Research Article

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Association between cytokines and functional, hemodynamic parameters, and clinical outcomes in pulmonary arterial hypertension

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Abstract

To assess the relationship of cytokines with functional and clinical outcomes in pulmonary arterial hypertension (PAH). Endothelial dysfunction and vascular inflammation are characteristic of PAH. We investigated whether markers of angiogenesis and inflammation associated with functional, hemodynamic parameters, and clinical outcomes in PAH. PAH patients (n = 206) were pooled from two clinical trials: TRUST-1 and FREEDOM-C2. Baseline and post-treatment cytokine levels were correlated to baseline clinical and hemodynamic parameters, were assessed in clinical subgroups, and were associated with clinical outcomes. In 206 patients (mean age = 48 years; 74% women) with WHO group-1 PAH, most cytokine levels were higher in those with 6-min walking distance (6MWD) < median (335 m) vs. those above median, including Ang-1 (11.9 ± 10.1 vs. 5.9 ± 6.0 ng/mL), Ang-2 (14.3 ± 11.8 vs. 12.2 ± 11.2 ng/mL), and MMP-9 (221 ± 262.3 vs. 119 ± 171 ng/mL). Baseline 6MWD inversely correlated with Ang-1 ($r = -0.27$, $P < 0.0001$), Ang-2 ($r = -0.20$, $P = 0.004$), and MMP-9 ($r = -0.27$, $P < 0.0001$). MMP-9 levels differed significantly by NYHA functional class ($P = 0.001$) suggesting an association between MMP-9 and subjective PAH severity. Mean Ang-2 levels were higher in those with baseline right atrial pressure (RAP) > 15 mmHg compared to those with RAP < 15 mmHg (23,841 vs. 11,020 pg/mL). Baseline RAP was associated with change in MMP-9 levels ($r = -0.53$, $P = 0.03$). Finally, baseline Ang-1, VEGF and MMP-9 levels were associated with risk of death and hospitalization at 16-week follow-up. Inflammatory cytokines and vascular angiogenesis markers are associated with baseline functional, hemodynamic parameters in PAH, and predict death and hospitalization. Larger prospective studies are needed to confirm the utility of cytokines in PAH.

Keywords

pulmonary arterial hypertension, cytokines, inflammation, MMP-9, prostacyclin analogs

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Pulmonary arterial hypertension (PAH) is a progressive, debilitating, and often fatal disease characterized by a variety of physiologic changes in the distal pulmonary vasculature, ultimately resulting in a rise in pulmonary vascular resistance (PVR) and right ventricular afterload.[1,2] Smooth muscle cell proliferation, endothelial dysfunction, and vascular inflammation are some of the underlying mechanisms

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thought to be responsible for the pathogenesis of PAH.[3] In recent years, greater attention has been focused on vascular inflammation in patients with PAH, ranging from idiopathic PAH (IPAH) to connective tissue disease-associated PAH.[4,5]

Three major molecular pathways have already been implicated in the pathogenesis of PAH specifically involving endothelin-1, nitric oxide, and prostacyclin.[6] All currently available PAH-specific therapies target one of these pathways.[7] Among the available therapeutic agents for the treatment of PAH, prostacyclin analogs, such as Epoprostenol and Treprostinil, are potent agents that have proven efficacy with regard to symptomatic improvement and, in the case of intravenous Epoprostenol, a demonstrated survival benefit.[8–11] As novel treatments for this incurable disease are being developed, surrogate markers to evaluate treatment response are required; these may in turn shed light on the pathophysiology of the disease itself.[12] Emerging evidence suggests the potential for cytokine signatures as important circulating biomarkers in PAH pathology as well a means to measure treatment response.[13–16]

In this study, we sought to investigate the association between circulating markers of vascular angiogenesis and inflammatory cytokine levels with PAH severity at baseline and in response to treatment with a prostacyclin analog. To achieve this, we utilized the treatment arms of two previously published randomized trials of Treprostinil in PAH, TRUST-1[17] and FREEDOM-C2.[18] We hypothesized that serum markers of vascular angiogenesis, such as angiopoietin-2, and of vascular inflammation, such as IL-8, along with matrix metalloproteinases, such as matrix metalloproteinase-9 (MMP-9), would be associated with baseline clinical, hemodynamic, and functional parameters in PAH. Moreover, we sought to investigate the effect of oral and intravenous treprostinil therapy on the levels of these biomarkers. Finally, we examined whether baseline levels of these markers might be associated with clinical outcomes such as death and hospitalization after PAH therapy with oral and intravenous treprostinil.

