with a worse WHO functional class (OR per 50% increase in vWF, 1.56; 95% CI, 1.02–2.50) after adjustment for age, sex, and etiology of PAH (Table 2). Lower HDL cholesterol was associated with greater odds of having a more advanced WHO functional class, as were increased levels of TX (per 800-pg/ml increments). FMD and other biomarkers were not significantly associated with WHO functional class after adjustment for covariates.

Higher NT-pro-BNP levels at baseline were associated with a greater risk of death or transplant during the study period (hazard ratio [HR] per 500-pg/ml increase in NTpro-BNP, 1.13; 95% CI, 1.05–1.21; $P < 0.001$) after adjustment for age, sex, etiology of PAH, and the 6MWD

Table 1. Baseline characteristics of study subjects

	Study Subjects (*N* = 65)
Age, yr	51 ± 14
Female sex, n (%)	56 (86)
Body mass index, kg/m^2	27.9 ± 6.9
Race/ethnicity, n (%)	
White (non-Hispanic)	39 (60)
Hispanic or Latino	9 (14)
Black	13 (20)
Asian	3 (5)
Other	1 (1)
PAH diagnosis, n (%)	
Idiopathic	33 (51)
Heritable	3 (5)
Congenital systemic to pulmonary shunt	6 (9)
Systemic sclerosis	12 (18)
Other connective tissue disease	9 (14)
Drugs/toxins	2 (3)
Concomitant medications, n (%)	
Ambrisentan	18 (28)
Bosentan	18 (28)
Epoprostenol	15 (23)
Iloprost, inhaled	9 (14)
Sildenafil	42 (65)
Treprostinil, intravenous	5 (8)
Combination therapy	40 (62)
Warfarin	51 (78)
WHO functional classification, n (%)	
Class I	5 (8)
Class II	41 (63)
Class III	19 (29)
6-min walk distance, m	433 ± 116
Biomarkers	
NT-pro-BNP, pg/ml	204 (82–1,451)
Total cholesterol, mg/dl	181 ± 37
HDL cholesterol, mg/dl	50 ± 16
vWF, %	142 ± 69
FMD, % (n = 56)	6 ± 5
CRP, μg/ml	2.8 (1.1–6.3)
TNF, pg/ml (n = 62)	3.6 (1.9–6.2)
Triglycerides, mg/dl	120 ± 52
sP selectin, ng/ml	55.3 ± 26.1
BTG, IU/ml	50.7 (26.7–110.4)
TX, pg/ml	1,315 (401–3,775)
IL-6, pg/ml (n = 62)	3.3 (2.1–5.5)

Definition of abbreviations: BTG = β-thromboglobulin; CRP = C-reactive protein; FMD = flow-mediated dilation; HDL = high-density lipoprotein; NT-pro-BNP = N-terminal pro-brain natriuretic peptide; PAH = pulmonary arterial hypertension; sP selectin = soluble P selectin; TNF = tumor necrosis factor; TX = thromboxane B_2; vWF = von Willebrand factor; WHO = World Health Organization. Continuous variables presented as either mean ± SD or median (interquartile range). Categorical variables presented as n (%).

Table 2. Relationship between biomarker levels and World Health Organization functional class (n = 65)

Biomarker	OR*	95% CI	*P* Value
NT-pro-BNP (per 500-pg/ml increase)	1.05	0.95–3.44	0.48
vWF (per 50% increase)	1.56	1.02–2.50	0.049
Total cholesterol (per 50-mg/dl decrease)	1.49	0.74–3.06	0.27
HDL cholesterol (per 15-mg/dl decrease)	2.11	1.23–3.83	0.009
FMD (per 1% increase)†	0.99	0.89–1.09	0.78
CRP (per 1-μg/ml increase)	0.97	0.92–1.03	0.39
TNF (per 1-pg/ml increase)‡	1.07	0.99–1.33	0.54
Triglycerides (per 50-mg/dl decrease)	0.70	0.41–1.16	0.17
sP-selectin (per 25-ng/ml increase)	0.97	0.58–1.61	0.91
BTG (per 40-IU/ml increase)	1.18	0.81–1.73	0.37
TX (per 800-pg/ml increase)	1.11	1.01–1.23	0.041
IL-6 (per 1-pg/ml increase)‡	1.22	1.04–1.60	0.10

