Elevated Levels of Inflammatory Cytokines Predict Survival in Idiopathic and Familial Pulmonary Arterial Hypertension

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Background—Inflammation is a feature of pulmonary arterial hypertension (PAH), and increased circulating levels of cytokines are reported in patients with PAH. However, to date, no information exists on the significance of elevated cytokines or their potential as biomarkers. We sought to determine the levels of a range of cytokines in PAH and to examine their impact on survival and relationship to hemodynamic indexes.

Methods and Results—We measured levels of serum cytokines (tumor necrosis factor-α, interferon-γ and interleukin-1β, -2, -4, -5, -6, -8, -10, -12p70, and -13) using ELISAs in idiopathic and heritable PAH patients (n=60). Concurrent clinical data included hemodynamics, 6-minute walk distance, and survival time from sampling to death or transplantation. Healthy volunteers served as control subjects (n=21). PAH patients had significantly higher levels of interleukin-1β, -2, -4, -6, -8, -10, and -12p70 and tumor necrosis factor-α compared with healthy control subjects. Kaplan-Meier analysis showed that levels of interleukin-6, 8, 10, and 12p70 predicted survival in patients. For example, 5-year survival with interleukin-6 levels of >9 pg/mL was 30% compared with 63% for patients with levels ≤9 pg/mL (P=0.008). In this PAH cohort, cytokine levels were superior to traditional markers of prognosis such as 6-minute walk distance and hemodynamics.

Conclusions—This study illustrates dysregulation of a broad range of inflammatory mediators in idiopathic and familial PAH and demonstrates that cytokine levels have a previously unrecognized impact on patient survival. They may prove to be useful biomarkers and provide insight into the contribution of inflammation in PAH. **(*Circulation*. 2010;122:920-927.)**

Key Words: cytokines ■ inflammation ■ interleukins ■ pulmonary heart disease ■ pulmonary hypertension

Idiopathic pulmonary arterial hypertension (PAH) is a devastating condition characterized by remodeling of small to medium-sized pulmonary arteries, leading to elevated pulmonary arterial pressure and ultimately to right heart failure and death. Our knowledge of the pathogenesis and progression of idiopathic PAH is still limited. Mutations in the bone morphogenetic protein receptor type II (BMPR-II) gene are linked to susceptibility to both familial and sporadic forms of PAH.[1,2] However, because the penetrance of disease for known *BMPR2* mutations ranges from 15% to 80%,[3] additional "hits" must be required for initiation of disease. The exact nature of these remains unclear, but inflammation has been widely implicated.

Clinical Perspective on p 927

There is substantial evidence supporting a role for inflammatory cytokines in the development of idiopathic PAH. In patients with idiopathic PAH, increased circulating levels of monocyte chemoattractant protein-1, tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6[4,5] are observed. Inflammatory infiltrates, consisting of T and B lymphocytes and macrophages, are described in the plexiform lesions[6] characteristic of PAH. Animal models also support the role of inflammatory cytokines in the initiation and progression of PAH. For example, IL-6 is consistently elevated in animal models of experimental PAH.[7,8] Transgenic overexpression of IL-6 leads to severe pulmonary hypertension in mice,[9] and IL-6–deficient mice are protected from hypoxia-driven experimental pulmonary hypertension.[10] There is now increasing evidence for an interaction between BMPR-II and IL-6 in both in vitro and in vivo models. For example, transgenic mice overexpressing a dominant-negative BMPR-II demonstrated increased lung IL-6 expression and enhanced suscep-

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tibility to pulmonary hypertension.[11] In addition, increased IL-6 suppresses the expression of BMPR-II via induction of a micro-RNA cluster pathway.[12] Interestingly, adenoviral overexpression of IL-10 protects against monocrotaline-induced pulmonary hypertension in rats.[13] Here, we determined the profile of a broad range of cytokines in patients with idiopathic and heritable PAH and examined the relationship between these cytokines and the hemodynamics, functional capacity, and survival of these patients.

Methods

Study Subjects

Sixty patients with a confirmed diagnosis of idiopathic or heritable PAH were prospectively recruited at Papworth Hospital, Cambridge, UK, from 2001 to 2007. Idiopathic PAH was defined by mean pulmonary artery pressure of >25 mm Hg at rest with a pulmonary capillary wedge pressure of ≤15 mm Hg with no underlying cause for PAH (Venice classification 1a).[14] All patients were screened for mutations in BMPR-II. Eleven patients had a confirmed mutation in BMPR-II. One other patient had a family history of PAH but no mutation in BMPR-II. These 12 were classified as having heritable PAH and analyzed as a subset. In addition to basic demographics, the following data were gathered: hemodynamic measurements from the right heart catheterization (RHC) performed closest to the time of blood sampling for cytokines; exercise tolerance as measured by the 6-minute walk distance (6MWD) test; survival status at December 31, 2008, and survival time from sampling for cytokines to either death or transplantation; and exposure to PAH therapy, especially if the patients were on prostacyclin or prostaglandin analogs.

Sampling for cytokines and measurement of hemodynamics and 6MWD were undertaken after a diagnosis of idiopathic or heritable PAH was established. Sampling was performed once in each subject and at various times from diagnosis (mean, 1.42±2.4 years from diagnosis). The present study thus represents a series of point measurements of cytokine levels and clinical parameters in PAH patients who were at different stages of their disease. Survival time was calculated individually for each patient as the time period from blood sampling or clinical measurement to either death or transplantation.