Methods

Data collection

Data were pooled from the treatment arms of two clinical trials assessing the effect of treprostinil on PAH, TRUST-1[17] and FREEDOM-C2.[18] Specific details of the inclusion and exclusion criteria as well as the study results have been previously reported. Baseline data from 206 patients (28 patients from TRUST-1 and 178 from FREEDOM-C2) were pooled while follow-up data were available for a total of 193 patients (16 from TRUST-1 and 177 from FREEDOM-C2).

In brief, the TRUST-1 trial comprised 28 WHO Group I PAH patients who were evaluated at baseline and after 12 weeks of treatment with IV treprostinil with 6-min walking distance (6MWD), New York Heart Association (NYHA) functional class (FC) assessment, and N-terminal pro-B type natriuretic peptide (NT-proBNP). These patients all had invasive hemodynamic assessment at baseline before starting IV treprostinil. Similarly, data from 178 patients enrolled in the treatment arm of the FREEDOM-C2 trial were available for analysis. These patients underwent clinical assessment for 6MWD, NYHA FC, and NT-proBNP at baseline and at 16-week follow-up visits after treatment with oral treprostinil.

Cytokine levels were measured for all patients at baseline and when possible at 12- or 16-week follow-up (TRUST-1 and FREEDOM C-2, respectively). Biomarkers were assessed in EDTA plasma using a multiplexed immunoassay (DiscoveryMAP® v. 3.0 assay, Myriad RBM, Inc., Austin, TX, USA). Previous publications of these data have included detailed information regarding biomarker measurements and the assays used.[17,18] Pre-specified cytokines that were measured included angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2), basic fibroblast growth factor (BFGF), interleukin-1 beta (IL-1b), IL-6, IL-8, IL-13, MMP-9, and vascular endothelial growth factor (VEGF). In addition, in a subset of 95 patients, levels of NT-proBNP levels were also measured.

Statistical analysis

Descriptive analyses were performed to summarize the baseline characteristics of the combined group. Normality of the data was assessed by considering skewness and kurtosis. Continuous variables were reported as mean $\pm$ SD or median and range for parametric and non-parametric descriptive summaries, respectively. Categorical variables were represented as frequencies. Stratified analyses based on baseline 6MWD, baseline NYHA FC, death, and hospitalization were performed, in which differences in continuous variables were assessed using the Student's t-test for normally distributed data whereas non-normal data were compared between stratified groups utilizing the Mann–Whitney U test. Categorical variable comparisons were performed utilizing Pearson's chi-square test. The Holm-Bonferroni step-down procedure to test the significance of multiple comparisons of analysis of biomarkers was performed. Furthermore, Spearman correlation analyses were carried out to examine correlations between baseline cytokine levels and baseline 6MWD as well as between change in cytokine levels and baseline 6MWD. ANOVA models were built to evaluate associations between cytokines and NYHA FC. Finally, Kaplan–Maier analysis and log-rank testing was used to determine the association between baseline cytokine levels and subsequent composite outcome including death and/or hospitalization. P values ≤ 0.05 were considered statistically significant. All analyses were carried out using SAS statistical software, version 9.4.

