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Cytokines as Prognostic Biomarkers in Pulmonary Arterial Hypertension

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Running title: Prognostic biomarkers in Pulmonary Arterial Hypertension

Abstract

Introduction:

Risk stratification and assessment of disease progression in patients with pulmonary arterial hypertension (PAH) are challenged by the lack of accurate disease-specific and prognostic biomarkers. To date, B-type natriuretic peptide (BNP) and/or its N-terminal fragment (NT-proBNP) are the only marker for right ventricular dysfunction used in clinical practice, in association with echocardiographic and invasive hemodynamic variables to predict outcome in patients with PAH.

Methods:

This study was designed to identify an easily measurable biomarker panel in the serum of 80 well-phenotyped PAH patients with idiopathic, heritable, or drug-induced PAH at baseline and first follow-up. The prognostic value of identified cytokines of interest was secondly analysed in an external validation cohort of 125 PAH patients.

Results:

Among the 20 biomarkers studied with the multiplex Ella™ platform, we identified a 3-biomarker panel composed of β -NGF, CXCL9 and TRAIL that were independently associated with prognosis both at the time of PAH diagnosis and at the first follow-up after initiation of PAH therapy. β -NGF and CXCL9 were predictors of death or transplantation, whereas high levels of TRAIL were associated with a better prognosis. Furthermore, prognostic value of the three cytokines was more powerful for predicting survival than usual non-invasive variables (functional class, 6-minute walking distance and BNP/NT-proBNP). The results were validated in a fully independent external validation cohort.

Conclusion:

The monitoring of β -NGF, CXCL9 and TRAIL levels in serum should be considered in the management and treatment of patients with PAH to objectively guide therapeutic options.

Keywords:

Hypertension, pulmonary; Pulmonary arterial hypertension; Cytokines; Biomarker; Survival analysis.

INTRODUCTION

Pulmonary arterial hypertension (PAH) is a heterogeneous group of incurable cardiopulmonary disorders characterized by occlusive remodelling of precapillary pulmonary arteries, leading to right heart failure and premature death. Hemodynamically, PAH is defined by a resting mean pulmonary artery pressure (mPAP) >20 mmHg in the presence of abnormal pulmonary vascular resistance (PVR) ≥ 3 Wood Units and normal left heart filling pressure (pulmonary artery wedge pressure ≤ 15 mmHg) [1]. Endothelin receptor antagonists, phosphodiesterase type-5 inhibitors, and prostacyclin analogs represent the currently approved PAH medications that have markedly improved overall quality of life, exercise capacity, and long-term outcomes [2–6]. However, the 5-year survival rate for patients suffering from PAH remains low (around 60%) [7–9], and lung transplantation remains an important treatment option for eligible patients with severe PAH if medical treatment fails [10].

Risk assessment is of paramount importance in the management of patients with PAH [5, 6, 11]. European guidelines have proposed a risk stratification table based on the evaluation of a panel of clinical, functional, biological and hemodynamic variables to determine the risk of mortality at one year [5, 6]. Other methods of risk stratification have also been proposed including REVEAL risk scores [12, 13]. Regardless of the method utilized, repeated assessment of risk is essential to determine treatment strategy and to guide PAH therapy [2–6]. The accepted clinical tools to assess severity, determine prognosis and response to therapy include NYHA functional class (FC), 6-minute walk test (6-MWT), echocardiography, right heart catheterization (RHC) and circulating levels of B-type natriuretic peptide (BNP) or its N-terminal fragment (NT-proBNP) [2–6]. Plasma proteins, metabolites and whole blood transcriptomics have been studied for prognostication in PAH patients, as well as circulating endothelial cells, microRNAs and cell-free DNA [14–18]. However, none has to date replaced BNP/NT-proBNP as a prognostic marker or in the assessment of treatment effect in routine clinical practice.

Although the exact pathogenic mechanisms of PAH are complex and poorly understood, pulmonary vascular lesions occurring in patients with PAH as well as in animal models of pulmonary hypertension (PH) are characterized by varying degrees of perivascular inflammatory infiltrates, comprising T- and B-lymphocytes, macrophages, dendritic cells and mast cells [19–22]. In addition, circulating levels of certain cytokines and chemokines are abnormally elevated in human PAH [i.e., interleukin (IL)-1 α and β , IL-6, IL-8, IL-10, IL-12, CCL2, CCL5 and tumor necrosis factor (TNF)- α], and some have been reported to correlate with a worse clinical outcome [22–24]. More recently, four PAH clusters corresponding to different PAH immune phenotypes with different clinical risks were identified in a cohort of 281 patients with PAH (mostly prevalent) using a panel of 48 circulating factors, including several inflammatory mediators and growth factors [25]. The cluster of PAH patients with the worst prognosis was characterized by high levels of TNF-factor-related apoptosis-inducing ligand (TRAIL), CC motif chemokine ligand 5 (CCL5), CCL7, CCL4, CXC motif chemokine ligand 9 (CXCL9), IL-18 and macrophage migration inhibitory factor (MIF). These results were validated in a UK (Sheffield) cohort including 104 incident patients with PAH [25]. However, there is a need to evaluate the prognostic value of cytokines at both baseline and follow-up to include them into clinical practice. To facilitate the incorporation of multiplex immunoassays within routine clinical diagnosis, the new generation of multiplex platforms should offer the possibility to test few samples with robust automation to minimize time and operational costs. Compared to the other commercialized multiplex platforms, the EllaTM

microfluidic platform (Protein Simple, CA, USA) offers advantage in ease and time of completion, number of samples per assay, and dynamic concentration range [26].

We hypothesized that the assessment of inflammatory biomarker panels in serum using an Ella™ automated immunoassay platform could represent simple, accessible, and easily measurable biomarkers for the evaluation of the disease at diagnosis as well as the determination of prognosis. We investigated a panel of circulating cytokine/chemokine/adipokine levels in the serum of well-phenotyped PAH patients with idiopathic, heritable, or drug-induced PAH at the time of first presentation (baseline) and at the first follow-up.

METHODS

Cohort data collection

The discovery study used the EFORT (Evaluation of prognostic FactORs and Treatment goals in PAH) cohort (NCT 01185730) and was conducted in accordance with the Declaration of Helsinki with informed consent obtained for each patient prior to their enrolment.

EFORT^c is a prospective study that was designed to assess prognostic factors at both baseline and follow up in a French cohort of incident (i.e., newly diagnosed) patients with PAH. All incident patients entered in the French Registry between January 2011 and December 2013 with a diagnosis of idiopathic, heritable or anorexigen-induced PAH were enrolled in the EFORT study.

