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Plasma receptor tyrosine kinase RET in pulmonary arterial hypertension diagnosis and differentiation

Joanna Säleby[1,2], Habib Bouzina[1,2], Salaheldin Ahmed[1,2], Jakob Lundgren[1,2] and Göran Rådegran[1,2]

Affiliations: [1]Dept of Clinical Sciences Lund, Cardiology, Faculty of Medicine, Lund University, Lund, Sweden. [2]The Hemodynamic Lab, The Section for Heart Failure and Valvular Disease, VO Heart and Lung Medicine, Skåne University Hospital, Lund, Sweden.

Correspondence: Joanna Säleby, The Hemodynamic Lab, The Section for Heart Failure and Valvular Disease, VO Heart and Lung Medicine, Skåne University Hospital, Getingevägen 4, 221 84 Lund, Sweden.
E-mail: joanna.saleby@med.lu.se

ABSTRACT

Background: Pulmonary arterial hypertension (PAH) is a serious disease exhibiting unspecific symptoms, as a result of which diagnosis is often delayed and prognosis is poor. The underlying pathophysiology includes vasoconstriction and remodelling of small pulmonary arteries. As receptor tyrosine kinases (RTKs) and their ligands have been shown to promote PAH remodelling, our aim was to evaluate if their plasma levels may be utilised to differentiate between various causes of pulmonary hypertension.

Methods: 28 biomarkers involved in RTK signalling were measured using proximity extension assays in venous plasma from patients with PAH (n=48), chronic thromboembolic pulmonary hypertension (CTEPH) (n=20), pulmonary hypertension due to diastolic (n=33) or systolic (n=36) heart failure and heart failure patients without pulmonary hypertension (n=15), as well as healthy controls (n=20).

Results: Plasma proto-oncogene tyrosine-protein kinase receptor Ret (RET) was decreased ($p<0.04$) in PAH compared with all disease groups and controls. RET generated a sensitivity of 64.6% and a specificity of 81.6% for detecting PAH from other disease groups. PAH and the other pulmonary hypertension groups showed elevated plasma tyrosine-protein kinase MER ($p<0.01$), vascular endothelial growth factor (VEGF)-A ($p<0.02$), VEGF-D ($p<0.01$), placental growth factor ($p<0.01$), amphiregulin ($p<0.02$), hepatocyte growth factor ($p<0.01$) and transforming growth factor-α ($p<0.05$) and decreased VEGF receptor-2 ($p<0.04$) and epidermal growth factor receptor ($p<0.01$) levels compared with controls.

Conclusion: Plasma RET differentiates patients with PAH from those with CTEPH, systolic or diastolic heart failure with or without pulmonary hypertension as well as healthy controls. Future studies would be of value to determine the clinical usefulness of RET as a biomarker and its link to PAH pathophysiology.

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Receptor tyrosine kinases have been shown to promote PAH remodelling. Plasma RET differentiates PAH from other causes of PH. RET could have the potential to be used as a future diagnostic biomarker. http://bit.ly/2LChPUS

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Introduction

Pulmonary hypertension is defined as an elevated mean pulmonary arterial pressure (mPAP) ⩾25 mmHg at rest and is classified into five groups, including 1) pulmonary arterial hypertension (PAH), 2) pulmonary hypertension due to left heart disease, 3) pulmonary hypertension due to lung disease and/or chronic hypoxia, 4) chronic thromboembolic pulmonary hypertension (CTEPH) and 5) pulmonary hypertension due to unclear and/or multifactorial mechanisms [1]. At the World Symposium on Pulmonary Hypertension in Nice 2018, a new definition of pulmonary hypertension was suggested [2]. However, the new proposed breakpoint mPAP value of 20 mmHg has not yet been adopted in European guidelines.

PAH is a serious condition where time to diagnosis and introduction of targeted therapy is crucial to improve outcome [1]. However, due to diffuse and unspecific symptoms, such as dyspnoea and fatigue, PAH diagnosis is often set late [1, 3]. Despite current vasoactive therapy, prognosis still remains poor [4, 5]. PAH is related to an imbalance of vasoactive compounds, with pathological changes located to distal pulmonary arteries, comprising vasoconstriction and vessel wall proliferation of the intima, media and adventitia, formation of plexiform lesions, and *in situ* thrombosis [1, 6, 7]. The precise underlying mechanistic causes of these changes are not completely understood. CTEPH is characterised by organised and fibrotic thromboembolism in the pulmonary arteries, resulting in obstruction and subsequent remodelling [1, 8, 9], partly similar to that seen in PAH. Moreover, pulmonary hypertension due to left heart disease, comprising the largest group of pulmonary hypertension patients [1], may result in endothelial damage and dysfunction, excessive vasoconstriction, followed by vascular remodelling. If such changes become unresponsive to medical vasoactive therapy, it may constitute a contraindication for cardiac transplantation [10, 11].

At present, there is no curative medical treatment available for PAH or for other causes of pulmonary hypertension. Medical vasoactive therapies are, however, well established in PAH and may decrease morbidity as well as mortality. As pulmonary hypertension is a negative prognostic factor in both left heart and pulmonary diseases [1, 11–13], it is of great importance to develop new diagnostic methods to identify and differentiate pulmonary hypertension, and especially PAH, patients at an earlier stage, to enable earlier treatment initiation that may stabilise or move the patient's "risk strata" into the low-risk zone.

Receptor tyrosine kinases (RTKs) and their ligands are involved in cell cycle regulation and of great interest in PAH development, since cellular changes involving their signalling pathways have been shown to be affected [14–16]. Such changes are suggested to be involved in PAH remodelling, including characteristics that are shared with cancer pathology, with dysregulated proliferation, apoptosis resistance and abnormal metabolism [15]. Antineoplastic drugs have therefore become of great interest. The tyrosine kinase inhibitor imatinib, a platelet-derived growth factor (PDGF) receptor inhibitor, was evaluated in the Imatinib in Pulmonary Arterial Hypertension, a Randomised, Efficacy Study (IMPRES), where patients showed improved physical capacity and haemodynamics. However, serious adverse events occurred [17].