Results

Characteristics of the overall study group

Baseline characteristics of the pooled cohort are reported in Table 1. The pooled cohort comprised 206 patients with a mean age of 48 years and was predominantly female (74%). Most patients had IPAH (67%) or connective tissues associated PAH (30%). Most patients in the pooled cohort (79%) had NYHA FC III symptoms at baseline with a median 6MWD of 335 m. While only 15% patients were previously on an endothelin receptor antagonist, almost 44% were on phosphodiesterase-5 inhibitors as background therapy. Notably, no patients in TRUST-1 were on immunosuppressive agents during the study and only 31 patients in FREEDOM-C2 were on any of these agents over the course of the study. Given the small number of patients on immunosuppressive agents, we were not able to demonstrate any significant differences in 6MWD or cytokine levels in these patients vs. the entire cohort.

Stratified analyses of baseline characteristics

We performed stratified analyses of baseline cytokine characteristics based upon following characteristics: (1) median 6MWD in the pooled cohort (335 m); (2) whether the patient died during the study period; (3) whether the patient was hospitalized during the study period; and (4) baseline NYHA FC (class II vs. classes III and IV). Strikingly all cytokine levels were significantly higher in those with baseline 6MWD $\leq$ 335 m compared to those with 6MWD > 335 m, with the exceptions of BFGF and IL-8. Elevation in IL-8 was statistically non-significant while BFGF levels were increased in those with higher 6MWD values (Table 2). Additionally, these analyses held true even after the Holm–Bonferroni correction for multiple comparisons. Furthermore, patients who were alive at the end of both studies had significantly lower baseline values of Ang-1, BFGF, IL-1b, IL-6, IL-13, MMP-9, and VEGF compared to those who died (Table 3). Moreover, IL-8 was one of the few cytokines that was significantly elevated among those who were hospitalized during the study compared to those who did not require hospitalization. Additionally, Ang-1, IL-1b, and IL-13 were also elevated among those hospitalized (Table 4). Finally, patients with worse baseline NYHA FC including NYHA classes III and IV had elevated levels of pro-inflammatory cytokines comprising IL-1b, IL-6, IL-13, and MMP-9 (Table 5). All analyses were repeated in a sensitivity analysis after removing any patients with previous use of anti-inflammatory medications. The sensitivity analyses revealed results similar to the results for the overall cohort.

Table 1. Baseline characteristics of the combined patient population (n = 206).

Parameter	Value
Age (years)	48.0 ± 15.9
Female (n (%))	152 (74)
Etiology of pulmonary hypertension (n (%))	
Idiopathic/Familial	137 (66.5)
Collagen vascular disease	62 (30.1)
HIV infection	4 (1.9)
Congenital heart defect	2 (1.0)
Other	1 (0.5)
Baseline NYHA functional class (n (%))	
Class II	41 (20)
Class III	162 (79)
Class IV	2 (1)
Baseline 6-minute walk distance (m)	335 (75–422)
Body mass index (kg/m^2)	26.1 ± 6.6
Borg scale	4 (0–10)
Background therapy (n (%))	
ERA	27 (15.2)
PDE-5i	79 (44.4)
ERA+PDE-5i	72 (40.4)

Values are represented as mean ± SD or median (range) for continuous variables and as n (%) for categorical variables.
HIV, human immunodeficiency virus; ERA, endothelin receptor antagonist; PDE-5i, phosphodiesterase-5 inhibitor.

Spearman correlation reveals significant inverse associations between pro-inflammatory cytokine levels and baseline 6MWD

Spearman correlation analyses were performed to assess the association between baseline cytokines and baseline 6MWD (Table 6). These analyses revealed a significant inverse correlation between baseline 6MWD and Ang-1 ($r = -0.27$, $P < 0.0001$), Ang-2 ($r = -0.20$, $P = 0.004$), IL-1b

Table 2. Baseline cytokine profile comparison between groups stratified by 6-min walk distance (6MWD).