Assessments were performed at baseline (i.e., time of PAH diagnosis), 3-4 months after treatment initiation or treatment change, and then once a year until a 3-year follow up for patients included into the study in the first two years (2011-2012) and until a 2-year follow up for patients included in the last year (2013). Serial assessments included NYHA FC, non-encouraged 6-MWT, RHC, echocardiography and biomarkers (BNP/NT-pro-BNP, uric acid, creatinine, sodium). All blood samples were collected peripherally. Serum samples were available from 80 patients at both baseline (i.e., time of PAH diagnosis) and first follow-up visit for cytokines measurements. These 80 patients constituted the study discovery population. All samples were prepared in the same conditions and stored at -80°C in the Biological Resource Centre (CRB) of the Université Paris-Saclay.

Sixteen healthy blood donors with serum samples constituted the control population. The validation cohort comprised serum samples from 125 incident patients with PAH followed up at Imperial College of London, UK (under UK Research Ethics Committee approval 17/LO/0563). A sample was available at the time of diagnosis in all patients. In addition, another sample was withdrawn in 33 of these patients at follow-up.

Ella™ microfluidic platform

Reagents for the SimplePlex Ella™ microfluidic platform (Protein Simple, CA, USA) were custom developed. Twenty biomarkers were selected according to results from previous studies [22–25] and divided into 6 panels based on relative serum abundance and assay

dynamic range. Panel 1 included the following 3 high-abundance biomarkers tested at a dilution of 1:10: leptin, MIF, CCL5. Five other panels included the following low-abundance biomarkers: 1:2: β -NGF, G-CSF, IL-6, IL-10 (Panel 2); 1:2: CXCL9, TRAIL, VEGF-A (Panel 3); 1:2: CCL2, CCL4, CXCL10 (Panel 4); 1:2: IL-12p70, IL-15, IL-18, IL-17 (Panel 5); 1:2: IL-8, IL-1 α , IL-4 (Panel 6). For the external validation cohort, three panels have been used: 1:2: β -NGF (panel 1); CXCL10, IL-18, IL-6, TRAIL (Panel 2); CXCL9, IL-1 α (panel 3). Assays were performed in a single centre at once according to the manufacturer's protocol. Briefly, 50 μ L of diluted serum was added to the appropriate cartridge, followed by placement into the Ella instrument requiring no further user intervention. Each cartridge included a built-in lot-specific standard curve and samples were run as internal triplicates. Detection and washing steps were automatically performed by the instrument. Raw data were analysed using the SimplePlex Explorer software Version 3.7.2.0. CA, USA.

Statistical analysis

Data were collected from the web-based French PH Registry (PAHTool®; Inovultus Ltd. Santa Maria da Feira, Portugal). Statistical analysis was performed using SPSS Statistics version 26 (SPSS Inc., Chicago, IL). Continuous variables are expressed as the means \pm SD or medians (interquartile range, 25% to 75% [IQR]) according to the data distribution. Levels of cytokines in PAH patients were compared with the serum of healthy controls (blood donors) by Mann–Whitney U tests. Comparisons between levels of cytokines at baseline and the first follow-up were performed by paired t test or nonparametric test according to the data distribution.

The date of diagnostic RHC was used as the starting point to determine the length of survival. The cut-off date was December 31, 2020. Transplant-free survival was represented using the Kaplan–Meier method. Univariable and multivariable forward stepwise Cox proportional hazards regression models were performed to determine the risk of event (death or transplantation) according to baseline and first follow-up visit variables. A p value threshold of <0.10 was used for entry into the multivariable model, and $p>0.05$ was the threshold for variable removal. All comparisons were two-sided, and a p value <0.05 was considered statistically significant.

Variables identified in univariable analysis to be significantly associated with the prognosis at both baseline and follow-up were considered cytokines of interest. For each of them, ROC curves were performed at baseline to determine the best threshold of transplant-free survival by Youden's index.

Patients with idiopathic, heritable or anorexigen-induced PAH from the UK in whom blood samples were collected at the time of PAH diagnosis and during follow-up were used for external validation. In this validation cohort, survival analyses were performed using the Kaplan–Meier method.

RESULTS

Patient demographics

Eighty PAH patients constituted our study population (71% female, mean age 51 ± 19 years, 66% idiopathic PAH, 20% heritable PAH and 14% anorexigen-induced PAH). No patient received immunomodulatory drugs. Baseline characteristics and initial treatment strategies are summarized in Table 1.

Cytokine measurements

Levels of leptin, G-CSF, MIF, CXCL9, CXCL10, IL-1, IL-4, IL-6, IL-8 and IL-15 were significantly increased in the PAH group as compared with healthy subjects whereas levels of TRAIL and IL-10 were significantly decreased. Details of these results are presented in Supplementary Figure 1.

Levels of TRAIL and IL-17 were significantly increased at follow-up compared to baseline levels, whereas levels of G-CSF, CXCL10, CCL2, CCL4, \square -NGF, IL-6, IL-8, IL-15 and IL-18 were significantly decreased between baseline and follow-up (Supplementary Figure 1).

Survival analysis

After a median follow-up of 69 (50-81) months, 21 patients had died and 5 underwent lung transplantation. Among the 21 patients who died, 6 received intravenous (IV) or subcutaneous (SC) prostacyclin at the time of death (2 on IV epoprostenol, 3 on SC treprostinil and 1 on IV treprostinil). All of them achieved a therapeutic dose of prostacyclin. The main cause of death was right heart failure (n=12), followed by sudden death (n=2), cancer (n=1) and stroke (n=1). The cause of death remained unknown in five patients.

Univariable analysis was performed with the 20 cytokines at baseline and at first follow-up (4.6 (3.9 – 7.3) months after PAH diagnosis). Significant results of univariable analyses are presented in Table 2. At baseline, prognostic cytokines were β -NGF, CXCL9, TRAIL and IL-18, whereas β -NGF, CXCL9, TRAIL, CXCL10 and IL-6 were associated with survival at the first follow-up. In univariable analysis, 3 cytokines were associated with transplant-free survival at both baseline and follow-up: β -NGF, CXCL9, which were both associated with poor prognosis, and TRAIL, which was associated with good outcomes. The relationship between each cytokine and survival persisted in multivariable models adjusted for age and sex, as well as for BMI.

Determination of discriminating thresholds

For each identified cytokine at both baseline and follow-up (\square -NGF, CXCL9 and TRAIL), ROC curve analysis was performed to determine the best threshold of transplant-free survival at baseline: <3.65 pg/mL for \square -NGF, <625.5 pg/mL for CXCL9 and >52.65 pg/mL for TRAIL. The ROC curves from which the thresholds were identified are presented in Supplementary Figure 2. Kaplan–Meier survival curves according to thresholds of β -NGF, CXCL9 and TRAIL are presented in **Figure 1**.

In univariable analysis, the hazard ratios (HR) of cytokines expressed as dichotomous variables (according to thresholds previously determined) at diagnosis were: β -NGF HR 9.866 (95%CI 2.906 – 33.494), CXCL9 HR 6.429 (95%CI 2.531 – 16.335) and TRAIL HR 0.200 (95%CI 0.069 – 0.581), and at follow-up: β -NGF HR 10.811 (95%CI 4.266 – 27.396), CXCL9 HR 3.875 (95%CI 1.615 – 9.299) and TRAIL HR 0.169 (95%CI 0.064 – 0.450).