Moreover, the vascular endothelial growth factor (VEGF) family is an intriguing group of tyrosine kinases that have been widely investigated in pulmonary hypertension. The VEGF ligands and their receptors have been shown to be altered in earlier studies, indicating dysregulated angiogenesis in pulmonary hypertension pathology [18–21]. In a rat model, monocrotaline-induced pulmonary hypertension was shown to be associated with reduced VEGF expression [22]. Overexpression of the angiogenic factor VEGF-A has furthermore been suggested to be protective in pulmonary hypertension [23]. PDGF has additionally been reported to mediate smooth muscle proliferation in pulmonary vascular remodelling in rats [24] and to be upregulated in PAH patients [25]. These findings indicate the complexity in pulmonary hypertension pathophysiology and the value of further research to characterise the specific roles of tyrosine kinases in pulmonary hypertension development.

New circulating plasma biomarkers, such as tyrosine kinases, could have the potential to be used for earlier diagnosis and differentiation of pulmonary hypertension aetiologies, for risk stratification, as well as in decisions for listing patients for lung and/or heart transplantation. We therefore investigated a broad range of blood-borne plasma biomarkers related to RTKs and associated proteins, in relation to different causes of pulmonary hypertension.

Methods

Study design and population

28 circulating plasma biomarkers related to RTK signalling were analysed from mixed venous blood plasma samples of 152 patients, separated into PAH (n=48), CTEPH (n=20), pulmonary hypertension due to diastolic heart failure (preserved ejection fraction, n=33), pulmonary hypertension due to systolic heart failure (reduced ejection fraction, n=36) and heart failure without pulmonary hypertension (n=15), as well as from peripheral venous plasma samples from healthy control subjects (n=20). All samples from the patient groups were obtained from the same location of venous introducers during standard right heart

catheterisation (RHC) procedures between September 2011 and March 2017 at diagnosis, before initiation of any disease-specific treatments in PAH patients. The samples were stored in and retrieved from the Lund Cardio Pulmonary Register (LCPR), a cohort in the Biobank of Region Skåne (Sweden). Data on patient characteristics, medications, haemodynamics and clinical parameters, including World Health Organization Functional Classification (WHO FC) and 6-min walk distance (6MWD), assessed in connection with blood sampling, were retrieved retrospectively from medical records. All patients and controls gave written informed consent for storage and analysis of their blood samples in the LCPR. The study was approved by the local ethics board in Lund (2010/114, 2010/442, 2011/368, 2011/777 and 2015/270) and performed in accordance with the Declaration of Helsinki.

Right heart catheterisation

RHCs were performed as a part of the routine clinical investigation for all patients, using a Swan–Ganz catheter (Baxter Health Care, Santa Ana, CA, USA), inserted predominantly *via* an introducer in the right internal jugular vein, at The Hemodynamic Lab, The Section for Heart Failure and Valvular Disease, Skåne University Hospital (Lund, Sweden). Haemodynamic parameters including mPAP, mean right atrial pressure (mRAP), pulmonary arterial wedge pressure (PAWP), diastolic pulmonary arterial pressure, systolic pulmonary arterial pressure and mean arterial pressure (mAP) were measured during the procedure. Cardiac output (CO) was estimated using thermodilution.

Cardiac index (CI), pulmonary vascular resistance (PVR), left ventricular stroke work index (LVSWI) and right ventricular stroke work index (RVSWI) were calculated using the following formulae: CI=CO/body surface area (BSA), PVR=(mPAP−PAWP)/CO, LVSWI=(mAP−PAWP)×stroke volume (SV)/BSA and RVSWI=(mPAP−mRAP)×SV/BSA.

Biomarker analyses

Blood samples were centrifuged and stored at −80°C in the LCPR. Biomarkers were analysed using the proximity extension assay (PEA) technique followed by PCR (Olink Bioscience, Uppsala, Sweden), as previously described [26]. A subgroup of biomarkers involved in RTK signalling was analysed using Proseek Multiplex CVD II, CVD III and Oncology II 96-plex immunoassay panels (Olink Bioscience), including members of 1) TAM receptor kinase family (tyrosine-protein kinase MER (MERTK) and tyrosine-protein kinase receptor UFO (AXL)); 2) VEGF family (VEGF receptor (VEGFR)-2 and -3, VEGF-A and -D, and placental growth factor (PlGF)); 3) epidermal growth factor (EGF) family (EGF receptor (EGFR), human EGFR (HER) 2, HER3, HER4, pro-EGF, amphiregulin, heparin-binding EGF-like growth factor (HB-EGF) and transforming growth factor (TGF)-α); 4) TIE family of angiopoietin receptors (angiopoietin receptor TEK tyrosine kinase (Tie2) and angiopoietin 1 (Ang-1)); 5) PDGF family (PDGF subunit A and B); 6) non-RTK family (proto-oncogene tyrosine-protein kinase Src (SRC), tyrosine-protein kinase Lyn (LYN) and tyrosine-protein kinase ABL1 (ABL-1)); 7) ephrin receptor family (ephrin type A receptor 2 (EPHA2), ephrin type B receptor 4 (EPHB4)) and 8) other tyrosine kinases (proto-oncogene tyrosine-protein kinase receptor Ret (RET), stem cell factor (SCF), hepatocyte growth factor (HGF) and fibroblast growth factor-binding protein 1 (FGF-BP1)). N-terminal pro-brain natriuretic peptide (NT-proBNP) levels were analysed using the same technique. Plasma levels of these biomarkers were measured in normalised protein expression values, which is an arbitrary unit on a $\log_2$ scale. Validation documents of the Olink PEA and PCR analyses can be found at: www.olink.com/resources-support/document-download-center.

