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Circulating angiopoietins in idiopathic pulmonary arterial hypertension

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Aims To determine the diagnostic utility of circulating angiopoietin-1 (Ang-1) and its antagonist angiopoietin-2 (Ang-2) as potential biomarkers of disease severity or response to treatment in idiopathic pulmonary arterial hypertension (IPAH). Imbalances in angiogenic factors including vascular endothelial cell growth factor (VEGF) and the angiopoetin–Tie2 receptor system have been implicated in the pathogenesis of IPAH.

Methods and results Plasma Ang-1, Ang-2, soluble Tie2 (sTie2), and VEGF were determined by in-house immunoassays in two cohorts of IPAH patients: a retrospective cohort ($n = 81$) and a prospective cohort ($n = 25$). Ten patients with normal pulmonary artery pressures and 14 apparently healthy subjects served as controls. Plasma levels of all angiogenic factors were elevated in IPAH patients compared with controls (all $P < 0.005$). Angiopoietin-2, but not Ang-1, sTie2, and VEGF correlated with cardiac index ($r = -0.53$, $P < 0.001$), pulmonary vascular resistance (PVR) ($r = 0.60$, $P < 0.001$), and mixed venous oxygen saturation (SvO_2) ($r = -0.63$, $P < 0.001$). In multivariate analysis, elevated Ang-2 was an independent risk factor of mortality ($P = 0.004$). The patients in the prospective cohort were studied longitudinally at baseline and 3 months after initiation of therapy. Changes in Ang-2 after initiation of therapy correlated with changes in mean right atrial pressure ($r = 0.6$, $P = 0.008$), PVR ($r = 0.51$, $P = 0.04$), and inversely related to changes in SvO_2 ($r = -0.75$, $P < 0.001$). Histological studies showed that the expression of Ang-2 mRNA and protein was up-regulated in plexiform lesions from IPAH lung tissue samples.

Conclusion Ang-2 may be involved in the pathogenesis of IPAH, and plasma Ang-2 might serve as a promising new biomarker of disease severity and response to treatment in patients with IPAH.

Keywords Pulmonary hypertension • Endothelium-derived factors • Angiopoietins • Biomarker • Remodelling • Smooth muscle cells

Introduction

Idiopathic pulmonary arterial hypertension (IPAH) is a devastating chronic disease caused by progressive pulmonary vascular remodelling, which results in right ventricular overload and failure if not treated effectively.[1,2] Among the molecular mechanisms involved in the pathogenesis of IPAH, the angiopoietin–Tie2 ligand-receptor system has been recognized as a major signalling pathway that controls vascular remodelling and stabilization in a non-redundant manner.[3–8]

Angiopoietins are angiogenic factors essential for vascular development and maturation.[9] As circulating or matrix-bound molecules, angiopoietin-1 (Ang-1) and its antagonist angiopoietin-2 (Ang-2) bind to the extracellular domain of the tyrosine kinase receptor Tie2 which is almost exclusively expressed on endothelial cells (ECs).[10–12] Produced by vascular smooth muscle cells

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(SMCs) and precursor pericytes, Ang-1 stabilizes the development of newly formed blood vessels by recruiting mural cells and promotes quiescence and structural integrity of mature vessels.[13] The importance of operational Ang-1/Tie2 signalling for developmental angiogenesis is illustrated by either Ang-1$^{-/-}$ and Tie2$^{-/-}$ knockout mice which die *in utero* owing to severe vascular remodelling defects causing perturbed vascular integrity.[14,15] In the adult vasculature, constitutive Ang-1 expression and low-level Tie2 phosphorylation probably represent a control pathway to maintain vascular quiescence by antiapoptotic and anti-inflammatory effects, thus protecting the endothelium from excessive activation by cytokines and growth factors.[13,16,17]

Ang-2 is expressed in ECs, where it is stored in granules, the so-called Weibel–Palade bodies.[18] The release of Ang-2 upon activation of the endothelium with for instance thrombin, histamine, or hypoxia disrupts the constitutive Ang-1/Tie2 signalling by preventing Ang-1 from binding to the receptor.[10,18–20] Consequently, loss of Tie2 signalling destabilizes the endothelium and induces an angiogenic response in the presence of vascular endothelial growth factor (VEGF), whereas in the absence of VEGF, Ang-2 induces EC death and vessel regression.[21] Hence, Ang-2 functions as a dynamic autocrine-negative regulator of the quiescent resting endothelium.[13,16]

Several lines of evidence suggest that aberrant activation of Tie2 is causally involved in PAH pathophysiology, although the complex interaction between these angiogenic and anti-angiogenic molecules remains incompletely understood. Tie2 activation attenuates bone morphogenetic protein signalling and increases the concurrent release of serotonin, a potent stimulator of SMC proliferation in human pulmonary artery ECs. Both mechanisms have already been implicated in PAH pathophysiology.[5,8] Further support for the causative role of Ang-1 in PAH comes from the fact that overexpression of Ang-1 in rodents results in a PAH-like phenotype.[3,8] Consistently, overexpression of a soluble Tie2 (sTie2) ectodomain, which sequesters Ang-1, suppresses the pulmonary hypertension (PH) phenotype in Ang-1-induced PH.[22] On the other hand, Stewart and colleagues[23,24] reported that operative Ang-1/Tie2 signalling protects from endothelial apoptosis, capillary rarefication, and finally the development of PH.

The present study was performed to investigate the possible role of members of the Ang–Tie2 family as biomarkers in patients with IPAH. Therefore, plasma levels of Ang-1, Ang-2, sTie2, and VEGF were correlated with haemodynamics, disease severity, response to treatment, and outcome in patients with IPAH.

Methods

Patients and study design

In all patients, the diagnosis of IPAH was based on standard criteria[25] with confirmation by right heart catheterization and exclusion of other forms of PH by various laboratory studies, echocardiography, pulmonary function testing, chest X-ray, ventilation–perfusion scanning, chest computed tomography angiography, and/or pulmonary angiography, if necessary.[25] All catheter examinations were done for clinical reasons unrelated to this study. All patients gave written, informed consent to the storage and later analysis of the blood samples for scientific purposes. The study was performed in accordance with the declaration of Helsinki and approved by the institutional review board.