Parameter	6MWD $\leq$ 346 (n = 103)	6MWD > 346 (n = 103)	*P* value
Ang-1 (ng/mL)	7.6 (1.3–47.1)	5.0 (1.3–53.0)	<0.001
Ang-2 (ng/mL)	10.0 (2.1–81.1)	7.1 (1.8–52.0)	0.010
Basic fibroblast growth factor (pg/mL)	10.5 (3.2–48.1)	10.5 (10.5–115)	0.009
IL-1 beta (pg/mL)	1.6 (1.6–38.5)	1.6 (1.6–4.2)	<0.001
IL-6 (pg/mL)	2.2 (2.2–173)	2.2 (2.2–10)	<0.001
IL-8 (pg/mL)	9.5 (2.1–1293)	7.9 (2.1–124)	0.05
IL-13 (pg/mL)	4.6 (4.6–1207)	4.6 (4.6–4.6)	<0.001
VEGF (pg/mL)	65 (11.5–1934)	49 (11.5–658)	0.001
MMP-9 (ng/mL)	109 (28–1041)	73 (11–1550)	0.002

Between-group comparisons were performed by Mann–Whitney U-test.

Table 3. Baseline cytokine profile comparison between patients who died during the study and patients who were alive at the end of the study.

Parameter	Patients alive (n = 200)	Patients who died (n = 6)	*P* value
Ang-1 (ng/mL)	5.9 (1.3–53)	22.3 (7.6–30.5)	0.001
Ang-2 (ng/mL)	8.7 (1.8–52)	8.2 (2.1–81.1)	0.81
Basic fibroblast growth factor (pg/mL)	10.5 (3.2–115)	14.3 (3.9–48.1)	0.03
IL-1 beta (pg/mL)	1.6 (1.6–38.5)	5.0 (5.0–6.9)	<0.001
IL-6 (pg/mL)	2.2 (2.2–173)	5.0 (5.0–5.0)	<0.001
IL-8 (pg/mL)	8.7 (2.1–1293)	5.0 (5.0–367)	0.68
IL-13 (pg/mL)	4.6 (4.6–1207)	156 (156–156)	<0.001
VEGF (pg/mL)	56 (11.5–1934)	160 (15.7–753)	0.03
MMP-9 (ng/mL)	85.5 (11–1550)	635 (195–901)	<0.001

Between-group comparisons were performed by Mann–Whitney U-test.

Table 4. Baseline cytokine profile comparison between patients who were hospitalized during study and those who were not.

Parameter	Not hospitalized (n = 173)	Hospitalized (n = 33)	*P* value
Ang-1 (ng/mL)	5.7 (1.3–53)	7.2 (1.3–29.9)	0.04
Ang-2 (ng/mL)	8.2 (1.8–81.1)	10.0 (4.6–32.6)	0.3
Basic fibroblast growth factor (pg/mL)	10.5 (3.2–115)	10.5 (3.3–48.1)	0.99
IL-1 beta (pg/mL)	1.6 (1.6–38.5)	1.6 (1.6–6.9)	0.02
IL-6 (pg/mL)	2.2 (2.2–57)	2.2 (2.2–173)	0.07
IL-8 (pg/mL)	8.2 (2.1–133)	12.0 (4.6–1293)	0.01
IL-13 (pg/mL)	4.6 (4.6–1207)	4.6 (4.6–156)	0.01
VEGF (pg/mL)	56 (11.5–1934)	63 (11.5–1397)	0.15
MMP-9 (ng/mL)	84 (11–1550)	118 (28–835)	0.15

Between-group comparisons were performed by Mann–Whitney U-test.

Table 5. Baseline cytokine profile comparison between patients with baseline NYHA FC 2 and those with NYHA FC 3/4.

Parameter	Functional class 2 (n = 41)	Functional class 3 or 4 (n = 164)	*P* value
Ang-1 (ng/mL)	4.9 (1.3–25)	6.1 (1.3–53)	0.06
Ang-2 (ng/mL)	7.9 (2.3–45)	9.0 (1.8–81.1)	0.62
Basic fibroblast growth factor (pg/mL)	10.5 (10.5–35.0)	10.5 (3.2–115)	0.23
IL-1 beta (pg/mL)	1.6 (1.6–3.4)	1.6 (1.6–38.5)	0.02
IL-6 (pg/mL)	2.2 (2.2–8.9)	2.2 (2.2–173)	0.005
IL-8 (pg/mL)	7.9 (2.1–58)	8.7 (2.1–1293)	0.37
IL-13 (pg/mL)	4.6 (4.6–4.6)	4.6 (4.6–1207)	0.005
VEGF (pg/mL)	49 (11.5–201)	59 (11.5–1934)	0.09
MMP-9 (ng/mL)	62 (11–386)	93.5 (11–1550)	0.001

Between-group comparisons were performed by Mann–Whitney U-test.

