A systematic review with meta-analysis of biomarkers for detection of pulmonary arterial hypertension

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Shareable abstract (@ERSpublications)
Meta-analysis of 26 biomarkers yielded 17 differentially expressed biomarkers in PAH. NT-proBNP had the highest diagnostic accuracy but had a low specificity for PAH. Other markers, including IL-6, RDW, LDL-c, D-dimer and UA, lacked clinical validation. https://bit.ly/3J4YAyC

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Abstract

Rationale The blood is a rich source of potential biomarkers for the diagnosis of idiopathic and hereditary pulmonary arterial hypertension (iPAH and hPAH, referred to as "PAH"). While a lot of biomarkers have been identified for PAH, the clinical utility of these biomarkers often remains unclear. Here, we performed an unbiased meta-analysis of published biomarkers to identify biomarkers with the highest performance for detection of PAH.

Methods A literature search (in PubMed, Embase.com, Clarivate Analytics/Web of Science Core Collection and Wiley/Cochrane Library) was performed up to 28 January 2021. Primary end points were blood biomarker levels in PAH *versus* asymptomatic controls or patients suspected of pulmonary hypertension (PH) with proven normal haemodynamic profiles.

Results 149 articles were identified by the literature search. Meta-analysis of 26 biomarkers yielded 17 biomarkers that were differentially expressed in PAH and non-PH control subjects. Red cell distribution width, low density lipid-cholesterol, d-dimer, N-terminal prohormone of brain natriuretic protein (NT-proBNP), interleukin-6 (IL-6) and uric acid were biomarkers with the largest observed differences, largest sample sizes and a low risk of publication bias. Receiver operating characteristic curves and sensitivity/specificity analyses demonstrated that NT-proBNP had a high sensitivity, but low specificity for PAH. For the other biomarkers, insufficient data on diagnostic accuracy with receiver operating characteristic curves were available for meta-analysis.

Conclusion This meta-analysis validates NT-proBNP as a biomarker with high sensitivity for PAH, albeit with low specificity. The majority of biomarkers evaluated in this meta-analysis lacked either external validation or data on diagnostic accuracy. Further validation studies are required as well as studies that test combinations of biomarkers to improve specificity.

Introduction

Pulmonary arterial hypertension (PAH) is a cardiovascular condition in which progressive occlusive remodelling leads to increased pulmonary vascular resistance and ultimately right ventricular failure. PAH can be hereditary (hPAH) or idiopathic (iPAH) after exclusion of significant comorbidity [1], referred to as "PAH" throughout this study. The diagnosis of PAH is a complex, specialist process, attributing to a mean time to diagnosis of 17–24 months [2]. Availability of noninvasive biomarkers for faster diagnosis and initiation of treatment prior to the development of right heart failure may improve survival and quality of life [3].

Until now, N-terminal prohormone of brain natriuretic protein (NT-proBNP) remains the most useful clinical marker of myocardial strain and is employed for risk stratification of patients in guidelines and

clinical practice [1]. However, improved understanding of the pathways leading to PAH, which include endothelial dysfunction, immunity and altered cellular metabolism, may result in the emergence of novel biomarkers that can detect proliferation and occlusive remodelling of the vascular wall with higher specificity. With the ongoing interest to develop biomarkers that help noninvasive diagnosis of PAH, new biomarkers have been proposed. Yet, many of these biomarkers lack external validation, leaving the performance of these biomarkers – in terms of reproducibility and clinical utility – unclear. We used unbiased meta-analysis to identify biomarkers with robust sensitivity and specificity to detect PAH.

We conducted a systematic review and meta-analysis of the literature on published biomarkers of PAH in blood or urine. Here we show: 1) biomarkers differentially expressed in iPAH and hPAH compared to non-pulmonary hypertension (PH) controls; and 2) available evidence supporting the suitability of these biomarkers for clinical implementation, including calculation of diagnostic accuracy employing receiver operating curve analyses.

Materials and methods

Search strategy

The conduct and reporting of this review adhere to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA)-statement (www.prismastatement.org) [4] and is registered in PROSPERO (CRD42020215820).

Four bibliographic databases (PubMed, Embase.com, Clarivate Analytics/Web of Science Core Collection and Wiley/Cochrane Library) were searched for relevant literature from inception to 28 January 2021. Searches were constructed in collaboration with a medical information specialist (K.A.Z.). Search terms including synonyms, closely related words and keywords were used as index terms or free-text words. The searches contained no methodological search filter, date or language restrictions that would limit results to specific study designs, date or language (detailed search; supplementary table S1). Duplicate articles were excluded using Endnote (X9.3.3), Amsterdam Efficient Deduplication-method and Bramer-method [5]).

Two reviewers (A.J.S. and L.B.) independently screened all potentially relevant titles and abstracts for eligibility using Rayyan. If necessary, the full text article was checked for the eligibility criteria. Differences in judgement were resolved through: 1) discussion among reviewers (A.J.S. and L.B.); 2) arbitration of a third reviewer (J.A.); or 3) contacting the author. Studies were included if they met the following criteria: 1) analysis of potential blood and urine biomarkers in any form, including growth factors, inflammatory mediators, circulating cells, protein, (micro)RNA, or microvesicles; and 2) involved group 1 PAH, provided that iPAH or hPAH patients were included. The following studies were excluded: 1) animal studies; 2) studies involving subjects <18 years of age; 3) studies that did not report biomarker levels for group 1 PAH, or lacked inclusion of iPAH or hPAH patients; 3) studies that lacked a control group, or included a control group suspected of PH without measurement of haemodynamics; and 4) certain publication types: editorials, letters, legal cases, interviews, *etc*. The full text of the selected articles was obtained for further review and data extraction. In a minority of articles data were estimated from figures. Biomarker levels were conversed to a uniform unit of measurement. Two reviewers (A.J.S. and L.B.) independently evaluated the methodological quality of the full text papers using QUADAS-2 [6]. Articles were scored as low, unclear or high on domains "patients inclusion (P)", "index test (I)", "reference test (R)" and "flow and timing (T)" [6]. The risk of bias assessment tool was optimised by A.J.S. and L.B. from a pilot of 10 studies and are presented in supplementary table S2.