Statistics

Statistical analyses as well as figures were made using Prism version 7.00 for Mac (www.graphpad.com) or R version 3.5.1 (www.r-project.org). Comparisons of plasma biomarker level differences between PAH, CTEPH, pulmonary hypertension due to diastolic heart failure (preserved ejection fraction), pulmonary hypertension due to systolic heart failure (reduced ejection fraction) with pulmonary hypertension and heart failure patients without pulmonary hypertension, as well as controls, were analysed using the Kruskal–Wallis test. False discovery rates (FDRs) were determined for all Kruskal–Wallis tests comparing biomarkers in the studied groups. FDR was set to 5%. The number of statistical tests was 273 based on the biomarkers included in all three panels. *Post hoc* analyses using Dunn's multiple comparison test was performed for tests with significant adjusted Kruskal–Wallis p-values.

To assess the performance of RET as a predictor of PAH among other disease groups, the area under the curve (AUC) of the receiver operating characteristics (ROC) curve was calculated. The best cut-off was determined according to Youden's index. Correlation analyses between RET levels and mPAP, mRAP, CI, PVR as well as S_{vO_2}, 6MWD and NT-proBNP were performed using Spearman's coefficient.

Results are presented as median (interquartile range (IQR)) for continuous variables and absolute or percentage for categorical variables, unless otherwise stated. Statistical significance was accepted at p<0.05.

Results

Population and haemodynamic characteristics

Study population characteristics are shown in table 1. In PAH, 21 patients (43.8%) were diagnosed with idiopathic PAH, two patients (4.2%) with familial PAH, 17 patients (35.4%) with systemic sclerosis (SSc)-associated PAH, four patients (8.3%) with SSc-associated PAH without interstitial lung disease and four patients (8.3%) with other connective tissue disease-associated PAH. Median (IQR) 6MWD at diagnosis was 242 (172.5–349) m in PAH and 300 (220–337.5) m in CTEPH. 6MWD data were missing for two PAH patients and three CTEPH patients. In PAH, one patient (2.1%) was classified in WHO FC 1, nine patients (18.8%) in WHO FC 2, 28 patients (58.3%) in WHO FC 3 and two patients (4.2%) in WHO FC 4. In CTEPH, six patients (30.0%) were classified in WHO FC 2 and 13 patients (65.0%) in WHO FC 3. WHO FC data were missing for eight PAH patients and one CTEPH patient. The controls had no events of ischaemic heart disease, atrial fibrillation, stroke or diabetes mellitus.

Biomarker levels

RET plasma levels are lower in PAH

RET plasma levels were lower in PAH compared with all the other disease groups (p<0.038) and controls (p<0.001) (table 2 and figure 1). ROC analyses for plasma RET generated an AUC of 0.747 (95% CI 0.659–0.834) (p<0.001) with a sensitivity of 64.6% and a specificity of 81.6% for PAH (figure 1). No specific correlations were, however, detected between plasma RET and mPAP, mRAP, CI, PVR, S_{vO_2}, 6MWD and NT-proBNP (all nonsignificant).

Plasma VEGF ligands are higher and VEGFR-2 is lower in pulmonary hypertension

Controls had lower levels of VEGF-A (p<0.013), VEGF-D (p<0.010) and PlGF (p<0.001) and higher levels of VEGFR-2 (p<0.036) compared with the pulmonary hypertension patient groups (table 2 and figure 2).

TABLE 1 Population characteristics

	Control	PAH	CTEPH	PH-HFpEF	PH-HFrEF	Non-PH-HF
Subjects n (% female)	20 (55)	48 (83)	20 (65)	33 (64)	36 (19)	15 (53)
Age years	41 (26.8–50.5)	71.5 (64.0–76.0)	75 (70.8–77.8)	75.0 (68.5–83.0)	54.0 (47.3–59.5)	60.0 (46.0–76.0)
BSA m^2	1.9 (1.8–2.0)	1.7 (1.6–2.0)	1.8 (1.8–2.0)	1.9 (1.7–2.1)	2.0 (1.9–2.1)	2.0 (1.7–2.1)
mAP mmHg	89.0 (95.0–100.0)	96.0 (89.4–104.0)	98.5 (94.0–110.3)	98.0 (91.5–104.5)	79.5 (75.3–88.8)	89.0 (80.0–96.0)
mPAP mmHg		43.0 (37.0–54.8)	42.0 (35.0–54.3)	34.0 (28.5–46.0)	34.5 (29.0–40.8)	20.0 (17.0–22.0)
PAWP mmHg		8.0 (6.0–11.0)	9.5 (7.0–13.0)	18.0 (16.0–22.5)	25.0 (19.0–28.0)	15.0 (9.0–18.0)
mRAP mmHg		7.0 (4.0–11.0)	5.5 (3.3–8.0)	10.0 (6.5–14.0)	14.5 (9.0–17.0)	6.0 (2.0–16.0)
CI $L{\cdot}min^{-1}{\cdot}m^{-2}$		2.2 (1.8–2.8)	2.3 (1.9–2.5)	2.4 (2.1–2.8)	1.6 (1.4–1.9)	1.9 (1.6–2.2)
PVR WU		9.5 (6.2–11.8)	9.3 (5.9–10.8)	3.6 (2.4–4.9)	3.0 (2.3–3.7)	1.5 (1.0–2.0)
LVSWI $mmHg{\cdot}mL{\cdot}m^{-2}$		2488.0 (2045.0–3213.0)	2508.0 (2330.0–3187.0)	2664 (2189.0–3308.0)	1152.0 (957.0–1636.0)	2168.0 (1650.0–2716.0)
RVSWI $mmHg{\cdot}mL{\cdot}m^{-2}$		990.5 (807.2–1246.0)	1111.0 (844.5–1298.0)	831.5 (670.7–1140.0)	439.6 (305.8–649.3)	382.4 (195.5–494.5)
S_{vO_2} %		60.5 (51.6–66.6)	62.5 (54.9–67.9)	64.1 (57.8–66.8)	50.3 (46.5–55.2)	61.2 (58.5–69.2)
NT-proBNP AU		3.1 (2.1–3.8)	2.6 (1.0–4.2)	2.9 (2.4–3.3)	4.9 (4.1–5.4)	3.2 (1.3–4.4)
Creatinine $\mu mol{\cdot}L^{-1}$		90.0 (70.8–113.5)	88.0 (73.0–122.5)	99.0 (79.0–117.0)	121.0 (90.0–145.0)	93.0 (80.5–123.0)
Hypertension		17 (35)	11 (55)	22 (67)	7 (19)	7 (47)
Diabetes mellitus		12 (25)	0 (0)	11 (33)	4 (11)	3 (20)
Atrial fibrillation		4 (8)	3 (15)	25 (76)	15 (42)	8 (53)
Stroke		2 (4)	1 (5)	6 (18)	4 (11)	2 (13)
Ischaemic heart disease		7 (15)	1 (5)	6 (18)	6 (17)	6 (40)
Thyroid disease		11 (23)	1 (5)	2 (6)	3 (8)	3 (20)
ACEi		10 (21)	2 (10)	12 (36)	19 (53)	3 (20)
β-blockers		16 (33)	9 (45)	25 (76)	35 (97)	11 (73)
ARB		4 (8)	7 (35)	10 (30)	14 (39)	5 (33)
MRA		11 (23)	3 (15)	9 (27)	21 (58)	7 (47)