Twenty-one control subjects were selected from a cohort of healthy volunteers. The mean age of control subjects was 42.0±13 years, and the ratio of men to women was 1:3. The study was approved by the local research ethics committee, and all participants gave informed written consent.

BMPR2 Mutation Analysis

The mutation status of PAH patients was determined as previously described.[15] Briefly, DNA was isolated by standard protocols, and the protein coding sequence for *BMPR2* was amplified from genomic DNA, together with intron-exon boundaries. Polymerase chain reaction products were purified with the QIAquick purification kit (Qiagen, Valencia, Calif) and cycle sequenced with ABI Big Dye terminator on an ABI PRISM 377 (Applied Biosystems, Foster City, Calif).

Serum Samples and Cytokine Levels

Peripheral venous blood samples were collected, kept on ice, and centrifuged within 30 minutes. Serum samples were stored at −20°C until analyzed. The system used was single-plex and multiplex assays manufactured by Meso Scale Discovery (Gaithersburg, Md). Serum from PAH patients (n=60) and control subjects (n=21) was incubated on a multiplex 96-well plate-based assay that contained antibodies to interferon-γ, TNF-α, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p70, and IL-13 or a single-plex 384-well plate-based assay with the IL-6 antibody. Briefly, the Meso Scale Discovery plex assays were run as follows. Calibration curves were prepared in the supplied assay diluent for human serum, with a range of 40 000 to 1.2 pg/mL, depending on the cytokine. Arrays were preincubated with 25 μL per well of assay diluent for 30 minutes. After the preincubation, 25 μL sample or calibrator was added in duplicate to the appropriate wells. The array was then incubated at room temperature for 2 hours. The array was washed with PBS plus 0.05% Tween 20, and 25 μL detection antibody reagent was added. After 2 hours of incubation at room temperature, the array was washed and the detection buffer was added. Results were read with a Meso Scale Discovery Sector Imager 6000. Sample cytokine concentrations were determined with Softmax Pro Version 4.6 software using curve fit models.

Table 1. Baseline Characteristics of All PAH Patients

	Control Subjects	IPAH		
		Total	BMPR2*mut*	BMPR2*wt*
N	21	58	12	46
Age, y	42.0 (13.0)	48.3 (15.3)	32.6 (10.5)*	52.4 (13.7)
Female:male	3:1	2.4:1	1.4:1	2.8:1
mPAP, mm Hg	. . .	48.6 (14.7)	60.2 (12.0)*	48.3 (14.8)
CI, $L \cdot min^{-1} \cdot m^{-2}$	. . .	2.0 (0.7)	1.9 (0.4)	2.0 (0.7)
PVR, Wood units	. . .	13.4 (7.6)	15.8 (8.5)	13.4 (7.2)
6MWD, m	. . .	332 (115)	416 (78)*	308 (113)

*$P<0.05$ versus BMPR*wt*.

Statistics

Values in tables are mean±SEM. Data were tested for adherence to a normal distribution with the Kolmogorov-Smirnov method. The Mann-Whitney test was used to compare nonparametric data, and the unpaired *t* test (with Welch correction) was used for parametric data. The Spearman test was performed for intergroup correlations. In graphs showing cytokine levels, median values are given. Statistical analysis, including Kaplan-Meier plotting, log-rank testing, and Cox proportional-hazard model analysis, was performed with SPSS version 16.0 and Prism 5.0 (SPSS Inc, Chicago, Ill). Kaplan-Meier curves were constructed on the basis of the quartile values of the parameter being measured. In addition, an interpolation process was undertaken to select a single value for cytokine level or hemodynamic parameter that demonstrated the largest difference for survival curves in between groups. In addition, the c index[16] was calculated to provide additional information on the most appropriate cutoff points for cytokines. Values of $P<0.05$ were considered statistically significant.

Results

Table 1 shows the demographics, hemodynamic parameters, and 6MWD for all patients.

Analysis of Cytokine Profile for All PAH Patients

Eight cytokines, IL-1β ($P=0.0004$), IL-2 ($P=0.0008$), IL-4 ($P=0.015$), IL-6 ($P<0.0001$), IL-8 ($P<0.0001$), IL-12p70 ($P=0.0489$), TNF-α ($P=0.0002$), and IL-10 ($P=0.014$), were significantly raised in the PAH group as a whole compared with healthy control subjects. No significant difference was observed in levels of IL-5, IL-13, or interferon-γ compared with healthy control subjects. Details of these results are presented in Table 2 and Figure 1. Two PAH patients were not included in the following analyses because their cytokine levels were >2 orders of magnitude greater than the mean for the group.

Impact of *BMPR2* Mutations on Clinical Characteristics and Cytokine Levels

Twelve patients had either a proven mutation in *BMPR2* or a family history of PAH, fitting the criteria for heritable PAH. They were analyzed as a subset. The patients with heritable

Table 2. Serum Cytokine Levels in PAH Patient Groups Versus Healthy Control Subjects