There were two patient groups; the first patient cohort consisted of 81 non-selected, treatment-naïve patients with IPAH referred to Hannover Medical School between 1999 and 2008 (*Table 1*). The second patient cohort included 25 consecutive patients with IPAH studied prospectively at Hannover Medical School between 2006 and 2009 (see *Table 4* below). In contrast to the first cohort, these patients had systematic follow-up right heart catheterizations at

Table 1 Baseline characteristics of the first cohort

	IPAH (n = 81)	Disease controls (n = 10)	P-value
Age (years)	54 (43-62)	56.5 (46–67)	0.45
Female (%)	65	57	0.58
Body mass index (kg/m^2)	24.4 (22.1–27.2)	25.7 (18.1–33.5)	0.52
6 minute walking distance (m)	370 (297–444)	—	—
WHO class	3.0 ± 0.5	—	—
I/II (n, %)	4 (4.3)	—	—
III (n, %)	60 (74.5)	—	—
IV (n, %)	17 (21.3)	—	—
Mean RAP (mmHg)	7.6 (6–9)	1.5 (1–3)	<0.001
Mean PAP (mmHg)	54 (51–58)	20 (17–23)	<0.001
Mean PCWP (mmHg)	6 (6-7)	4.6 (2.5–6.7)	0.28
Cardiac output (L/min)	3.9 (3.6–4.2)	5.4 (3.7–7.1)	0.001
Cardiac index (L/min/m^2)	2.2 (2–2.4)	2.9 (2.3–3.4)	0.007
PVR (dyn s/cm^5)	1.067 (960–1.174)	240 (168–312)	<0.001
SvO_2 (%)	64 (61–66)	75 (67–83)	<0.001

Except for gender (%) and WHO class (mean ± SD), data are shown as median (IQR). Differences between patients and controls were calculated by using Mann–Whitney U-test for continues variables and χ^2 test for nominal variables. Data on 6 min walking distance were available from n = 30 individuals. IPAH, idiopathic pulmonary arterial hypertension; WHO class, functional World Health Organization classification of heart failure; RAP, right atrial pressure; PAP, pulmonary arterial pressure; PCWP, pulmonary capillary wedge pressure; PVR, denotes pulmonary vascular resistance; SvO_2, mixed venous oxygen saturation.

baseline and 3 months after the introduction of PAH-targeted therapy (see Supplementary material online, *File 1* for details on PAH-targeted therapy).

Baseline blood samples were collected in both IPAH cohorts and disease controls at the time of the initial right heart catheterization before the initiation of any PAH-targeted therapy. In the prospective IPAH cohort, additional blood samples were obtained 3 months after the introduction of medical therapy at the time of follow-up right heart catheterizations.

Follow-up and outcome definitions

Patients from the first cohort were followed by regular outpatient assessments for a median of 38 months (range, 3–89 months). Forty-four patients reached the primary composite endpoint of death ($n = 41$) or lung transplantation ($n = 3$). Survival status was censored on 30 April 2009. Two patients were lost to follow-up. In the prospective cohort, patients were followed up for 3 months after initiating therapy; follow-up ended with a second right heart catheterization.

Controls

There were two control groups; the first one consisted of 10 patients referred to Hannover Medical School between 2007 and 2009 with suspected PH by echocardiography that was eventually excluded by right heart catheterization [mean pulmonary arterial pressure (PAPm) <25 mmHg] referred to as *disease controls* (*Table 1*). The second control group consisted of 14 apparently healthy volunteers without a history of chronic disease (referred to as *healthy controls*).

Blood sampling and laboratory analyses

All samples were collected into ethylenediaminetetraacetic acid tubes and were immediately placed on ice. Within 30 min of collection, samples were centrifuged at 3000 *g* for 10 min, divided into aliquots and stored at -80°C. Creatinine and uric acid were measured using standard laboratory techniques. N-terminal fragment of the B-type natriuretic peptide (NT-proBNP) was determined using a sandwich immunoassay on an Elecsys 2010 instrument with a detection limit of 20 ng/L (Roche Diagnostics, Mannheim, Germany). All measurements were performed by investigators blinded to patients' characteristics and outcome.

Quantification of circulating angiogenic factors

Plasma Ang-1 and Ang-2 were measured by in-house immunoluminometric assay methodology as previously reported by our group in detail.[26,27] In our hands, the assays had detection limits of 0.12 ng/mL (Ang-1) and 0.2 ng/mL (Ang-2). Inter- and intra-assay imprecision was $\leq$8.8 and 3.7% for Ang-1 and was $\leq$4.6 and 5.2% for Ang-2, respectively.

Plasma VEGF (biologically active VEGF-A_{121} and VEGF-A_{165}) and sTie2 were measured using commercially available sandwich ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. All assays were performed in duplicate by investigators blinded to patients' characteristics and outcome.

Lung tissue sampling and immunohistochemical staining

Tissue samples from lung explants were obtained from 13 additional patients with IPAH as well as from 4 brain-dead organ donors. Formalin-fixed and paraffin-embedded lung tissue sections were stained with Angiopoietin-2 antibody {dilution 1:15, goat anti-human Angiopoietin-2 Antibody, R&D System; secondary anti-mouse-polymer-antibody [Reagent 3, ZytoChem Plus (HRP) Polymer Kit, Zytomed Systems, Berlin, Germany]; chromogene substrate: DAB (DAB Substrate Kit High Contrast, Zytomed Systems)} and counterstained with Haemalaun (see Supplementary material online, *File 2* for details).

Microdissection and quantitative real-time polymerase chain reaction

Laser-assisted microdissection of distinct anatomical lung structures was performed as described earlier.[28,29] Ang-2 expression levels (real-time polymerase chain reaction) were analysed in plexiform lesions from IPAH patients and compared relatively with those in the unaffected adjacent arterioles within the same sample, as well as to arterioles, arteries, and alveolar septa in lung samples from brain-dead donors (see Supplementary material online, *File 2* for details).