Data are presented as median (interquartile range) or n (%), unless otherwise stated. PAH: pulmonary arterial hypertension; CTEPH: chronic thromboembolic pulmonary hypertension; PH-HFpEF: pulmonary hypertension due to diastolic heart failure (preserved ejection fraction); PH-HFrEF: pulmonary hypertension due to systolic heart failure (reduced ejection fraction); non-PH-HF: heart failure without pulmonary hypertension; BSA: body surface area; mAP: mean arterial pressure; mPAP: mean pulmonary arterial pressure; PAWP: pulmonary arterial wedge pressure; mRAP: mean right atrial pressure; CI: cardiac index; SV: stroke volume; PVR: pulmonary vascular resistance; WU: Wood Units; LVSWI: left ventricular stroke work index; RVSWI: right ventricular stroke work index; S_{vO_2}: mixed venous oxygen saturation; NT-proBNP: N-terminal pro-brain natriuretic peptide; AU: arbitrary units; ACEi: angiotensin converting enzyme inhibitor; ARB: angiotensin receptor blocker; MRA: mineralocorticoid receptor antagonist.

TABLE 2 Biomarker plasma levels

	Control	PAH	CTEPH	PH-HFpEF	PH-HFrEF	Non-PH-HF
TAM receptor kinase family						
MERTK	3.58 (3.47–3.90)	4.05 (3.82–4.36)[#]	4.32 (4.12–4.56)[#]	4.37 (4.13–4.62)[#]	4.15 (3.89–4.68)[#]	4.33 (4.19–4.57)[#]
AXL	7.35 (7.18–7.63)	7.35 (6.93–7.65)	7.23 (7.03–7.43)	7.47 (7.20–8.02)	7.68 (7.38–8.05)[¶,+]	7.50 (7.23–8.01)
VEGF family						
VEGFR-2	6.58 (6.38–6.68)	6.18 (5.96–6.41)[#]	6.18 (6.02–6.40)[#]	6.01 (5.86–6.18)[#]	6.18 (5.99–6.32)[#]	6.10 (5.88–6.34)[#]
VEGFR-3	5.74 (5.42–5.79)	5.74 (5.51–5.92)	5.80 (5.55–5.95)	5.77 (5.51–5.91)	5.88 (5.74–6.06)[#,¶]	5.73 (5.49–5.96)
VEGF-A	9.13 (8.94–9.43)	9.83 (9.45–10.22)[#]	9.87 (9.61–10.54)[#]	10.02 (9.78–10.45)[#]	9.76 (9.37–10.18)[#]	9.77 (9.45–10.02)[#]
VEGF-D	6.78 (6.56–6.92)	7.23 (7.01–7.48)[#]	7.47 (7.24–7.66)[#]	7.47 (7.20–7.74)[#]	7.97 (7.74 –8.05)[#,¶,+,§]	7.36 (6.65–7.74)[f]
PlGF	6.92 (6.78–7.26)	7.61 (7.43–8.08)[#]	7.64 (7.46–7.93)[#]	7.92 (7.57–8.31)[#]	7.62 (7.31–8.03)[#]	7.72 (7.42–7.89)[#]
EGF family						
EGFR	1.38 (1.26–1.56)	0.82 (0.65–1.08)[#]	1.04 (0.82–1.10)[#]	0.99 (0.73–1.15)[#]	1.08 (0.97–1.23)[#,¶]	1.06 (0.92–1.24)[#]
HER2	6.44 (6.20–6.69)	6.39 (6.09–6.55)	6.54 (6.37–6.72)	6.43 (6.16–6.71)	6.72 (6.63–6.92)[#,¶,§]	6.68 (6.47–6.74)
HER3	7.46 (7.31–7.54)	7.26 (7.11–7.44)	7.42 (7.27–7.63)	7.32 (7.08–7.38)	7.37 (7.20–7.62)	7.30 (7.20–7.56)
HER4	4.55 (4.24–4.65)	4.50 (4.36–4.66)	4.56 (4.38–4.67)	4.48 (4.33–4.68)	4.80 (4.63–5.00)[#,¶,§]	4.76 (4.27–4.93)
Pro-EGF	8.63 (7.36–9.48)	7.76 (6.57–8.62)	7.96 (6.84–8.61)	7.97 (7.16–8.85)	7.12 (5.93–8.05)[#]	7.85 (7.25–8.96)
Amphiregulin	1.43 (1.21–1.57)	2.43 (2.09–2.79)[#]	2.14 (1.99–2.49)[#]	2.30 (1.99–2.83)[#]	2.62 (2.02–3.15)[#]	1.99 (1.76–2.61)[#]
HB-EGF	5.90 (5.44–6.33)	5.77 (5.53–6.24)	6.52 (5.84–6.98)[¶]	6.13 (5.57–6.48)	5.75 (5.37–6.17)[+]	6.28 (5.75–6.70)
TGF-α	1.20 (0.93–1.39)	1.82 (1.62–2.15)[#]	2.07 (1.60–2.37)[#]	2.05 (1.67–2.48)[#]	2.06 (1.77–2.35)[#]	1.56 (1.48–2.09)[#]
TIE family of angiopoietin receptors						
Tie2	7.53 (7.35–7.64)	7.57 (7.40–7.82)	7.81 (7.59–7.98)[#]	7.72 (7.57–7.97)	8.09 (7.79–8.17)[#,¶,§]	7.90 (7.60–8.11)[#]
Ang-1	8.26 (7.68–8.97)	8.27 (7.84–9.03)	9.20 (8.09–9.75)	8.57 (7.87–9.36)	8.11 (7.32–8.81)[+]	9.00 (7.89–9.50)
PDGF family						
PDGF subunit A	2.89 (2.41–3.40)	2.89 (2.04–3.36)	3.57 (3.01–4.63)	3.12 (2.09–3.77)	2.70 (2.16–3.42)[+]	3.22 (2.30–3.90)
PDGF subunit B	9.48 (8.65–9.92)	9.17 (8.52–9.77)	9.55 (8.94–10.16)	9.50 (8.27–9.83)	8.82 (7.87–9.54)	9.45 (8.66–9.98)
Non-RTK family						
SRC	6.87 (6.77–7.11)	6.93 (6.51–7.17)	6.82 (6.52–7.12)	6.96 (6.68–7.14)	6.88 (5.95–7.22)	7.12 (6.64–7.28)
LYN	2.80 (2.13–3.21)	2.38 (1.89–2.97)	2.30 (2.11–2.54)	2.49 (2.17–3.11)	2.28 (1.87–2.75)	2.48 (2.13–3.32)
ABL-1	4.57 (3.05–5.31)	3.87 (3.24–4.99)	4.15 (3.93–4.71)	4.57 (4.08–5.33)[¶]	4.11 (3.71–4.88)	4.88 (4.04–5.37)
Ephrin receptor family						
EPHA2	1.50 (1.39–1.59)	1.89 (1.63–2.18)[#]	1.69 (1.52–1.97)	2.10 (1.83–2.38)[#]	1.67 (1.48–2.24)[#]	1.81 (1.70–2.10)[#]
EPHB4	1.44 (1.29–1.54)	1.50 (1.13–1.77)	1.54 (1.39–1.65)	1.72 (1.46–2.02)[#,¶]	1.72 (1.41–1.89)	1.62 (1.36–1.68)
Other tyrosine kinases						
RET	3.96 (3.59–4.32)	3.13 (2.77–3.53)[#]	3.75 (3.37–4.12)[¶]	3.58 (3.36–3.91)[¶]	3.71 (3.30–4.19)[¶]	3.73 (3.4–4.16)[¶]
SCF	8.45 (8.34–8.80)	8.01 (7.37–8.36)[#]	8.29 (8.00–8.68)	8.26 (7.78–8.48)	7.72 (7.31–8.08)[#,+,§]	8.11 (7.71–8.46)
HGF	5.50 (5.24–5.85)	6.35 (6.18–6.66)[#]	6.54 (6.06–6.81)[#]	6.66 (6.20–7.02)[#]	6.59 (6.33–7.38)[#]	6.60 (6.13–7.22)[#]
FGF-BP1	4.30 (4.19–4.34)	4.59 (4.38–4.89)[#]	4.61 (4.36–4.72)	4.5 (4.33–4.81)	4.83 (4.40–5.19)[#]	4.49 (4.26–4.81)