	Controls (n=21), pg/mL	IPAH (n=58), pg/mL	*BMPR2mut* (n=12), pg/mL	*BMPR2wt* (n=46), pg/mL
IL-1β	0.24 (0.05)	0.52 (0.08)†	0.46 (0.10)*	0.54 (0.09)†
IL-2	0.67 (0.08)	1.68 (0.40)†	1.40 (0.22)†	1.76 (0.50)†
IL-4	0.83 (0.07)	1.10 (0.64)*	1.40 (0.23)*	1.02 (0.08)
IL-5	1.37 (0.28)	1.97 (0.30)	2.25 (0.66)	1.89 (0.34)
IL-6	5.70 (0.40)	19.87 (7.45)†	14.56 (3.97)*	21.25 (9.36)†
IL-8	14.30 (1.08)	55.38 (22.18)†	40.04 (11.52)*	59.38 (27.85)†
IL-10	3.83 (1.07)	8.70 (1.66)*	10.00 (3.96)*	8.36 (1.85)*
IL-12	5.62 (1.99)	14.99 (5.42)*	13.85 (5.81)	15.29 (6.69)
IL-13	9.14 (2.58)	16.08 (3.39)	17.03 (6.52)	15.83 (3.95)
Interferon-γ	1.25 (0.14)	1.64 (0.17)	1.96 (0.36)	1.56 (0.19)
TNF-α	7.92 (0.34)	10.45 (0.55)†	9.85 (0.93)	10.61 (0.66)†

All values are expressed as mean (SEM).
*$P<0.05$, †$P<0.01$ versus control subjects.

PAH (known as *BMPR2mut*) were significantly younger than the patients free of mutations (*BMPR2wt*), and their mean pulmonary artery pressures (mPAPs) were higher (60.2±12.0 versus 48.3±14.8 mm Hg; $P=0.009$). There were no significant differences in either their CIs or PVRs (Table 1). As a group, heritable PAH patients demonstrated increased levels of IL-1β, IL-2, IL-4, IL-6, IL-8, and IL-10 (Table 2) compared with control subjects. There was no significant difference in cytokine levels between the heritable and *BMPR2wt* group.

Correlations Between Different Cytokines

Significant correlations were observed between individual cytokines. For example, levels of IL-8 demonstrated a weak correlation with TNF-α ($r=0.27$, $P<0.05$) and IL-2 ($r=0.34$, $P<0.01$), whereas IL-6 correlated strongly with levels of IL-8 ($r=0.94$, $P<0.0001$). Full results are shown in Table 3.

Correlations of Cytokine Levels With Hemodynamic Parameters

For the following analyses, heritable and idiopathic PAH patients were considered together. The median time from cytokine sampling to the closest RHC was 3.3 months (interquartile range, 0.7 to 7.2 months). We sought to determine whether cytokine levels correlated with the following hemodynamic parameters: mPAP, cardiac index (CI), and pulmonary vascular resistance (PVR). There was a negative correlation between IL-8 and CI ($r=-0.26$, $P=0.047$; see Table 3). However, there were no significant correlations between cytokine levels and mPAP or PVR (data not shown). When patients who had undergone RHC within 3 months of cytokine sampling were analyzed separately (n=31), there was a significant correlation between TNF-α and CI ($r=-0.39$, $P=0.03$). None of the other cytokines demonstrated any significant correlations with hemodynamic parameters in this subgroup.

Correlations of Cytokine Levels With 6MWD

We next questioned whether any of the cytokines levels correlated with exercise capacity, as measured by 6MWD. Only TNF-α showed a weak correlation with 6MWD at the time of sampling ($r=-0.24$), although this did not quite reach significance ($P=0.07$). None of the other cytokines measured correlated significantly with 6MWD.

Impact of Prostaglandin/Prostanoid Use on Cytokine Levels

Because the use of prostanoids in PAH has been shown to suppress levels of some cytokines,[16] we investigated the impact of these agents on circulating cytokine levels in our population. Of the 58 PAH patients, 30 were on prostanoid therapy of some kind (ie, epoprostenol, treprostinil, iloprost, or combinations of the above with agents such as sildenafil). Compared with the remaining 28 patients, prostanoid users exhibited significantly higher levels of IL-10 (4.72 versus 2.85 pg/mL in prostanoid-naive patients; $P=0.05$). There were also higher levels of IL-8 in prostanoid users, but this

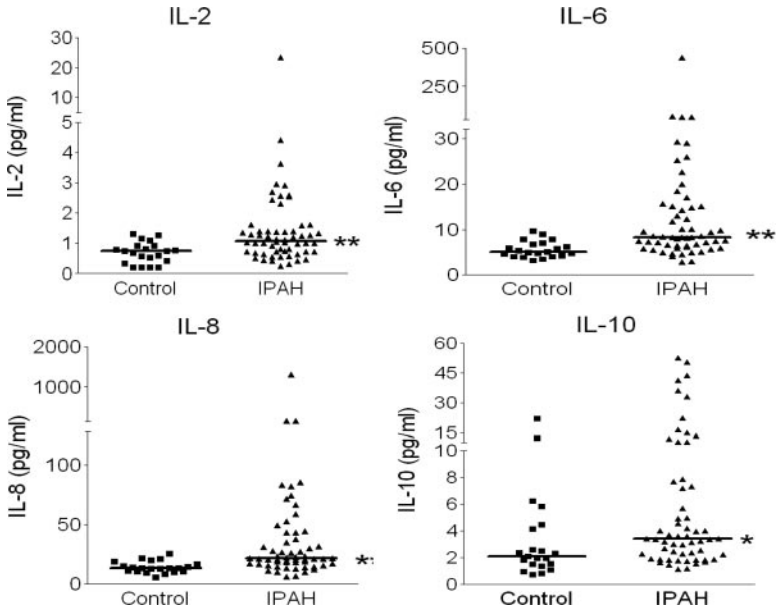


Figure 1. Graphs showing the distribution and medians of serum cytokine levels in PAH patients and control subjects. Graphs show IL-2, IL-6, IL-10, and IL-8. Note that axes are split on all graphs. Statistical significance was determined with the Mann-Whitney test. *$P<0.05$; **$P<0.01$.