Determination of the added value of cytokines to current risk stratification

The results of univariable analysis at baseline and follow-up are presented in **Table 3**. Several models of multivariable analysis were performed by testing each cytokine separately or together. In multivariable analysis, β -NGF, CXCL9 and TRAIL were more powerful for predicting survival than usual clinical variables (NYHA FC, 6MWD) and biomarkers (BNP and NT-proBNP) (Supplementary Tables 1 and 2).

Survival according to β -NGF, CXCL9 and TRAIL status

Transplant-free survival was associated with levels of β -NGF, CXCL9 and TRAIL at baseline and at follow-up. No deaths have occurred in patients with 3 —low-risk‖ statuses of cytokines (low levels of β -NGF and CXCL9, and high levels of TRAIL). On the other hand, patients without a —low-risk‖ profile of cytokines had a worse prognosis (**Figure 2**). Patients achieving 2 —low-risk‖ statuses of cytokines at the first follow-up had an excellent long-term survival similar to that of patients achieving 3 —low-risk‖ statuses of cytokines.

Validation cohort

The London validation cohort comprised 125 incident patients (69% female, mean age 59±17 years, 91% idiopathic PAH, 9% heritable PAH), with a mean pulmonary vascular resistance of 12±6 WU (**Table 1**). After a median follow-up of 49±29 months, 53 patients had died. None of the patients underwent lung transplantation. Among the 125 patients of the cohort, serum samples were available for all at baseline (i.e., time of PAH diagnosis), and in 33 at both baseline and follow-up. No samples were available at follow-up in the remaining 92 patients. Kaplan–Meier survival analysis in this cohort according to the status of β -NGF, CXCL9 and TRAIL confirmed the results previously observed in the French cohort (**Figure 3**).

DISCUSSION

In this study, we used a cohort of incident idiopathic, heritable, or anorexigen-associated PAH patients to examine the prognostic value of multiple cytokine markers in PAH. Twenty biomarkers were selected and measured by a multiplex Ella™ platform. This approach allowed the identification of three cytokines independently associated with prognosis both at the time of PAH diagnosis and at the first follow-up after PAH therapy initiation: β -NGF, CXCL9 and TRAIL. β -NGF and CXCL9 were predictors of death or transplantation, whereas high levels of TRAIL were associated with a better prognosis. Furthermore, we observed that the prognostic value of the three cytokines was more powerful for predicting survival than usual clinical and hemodynamic variables. These results were validated in a fully independent external validation cohort.

Inflammation and autoimmune disorders are common denominators of all forms of PAH, even in the absence of associated inflammatory or autoimmune disorders [19–22]. Consistent with this notion, circulating autoantibodies targeting endothelial cells and fibroblasts as well as high levels of certain cytokines, such as IL-6, TNF- α and IL-1 β , have previously been described in patients with idiopathic PAH [23–25, 27, 28]. Inflammation and immune disorders are detected in the circulating blood as well as within the pulmonary vascular lesions that characterize PAH, where they facilitate pulmonary vascular cell survival and growth [19–22]. Given the link between inflammation and pulmonary vascular remodelling, several studies have demonstrated the prognostic value of certain inflammatory mediators in PAH. However, these studies are limited by the fact that they mostly integrated incident and prevalent cases of PAH and different forms of PAH, including those associated with another disease or condition that can strongly influence the inflammatory profiles. In addition, data on the evolution of these biomarkers during follow-up are also lacking. As a result, the integration of inflammatory biomarkers into risk assessment tools and treatment response assessment has eluded clinicians.

The ability to predict PAH patients at risk of adverse outcomes is of value to clinicians to improve risk stratification. Currently, BNP and/or NT-proBNP are the only two biomarkers incorporated into several PAH risk stratification tools and screening algorithms to detect and monitor PAH [5, 6], even if other potential candidates have been identified such as growth and differentiation factor (GDF)-15 [29], red cell distribution width (RDW) [30–33], uric acid (UA) [34], creatinine [35], IL-6 [23–25, 27, 28] and angiopoietins [36]. While most of these individual biomarkers are closely linked with the level of right ventricular (RV) dysfunction, it is possible that characterizing risk assessment by profiling multiple factors involved in different disease components may be a more robust approach than only measuring impairment of cardiac function. Therefore, novel biological markers to predict response to therapies and outcome are needed to accelerate the development of precision medicine tools for PAH. In this context, the use of objective biomarkers and clinical trial end points are crucial as well as the selection of a reproducible, accurate, sensitive, and appropriate multiplex immunoassay platform. Since the past few years have seen significant progress and innovation and several multiplex assays that are now commercially available, we have chosen to use the EllaTM platform. Indeed, this microfluidic-based system is an easy-to-use and fully automated platform allowing the acquisition of both highly sensitive and reproducible results with minimal sample handling [26]. In contrast to the other multiplex platforms that are designed to analyse a large number of samples in a short time frame, the EllaTM microfluidic platform has the capacity to test fewer samples with fast turn-around (approximately 1.25 hours), making this platform amenable to clinical use. This high-throughput proteomic approach allows accurate measurement of different biomarkers that can have various circulating levels of expression and concentration. Our analysis confirmed the known association between PAH development and high levels of various cytokines and inflammatory mediators. As previously reported, some of these individual cytokines are predictors of survival at diagnosis and/or at first follow-up. To identify cytokines with the most powerful prognostic value, we chose to select only those that exhibited a circulating level independently associated with transplant-free survival at diagnosis and during follow-up.

Even if this 3-biomarker panel detected in the peripheral blood could be useful in clinical settings, it remains unclear how they reflect what is occurring in PAH lungs. Recent studies have emphasized the importance of β -NGF, CXCL9 and TRAIL in the development and progression of PAH, but much remains to be done in this area. It has been previously demonstrated that the increased expression of NGF and its receptor in human and