Statistical analysis

Data are presented as absolute numbers, percentages, means with corresponding standard deviations, or medians with corresponding 25th and 75th percentiles [inter-quartile range (IQR)]. Baseline characteristics of IPAH patients and control subjects and the differences in angiogenic factors between IPAH patients and controls were compared using the two-sided Mann–Whitney *U*-test. χ^2 analysis was used to compare gender. The relationship between the angiogenic factors and haemodynamic as well as 6 min walk distance was investigated using Pearson's product–moment correlation. In all parametric tests, preliminary analysis and transformation were performed to ensure no violation of the assumption of normality, linearity and homoscedasticity. The Kaplan–Meier plots were used to illustrate the timing of events during follow-up in relation to baseline Ang-2 levels and statistical assessment was performed by the log-rank test. The association of circulating Ang-2 with outcome was evaluated in adjusted Cox's proportional hazards regression models. Selection of variables to be included in the multivariable models was done a priori by determining probable confounders based on differences in baseline characteristics between patients with different Ang-2 levels and based on theoretical considerations. To fulfil the assumptions needed for the analysis, logarithmic (ln) transformation of NT-proBNP and Ang-2 was performed. For comparison of the prognostic values of Ang-2, uric acid, NT-proBNP, and selected haemodynamic parameters, receiver operating characteristic (ROC) curves were generated, and the areas under the curves (AUCs) were calculated. The Wilcoxon signed-rank test was used to compare changes in haemodynamic parameters, 6 min walking distance, and angiogenic factors over time. Changes in these parameters in relation to changes in Ang-2 over time were assessed by Spearman's rank correlation coefficients. All tests were two-sided and significance was accepted at $P < 0.05$. Data analysis was performed using SPSS (SPSS Inc., Chicago, IL, USA). Figures were prepared using the GraphPad Prism (GraphPad Prism Software Inc., San Diego, CA, USA).

Results

Patient's characteristics

The first (retrospective) patient cohort consisted of 81 patients (65% female) with a median (IQR) age of 54 (43–62) years. Control subjects (exclusion of PH by right heart catheterization) were not different with respect to age, gender, and body mass index, but showed significantly better haemodynamics and functional capacity compared with IPAH patients. Demographic, clinical, and biochemical characteristics of patients and controls are summarized in *Table 1*.

Plasma levels of angiogenic factors are elevated in pulmonary arterial hypertension

The median Ang-1 level was three-fold higher in patients with IPAH compared with apparently healthy controls [14.8 (11.8–17.9) vs. 4.4 (1.8–6.9) ng/mL; $P < 0.001$]. The same was true for Ang-2 (four-fold over controls) [6.5 (5.1–7.7) vs. 1.6 (0.8–2.3) ng/mL; $P = 0.001$], sTie2 [1.7 (1.5–1.8) vs. 1.2 (1.0–1.4) ng/mL; $P < 0.01$], and VEGF [232.4 (174.8–290.1) vs. 53.7 (30.6–76.7) ng/mL; $P < 0.001$] (*Figure 1*). No differences were detected between apparently healthy controls and patient controls (PAPm < 25 mmHg).

Only angiopoietin-2, but not angiopoietin-1, soluble Tie2, or vascular endothelial growth factor correlates with disease severity

Next, we investigated the relationship of circulating angiogenic factors and disease severity. Only Ang-2, but not Ang-1, sTie2, or VEGF showed a strong and consistent association with several clinicopathological variables in the first patient cohort (see Supplementary material online, *File 3* for details). The relation of Ang-2 to pulmonary haemodynamics was illustrated by a close correlation with mean right atrial pressure (mRAP) ($r = 0.53$, $P < 0.001$) and pulmonary vascular resistance (PVR) ($r = 0.6$, $P < 0.001$) (*Figure 2A*). Moreover, Ang-2 correlated positively with New York Heart Association (WHO) class ($r = 0.47$, $P < 0.001$) and was inversely correlated with cardiac index ($r = -0.53$, $P < 0.001$) and mixed venous oxygen saturation (SvO_2) ($r = -0.63$, $P < 0.001$) (*Figure 2B*). These findings were confirmed in the second (prospective) group (see below). Angiopoietin-1 solely correlated with VEGF ($r = 0.75$, $P < 0.001$), whereas Ang-2 solely correlated with sTie2 levels ($r = 0.49$, $P < 0.001$).

Elevated circulating angiopoietin-2 is associated with impaired haemodynamics, excess N-terminal pro-B-type natriuretic peptide levels and unfavourable outcome

Receiver operating characteristic curve analysis demonstrated that Ang-2 was a strong predictor of adverse outcome in the first patient cohort. Of the 81 patients in the retrospective group, 44 (54.3%) who died during follow-up had significantly higher levels of Ang-2 at baseline [7.7 (5.8–9.6) ng/mL], compared with patients who survived [3.7 (2.7–4.8) ng/mL; $P < 0.001$]. The best Ang-2 cut-off level for predicting outcome at 3 years was 2.9 ng/mL (sensitivity 85% and specificity 58%) with an AUC of 0.79 [95%