Table 3. Analysis of Correlations Between Significantly Elevated Cytokines in PAH Patients

CI	IL-1β	IL-2	IL-6	IL-8	IL-10	IL-12	TNF-α	
	0.05	−0.04	−0.19	−0.20	−0.27*	−0.20	−0.15	CI
		0.31*	0.14	0.12	0.30*	0.20	−0.22	IL-1β
			0.28*	0.33†	0.22	0.21	0.17	IL-2
				0.94‡	0.32*	0.09	0.24	IL-6
					0.26	−0.04	0.27*	IL-8
						0.66†	0.09	IL-10
							−0.06	IL-12
								TNF-α

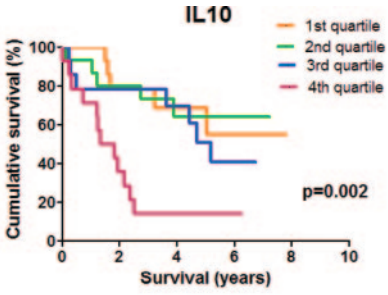
Correlation coefficients are determined by the Spearman method.
*$P<0.05$, †$P<0.01$, ‡$P<0.001$.

did not quite reach significance (26.65 versus 20.7 pg/mL in prostanoid-naive patients; $P=0.075$).

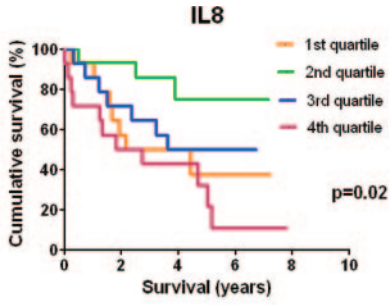
Correlations of Cytokine Levels With Survival Time

Kaplan-Meier analyses were undertaken for all significantly elevated cytokines for 57 of the 58 patients (1 patient had emigrated and was lost to follow-up). At the time of censoring, 25 patients had died without undergoing transplantation, and 5 had been transplanted. It was possible to stratify PAH patients into groups with significantly better and worse cumulative survival on the basis of levels of IL-2,IL-6, IL-8, IL-10, and IL-12p70.

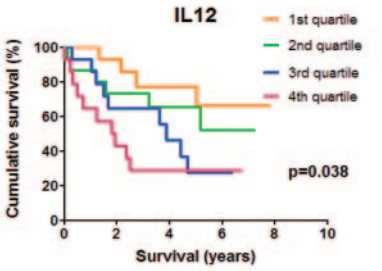
Initially, Kaplan-Meier curves were constructed through the use of the 4-quartile groups for each cytokine (Figure 2) and clinical parameter (Figure 3). The most discriminating survival curves were observed for IL-6, IL-8, IL-10, and IL-12p70. For example, the 1-year survival for patients in the



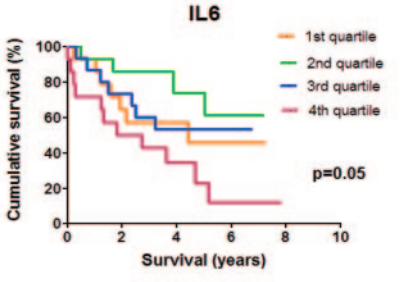
Patients at risk	0 yrs	2 yrs	4 yrs	6 yrs
1st quartile: 0-1.92 pg/ml	14	12	7	3
2nd quartile: 2.24-3.43	15	13	8	4
3rd quartile: 3.58-7.70	14	12	9	3
4th quartile: 7.85 – 52.72	14	6	3	2



Patients at risk	0 yrs	2 yrs	4 yrs	6 yrs
1st quartile: 0-17.21 pg/ml	14	9	5	2
2nd quartile: 17.42-21.98	15	15	8	3
3rd quartile: 22.04-43.38	14	11	8	5
4th quartile: 43.52-1300.23	14	8	6	2

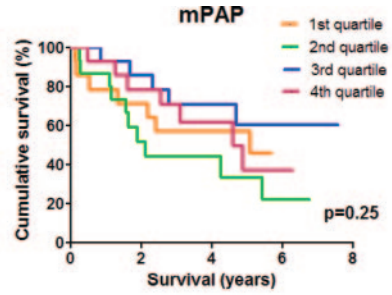


Patients at risk	0 yrs	2 yrs	4 yrs	6 yrs
1st quartile: 0-0.72 pg/ml	14	14	9	3
2nd quartile: 0.73-1.05	15	12	8	4
3rd quartile: 1.11- 1.41	14	10	6	3
4th quartile: 1.60-23.46	14	7	4	2

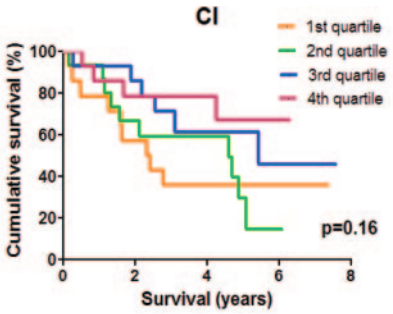


Patients at risk	0 yrs	2 yrs	4 yrs	6 yrs
1st quartile: 0-6.25 pg/ml	14	10	6	2
2nd quartile: 6.51-8.32	14	13	7	2
3rd quartile: 8.45-15.15	15	12	9	6
4th quartile: 15.19- 438.51	14	8	5	2