experimental PAH promotes pulmonary vascular cell proliferation and migration, pulmonary arterial hyper-reactivity, and secretion of pro-inflammatory cytokines [37]. Recently, the prognostic value of NGF was identified for the first time in a discovery cohort of 121 incident and prevalent PAH patients and a validation cohort of 76 patients [38]. High levels of NGF were associated with worse Mayo and Stanford scores independent of pulmonary vascular resistance or pressure in both cohorts [38]. The chemokine CXCL-9 (also known as MIG) is an interferon-inducible member of the CXC chemokine family that lack the tripeptide structure/function motif Glu-Leu-Arg (ELR) that is important in the chemoattraction of mononuclear cells including of activated T cells, B cells and natural killer (NK) cells. Thus, CXCL-9 could play a role in the infiltration of inflammatory cells into the perivascular area of pulmonary vessels in PAH. CXCL9 also demonstrates angiogenic activity via the receptor CXCR3 [39]. However, its potential implication in PAH development has never been studied. Recently, CXCL-9 was identified as a prognostic factor in the subpopulation for patients with chronic thromboembolic pulmonary hypertension (CTEPH) [40]. TRAIL, also known as Apo2L, is a member of the TNF superfamily of cytokines that can bind five different receptors to induce several biological processes including cell survival, migration and proliferation via kinase signalling pathways [41]. In our study, high circulating TRAIL levels were associated with better prognosis. Paradoxically, previous experimental studies reported that TRAIL was upregulated in idiopathic PAH and could be involved in PAH pathophysiology by inducing migration and proliferation of pulmonary artery smooth muscle cells (PA-SMCs) [42, 43]. Accordingly, administration of an anti-TRAIL antibody or its genetic deletion have been reported to prevent and reverse vascular remodelling in various models of PAH. However, the role of TRAIL in the pathogenesis of lung diseases can be divergent [41]. TRAIL also has the ability to function as either a pro-apoptotic or pro-survival signal depending on the cell types and receptor expression on local tissues to mediate either protective or pathogenic mechanisms. The exact mechanism by which TRAIL modulates these functions is not fully understood, although regulation of TRAIL, and its cleavage, as well as the expression of receptors by specific cell types, are clearly important in determining its effects. Further work is required to fully elucidate the divergent roles of TRAIL to gain a better understanding its role in underlying processes of lung disease and its potential as a therapeutic agent—or target—depending on disease context.

Our study confirmed the importance of non-invasive parameters in risk stratification, specifically at the first follow-up after PAH therapy initiation. BNP and NTproBNP are the only biomarkers that are currently used for risk stratification according to the ERS/ESC method [5, 6]. eGFR is also listed in the REVEAL score [12, 13], but cardio-renal syndrome is more frequently indicative of advanced PAH with severe right heart failure. Other candidate prognostic biomarkers have been identified [23–25, 27–36], but most often fail to demonstrate a sufficient power to replace or add value to the use of BNP or NT-proBNP alone. Our study clearly demonstrated that the prognostic value of β -NGF, CXCL9, and TRAIL remains strong and independent after adjustment for all other variables included in risk stratification. Interestingly, this multimarker approach allowed us to distinguish a subgroup of patients with a very good prognosis when the threshold established by the ROC analysis reached at least 2 biomarkers. These results suggest that the use of these three biomarkers should be useful for predicting patient survival and evaluating response to treatment independent of commonly used variables.

In the validation cohort, the patients were older and display a more severe disease as reflected by a lower 6MWD and higher FC. Despite these differences with the discovery cohort, the prognostic value of the three identified cytokines remains highly relevant, suggesting that the

use of these biomarkers as prognostic factors remains applicable irrespective of the initial PAH severity and management.

Our results emphasize the importance of including multiple inflammatory markers in PAH risk assessment. This is a large incident cohort that has been prospectively phenotyped at both baseline and during follow-up after PAH therapy initiation. The detailed clinical and biological data captured allow for multivariable modelling to adjust for key confounders. Moreover, the prognostic value of the three identified biomarkers was validated in an external cohort of PAH patients.

The main limitation of our study is the relatively small number of patients included in the discovery (n=80) and the validation (n=125) cohorts that might partly explain why we did not find in an association between baseline NYHA FC and transplant-free survival in the univariable analysis. In contrast to previous retrospective studies including larger numbers of patients [2–4], we did not find in multivariable analysis an association between important prognostic markers such as NYHA FC, 6-MWD and BNP/NT-proBNP at both baseline and follow-up. However, when discovery and validation cohorts were pooled, we found that those variables were associated with transplant-free survival, as previously shown [2–4]. After adding cytokines in multivariable models including NYHA FC, 6MWD and BNP/NT-proBNP at follow-up, we found that each cytokine was independently associated with outcomes. Only BNP/NT-proBNP criteria was also independently associated with transplant-free survival in multivariable models with β -NGF and CXCL9 (data not shown). However, due to the small number of subjects that comprised the two cohorts we cannot say that BNP/NT-proBNP is an inferior predictor than inflammatory biomarkers in PAH. Further studies are needed to confirm the utility of these biomarkers in the clinical setting.

As patients with PAH associated with connective tissue disease and congenital heart diseases were excluded from the original EFORT study, blood samples were only obtained from idiopathic, heritable and anorexigen-associated PAH. We think that a strength of the present study was to analyse a cohort of PAH patients without confounding inflammatory factors that could have influenced cytokine levels. However, it would be interesting in the future to extend the analysis to other types of PAH associated with inflammatory conditions such as connective tissue diseases, HIV infection or portal hypertension. Finally, to study the prognostic value of these cytokines at different time-point during follow-up would be also relevant.

In conclusion, our study identified a novel panel of three cytokines (β -NGF, CXCL9 and TRAIL) in serum independently associated with prognosis at both baseline and at the first follow-up after PAH therapy initiation. Subsequent analysis revealed that the prognostic value of this 3-biomarker panel was more powerful for predicting PAH survival than usual clinical and hemodynamic variables. The results were validated in a fully independent external validation cohort. Therefore, we propose that serum β -NGF, CXCL9 and TRAIL levels should be considered in the management and treatment of patients with PAH during follow-up to objectively identify patients with a high risk of death to adapt treatment (treatment escalation and/or lung transplantation).

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Table 1: Baseline characteristics

	Incident PAH patients from the EFORT cohort (n=80)	Incident PAH patients from the validation cohort (n=125)
Female, n (%)	57 (71)	86 (69)
Age, years	51 ± 19	59 ± 17
BMI, kg/m²	28.3 ± 7.1	NA
PAH etiology, n (%)		
Idiopathic	53 (66)	114 (91)
Heritable	16 (20)	11 (9)
Drug-induced PAH	11 (14)	0
NYHA functional class, n (%)		
I-II / III / IV	28 (35) / 45 (56) / 7 (9)	10 (8) / 94 (75) / 21(17)
6MWD, m	343 ± 143	230 ± 160
RHC		
RAP, mmHg	9 ± 5	10 ± 5
mPAP, mmHg	51 ± 11	50 ± 12
PAWP, mmHg	9 ± 3	9 ± 3
CO, L/min	4.5 ± 1.2	3.8 ± 1.4
CI, L/min/m ²	2.5 ± 0.6	2.1 ± 0.8
PVR, WU	10 ± 4	12 ± 6
SvO ₂ , %	63 ± 8	64 ± 11
BNP, ng/L	158 (54 – 312)	274 (81 – 544)
NT-proBNP, ng/L	2349 (106 – 4977)	NA
First line treatment, n (%)		
Calcium channel blocker	5 (6)	6 (5)
Oral monotherapy	34 (43)	75 (60)
Oral dual combination therapy	33 (41)	35 (28)
Dual combination therapy including IV prostacyclin	0 (0)	6 (5)
Triple combination therapy	8 (10)	3 (2)

Results are expressed as mean \pm SD, median (IQR25-75%) or n (%). All assessments were performed before any treatment was initiated. Oral dual combination therapy was defined by the association of an endothelin receptor antagonist (ERA) and a phosphodiesterase-type 5 inhibitor (PDE5-i). Triple combination therapy was defined by the association of an ERA, a PDE5-i and a parenteral prostacyclin or selexipag.