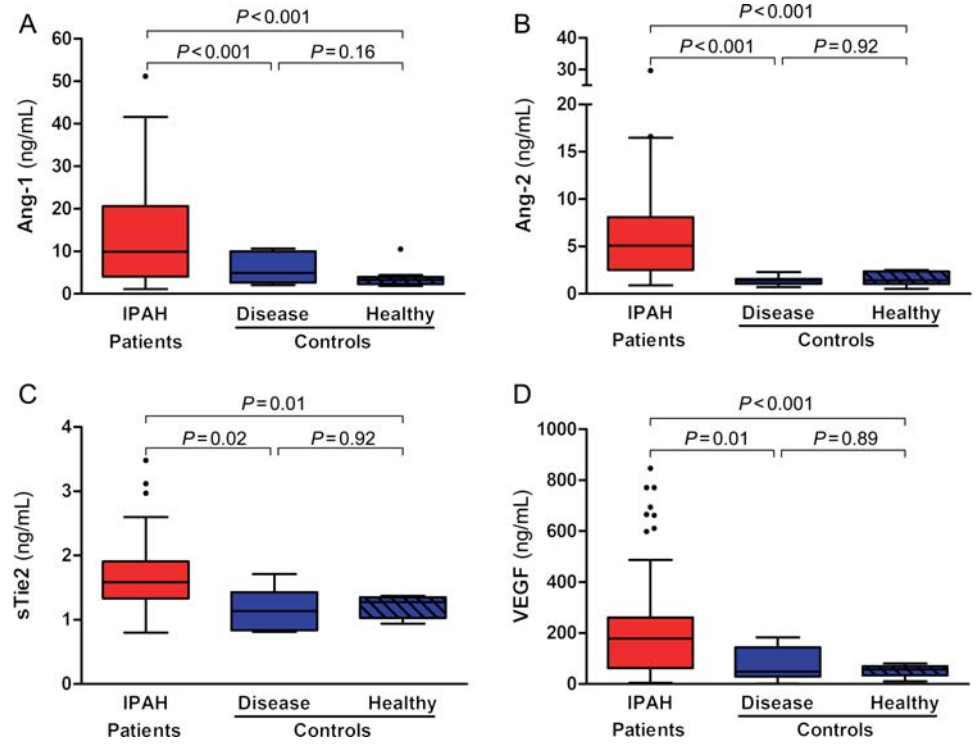

Figure 1 Angiogenic factors are elevated in IPAH. Box plots showing plasma levels of angiopoietin 1 (Ang-1, *A*) and 2 (Ang-2, *B*), soluble Tie-2 receptor (sTie2, *C*), and vascular endothelial growth factor (VEGF, *D*). The differences between patients with idiopathic pulmonary hypertension and control subjects were assessed by Mann–Whitney *U*-test. IPAH, idiopathic pulmonary arterial hypertension; PAP, pulmonary arterial pressure. Angiopoietin-1and Ang-2 were available from $n = 81$ individuals with IPAH, $n = 10$ patients with mean pulmonary arterial pressure (PAPm) <25 mmHg (disease controls) and $n = 14$ apparently healthy individuals (healthy controls), sTie and VEGF were available from $n = 60$ individuals with IPAH, $n = 10$ individuals with PAPm < 25 mmHg and $n = 14$ apparently healthy individuals.

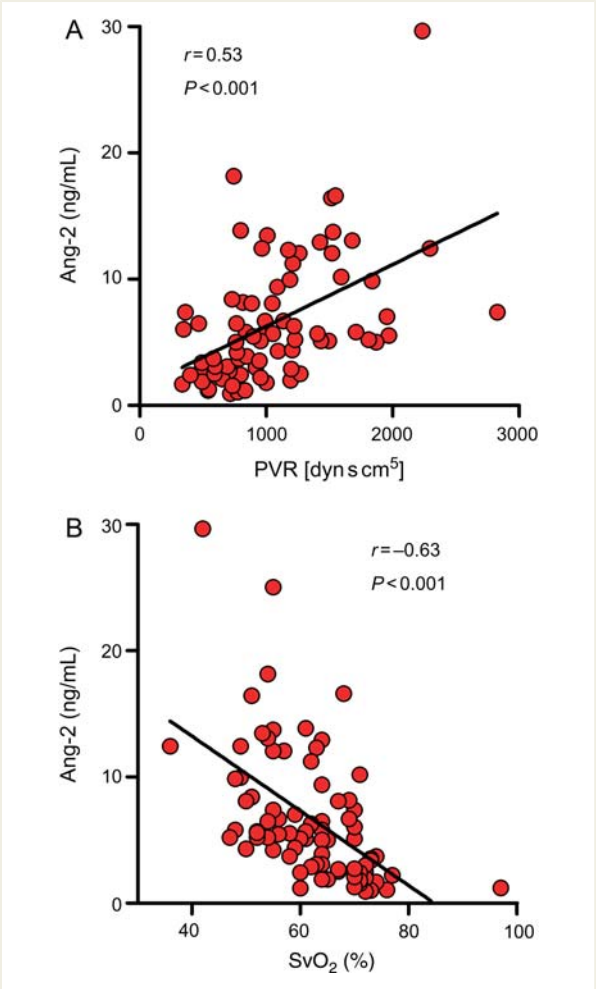

Figure 2 Circulating Ang-2 correlates with disease severity. Scatter plots showing the relation of angiopoietin-2 (Ang-2) to (*A*) pulmonary vascular resistance (PVR) and (*B*) mixed venous oxygen saturation (SvO_2) in the first patient cohort.

confidence interval (CI), 0.67–0.91] (*Figure 3*). Fifty-two patients (64% of the first cohort) had Ang-2 levels >2.9 ng/mL. These patients showed a more advanced disease stages as evidenced by higher WHO class, higher mRAP and PAP, and a higher PVR compared with patients with Ang-2 values ≤2.9 ng/mL) (*Table 2*). Moreover, patients with Ang-2 > 2.9 ng/mL had a lower cardiac index, lower mixed venous oxygen saturation, and more elevated concentrations of uric acid and NT-proBNP. No significant differences were observed with regard to age, gender, 6 min walking distance, and Ang-1 or VEGF levels (*Table 2*).

Survival rates at 1 year were 100% in patients with baseline Ang-2 ≤ 2.9 ng/mL compared with 78% in patients with Ang-2 > 2.9 ng/mL ($P < 0.001$). Survival rates during follow-up were 92 vs. 63% after 2 years, 88 vs. 54% after 3 years, and 88 vs. 46% after 4 years, respectively (all $P < 0.001$) (*Table 2*). The corresponding Kaplan–Meier curves are shown in *Figure 4* (log-rank test: $P = 0.001$). However, dividing these two Ang-2 subgroups by VEGF levels (> or < median) did not further improve risk prediction (data not shown).