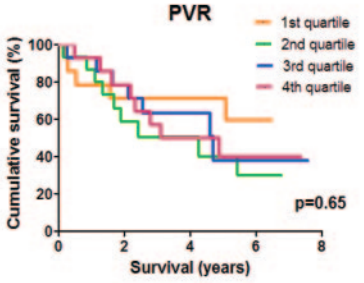
Figure 2. Kaplan-Meier analyses based on cytokine levels in PAH patients. Graphs show survival curves based on levels of IL-10, IL-8, IL-12, and IL-6. Note that these analyses were performed on 57 of 58 patients; 1 patient was lost to follow-up.



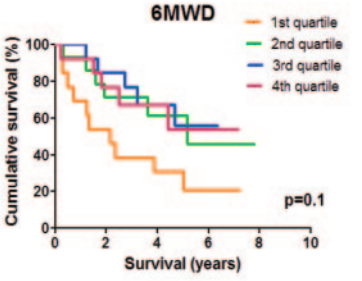
Patients at risk	0 yrs	2 yrs	4 yrs	6 yrs
1st quartile: 16-40 mmHg	14	11	8	1
2nd quartile: 41-53	15	8	5	2
3rd quartile: 54- 62	14	13	8	6
4th quartile: 63- 78	14	12	7	2



Patients at risk	0 yrs	2 yrs	4 yrs	6 yrs
1st quartile: 0.9- 1.5 l/min²	14	9	5	4
2nd quartile: 1.6-1.9	15	10	8	2
3rd quartile: 1.9-2.3	14	13	7	3
4th quartile: 2.3- 4.2	14	12	8	3



Patients at risk	0 yrs	2 yrs	4 yrs	6 yrs
1st quartile: 1.5-8.8 Wood units	14	11	9	2
2nd quartile: 8.9- 12.7	15	9	6	3
3rd quartile: 13.2- 18.3	14	12	6	3
4th quartile: 18.3- 36.9	14	12	7	3



Patients at risk	0 yrs	2 yrs	4 yrs	6 yrs
1st quartile: 50- 256m	13	8	5	3
2nd quartile: 262- 330	14	11	7	3
3rd quartile: 340- 400	13	12	8	3
4th quartile: 415- 660	13	11	6	3

Figure 3. Kaplan-Meier analyses based on traditional clinical parameters in PAH patients. Graphs show survival curves based on mPAP, CI, PVR, and 6MWD. Note that the analysis for the 6MWD graph was based on 53 of the 57 patients; 4 patients were unable to perform a 6MWD.

first quartile for IL-10 values was 100%, whereas the corresponding 1-year survival for patients in the fourth quartile was 71.4%. At 5 years, the cumulative survival for patients in the first quartile was 71.4%, whereas the cumulative survival for patients in the fourth quartile was 14.3%. The log-rank *P* value for comparing the 4-group curves for IL-10 was 0.002. For IL-12p70, the 1-year and 5-year cumulative survival rates for patients in the first quartile were 100% and 77.0%, whereas the 1-year and 5-year cumulative survival rates for patients in the fourth quartile were 64.3% and 28.6% (log-rank $P=0.038$).

In addition, we undertook a 2-group survival comparison based on a single cytokine level that provided the largest difference in survival curves. For example, the 1-year cumulative survival for patients with IL-10 ≤5 pg/mL was 92.3%, whereas it was 72.2% for patients with IL-10 levels of >5 pg/mL. These differences became more pronounced at 5 years, when the cumulative survival rates were 63.0% and 17.3%, respectively ($P=0.001$). A similar pattern was seen for IL-8; a serum level of ≤30 pg/mL was associated with a 1-year cumulative survival of 94.6%, whereas patients with a serum level of >30 pg/mL had a 1-year cumulative survival of 70.0%. The 5-year cumulative survival rates were 57.6% and 32.0%, respectively ($P=0.005$). PAH patients with levels of IL-6 ≤9 pg/mL had 1- and 5-year cumulative survival rates of 93.9% and 62.8%, respectively, whereas patients with levels >9 pg/mL had 1- and 5-year survival rates of 75.0% and 29.6% ($P=0.008$). PAH patients with levels of IL-12p70 of ≤7 pg/mL had 1- and 5-year cumulative survival rates of 92.3% and 61.3%, respectively, whereas IL-12p70 levels of >7 pg/mL had 1- and 5-year cumulative survival rates of 72.2% and 20.7% ($P=0.014$). These results are summarized in Table 4. Using the c index as another approach to determine the most discriminating level of each cytokine as a predictor of survival gave results very similar to the above analysis, except for IL-12, for which the median value of 3.85 pg/mL improved survival prediction slightly.