Levels are presented as median (interquartile range) of arbitrary units. PAH: pulmonary arterial hypertension; CTEPH: chronic thromboembolic pulmonary hypertension; PH-HFpEF: pulmonary hypertension due to diastolic heart failure (preserved ejection fraction); PH-HFrEF: pulmonary hypertension due to systolic heart failure (reduced ejection fraction); non-PH-HF: heart failure without pulmonary hypertension; MERTK: tyrosine-protein kinase MER; AXL: tyrosine-protein kinase receptor UFO; VEGF: vascular endothelial growth factor; VEGFR: VEGF receptor; PlGF: placental growth factor; pro-EGF: pro-epidermal growth factor; EGFR: EGF receptor; HER: human EGFR; HB-EGF: heparin-binding EGF-like growth factor; TGF: transforming growth factor; Tie2: angiopoietin receptor TEK tyrosine kinase; Ang-1: angiopoietin 1; PDGF: platelet-derived growth factor; RTK: receptor tyrosine kinase; SRC: proto-oncogene tyrosine-protein kinase Src; LYN: tyrosine-protein kinase Lyn; ABL-1: tyrosine-protein kinase ABL1; EPHA2: ephrin type A receptor 2; EPHB4: ephrin type B receptor 4; RET: proto-oncogene tyrosine-protein kinase receptor Ret; SCF: stem cell factor; HGF: hepatocyte growth factor; FGF-BP1: fibroblast growth factor-binding protein 1. [#]: significantly different compared with controls; [¶]: significantly different compared with PAH; [+]: significantly different compared with CTEPH; [§]: significantly different compared with PH-HFpEF; [f]: significantly different compared with PH-HFrEF.

Plasma TGF-α and amphiregulin are higher and EGFR is lower in pulmonary hypertension and heart failure

Controls had lower levels of TGF-α ($p<0.042$) and amphiregulin ($p<0.014$) as well as higher levels of EGFR ($p<0.008$) compared with pulmonary hypertension patient groups and heart failure without pulmonary hypertension (table 2 and figure 3).

Plasma MERTK and HGF levels are higher in pulmonary hypertension and heart failure

Controls had lower levels of MERTK ($p<0.009$) and HGF ($p<0.001$) (table 2) compared with pulmonary hypertension patient groups and heart failure without pulmonary hypertension. The p-values of multiple comparisons between controls and patient groups are shown in supplementary table S1.