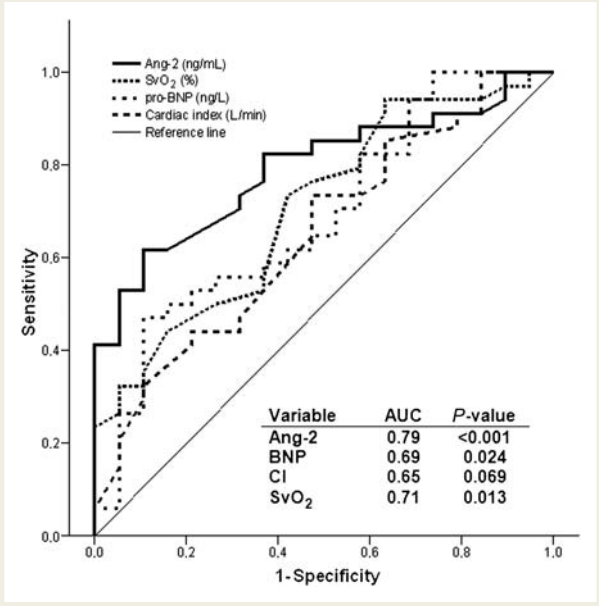

Figure 3 Angiopoietin-2 (Ang-2) in the context of other markers of adverse prognosis. Angiopoietin-2 receiver operator characteristic curves showing the prognostic sensitivity and specificity of Ang-2, N-terminal pro-B-type natriuretic peptide, cardiac index, and SvO_2 in the first patient cohort at baseline with regard to the composite endpoint at 3 years.

Angiopoietin-2 in the context of other markers of adverse prognosis

To test whether circulating Ang-2 could serve as an independent predictor of survival, we performed Cox's proportional hazards analyses. As a result, circulating Ang-2 was associated with the primary composite endpoint of death or lung transplantation {unadjusted hazard ratio (HR) 1.94 [95% confidence interval (CI), 1.50–2.51], $P < 0.001$}, even after adjustment for NT-proBNP and uric acid [HR 1.79 (95% CI, 1.36–2.30), $P = 0.034$], RAP, CI, and PVR [HR 1.85 (95% CI, 1.25–2.97), $P = 0.024$], or RAP, CI, PVR, and SvO_2 [HR 1.77 (95% CI, 1.29–2.12), $P = 0.043$] (*Table 3*).

Similarly, Ang-2 emerged as the only independent predictor of the composite endpoint when all variables found to be statistically significant at a 10% level in the univariate analysis (mean RAP, cardiac index, mean PVR, SvO_2, uric acid, NT-proBNP, and Ang-2) were concurrently subjected to forward stepwise multivariate Cox's regression analysis (see Supplementary material online, *File 4*).

Changes of angiopoietin-2 are closely related to treatment response

The second cohort consisted of 25 patients (63% female) with a median age of 54 (43–62) years. As in the first cohort, all angiogenic factors were elevated compared with controls, but only Ang-2 correlated with RAP ($r = 0.56$, $P = 0.002$), cardiac index ($r = -0.44$, $P = 0.02$), PVR ($r = 0.5$, $P = 0.01$), and mixed venous oxygen saturation (SvO_2) ($r = -0.67$, $P < 0.001$). Baseline characteristics are shown in *Table 4*. Three months after the

Table 2 Baseline characteristics in relation to plasma angiopoietin-2 level

Variables	Ang-2 ≤ 2.9 ng/mL (*n* = 27)	Ang-2 > 2.9 ng/mL (*n* = 52)	*P*-value
Age (years)	55 (48–62)	49 (44–55)	0.144
Female (%)	69	65	0.41
6 min walking distance (m)	381 (300–463)	315 (242–388)	0.218
WHO class	3.0 ± 0.5	3.4 ± 0.5	0.017
Mean RAP (mmHg)	4.1 (2.4–5.9)	9.4 (7.3–11.4)	0.001
Mean PAP (mmHg)	47 (41–52)	57 (50–63)	0.026
Cardiac output (L/min)	3.2 (2.8–3.6)	2.8 (2.2–3.5)	<0.001
Cardiac index (L/min/m^2)	2.9 (2.2–3.4)	1.8 (1.6–1.9)	<0.001
PVR (dyn s/cm^5)	698 (541–854)	1.263 (1.106–1.421)	<0.001
SvO_2 (%)	71 (67–76)	57 (54–60)	<0.001
Uric acid (mg/dL)	323 (273–372)	502 (452–552)	<0.001
NT-proBNP (ng/L)	259 (130–386)	2.321 (1.410–3.232)	<0.001
Ang-1 (ng/mL)	13.7 (7.2–20.1)	21.2 (15.9–26.3)	0.067
Ang-2 (ng/mL)	2.0 (1.7 -2.3)	9.2 (7.3–11.1)	<0.001
sTie2 (ng/mL)	1.4 (1.2–1.6)	1.9 (1.7–2.1)	<0.001
VEGF (pg/mL)	238 (125–351)	307 (227–387)	0.13
Survival			
12 months	100%	78%	<0.001
24 months	92%	63%	
36 months	88%	54%	
48 months	88%	46%	

Data are from the retrospective patient cohort. Except for gender, survival (%) and WHO class (mean ± SD), data are shown as median (IQR). Patients are divided by an ROC curve optimized Ang-2 cut-off of 2.9 ng/mL. Two patients with Ang-2 ≤ 2.9 ng/mL were lost to follow-up: *n* = 27. Differences were calculated by using Mann–Whitney *U*-test for continued variables, Fisher's exact test for gender comparison, and log-rank test comparison for survival comparison. Data on 6 min walking distance were available from *n* = 30 individuals. IPAH, idiopathic pulmonary arterial hypertension; WHO class, functional World Health Organization classification of heart failure; RAP, right atrial pressure; PAP, pulmonary arterial pressure; PVR, denotes pulmonary vascular resistance; SvO_2, mixed venous oxygen saturation; NT-proBNP, N-terminal fragment of the B-type natriuretic peptide; Ang-1, angiopoietin-1; Ang-2, angiopoietin-2; sTie2, soluble Tie-2-receptor; VEGF, vascular endothelial growth factor.