Table 6. Spearman correlation analyses between cytokines and 6-min walk distance (6MWD).

6MWD at baseline		
Cytokine at baseline (n = 206)	Spearman correlation coefficient	*P* value
Ang-1	−0.27143	<0.0001
Ang-2	−0.20055	0.0038
BFGF	0.18320	0.0084
IL-1b	−0.37048	<0.0001
IL-6	−0.40821	<0.0001
IL-8	−0.15693	0.0243
IL-13	−0.37443	<0.0001
MMP-9	−0.26912	<0.0001
VEGF	−0.20340	0.0034

(r = −0.37, $P < 0.0001$), IL-6 (r = −0.41, $P < 0.0001$), IL-8 (r = −0.16, $P = 0.02$), IL-13 (r = −0.37, $P < 0.0001$), MMP-9 (r = −0.27, $P < 0.0001$), and VEGF (r = −0.20, $P = 0.003$). Furthermore, a positive correlation was observed between baseline 6MWD and baseline BFGF (r = 0.18, $P = 0.008$).

Correlation between baseline hemodynamic parameters and cytokine levels

Among patients enrolled in TRUST-1, both Ang-2 and IL-8 levels were associated with baseline RA pressure > 15 mmHg vs. those with RA pressure < 15 mmHg (23,841 pg/mL vs. 11,020 pg/mL, $P = 0.065$) and (229 pg/mL vs. 14 pg/mL, $P = 0.03$), respectively. Conversely, baseline cardiac index (CI) (r = 0.69, $P = 0.005$) and PVR index (PVRI) (R = −0.68, $P = 0.005$) were associated with change from baseline Ang-2 levels with IV treprostinil therapy. Furthermore, baseline RA pressure was associated with change from baseline MMP-9 levels (R = −0.53, $P = 0.035$). Additionally, the change from baseline MMP-9 levels was associated with change from baseline CI and PVRI after IV treprostinil therapy (R = −0.57, $P = 0.054$ and R = 0.61, $P = 0.036$, respectively).

Correlation between baseline cytokine levels and NT-ProBNP

In a subset of 95 patients, NT-proBNP levels were measured at baseline and correlated to baseline cytokine levels. From this sub-dataset, there was a significant positive correlation between NT-proBNP and Ang-2 at baseline (R = 0.67, $P < 0.0001$). There was also significant positive correlation noted between baseline levels of IL8 and NT-proBNP

Table 7. ANOVA model derived significance levels for comparisons of cytokine levels among NYHA FCs.

NYHA class at baseline (n = 205)	
Cytokine at baseline (n = 205)	*P* value
Ang-1	0.0588
Ang-2	0.7290
BFGF	0.9874
IL-1b	0.2792
IL-6	0.6048
IL-8	0.7140
IL-13	0.1338
MMP-9	0.0109
VEGF	0.1498

Table 8. Baseline cytokine levels in patients with IPAH vs. those with collagen vascular disease-related PAH.

Parameter	IPAH (n = 137)	CVD PAH (n = 62)	*P* value
Ang-1 (ng/mL)	9.8 (1.3–53.0)	6.2 (1.3–30)	0.08
Ang-2 (ng/mL)	13.8 (2.1–81.1)	11.7 (1.8–43)	0.66
Basic fibroblast growth factor (pg/mL)	11.5 (3.2–115)	10.5 (10.5–10.5)	0.36
IL-1 beta (pg/mL)	2.6 (1.6–38.5)	1.6 (1.6–3.4)	<0.001
IL-6 (pg/mL)	3.6 (2.2–57)	6.2 (2.2–173)	0.7
IL-8 (pg/mL)	26.0 (2.1–1293)	13.9 (2.1–116)	0.08
IL-13 (pg/mL)	42.2 (4.6–1207)	4.6 (4.6–4.6)	<0.001
VEGF (pg/mL)	209 (11–1550)	79.1 (11–261)	<0.001
MMP-9 (ng/mL)	136 (11.5–1934)	67.3 (11.5–226)	0.93

IPAH, idiopathic/familial pulmonary arterial hypertension; CVD PAH, collagen vascular disease-related pulmonary arterial hypertension.