Kaplan-Meier analyses were also performed using classic parameters affecting prognosis, including hemodynamics derived from RHC (for all patients) and 6MWD (for 53 of 57 patients because 4 patients were unable to perform a 6MWD). First, we analyzed the relationship between hemodynamic parameters and survival using an approach similar to that used to determine cutoff levels of individual cytokines based on interpolation between the median and quartile values. By this analysis, a CI of <2 L · min^{-1} · m^{-2} and an mPAP of <50 mmHg proved to be significant discriminators for

Table 4. Cumulative Survival at 1-, 3-, and 5-Year Periods Based on Cytokine Levels and Classic Parameters

Cytokine	1-y Cumulative Survival, %	3-y Cumulative Survival, %	5-y Cumulative Survival, %	Log-Rank *P*
IL-1β ≤0.50 pg/mL	83.8	61.9	51.3	0.675
IL-1β >0.50 pg/mL	90.0	58.3	43.7	
IL-2 ≤1.50 pg/mL	88.4	69.4	56.0	0.021*
IL-2 >1.50 pg/mL	78.6	35.7	28.6	
IL-6 ≤9.00 pg/mL	93.9	75.8	62.8	0.008†
IL-6 >9.00 pg/mL	75.0	41.7	29.6	
IL-8 ≤30.0 pg/mL	94.6	72.5	57.6	0.005†
IL-8 >30.0 pg/mL	70.0	40.0	32.0	
IL-10 ≤5.00 pg/mL	92.3	79.5	63.0	0.001†
IL-10 >5.00 pg/mL	72.2	25.9	17.3	
IL-12 ≤7.00 pg/mL	92.3	71.4	61.3	0.014*
IL-12 >7.00 pg/mL	72.2	38.9	20.7	
TNF-α ≤12.0 pg/mL	86.8	59.5	51.0	0.879
TNF-α >12.0 pg/mL	84.2	63.2	47.4	
mPAP ≤65 mmHg	87.2	59.0	50.3	0.887
mPAP >65 mmHg	90.0	68.6	34.3	
CI $<2\ L \cdot min^{-1} \cdot /m^{-2}$	87.9	47.0	35.3	0.042*
CI $\geq 2\ L \cdot min^{-1} \cdot /m^{-2}$	87.5	79.2	66.8	
6MWD ≤332 m	81.5	55.3	46.1	0.125
6MWD >332 m	96.2	72.3	54.7	

*$P<0.05$, †$P<0.01$.

survival. Patients with a CI of $<2\ L \cdot min^{-1} \cdot m^{-2}$ had 1- and 5-year cumulative survival rates of 87.9% and 35.3%, respectively, whereas those with a CI $\geq 2\ L \cdot min^{-1} \cdot m^{-2}$ had 1- and 5-year cumulative survival rates of 87.5% and 66.8% ($P=0.04$). Patients with an mPAP of <50 mmHg had 1- and 5-year cumulative survival rates of 80.8% and 40.0%, respectively, and those with an mPAP of ≥50 mmHg had 1- and 5-year cumulative survival rates of 93.5% and 54.5% ($P=0.044$). A 6MWD of <330 m gave the best separation of the curves, but differences were not significant ($P=0.07$). Similarly, the optimal value for PVR did not achieve significant separation of the survival curves. We further analyzed 6MWD, mPAP, and CI based on predictive cutoff values reported in the literature. By this analysis, neither a 6MWD of ≤332 m (based on work by Miyamoto et al[17]) nor an mPAP of <65 mm Hg (based on work by Sitbon et al[18]) proved to be a significant discriminator for survival. Our cutoff value of CI ($<2\ L \cdot min^{-1} \cdot m^{-2}$) was identical to that determined by d'Alonzo et al.[19]

To further determine whether there was any contribution of hemodynamics to the ability of cytokine levels to predict survival, we analyzed hemodynamics in patients with "low" and "high" levels of IL-2, IL-6, IL-8, IL-10, and IL-12p70, broken down according to the most discriminatory cytokine levels used in the Kaplan-Meier analyses. There were no significant differences in mPAP, CI, or PVR for patients with either low or high levels of IL-2, IL-6, IL-8, and IL-12p70. However, for IL-10, the mean CI was higher in patients with low levels of IL-10 ($2.12\pm0.67\ L/min^2$) than in patients with high levels ($1.67\pm0.34\ L/min^2$; $P=0.003$). Using a Cox proportional-hazards model, we found that the additional consideration of hemodynamic indices did not significantly improve the predictive power of cytokine levels (Table I in the online-only Data Supplement). The addition of cytokine levels to a Cox model based on hemodynamic parameters did not significantly improve survival predictions based on hemodynamics (Table II in the online-only Data Supplement).

Discussion

This study is the first to simultaneously profile a broad range of cytokines in patients with idiopathic and heritable PAH. Our results indicate a significant dysregulation in levels of inflammatory cytokines in IPAH patients. Consistent with previous studies, we observed significantly elevated levels of IL-1β, IL-6, and TNF-α.[4,5] In addition, we demonstrated that the dysregulation of serum cytokines in PAH is more extensive than previously recognized and includes significantly increased serum levels of IL-2, IL-4, IL-8, IL-10, and IL-12p70 compared with normal control subjects.

A major strength of this study was the long-term follow-up of PAH patients at our center after serum cytokine measurement. This resource allowed us to assess for the first time the impact of a range of serum cytokines on survival. We have demonstrated that levels of IL-2, IL-6, IL-8, IL-10, and IL-12p70 were predictors of survival in this cohort. Furthermore, serum cytokine levels were more closely related to survival than classic indexes of right heart function measured at RHC and performed better than the widely used 6MWD. There was a lack of correlation between levels of cytokines and hemodynamic parameters as measured by RHC. This is interesting because it may mean that cytokines are potentially involved in the pathogenesis of PAH and are not just a reflection of right ventricular function. An earlier study by Humbert et al[4] that examined IL-1β and IL-6 levels in 29 patients with IPAH also did not show any correlation between cytokine levels and hemodynamic parameters.