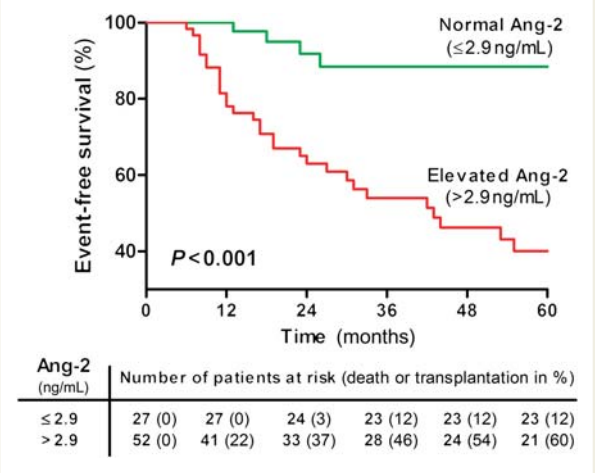

Figure 4 Elevated angiopoietin-2 (Ang-2) predicts poor outcome. The Kaplan–Meier curve showing the probability of event-free survival (death or transplantation) in the first patient cohort according to baseline Ang-2 levels above or below the previously defined reference value (2.9 ng/mL). The difference between the curves was determined by using a log-rank test.

initiation of PAH-targeted therapy, pulmonary haemodynamics had improved, as evidenced by a significant decrease in PVR ($P < 0.005$) (*Table 4*). Angiopoietin-2, but not Ang-1, sTie2, VEGF, or NT-proBNP changed significantly from baseline to follow-up in the prospective cohort [4.3 (3.2–5.4) at baseline vs. 3.4 (2.6–4.3) ng/mL after 3 months of therapy; $P = 0.04$] (*Table 4*). Changes in Ang-2 during follow-up were significantly correlated with changes in 6 min walking distance ($r = -0.72$; $P < 0.05$), mean RAP ($r = 0.6$; $P = 0.008$), and PVR ($r = 0.51$; $P = 0.04$) and were inversely related to changes in SvO_2 ($r = -0.75$; $P < 0.001$) (see Supplementary material online, *File 5*) (*Figure 5*). Changes of Ang-2 over time were not related to the therapeutic agent used (ANOVA with Bonferroni's correction: $P = 0.195$).

Angiopoietin-2 is expressed in plexiform lesions from idiopathic pulmonary arterial hypertension lung tissue samples

In order to clarify the cellular origin of elevated circulating Ang-2, we studied the protein expression of Ang-2 in lung tissue from IPAH patients. As a result, endothelial-specific Ang-2 protein expression was up-regulated in plexiform lesions (i.e. arterioles undergoing remodelling), but was not expressed in unaffected adjacent arterioles within the same IPAH lung tissue sample (*Figure 6A*

and *B*). Consistently, mRNA expression was significantly elevated in microdissected plexiform lesions compared with normal arterioles from both IPAH patients ($n = 13$, ANOVA with Bonferroni's correction: $P < 0.001$) and brain-dead organ donors ($n = 4$, $P < 0.001$).

Discussion

This is the first comprehensive study on circulating Ang-1, Ang-2, and sTie2 in IPAH. The decisive results are: (i) compared with healthy or disease controls, IPAH patients are characterized by an excess of circulating Ang-1, Ang-2, sTie2, and VEGF; (ii) of those, only Ang-2 correlated with disease severity; (iii) after adjustment for haemodynamic and biochemical variables, Ang-2 emerged as an independent predictor of outcome; (iv) changes in Ang-2 after initiation of medical therapy were closely related to changes in mean RAP, PVR, and inversely related to changes in cardiac output and SvO_2; (v) up-regulated Ang-2 mRNA and protein expression was an exclusive feature of plexiform lesions. These findings have implications for the role of angiopoietins in the pathogenesis of IPAH and they raise the questions whether circulating levels of Ang-2 may be useful as biomarkers in this patient population.

Table 3 Outcome in relation to clinical and biochemical variables at baseline

	HR (95% CI)	*P*-value
Ang-2 unadjusted	1.94 (1.50–2.51)	<0.001
Ang-2 adjusted for NT-proBNP, uric acid	1.79 (1.36–2.30)	0.034
Ang-2 adjusted for RAP, CI, PVR	1.85 (1.25–2.97)	0.024
Ang-2 adjusted for RAP, CI, PVR, SvO_2	1.77 (1.29–2.12)	0.043

Data are from the first patient cohort. Estimated hazard ratios (HR), 95% confidence intervals (CI), and *P*-values were calculated using adjusted Cox's proportional hazards regression models. Selection of variables to be included in the multivariable models was done a priori by determining probable confounders based on differences in baseline characteristics between patients with different Ang-2 levels and based on theoretical considerations. NT-proBNP and Ang-2 were not normally distributed and therefore ln transformed; NT-proBNP, N-terminal fragment of the B-type natriuretic peptide; RAP, right atrial pressure; CI, cardiac index; PVR, pulmonary vascular resistance; SvO_2, mixed venous oxygen saturation.