Abbreviations: 6MWD: 6-minute walk distance; BNP: brain natriuretic peptide; CI: cardiac index; CO: cardiac output; mPAP: mean pulmonary arterial pressure; NA: not available; NTproBNP: N-Terminal pro-B-Type Natriuretic Peptide; NYHA: New-York Heart Association; PAH: pulmonary arterial hypertension; PAWP: pulmonary artery wedge pressure; PVR: pulmonary vascular resistance; RAP: right atrial pressure; RHC: right heart catheterization; SvO₂: mixed venous oxygen saturation; WU: Wood unit

Table 2: Identification of cytokines associated with transplant-free survival by univariable analysis at diagnosis (A) and at first follow-up (B)

	Hazard-ratios	95%CI	p
A: at diagnosis			
β -NGF, ng/mL, per unit	1.353	1.122 – 1.632	0.002
TRAIL, ng/mL, per unit	0.965	0.942 – 0.989	0.004
CXCL9, ng/mL, per unit	1.001	1.000 – 1.001	0.002
IL-18, ng/mL, per unit	1.005	1.001 – 1.009	0.018
B: at first follow-up			
β -NGF, ng/mL, per unit	1.786	1.472 – 2.168	<0.001
TRAIL, ng/mL, per unit	0.959	0.938 – 0.980	<0.001
CXCL9, ng/mL, per unit	1.000	1.000 – 1.001	0.042
CXCL10, ng/mL, per unit	1.001	1.000 – 1.002	0.005
IL-6, ng/mL, per unit	1.076	1.023 – 1.131	0.004

Cytokines are expressed as continuous variables

Abbreviations: beta-NGF: beta-nerve growth factor; CI: confidence interval; CXCL: chemokine (C-X-C motif) ligand; IL interleukin; TRAIL: tumor-necrosis-factor related apoptosis inducing ligand; VEGFR2: vascular endothelial growth factor receptor-2;

Table 3: Univariable Cox regression analysis of usual low risk variables and three cytokines at diagnosis (A) and at first follow-up (B).

A – At diagnosis

	Hazard-ratios	95%CI	p
NYHA functional class I-II	0.824	0.355 – 1.913	0.653
6MWD > 440 m	0.254	0.074 – 0.869	0.029
RAP < 8 mmHg	0.428	0.183 – 0.998	0.050
CI > 2.5 L/min/m ²	0.827	0.373 – 1.835	0.640
BNP < 50 or NT-proBNP < 300 ng/L	0.199	0.046 – 0.857	0.030
β-NGF high	9.866	2.906 – 33.494	<0.001
CXCL9 high	6.429	2.531 – 16.335	<0.001
TRAIL high	0.200	0.069 – 0.581	0.003

B – At first follow-up

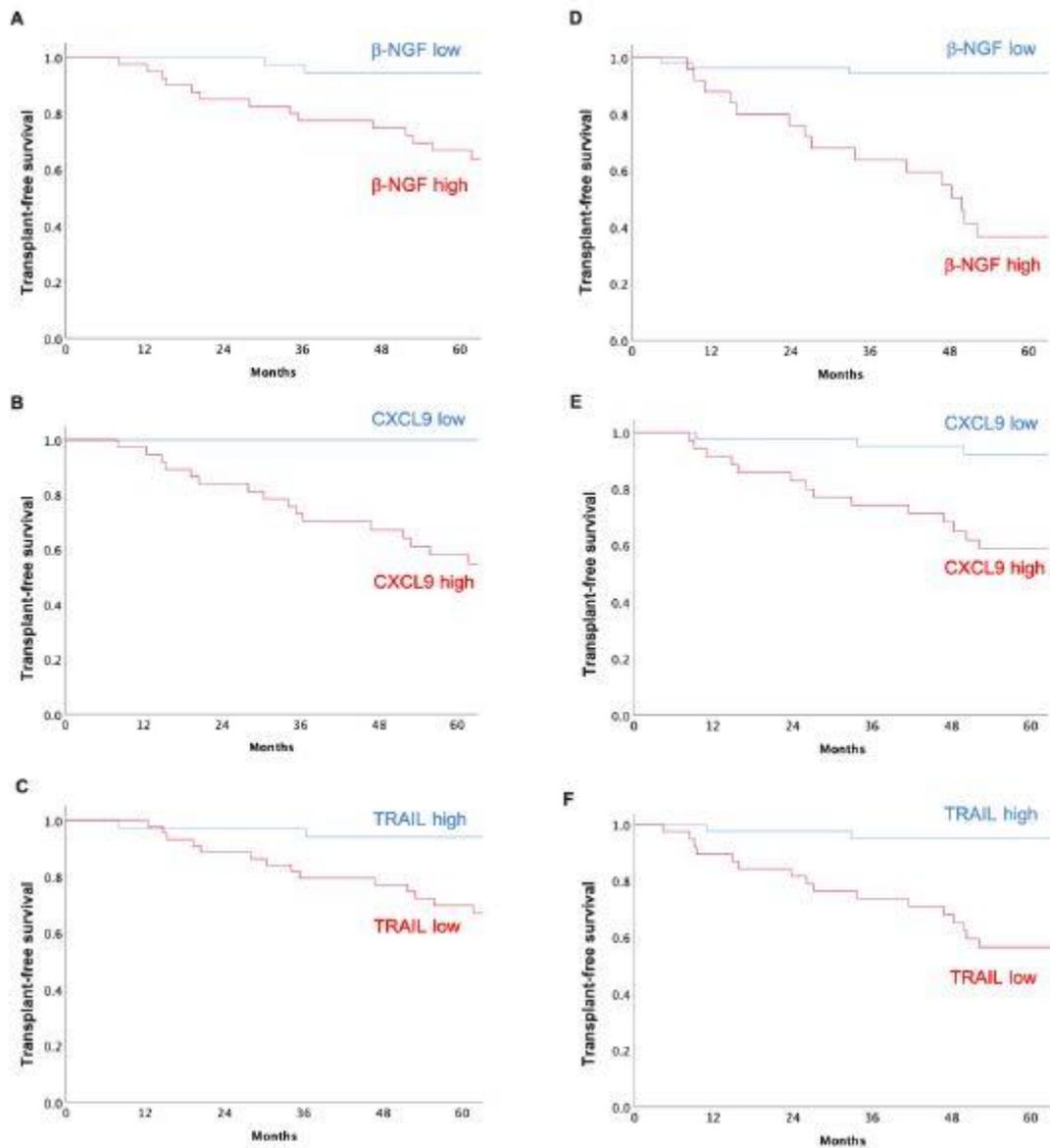
	Hazard-ratios	95%CI	p
NYHA functional class I-II	0.280	0.129 – 0.608	0.001
6MWD > 440 m	0.197	0.068 – 0.575	0.003
RAP < 8 mmHg	0.304	0.129 – 0.715	0.006
CI > 2.5 L/min/m ²	0.560	0.238 – 1.322	0.186
BNP < 50 or NT-proBNP < 300 ng/L	0.180	0.062 – 0.524	0.002
β-NGF high	10.811	4.266 – 27.396	<0.001
CXCL9 high	3.875	1.615 – 9.299	0.002
TRAIL high	0.169	0.064 – 0.450	<0.001

Cytokines are expressed as dichotomous variables (according to thresholds determined by ROC curves: β-NGF high: > 3.65 pg/mL; CXCL9 high: > 625.5 pg/mL, TRAIL high: > 52.65 pg/mL).