($R = 0.30$, $P = 0.0027$) as well as baseline levels of MMP-9 and NT-proBNP ($R = 0.26$, $P = 0.012$).

ANOVA model demonstrates significant dose response in MMP-9 levels in association with worsening NYHA class

We assessed whether cytokine levels differed among patients with various NYHA FCs (Table 7) using ANOVA modeling. This analysis revealed that baseline MMP-9 levels differed significantly based on the baseline NYHA FC of these patients ($P = 0.01$). However, no cytokine change over the stipulated study period significantly associated with NYHA FC.

Differentiation between cytokines in patients with collagen vascular disease versus IPAH

Differences in baseline cytokine levels among patients with collagen vascular disease versus those with IPAH are noted in Table 8. At baseline, levels of MMP-9, IL-1b, and IL-13 were all significantly higher in patients with IPAH (n = 137) versus those with collagen vascular disease (n = 62). There was no significant difference between change from baseline cytokine levels in patients with IPAH versus those with collagen vascular disease.

Baseline cytokine levels are associated with composite outcome of death and hospitalization

Finally, we assessed the association between baseline cytokine levels and the composite outcome of death and/or hospitalization in the two studies using Kaplan–Maier analysis (Fig. 1). As such, we found that higher than median levels of Ang-1 (log rank $P < 0.001$), VEGF (log rank $P = 0.001$), and MMP-9 (log rank $P < 0.001$) were associated with higher rates of hospitalizations and death. However, Ang-2 failed to show association with these outcomes.

Discussion

In this study, pooling data from the treatment arms of two randomized clinical trials of treprostinil therapy in PAH, we presented several important findings: (1) in cross-sectional analysis, we showed that increased levels of these cytokine signatures were associated with lower 6MWD, hospitalizations, and death; (2) further, we demonstrated that baseline 6MWD correlated inversely with multiple cytokines potentially involved in the pathophysiology of PAH; (3) additionally, we demonstrated an association between markers of inflammation including Ang-2 and MMP-9 and hemodynamic characteristics in patients with PAH, and revealed a direct relationship between baseline MMP-9 levels and NYHA FC in PAH patients; and (4) we demonstrated that baseline levels of pro-inflammatory cytokines were associated with subsequent risk of death and hospitalizations over a short-term follow-up of 16 weeks. Collectively, these findings demonstrate the potential utility of these cytokines as biological markers in PAH patients which may correlate with functional capacity and PAH-related hospitalizations and deaths.

PAH is an often-fatal disease characterized by increased PVR associated with endothelial dysfunction and vascular inflammation.[3] The three known pathways implicated in the pathophysiology of PAH are endothelin-1, nitric oxide, and prostacyclin. With the progress in our understanding of the complex interplay at the molecular between these pathways and the structural impact of this interplay, resulting in increases in PA pressure leading to PAH, research investigating the role of pro-inflammatory cytokines is gaining greater attention.[13,15,16] In view of these recent advances, our findings that elevated levels of markers of vascular angiogenesis and pro-inflammatory cytokines are associated with both surrogate outcome measures such as 6MWD and clinical outcomes such hospitalizations are of importance.