Definition of abbreviations: BTG = β-thromboglobulin; CI = confidence interval; CRP = C-reactive protein; FMD = flow-mediated dilation; HDL = high-density lipoprotein; NT-pro-BNP = N-terminal pro-brain natriuretic peptide; OR = odds ratio; sP selectin = soluble P selectin; TNF = tumor necrosis factor; TX = thromboxane B_2; vWF = von Willebrand factor
*Adjusted for age, sex and etiology of pulmonary arterial hypertension.
†n = 56.
‡n = 62.

(Table 3). A 50 mg/dl lower total cholesterol was associated with a greater risk of death or transplant after adjustment for the same covariates (HR, 2.35; 95% CI, 1.26–4.40; $P < 0.001$). A 50% absolute decrease in vWF activity was also associated with worse outcome (HR, 1.32; 95% CI, 1.00–1.75; $P = 0.05$). FMD and other biomarkers were not associated with transplant-free survival.

Further adjustment for warfarin use had no impact on the effect estimates of the associations of the biomarkers with WHO functional class or transplant-free survival (Tables E2 and E3).

Discussion

In a clinical trial population of patients with PAH, lower levels of HDL cholesterol were associated with worse functional class. Higher levels of NT-pro-BNP and lower total cholesterol levels were associated with higher risk of death or lung transplantation after adjustment for age, sex, etiology of PAH, and the 6MWD. Although higher plasma vWF activity was associated with worse WHO functional class, it was also surprisingly associated with better transplant-free survival. FMD, IL-6, TNF, and CRP were not associated with WHO functional class or transplant-free survival in adjusted analyses.

Cholesterol and lipoproteins have recently been implicated as markers of inflammation. Low cholesterol levels have been associated with an increased risk of sepsis in critically ill as well as surgical patient populations (23, 24). Low cholesterol levels have also been associated with poor survival in several patient populations, including the elderly (25) and patients with congestive heart failure (26–28), rheumatoid arthritis (29), and end-stage renal disease (30, 31). Previously, patients with PAH were found to have lower HDL cholesterol levels than age- and sex-matched control subjects (32), and lower HDL cholesterol levels were associated with worse survival independent of other potential confounders (33, 34). The prognostic significance of HDL cholesterol was not confirmed in a multicenter prospective cohort study of incident patients with PAH (35).

In the current study of patients with prevalent PAH, we demonstrate an association between low serum cholesterol (at baseline, before administration of simvastatin or placebo) and higher risk of death or lung transplantation. Lower cholesterol levels could reflect a malnourished state in the setting of malabsorption due to chronic bowel wall edema and decreased hepatic synthetic function leading to bacterial translocation and a chronic inflammatory response (31, 36). We also found that lower HDL cholesterol levels were associated with worse WHO functional class but not with survival (33, 34). Inflammation is believed to influence levels of cholesterol. However, inflammatory markers were not associated with outcomes in our study, as they have been in other studies (7).

Our study confirms previous findings that higher levels of NT-pro-BNP are associated with higher risk of death or lung transplantation (12, 37–39). NT-pro-BNP is secreted predominantly from ventricular tissue in response to stretch and overload. BNP levels have been shown to correlate with RV parameters, such as

Table 3. Relationship between biomarker levels and transplant-free survival (n = 65)