Abbreviations: 6MWD: 6-minute walk distance; BNP: brain natriuretic peptide; CI: cardiac index; CI: confidence interval; NTproBNP: N-Terminal pro-B-Type Natriuretic Peptide; NYHA: New-York Heart Association; RAP: right atrial pressure; beta-NGF: beta-nerve growth factor; CXCL: chemokine (C-X-C motif) ligand; TRAIL: tumor-necrosis-factor related apoptosis inducing ligand.

FIGURE LEGENDS

FIGURE 1: Kaplan–Meier survival curves according to levels of β -NGF, CXCL9 and TRAIL at the diagnosis (A, B, C) and at the follow-up (D, E, F): Cytokines are expressed as dichotomous variables according to thresholds determined by ROC curves: 3.65 pg/mL for beta-NGF, 625.5 pg/mL for CXCL9 and 52.65 pg/mL for TRAIL. Log rank $p < 0.005$ for both analyses.



Abbreviations: beta-NGF: beta-nerve growth factor; CXCL: chemokine (C-X-C motif) ligand; TRAIL: tumor-necrosis-factor related apoptosis inducing ligand.

FIGURE 2: Kaplan–Meier survival curves according to the number of low-risk status of β -NGF, CXCL9 and TRAIL at the diagnosis (A) and at the follow-up (B) in the French cohort: beta-NGF < 3.65 pg/mL; CXCL9 < 625.5 pg/mL, TRAIL > 52.65 pg/mL. Log rank $p < 0.001$ for both analyses.