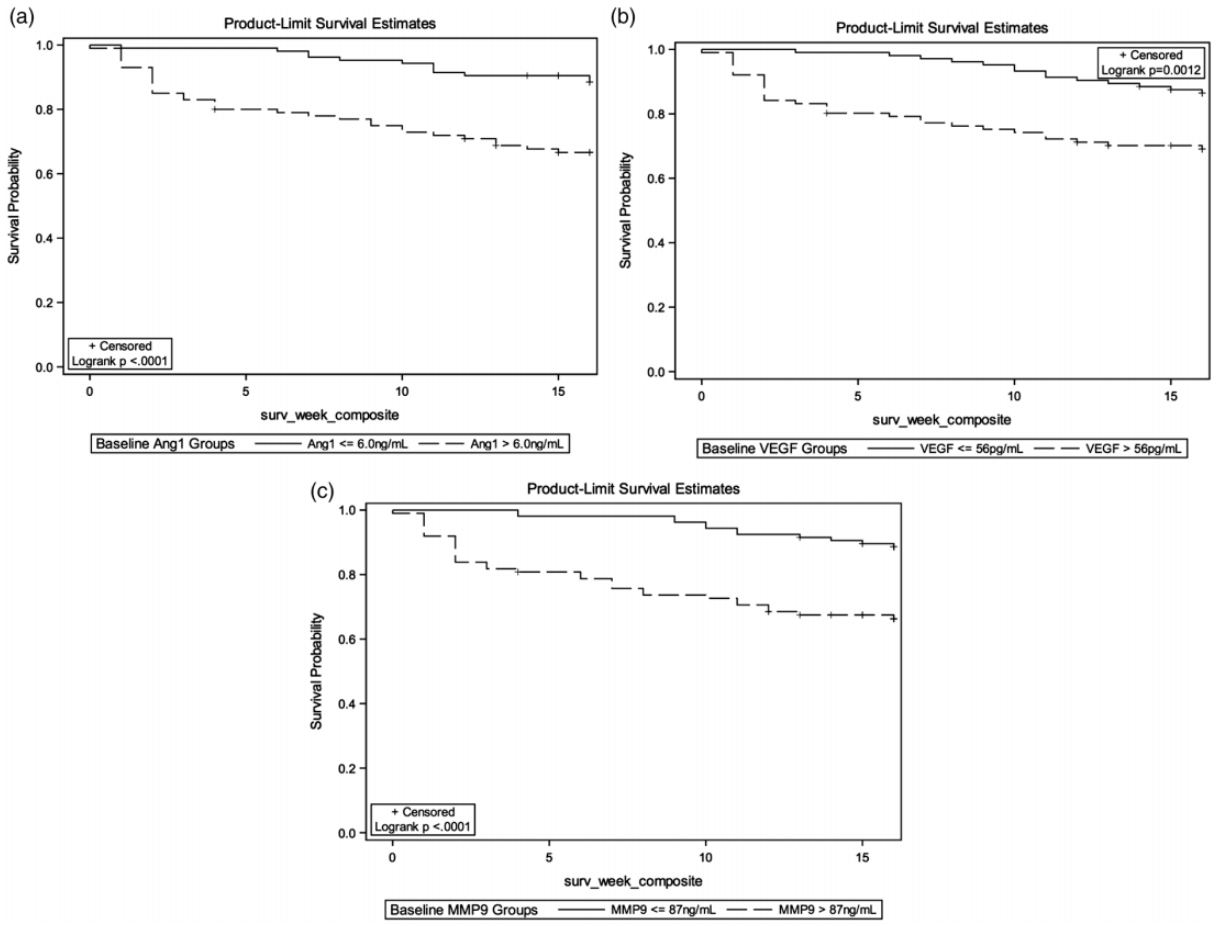

Fig. 1. Kaplan–Maier curves showing association between baseline Ang-1 levels (a), VEGF levels (b), mmp9 levels (c), and composite of death and hospitalization at 16 weeks.

Together, these results suggest that these cytokines may have clinical and subclinical implications as biomarkers of baseline assessment, gauging treatment response, and may suggest future outcomes in patients with PAH.