Angiopoietin-1

We provide evidence for a robust elevation of plasma Ang-1 in two independent cohorts of patients with IPAH. These findings are in accordance with results from the group of Thistlethwaite,[5,30] who elegantly showed that Ang-1 expression is confined to the cytoplasm of SMC within the wall of small pulmonary arteries and arterioles in patients with PAH, but cannot be detected in normal human lung samples. Although this has not been tested formally, it is conceivable to assume that SMC-derived Ang-1 might account for elevated Ang-1 plasma levels in our patients. Stewart and colleagues[31] did not detect any difference in Ang-1 expression or Tie2 activation in lung tissue from patients with PAH or controls, respectively. Dewachter *et al.*,[4] in contrast, detected fourfold higher Tie2 receptor expression and phosphorylation level in lung homogenates and cultured pulmonary ECs from patients with IPAH, whereas Ang-1 and Ang-2 expression did not differ compared with controls. Of note, Ang-1 levels were not increased in patients with congestive heart failure.[32] Thistlethwaite *et al.*[30] and Du *et al.*[5] detected a strong linear correlation of both Ang-1 expression and activation (i.e. phosphorylation) of Tie2 in lung samples from PAH patients with the degree of PVR, irrespective of the cause of the disease (i.e. idiopathic PAH vs. other forms of PH), leading them to conclude that Ang-1 expression is a marker for the severity of PAH. Contrary to that, there was no association between plasma Ang-1 levels and measures of

Table 4 Characteristics of the prospective cohort at baseline and after 3 months of follow-up

	Baseline ($n = 25$)	Follow-up ($n = 25$)	*P*-value
6 min walking distance (m)	367 (304–431)	381 (329–434)	0.42
mean RAP (mmHg)	6.8 (4.5–9.2)	5.7 (3.2–8.2)	0.72
mean PAP (mmHg)	47 (42–52)	42 (38–48)	0.18
Cardiac output (L/min)	4.0 (3.5–4.5)	4.5 (3.7–5.4)	<0.001
Cardiac index (L/min/m^2)	2.3 (2–2.5)	2.6 (2.2–3)	<0.001
PVR (dyn s/cm^5)	839 (677–1,002)	717 (512–922)	0.005
SvO_2 (%)	65 (60–68)	66 (64–69)	0.34
NT-proBNP (ng/L)	585 (422–1,911)	509 (506–1,980)	0.26
Ang-1 (ng/mL)	8.6 (5.2–12)	5.1 (2.8–7.4)	0.42
Ang-2 (ng/mL)	4.3 (3.2–5.4)	3.4 (2.6–4.3)	0.04
sTie2 (ng/mL)	1.3 (1.1–1.5)	1.3 (1–1.5)	0.68
VEGF (pg/mL)	257 (63–451)	224 (52–500)	0.77

Data are from the prospective cohort. Data are shown as median (IQR). Changes from baseline to 3-month follow-up were assessed by Wilcoxon's signed-rank test. Data on 6 min walking distance were available from $n = 18$ individuals. Data on sTie2 and VEGF were available for 10 individuals. RAP, right atrial pressure; PAP, pulmonary arterial pressure; PVR, pulmonary vascular resistance; SvO_2, mixed venous oxygen saturation. Ang-1, angiopoietin-1; sTie2, soluble Tie-2-Receptor; VEGF, vascular endothelial growth factor; NT-proBNP, N-terminal fragment of the B-type natriuretic peptide.

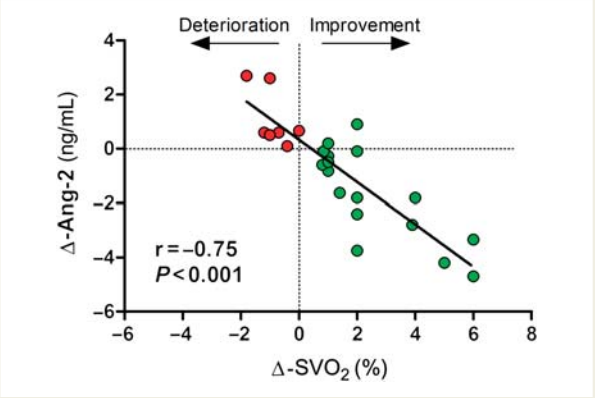

Figure 5 Changes of angiopoietin-2 (Ang-2) are closely related to treatment response. Scatter plot showing the relation of changes in Ang-2 (Δ-Ang-2) to changes in SvO_2 (Δ-SvO_2) after 3 months in patients from the prospective cohort ($n = 25$).

pulmonary haemodynamics in our study population. The reason for this discrepancy may lie in the different materials used for analyses. However, our data reveal an important limitation for circulating Ang-1 as a marker for disease severity: elevated circulating Ang-1 might be a surrogate parameter for increased vascular SMC burden *per se*, but it does not correlate with PVR.

Angiopoietin-2

The somewhat surprising finding of elevated Ang-2 levels in the plasma from IPAH patients is difficult to reconcile because little attention has been paid to Ang-2 after initial studies showed no significant differences in Ang-2 expression between lung tissues from PAH patients and healthy controls.[5,31] As a key-destabilizing signal involved in initiating angiogenic remodelling, Ang-2 expression is tightly controlled.[33] Hence, Ang-2 mRNA is almost undetectable in the quiescent vasculature and is detected only at those sites undergoing remodelling (for instance, in sprouting tumour blood vessels).[13,34,35] As a Weibel–Palade body-stored molecule,