Discussion

The present study indicates that RET plasma levels may be of use to identify patients with PAH among other conditions presenting with similar symptoms, such as heart failure with or without pulmonary

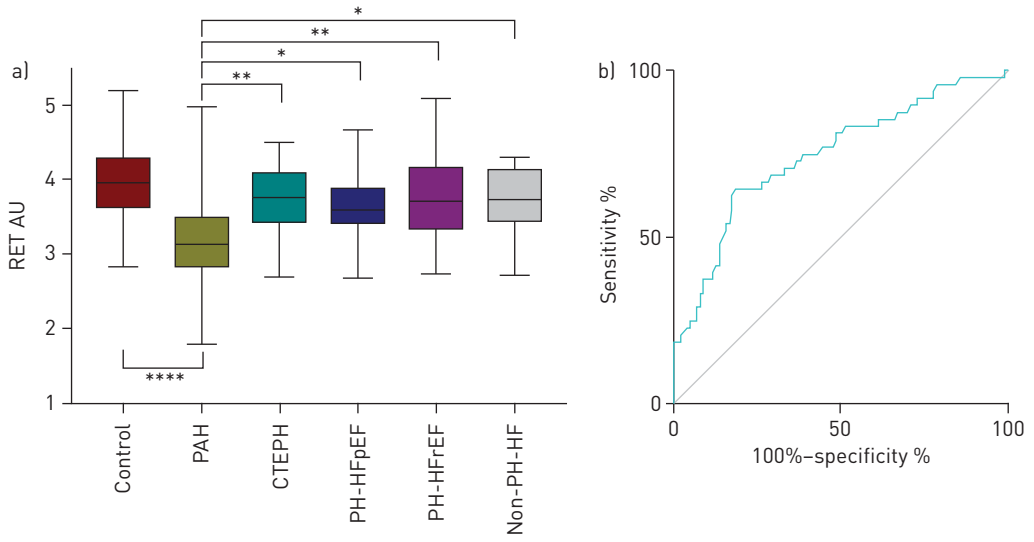

FIGURE 1 a) Proto-oncogene tyrosine-protein kinase receptor Ret (RET) in controls and disease groups. AU: arbitrary units; PAH: pulmonary arterial hypertension; CTEPH: chronic thromboembolic pulmonary hypertension; PH-HFpEF: pulmonary hypertension due to diastolic heart failure (preserved ejection fraction); PH-HFrEF: pulmonary hypertension due to systolic heart failure (reduced ejection fraction); non-PH-HF: heart failure without pulmonary hypertension. RET was reduced in plasma in PAH compared with the other disease groups and controls. *: p<0.05; **: p<0.01; ****: p<0.0001. b) Receiver operating characteristics of RET as a predictor of PAH among other pulmonary hypertension groups.

hypertension as well as CTEPH. Plasma RET furthermore differentiates PAH patients from healthy controls. Previous studies have shown RTKs to be dysregulated in PAH pathology, involving cellular pathways of proliferation, inhibition of apoptosis and angiogenesis, with characteristics similar to cancer pathology [14–16, 27]. However, to the best of our knowledge, RET as a plasma biomarker has not previously been investigated in the field of pulmonary hypertension. The present study identifies a potential link between RTKs and PAH pathophysiology, which may pave the way for new diagnostic approaches in the future.

Although the list of investigated biomarkers in pulmonary hypertension is constantly growing [1], so far NT-proBNP and its precursors, which reflect myocardial stress, are the only circulating biomarkers widely used clinically in pulmonary hypertension [1]. This conveys the need for further research to understand the pathophysiology of various causes of pulmonary hypertension, and to enable the development of new diagnostic methods and novel targets for therapy.

RET is a transmembrane tyrosine kinase with ligands of the glial cell line-derived neurotrophic factor (GDNF) family, which requires GDNF family receptor α co-receptors to exert its functions [28]. Mutations in the RET gene have been linked to human cancers, including papillary and medullary thyroid carcinoma, multiple endocrine neoplasia types 2A and 2B, and Hirschsprung's disease [28, 29]. RET has been reported to initiate signalling pathways involved in cell survival, differentiation, proliferation and migration [28, 29], and could thus also be involved in the vascular remodelling in PAH. In the present study, however, no correlations were detected between haemodynamic parameters and RET. This may indicate that RET is not involved in the disease progression. Pathological changes in RET systems may instead drive the initial changes seen in PAH. RET may consequently be of use as a biomarker in PAH diagnosis, but potentially not when evaluating disease severity or in risk stratification.

In this study, deranged levels of both growth factors and their receptors were seen in the pulmonary hypertension groups. In pulmonary hypertension, plasma VEGF-A, VEGF-D and PlGF levels were higher and VEGFR-2 levels were lower compared with controls. Elevated levels of VEGF family growth factors have previously also been observed in pulmonary hypertension when compared with control subjects [19–21], in line with our findings. Partovian *et al.* [30] have suggested that elevated levels of VEGFs are protective in hypoxic pulmonary hypertension in rats. Furthermore, VEGFR-2 has been reported to be upregulated in plexiform lesions in pulmonary hypertension [18].

In the EGF family, deranged levels of both growth factors and their receptors were also seen, with higher levels of the ligands TGF-α and amphiregulin, as well as lower levels of EGFR in pulmonary hypertension patients compared with controls. Le Cras *et al.* [31] showed that mice overexpressing TGF-α developed