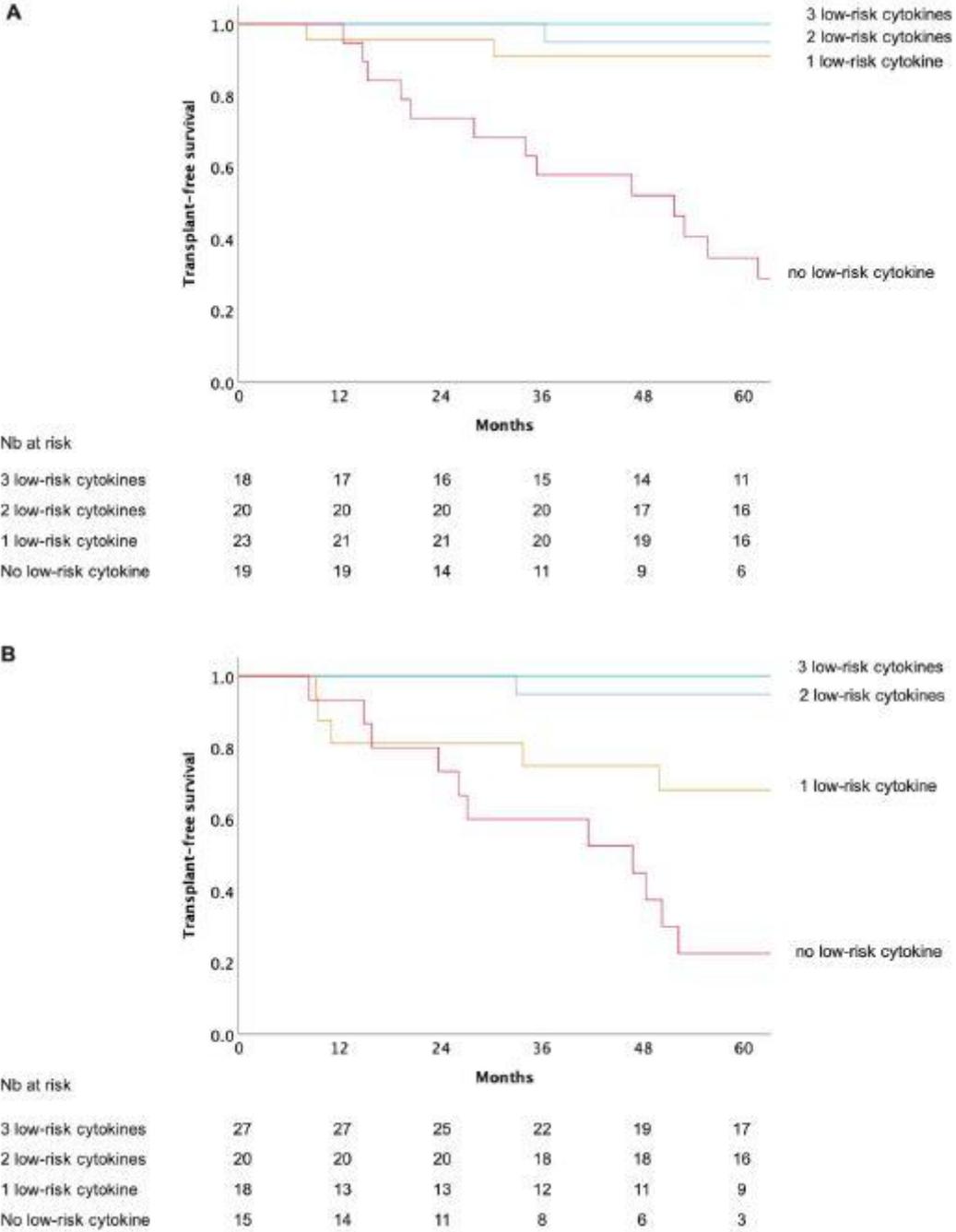
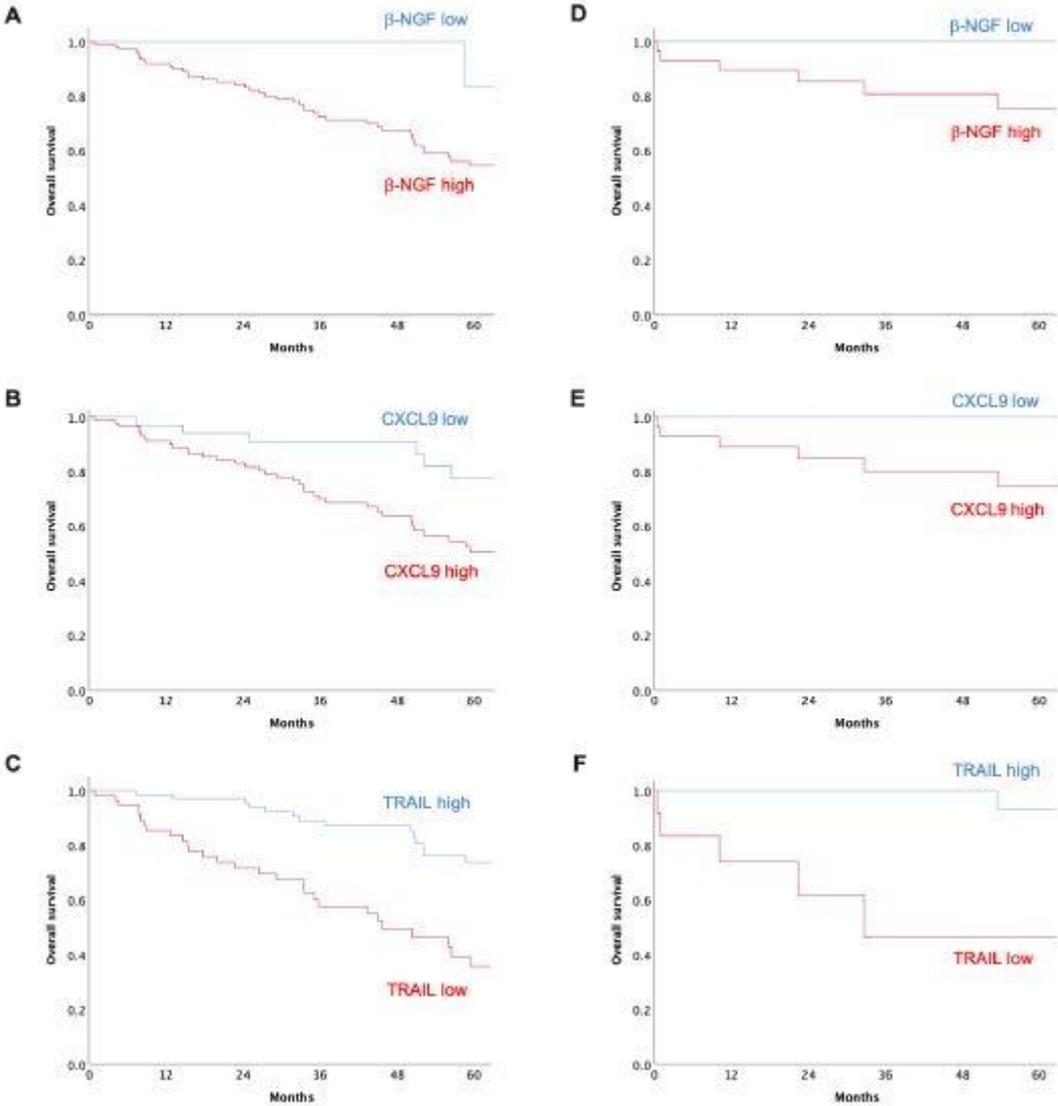
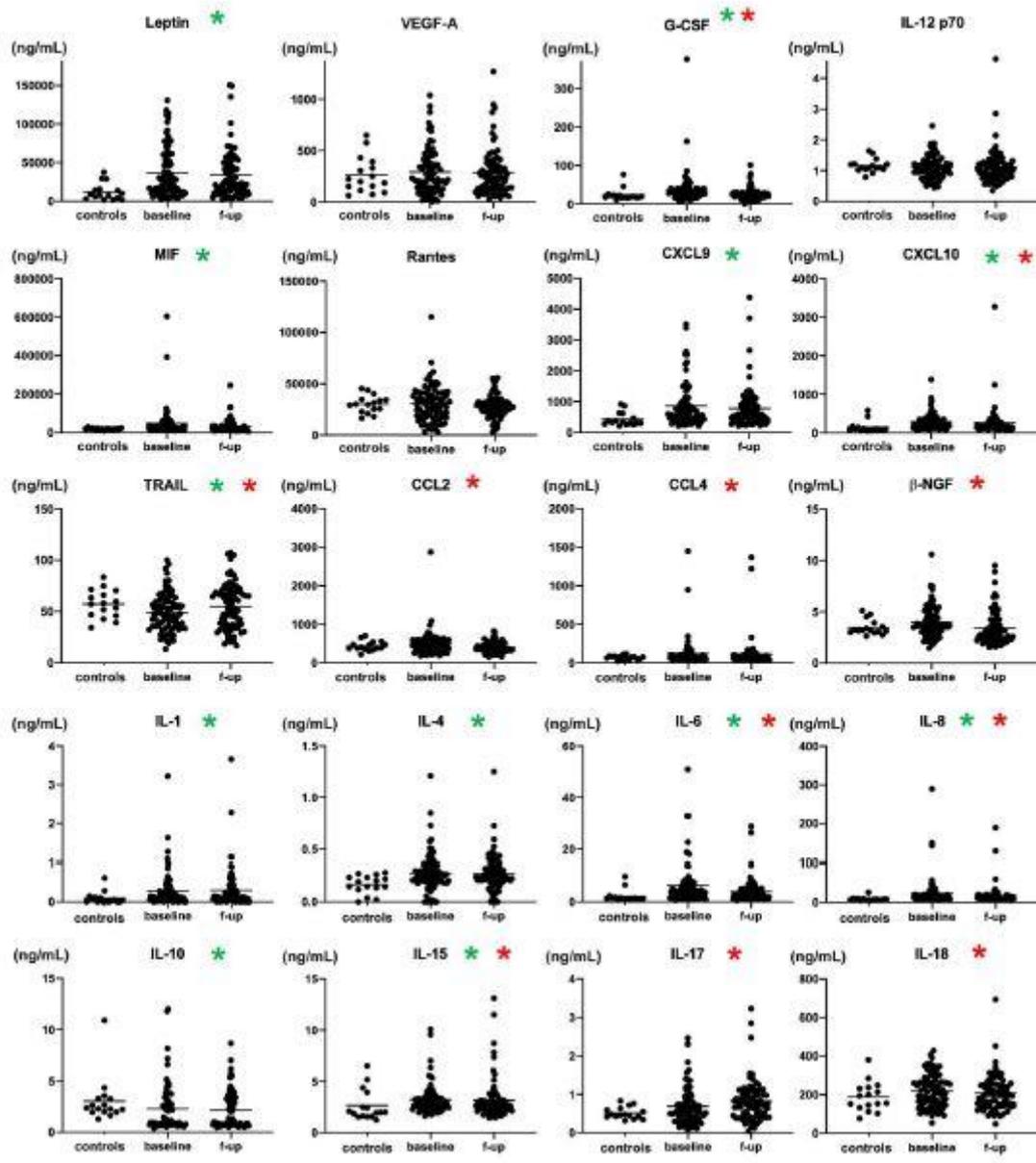


FIGURE 3: Kaplan-Meier survival curves according to levels of β -NGF, CXCL9 and TRAIL at the diagnosis (A, B, C) and at the follow-up (D, E, F) in the validation cohort: Cytokines are expressed as dichotomous variables according to thresholds determined by ROC curves in the study population: 3.65 pg/mL for β -NGF, 625.5 pg/mL for CXCL9 and 52.65 pg/mL for TRAIL. Log rank $p < 0.001$ for both analyses.