Biomarker	HR*	95% CI	*P* Value
NT-pro-BNP (per 500-pg/ml increase)	1.13	1.05–1.21	<0.001
vWF (per 50% decrease)	1.32	1.00–1.75	0.05
Total cholesterol (per 50-mg/dl decrease)	2.35	1.26–4.40	<0.001
HDL cholesterol (per 15-mg/dl decrease)	1.42	0.78–2.60	0.25
FMD (per 1% increase)†	0.98	0.87–1.10	0.74
CRP (per 1-μg/ml increase)	0.97	0.90–1.05	0.43
TNF (per 1-pg/ml increase)‡	0.99	0.95–1.02	0.48
Triglycerides (per 50-mg/dl decrease)	1.16	0.75–1.78	0.51
sP-selectin (per 25-ng/ml increase)	0.86	0.53–1.41	0.56
BTG (per 40-IU/ml increase)	1.07	0.77–1.47	0.69
TX (per 800-pg/ml increase)	1.01	0.93–1.09	0.85
IL-6 (per 1-pg/ml increase)‡	1.00	0.95–1.05	0.97

Definition of abbreviations: BTG = β-thromboglobulin; CI = confidence interval; CRP = C-reactive protein; FMD = flow-mediated dilation; HDL = high-density lipoprotein; HR = hazard ratio; NT-pro-BNP = N-terminal pro-brain natriuretic peptide; sP selectin = soluble P selectin; TNF = tumor necrosis factor; TX = thromboxane B_2; vWF = von Willebrand factor.
*Adjusted for age, sex, etiology of pulmonary arterial hypertension, and 6-min-walk distance.
†n = 56.
‡n = 62.

RV ejection fraction and RV end-diastolic pressure (12). Although BNP and NT-pro-BNP levels have been associated with the WHO functional class (40), we did not find such an association in this cohort. In patients with PAH associated with systemic sclerosis, despite similar hemodynamics, levels of NT-pro-BNP are higher than in patients with idiopathic PAH (41). The inclusion of patients with connective tissue disease–related PAH may account for the lack of association between WHO functional class and NT-pro-BNP in our study.

vWF plays a pivotal role in homeostasis and thrombogenesis by initiating platelet adhesion and aggregation and stabilizing factor VIII. Higher vWF activity has been associated with worse survival in patients with PAH (9, 42–44). In the current study, lower vWF activity was associated with worse survival and/or need for lung transplantation, and these results were independent of warfarin use. One possible explanation is that in a cohort of patients receiving background PAH therapy, persistently low vWF activity may reflect a state of persistent shear stress–induced proteolysis of vWF that did not improve with PAH treatment (45). Low vWF activity perhaps indicates ongoing endothelial perturbation in patients with more severe PAH, portending worse survival. vWF has also been shown to be a potent angiogenesis inhibitor (46). It is possible that in the presence of low vWF activity there is increased angiogenesis leading to a more aggressive PAH phenotype and worse survival.

FMD impairment is believed to result from a decrease in the activity of the L-arginine–NO synthetic pathway and increased NO degradation in vascular smooth muscle cells (47, 48). A reflection of endothelial function, brachial artery FMD is associated with cardiac index and RV performance in children with idiopathic PAH (13) and with pulmonary vasoreactivity in adults with idiopathic PAH (49). Brachial artery FMD is also associated with increased mortality in patients with congestive heart failure (50). In the current study, FMD was not associated with WHO functional class or transplant-free survival.

Our study has several strengths, including the prospective and multicenter design, rigorous standardized blood collection and analysis procedures with blinding to other information, and near complete long-term follow up. There are some limitations, however. The study sample was drawn from a clinical trial (with several inclusion/exclusion criteria), so that the findings may not be generalizable to the larger population of patients with PAH. In addition, biomarkers were assessed while on background PAH-specific therapy in a prevalent (rather than incident) population. We are therefore unable to speculate whether the associations we found would hold at the time of diagnosis before initiation of PAH-specific therapy. The study was designed and powered to detect a difference in the 6MWD between the active treatment groups and placebo; the results should be interpreted in the context of hypothesis generation. Although the small number of events provides limited power, the lack of association between some of the biomarkers and outcome is supported by effect estimates close to the null value with reasonably narrow 95% CIs.

Conclusions

In the ASA-STAT study, lower vWF activity and cholesterol levels and higher NT-pro-BNP levels at baseline were associated with increased risk of death or transplantation in patients with PAH. FMD was not associated with functional class or transplant-free survival. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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