Our study does not directly address the mechanism and pathological role of increased cytokines in PAH. Inflammation and inflammatory cytokines such as IL-6 and IL-8 may be an important part of the initial response to injury contributing to the process of vascular remodeling in PAH. Elevated levels of antiinflammatory cytokines such as IL-10 may serve as compensatory mechanisms. The patients in our population were on various approved treatments for PAH, often in combination. Almost half of the patients were on prostanoid therapy. A previous study has shown that administration of epoprostenol led to a significant decline in elevated circulating levels of monocyte chemoattractant protein-1 in PAH patients.[20] In our PAH population, prostanoids did not ameliorate the elevated cytokine levels observed in PAH patients. Indeed, there was a trend toward an increase in IL-10 and IL-8. We suspect that this was due to the presence of more advanced disease. Because prostanoids are indicated for severe disease, it is consistent with our findings that elevated levels of IL-10 and Il-8 are found in patients prescribed prostanoids.

Animal studies strongly support a pathological role for IL-6 in PAH. Overexpression of IL-6 induces PAH and vascular remodeling in rodents and further augments

hypoxia-driven pulmonary hypertension.[8–10] Interestingly, levels of IL-6 have a significant impact on survival despite having no significant correlation with hemodynamic parameters or exercise tolerance. Additionally, IL-8 levels correlated only weakly with CI. Previous studies support a wider role for IL-6 and IL-8 than modulation of immune responses. Indeed, IL-6 and IL-8 have been demonstrated to modulate smooth muscle and endothelial cell function and are strongly implicated in vascular remodeling.[21–25] Furthermore, Brock et al[12] recently described a novel pathway by which IL-6 can affect micro-RNAs and signaling via BMPR-II. Collectively, these studies support the notion that IL-6 and IL-8 can directly affect vascular remodeling.

The increased levels of the antiinflammatory cytokine IL-10 observed in patient sera may represent a compensatory mechanism antagonizing the inflammatory response. Adenoviral expression of IL-10 protects against monocrotaline-induced pulmonary hypertension in rats.[13] Furthermore, IL-10 is a potent immunomodulator that inhibits the secretion of various proinflammatory cytokines, including IL-6 and IL-8.[13,26] Collectively, this supports the notion that elevated serum levels of IL-10 in IPAH patients represent a compensatory mechanism to prevent inflammatory cell infiltration, chemokine expression, and smooth muscle cell proliferation. Studies to define the precise mechanisms by which these inflammatory cytokines act in vivo are currently ongoing.

Consistent with previous studies, we found IL-1β to be elevated in patient serum. Circulating levels of IL-2, like IL-1β, are also elevated in chronic left heart failure. A major role for IL-2 is to promote T-cell proliferation. Indeed, infusion of recombinant IL-2 has been used to treat some cancers. A noted side effect of this treatment is pulmonary edema. Administration of IL-2 in animal studies has demonstrated that IL-2 promotes pulmonary edema via enhanced vascular permeability. These same studies noted that IL-2 promoted vasoconstriction and pulmonary hypertension.[27,28] Although the pathological mechanisms by which IL-2 promotes these effects remains unclear, IL-2 induces expression of endothelin-1, a key pathological factor in IPAH.[29]

Elevated TNF-α in serum has previously been shown to correlate with mortality in left heart failure patients.[30] Surprisingly, levels of TNF-α, although significantly elevated in IPAH patients, did not correlate with survival or hemodynamic parameters in our cohort. Recent anti–TNF-α trials in heart failure patients failed to demonstrate efficacy,[31,32] suggesting a more complex role for TNF-α. One potential limitation of the present study is that hemodynamic data sampling and serum collection did not coincide in some of our patients. The median time from cytokine sampling to RHC was 3.3 months. However, when we analyzed only those patients in whom sampling and RHC occurred within 3 months of each other, we did find a significant correlation between CI and TNF-α. No other cytokine showed a correlation with hemodynamics in this subgroup.

The strong impact of circulating levels of IL-6, IL-8, and IL-10 on mortality suggests that these cytokines may prove to be important tools in risk stratifying IPAH patients. In this particular cohort, cytokine levels appeared to be more useful than traditional parameters such as hemodynamics and 6MWD. Focused studies to define the temporal changes of these cytokines during disease progression and treatment are now warranted. Finally, the finding that elevated levels of cytokines have an impact on mortality supports the need for further studies to define the potential for targeting these cytokines for therapeutic intervention in PAH.

Conclusions

The present study identifies, for the first time, elevated serum levels of IL-2, IL-4, IL-8, IL-10, and IL-12p70 in patients with idiopathic and familial PAH. We have also confirmed that IL-1β, IL-6, and TNF-α are elevated in PAH, confirming the results of previous smaller studies. Circulating levels of IL-2, IL-6, IL-8, IL-10, and IL-12p70 had a significant impact on survival and may prove to be important biomarkers in risk stratifying these patients.