Abbreviations: beta-NGF: beta-nerve growth factor; CXCL: chemokine (C-X-C motif) ligand; TRAIL: tumor-necrosis-factor related apoptosis inducing ligand

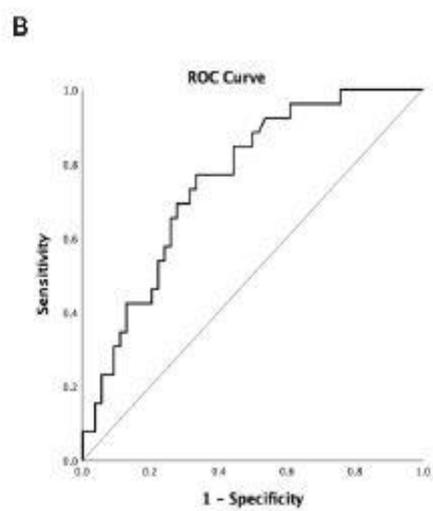
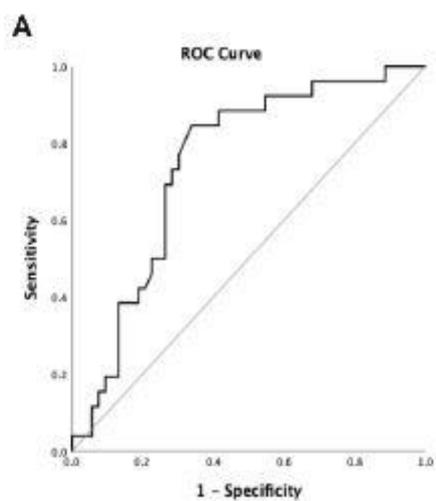
Supplementary FIGURE 1: Comparisons of serum cytokines levels concentrations between controls and PAH at the diagnosis, and in PAH between baseline and the first follow-up



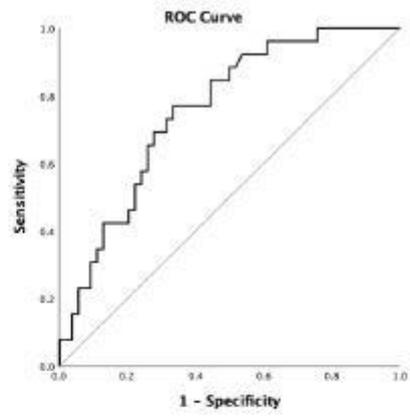
* Significant difference between controls and PAH at the time of diagnosis

* Significant difference in PAH between baseline and first follow-up (f-up)

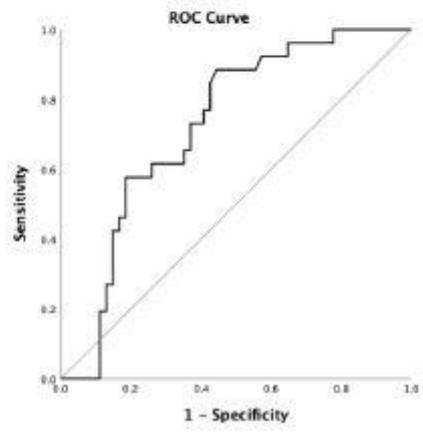
Supplementary FIGURE 2: ROC curves of transplant-free survival of β -NGF (A), CXCL9 (B) and TRAIL (C) at the time of diagnosis



B



C



Supplementary Table 1: Multivariable Cox regression analysis including the 3 non-invasive low risk variables and the three selected cytokines assessed at the time of diagnosis (baseline) in the discovery EFORT cohort.

At the time of diagnosis (baseline)	Model 1			Model 2			Model 3			Model 4		
	Hazard-ratio	95%CI	p-value	Hazard-ratio	95%CI	p-value	Hazard-ratio	95%CI	p-value	Hazard-ratio	95%CI	p-value
NYHA functional class I-II			NS			NS			NS			NS
6MWD >440 m			NS			NS			NS			NS
BNP <50 or NT-proBNP <300 ng/L			NS			NS			NS			NS
β -NGF high	7.768	2.294 – 26.307	0.001							5.001	1.358 – 18.417	0.016
CXCL9 high				8.622	3.034 – 24.499	<0.001				6.345	2.159 – 18.649	0.001
TRAIL high							0.212	0.071 – 0.631	0.005	0.203	0.054 – 0.772	0.019

β -NGF high: > 3.65 pg/mL; CXCL9 high: > 625.5 pg/mL, TRAIL high: > 52.65 pg/mL.

Abbreviations: 6MWD: 6-minute walk distance; BNP: brain natriuretic peptide; CI: cardiac index; CI: confidence interval; NTproBNP: N-Terminal pro-B-Type Natriuretic Peptide; NYHA: New-York Heart Association; RAP: right atrial pressure; beta-NGF: beta-nerve growth factor; CXCL: chemokine (C-X-C motif) ligand; TRAIL: tumor-necrosis-factor related apoptosis inducing ligand

Supplementary Table 2: Multivariable Cox regression analysis including the 3 non-invasive low risk variables and the three selected cytokines assessed at follow-up in the discovery EFORT cohort.

At the first follow-up	Model 1			Model 2			Model 3			Model 4		
	Hazard-ratio	95%CI	p-value	Hazard-ratio	95%CI	p-value	Hazard-ratio	95%CI	p-value	Hazard-ratio	95%CI	p-value
NYHA functional class I-II			NS			NS			NS			NS
6MWD >440 m			NS			NS			NS			NS
BNP <50 or NT-proBNP <300 ng/L			NS	0.242	0.073 – 0.809	0.021			NS			NS
β -NGF high	6.504	2.413 – 17.533	<0.001							4.945	1.438 – 17.000	0.011
CXCL9 high				4.231	1.669 – 10.727	0.002						NS
TRAIL high							0.261	0.095 – 0.716	0.009			NS

β -NGF high: > 3.65 pg/mL; CXCL9 high: > 625.5 pg/mL, TRAIL high: > 52.65 pg/mL.

Abbreviations: 6MWD: 6-minute walk distance; BNP: brain natriuretic peptide; CI: cardiac index; CI: confidence interval; NTproBNP: N-Terminal pro-B-Type Natriuretic Peptide; NYHA: New-York Heart Association; RAP: right atrial pressure; beta-NGF: beta-nerve growth factor; CXCL: chemokine (C-X-C motif) ligand; TRAIL: tumor-necrosis-factor related apoptosis inducing ligand