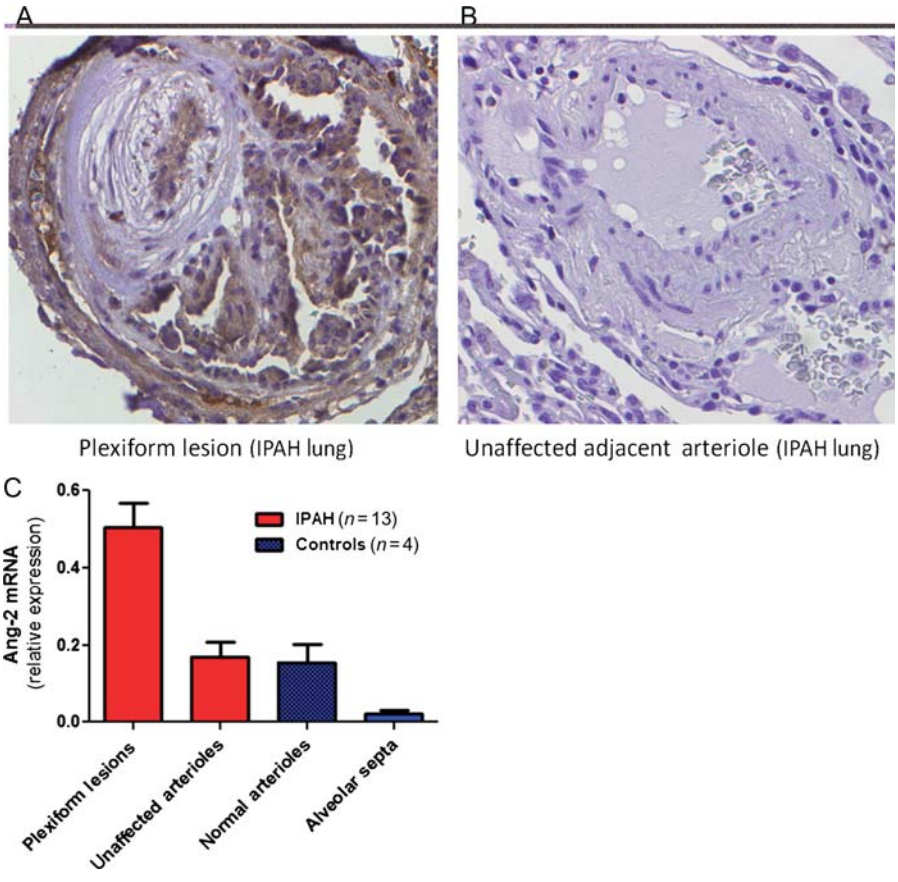

Figure 6 Compartments-specific angiopoietin-2 (Ang-2) expression in lung tissue samples from idiopathic pulmonary arterial hypertension (IPAH) patients and brain-dead donors. (*A*) Angiopoietin-2 protein expression of a plexiform lesion in an IPAH lung detected by immunohistochemical staining. The prominent luminal endothelial cells show a strong positivity for Ang-2, whereas the interstitial fibrocytes and smooth muscle cells do not stain. Horseradish peroxidase, brown. Original magnification ×400. (*B*) Angiopoietin-2 protein expression of an unremodelled small pulmonary artery in an IPAH lung. Neither the regular endothelial cells nor the smooth muscle cells of the vascular wall show positivity for Ang-2. Horseradish peroxidase, brown. Original magnification ×400. (*C*) Compartment-specific expression of Ang-2 mRNA levels assessed by quantitative real-time polymerase chain reaction in lung tissue from patients with IPAH ($n = 13$) after laser-assisted microdissection. Lung tissue from brain-dead organ donors ($n = 4$) served as a control.

however, Ang-2 protein is rapidly released and induced upon various stimuli by a multitude of factors, including cytokines, thrombin, activated platelets, and leucocytes, and changes in blood flow or oxygenation.[13,18,20,36,37]

Excessive EC proliferation, along with concurrent neoangiogenesis, is a common pathological feature in PAH.[38,39] Consistent with these findings, endothelial Ang-2 protein and mRNA expression was up-regulated in plexiform lesions, but entirely absent from unaffected adjacent arterioles within the same IPAH lung tissue sample.

Moreover, circulating Ang-2 levels were closely associated with tissue hypoxia, as evidenced by a relatively tight linear correlation of Ang-2 with SvO_2. From a biological perspective, it is entirely possible that Ang-2 is involved in remodelling of the pulmonary vessels. A larger comparative study investigating circulating Ang-2 levels together with Ang-2 tissue expression would be desirable.

Beyond its potential role as a facilitator/mediator, circulating Ang-2 seems to fulfil several prerequisites of a useful biomarker: it correlates relatively tightly with established haemodynamic markers of disease severity and seems to be an independent predictor of survival. Of note, Ang-2 changes with therapy and tightly reflects changes in PVR, RAP, and functional capacity (6 min walking distance). These findings are supported by Hiremath *et al.*[40] who recently found an inverse correlation between improvement in 6 min walking distance and a decrease in circulating Ang-2 levels after a 3-month treatment period with treprostinil in 12 IPAH patients. We have previously shown that circulating angiopoietins (stable for 24 h at room temperature and for at least 4 freeze-thaw cycles) can be readily quantified by immunoassay methodology with excellent intra- and inter-assay imprecision.[27] Together, these findings indicate that Ang-2 might emerge as a valuable biomarker for non-invasive monitoring of treatment response in IPAH.

N-terminal pro-B-type natriuretic peptide has been validated as a marker of right ventricular dysfunction in PAH.[39,41] In the present study, Ang-2 was much closer related to haemodynamic impairment than NT-proBNP both in the retrospective and in the prospective group of patients. Angiopoietin-2 did also perform better than NT-proBNP in terms of predicting survival. However, further prospective studies are needed to compare the performance of these two biomarkers. Another biomarker that has recently been shown to be of prognostic importance in IPAH is growth differentiation factor 15 (GDF-15), overexpression of which appears to be related to tissue hypoxia.[42] Thus, Ang-2, NT-proBNP, and GDF-15 may reflect various aspects of the haemodynamic compromise in patients with IPAH and it may therefore be useful to evaluate panels of these biomarkers in future studies.

The most important limitations of the present study are the single-centre design, the retrospective nature of large parts of the study and the relatively small sample size of the prospective cohort. On the other hand, the patient population was homogeneous and well defined and the results seem to be robust as the findings obtained in the retrospective group were confirmed in the prospective cohort. Unfortunately, data on 6 min walking distance were not available in the majority of cases, probably accounting for the lack of significant difference among subgroups. Another limitation is the descriptive nature of our study which precludes firm cause–effect conclusions. However, our study is hypothesis generating since human data on the Ang–Tie2 system in PAH are rare; especially since experimental animal studies by two groups with long expertise in the field have led to entirely antithetical conclusions on the role of Ang-1 in PAH.[3,8,22–24,31]

In summary, circulating Ang-2 is associated with haemodynamic compromise and outcome of patients with IPAH and might thus serve as a promising new biomarker of diseases severity and response to treatment in patients with IPAH.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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Conflict of interest: P.K., N.N., and M.M.H. have filed a patent for the use of circulating Ang-1 and Ang-2 as biomarkers in PH.

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