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Disclosures

Dr Soon has received travel awards from GlaxoSmithKline and Pfizer and a research grant from Pfizer. Dr Holmes is an employee of Novartis Institutes for BioMedical Research. C. Treacy has received travel awards from GlaxoSmithKline and Pfizer. N. Doughty has received an educational grant from Actelion and travel awards from Pfizer and Actelion and is a member of the nurse advisory board for United Therapeutics, Actelion, and GlaxoSmith-Kline. Dr Barker is a former employee of Novartis. Drs Jennings, Nicklin, and Budd are employees of Novartis. Dr Walker was an employee of Novartis. Dr Pepke-Zaba has received honoraria from Actelion, Pfizer, GlaxoSmithKline, Encysive, and Schering for speaking at conferences. She also holds a joint research grant between Actelion, Pfizer, Schering, and United Therapeutics. Dr Morrell has received a research grant from Novartis. The other authors report no conflicts.

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CLINICAL PERSPECTIVE

Inflammation is known to be a feature of pulmonary arterial hypertension (PAH), and increased circulating levels of cytokines have been reported in patients with PAH. A significant number of animal studies have also highlighted the importance of cytokines in the pathogenesis of PAH. However, to date, no information exists on the significance of elevated cytokines. We sought to determine the levels of a range of cytokines in PAH and to examine their impact on survival and their relationship to hemodynamic indexes. We measured levels of tumor necrosis factor-α, interferon-γ, and interleukins-1β, -2, -4, -5, -6, -8, -10, -12p70, and -13 using ELISAs in idiopathic and heritable PAH patients. Concurrent clinical data included hemodynamics, 6-minute walk distance, and survival time from sampling to death or transplantation. PAH patients had significantly higher levels of interleukin-1β, -2, -4, -6, -8, -10, and -12p70 and tumor necrosis factor-α compared with healthy control subjects. Kaplan-Meier analysis showed that levels of interleukin-6, -8, -10, and -12p70 predicted survival in patients. For example, 5-year survival with interleukin-6 levels of $>$9 pg/mL was 30% compared with 63% for patients with levels $\leq$9 pg/ml ($p=0.008$). In this cohort, cytokine levels were superior to traditional markers of prognosis such as hemodynamics. This study illustrates dysregulation of a broad range of inflammatory mediators in idiopathic PAH and demonstrates that cytokine levels have a previously unrecognized impact on patient survival. They may prove to be useful biomarkers of prognosis once validated prospectively. They may also provide insight into the contribution of inflammation in PAH.

Elevated Levels of Inflammatory Cytokines Predict Survival in Idiopathic and Familial Pulmonary Arterial Hypertension

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Supplementary Table 1: Cox regression modeling based on cytokine levels with the addition of hemodynamic parameters

	Parameter alone	Parameter + mPAP	Parameter + cardiac index	Parameter + PVR	Parameter + mPAP + CI + PVR
IL-2					
HR (per pg/ml)	1.098	1.104	1.083	1.094	1.091
95% CI	1.012-1.190	1.016-1.200	0.999-1.175	1.007-1.189	1.003-1.186
p-value	0.025*	0.019*	0.054	0.033*	0.042*
IL-6					
HR (per pg/ml)	1.005	1.005	1.004	1.005	1.004
95% CI	1.000-1.009	1.001-1.010	0.999-1.008	1.000-1.009	1.000-1.009
p-value	0.038*	0.029*	0.084	0.049*	0.055
IL-8					
HR (per pg/ml)	1.002	1.002	1.001	1.002	1.001
95% CI	1.000-1.003	1.000-1.003	1.000-1.003	1.000-1.003	1.000-1.003
p-value	0.035*	0.028*	0.081	0.044*	0.062
IL-10					
HR (per pg/ml)	1.025	1.026	1.022	1.025	1.023
95% CI	1.003-1.047	1.004-1.048	0.99-1.044	1.002-1.048	0.999-1.047
p-value	0.025*	0.021*	0.056	0.035	0.063
mPAP					
HR (per mmHg)	0.991	-	0.978	0.974	0.973
95% CI	0.966-1.016	-	0.950-1.007	0.940-1.009	0.937-1.011
p-value	0.472	-	0.14	0.14	0.163
Cardiac index					
HR (per l/min/m^2)	0.495	0.42	-	0.424	0.457
95% CI	0.232-1.057	0.193-0.912	-	0.171-1.054	0.193-1.082
p-value	0.069	0.028*	-	0.065	0.075
PVR					
HR (per Wood unit)	1.013	1.045	0.982	-	1.015
95% CI	0.968-1.060	0.986-1.108	0.928-1.040	-	0.946-1.088
p-value	0.569	0.142	0.535	-	0.682

Key:
*$p<0.05$

HR: hazard ratio
CI: confidence intervals

Supplementary Table 2: Cox regression modeling based on hemodynamic parameters with additional cytokine parameters

	Parameter alone	Parameter + IL-6	Parameter + IL-8	Parameter + IL-10	Parameter + IL-12
mPAP					
HR (per mmHg)	0.991	0.988	0.989	0.989	0.991
95% CI	0.996-1.016	0.963-1.014	0.963-1.015	0.963-1.015	0.965-1.016
p-value	0.472	0.370	0.396	0.384	0.468
Cardiac index					
HR (per l/min/m2)	0.495	0.526	0.532	0.528	0.493
95% CI	0.232-1.057	0.246-1.124	0.242-1.169	0.247-1.130	0.231-1.054
p-value	0.069	0.097	0.116	0.100	0.068
PVR					
HR (per Wood unit)	1.013	1.009	1.009	1.003	1.013
95% CI	0.968-1.060	0.963-1.058	0.963-1.058	0.955-1.053	0.968-1.060
p-value	0.569	0.701	0.694	0.915	0.571

HR: hazard ratio
CI: confidence intervals