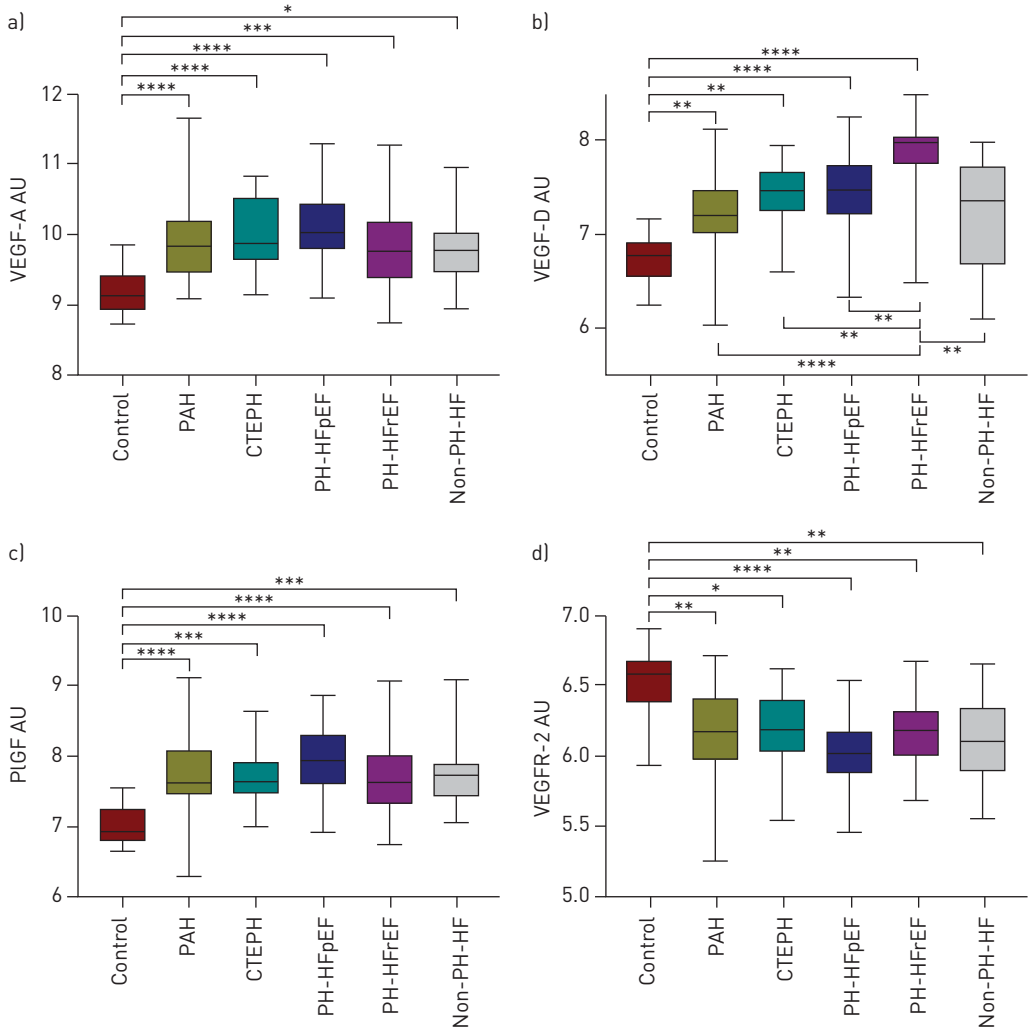

FIGURE 2 Vascular endothelial growth factor (VEGF) ligands a) VEGF-A, b) VEGF-D and c) placental growth factor (PlGF) are upregulated and d) tyrosine kinase receptor VEGF receptor (VEGFR)-2 is downregulated in pulmonary hypertension compared with controls. AU: arbitrary units; PAH: pulmonary arterial hypertension; CTEPH: chronic thromboembolic pulmonary hypertension; PH-HFpEF: pulmonary hypertension due to diastolic heart failure (preserved ejection fraction); PH-HFrEF: pulmonary hypertension due to systolic heart failure (reduced ejection fraction); non-PH-HF: heart failure without pulmonary hypertension. *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001.

severe pulmonary hypertension with extensive pulmonary vascular remodelling. Interestingly, reduced VEGF-A protein levels were observed in the lungs of these TGF-α mice. Amphiregulin has been shown to be a potent mitogen of vascular smooth muscle cells [32] and elevated levels in pulmonary hypertension patients could therefore potentially be involved in pulmonary vascular remodelling.

The plasma levels of another tyrosine kinase receptor, MERTK, were also shown to be altered, with lower levels in the control group compared with pulmonary hypertension groups and heart failure without pulmonary hypertension. MERTK is expressed on phagocytic cells and platelets, and has been shown to be involved in many cellular processes, including inflammation, and in both tethering and phagocytosis of apoptotic cells [33, 34]. Thorp *et al.* [35] showed that mice with mutated MERTK exhibited increased apoptotic cell accumulation and formation of necrotic plaques. The expression of MERTK has also been shown to be reduced in hypoxic mice, with subsequent repression of efferocytosis [36].

Defective clearance of apoptotic bodies with accumulation of cellular debris leading to pro-inflammatory stimulation and presence of autoantigens has been suggested to be the underlying mechanism in systemic autoimmune diseases [37]. Of interest, PAH may be related to autoimmune diseases, such as SSc and systemic lupus erythematosus [1]. Defective MERTK, leading to reduced efferocytosis, could therefore potentially be involved in PAH pathogenesis. Furthermore, in PAH, deranged levels of cytokines, as well as