As the molecular pathophysiology of PAH becomes further elucidated, more surrogate outcomes to evaluate disease severity and treatment response are required.[10] We demonstrated that Ang-2 levels were inversely associated with baseline 6MWD. Building upon the previous evidence suggesting Ang-2 as a biomarker for PAH,[17,19] we showed further potential for Ang-2 in a larger sample set. Additionally, matrix metalloproteinases are zinc-dependent, multi-functional, soluble, or cell-membrane anchored proteases.[20] Out of this family of proteolytic enzymes, MMP-9 may play a crucial role in the initiation, development, and progression of PAH. Specifically, MMP-9 plays an important role in endothelial cell migration, smooth muscle cell migration and proliferation, adventitial fibroblast trans-differentiation, and inflammatory cell recruitment and infiltration.[20] Through its multitude of effects, MMP-9 facilitates the process of pulmonary vascular remodeling thereby leading to PAH. Our study revealed that MMP-9 was the only biomarker associated with baseline NYHA FC, further supporting its potential role as a composite biomarker of PAH.

Invasive hemodynamic assessment via right heart catheterization is the gold standard for diagnosis of pulmonary hypertension (PH). As such, using right-sided invasive hemodynamic measures, we demonstrated an association of Ang-2, IL-8, and MMP-9 with CI as well as PVRI. Furthermore, utilizing a composite outcome of death and hospitalization, we established predictive values for Ang-1, VEGF, and MMP-9. Taken together, these results suggest that measurement of these inflammatory cytokines at baseline may be useful to incorporate into future risk stratification algorithms to assess the severity of PAH and risk for future hospitalization and death. However, our observations with regards to associations between cytokines and hospitalization/death should be cautiously interpreted since they are over a short time period and obviously are non-randomized. However, they may provide a "proof of concept" and should be evaluated further in larger studies with longer duration of follow-ups.

Recently, a study analyzing the role of circulating Ang-1 as a biomarker of disease severity in PAH[21] suggested that

Ang-1 was not reliable. However, this study had patients with PH secondary to multiple underlying pathophysiological conditions, rendering the results less generalizable. While the results from our study are somewhat at odds with these recently published data, we had a larger sample size, all of whom had WHO Group I PAH. Nonetheless, since both these studies were cross-sectional and observational in nature, larger population-based studies are required to elucidate the role of Ang-1 in detail.

Strengths and limitations

One of the strengths of this study is that it included a large dataset of rigorously characterized WHO Group I patients who were assessed with biomarker assessment at baseline and at a follow-up after treprostinil therapy. Regardless of the size of this study, the nature of these analyses remains cross-sectional and observational, leaving the possibility for residual confounding, which is a limitation of all observational studies. Furthermore, despite including over 200 WHO Group I PAH patients, our study sample size is still limited and future population-based studies are necessary to examine the role of these inflammatory biomarkers in PAH pathophysiology. Though we did have access to baseline hemodynamic data from TRUST-1, neither trial included right heart imaging data with echocardiography or cardiac MRI and this we could not correlate cytokine levels with right ventricular function. Finally, given the cross-sectional nature of most of our study analyses, we cannot evaluate causality and hence future research should focus on studying the mechanistic roles of these cytokines in PAH. However, despite these limitations, our study is the largest study to date to assess the role of inflammatory cytokines and markers of angiogenesis in PAH and to inspect their relationships with the characteristics of PAH disease severity in a systematic manner.

In summary, utilizing the pooled biomarker data from two PAH clinical trials, we conclude that the cytokine levels and markers of vascular angiogenesis are associated with baseline 6MWD in patients with PAH and with death and hospitalizations. Moreover, we established an association between markers of inflammation and hemodynamic characteristics of the patients with PAH. Finally, we established that baseline levels of Ang-1 and MMP-9 helped assess future risk for hospitalization and deaths. As such, inflammatory cytokines may be useful biomarkers in PAH, but larger prospective studies are required to confirm the utility of these findings in future risk prediction algorithms.

Conflict of interest

AR receives research support and is a principal investigator with United Therapeutics. AR is also a principal investigator for Actelion and Bellerophon, a speaker for Bayer, and consultant for Sr. Jude. RB is a PI and has grant support from Bellerophon, Actelion, Eiger, St Jude, and Bayer. YR and KS are United Therapeutics employees.

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