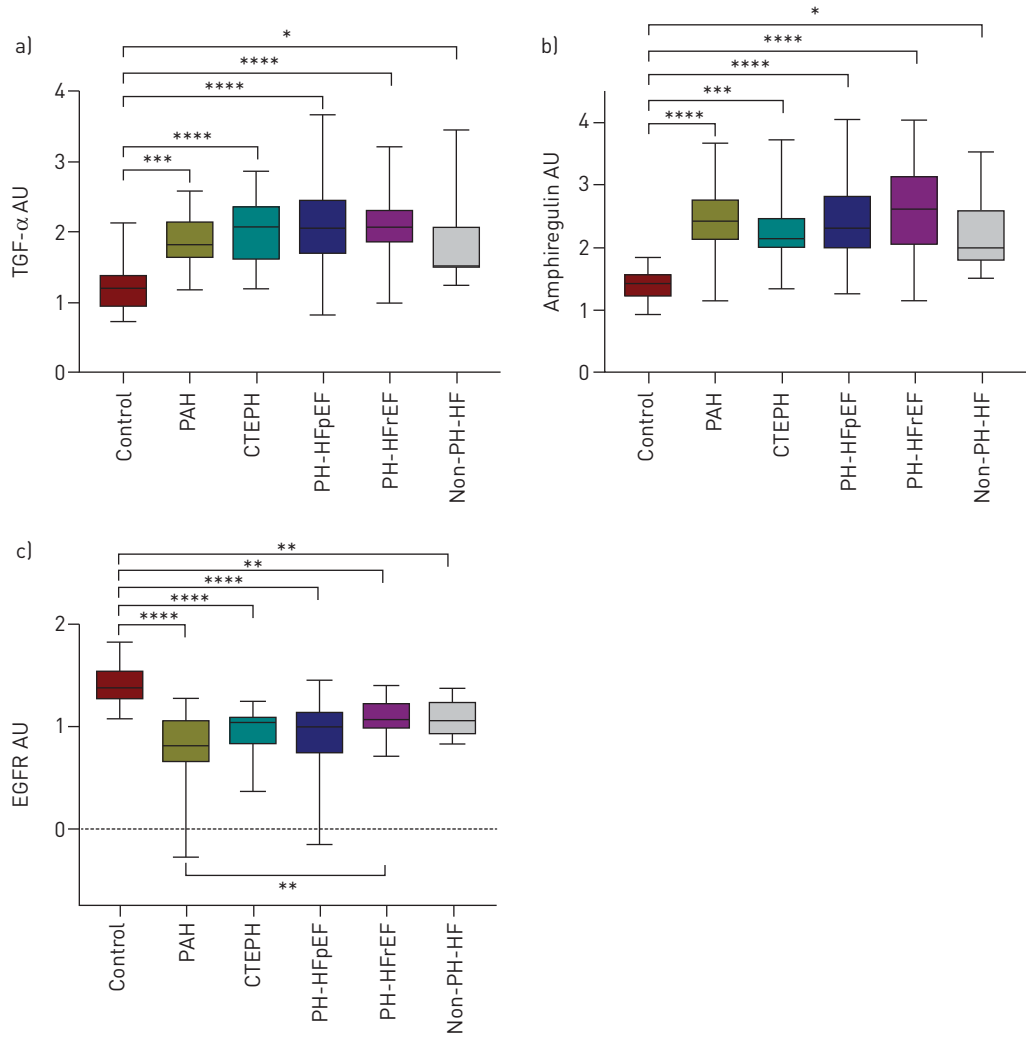

FIGURE 3 a) Transforming growth factor (TGF)-α and b) amphiregulin are upregulated and c) receptor tyrosine kinase epidermal growth factor receptor (EGFR) is downregulated in pulmonary hypertension plasma. AU: arbitrary units; PAH: pulmonary arterial hypertension; CTEPH: chronic thromboembolic pulmonary hypertension; PH-HFpEF: pulmonary hypertension due to diastolic heart failure (preserved ejection fraction); PH-HFrEF: pulmonary hypertension due to systolic heart failure (reduced ejection fraction); non-PH-HF: heart failure without pulmonary hypertension. *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001.

perivascular inflammatory infiltrates, have been reported, supporting disturbed inflammation as a part of the pathological process [19, 21, 38].

HGF plasma levels were observed to be higher in pulmonary hypertension groups compared with controls. In a previous study, HGF levels were shown to be elevated in PAH compared with controls, with higher levels observed in severe PAH compared with mild and moderate disease severity [39]. However, in a PAH rat model, 14 days administration of recombinant human HGF resulted in vascular lumen enlargement, decreased pulmonary arterial pressure and ultimately prolonged survival [40], emphasising the complexity of pulmonary hypertension pathophysiology.

Herein, we demonstrate that several circulating tyrosine kinases and their related ligands are of interest in the field of pulmonary hypertension. However, the results should be interpreted with caution and study limitations should be considered. First, this is an exploratory study evaluating a wide range of circulating biomarkers, with the bias of multiple comparisons and potential false-positive results. Thus, further studies are encouraged to outline the precise mechanisms of these plasma biomarkers, especially RET, in PAH. Moreover, the understanding of the pathological role of the investigated biomarkers is complex. In this study, biomarkers were analysed isoform independently. Soluble receptors may exert antagonistic or synergistic functions of the main cell membrane-bound isoform [41]. It is consequently important to

clarify differences between these forms, and when and where they are secreted. The influence of medications and other factors, such as food intake or daily variation, also remain to be investigated. Second, the somewhat unmatched control group and the low number of patients may have influenced the results. Most importantly, however, we show that several markers in the tyrosine kinase domain indeed differ between different disease aetiologies and may have the potential to be used as biomarkers in pulmonary hypertension differentiation. Therefore, multicentre studies are encouraged to further investigate the role of these biomarkers in pulmonary hypertension pathophysiology and assess their possible diagnostic potential.

Conclusions

The present study indicates that plasma RET may be of future use to identify and differentiate PAH patients from other groups of patients with pulmonary hypertension, including CTEPH, systolic or diastolic heart failure with pulmonary hypertension, as well as heart failure without pulmonary hypertension and healthy controls. MERTK, VEGF-A, VEGF-D, PlGF, VEGFR-2, amphiregulin, EGFR, HGF and TGF-α may, in addition to RET, also be involved in the complex nature of pulmonary hypertension pathophysiology, which encourages future international collaborative multicentre studies.

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Conflict of interest: J. Säleby reports grants from the Swedish Society of Pulmonary Hypertension on behalf of GlaxoSmithKline and lecture fees from Actelion, outside the submitted work. H. Bouzina reports an unrestricted research grant from Actelion during the conduct of the study; and speakers fees from Actelion, grants from the Swedish Society of Pulmonary Hypertension on behalf of GlaxoSmithKline and nonfinancial support from GlaxoSmithKline, outside the submitted work. S. Ahmed has nothing to disclose. J. Lundgren reports personal lecture fees from Actelion Pharmaceuticals Sweden AB, outside the submitted work. G. Rådegran reports an unrestricted research grant from Actelion, during the conduct of the study; and speaker fees from Actelion and Orion Pharma, advisory boards for Actelion and MSD, and that he was a principal investigator for Actelion, GlaxoSmithKline, Bayer and Novartis, outside the submitted work.

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