A similar search strategy was adopted in identical databases to identify "omics" studies performed in patients with iPAH and hPAH, compared to non-PH control subjects. Studies were included if they met the following criteria: 1) adopted an "omics" technology, including transcriptomics, proteomics, metabolomics, glycomics or lipidomics in blood or urine; and 2) involved patients with group 1 PAH, including iPAH or hPAH. Equal exclusion criteria applied as described above.

Data extraction

The following data were extracted from each publication: mean with standard deviation (SD) and the number of patients for each group (PAH *versus* non-PAH controls), area under the receiving-operating-curve (AUC/ROC), cut-off values, as well as sensitivity and specificity of a given biomarker for the diagnosis of PAH.

Statistics

Primary outcomes were biomarker concentrations in PAH and asymptomatic controls. Meta-analyses were performed when original data (expressed as mean±SD) were available from a minimum of three

publications using Review Manager 5.3.5 software (The Nordic Cochrane Center, Copenhagen, Denmark). A randomised model for continuous data was adopted, due to possible risk of bias. Based on population size, mean and standard deviation, the standardised mean difference, mean difference and odds ratio of biomarker levels in patients with PAH and non-PH controls were calculated. Mean and standardised mean differences are represented as mean with 95% confidence intervals (95% CI), or odds ratio with 95% CI. Biomarkers were ranked according to effect size and statistical significance. I^2 and Tau^2 statistics were performed to assess heterogeneity among studies, and explainable heterogeneity was solved by exclusion of the aberrant publication.

Publication bias was assessed in Comprehensive Meta-Analysis software V3 (Biostat, Englewood, NJ, USA) using funnel plots, Egger's regression test ($p<0.10$), Duval and Tweedie's trim and fill, and Orwin's Fail-safe number-test. The Fail-safe number estimates the number of unpublished studies required to turn the meta-analysis result into a clinically insignificant value. The clinically insignificant value was arbitrarily set at a standardised mean difference of <-0.25 or 0.25.

Selection of biomarkers for clinical implementation

We made a selection of differentially expressed biomarkers based on statistical significance ($p<0.05$) of the observed difference, sample size and quality of validation outside the discovery cohort by means of calculation of sensitivity and specificity values using ROC analyses in an independent validation cohort. Additionally, we selected for a negligible risk for publication bias, defined by Egger's regression $p>0.10$, Duval and Tweedie's trim and fill ($p<0.05$), and a minimum of five publications predicted to bring the result to a clinically insignificant value (standardised mean difference – 0.25, 0.25).

All biomarkers were grouped in six pathobiological domains: haematological, metabolic, coagulation, inflammatory, cardiac and renal. In each domain, we selected one preferred biomarker on the basis of observed difference, sample size, quality of external validation and risk of publication bias (see supplementary table S1).

Results

Inclusion and selection of publications

The literature search yielded a total of 3456 references: 887 in PubMed, 1506 in Embase.com, 976 in Clarivate Analytics/Web of Science Core Collection and 87 in Wiley/Cochrane Library. After removal of duplicates 1356 remained. 1207 full text articles were excluded based on inclusion and exclusion criteria (figure 1a). 149 publications remained eligible for data extraction. 45 publications were identified that describe biomarkers meeting criteria for meta-analysis and risk of publication bias assessment. A detailed overview of biomarker origin (whole blood, plasma or serum), location of blood draw (peripheral or central (RHC) blood draw), demographic criteria, treatment and concerns regarding inclusion procedure of these publications is provided in supplementary table S3. Risk of bias, attributable to the procedure of patient selection, index and reference test, as well as timing of the biomarker blood draw (see supplementary table S2), was systematically assessed using QUADAS-2 [6] and is reported in supplementary figure S1.

Exclusion of urine and non-protein blood biomarkers

In several publications, biomarker expression was studied on circulating platelets [7, 8], immune cells [9–12] and progenitor cells [13–16]. Heterogeneity in measurement methods, characterisation and flow cytometry (FACS) gating precluded meta-analysis of these publications.

Three publications reported on different types of extracellular vesicles as biomarker [17, 18] and three on different types of miRNA as biomarker [19–22]. A single publication reported on a urine biomarker [23]. These publications did not meet the criteria for meta-analysis.

Selection of eligible biomarkers

26 biomarkers were eligible for meta-analysis (table 1). A significant difference in expression was detected for 17 biomarkers in six pathobiological domains. In the haematological domain, these were red blood cell distribution width (RDW), platelet distribution width (PDW), mean platelet volume (MPV) and thrombocytes; in the metabolic domain, total cholesterol, low density lipid-cholesterol (LDL-c), triglycerides and fasting glucose. In the coagulation domain, d-dimer was differentially expressed. In the inflammatory domain, interleukin-6 (IL-6), C-reactive protein (CRP), soluble vascular adhesion molecule-1 (sVCAM-1), C-X-C motif chemokine ligand-10 (CXCL-10) and tissue inhibitor metalloproteinase-1 (TIMP1) were differentially expressed. In the cardiac domain, NT-proBNP, and in the renal domain, uric acid (UA) and blood urea nitrogen (BUN) were differentially expressed. Biomarkers described in fewer

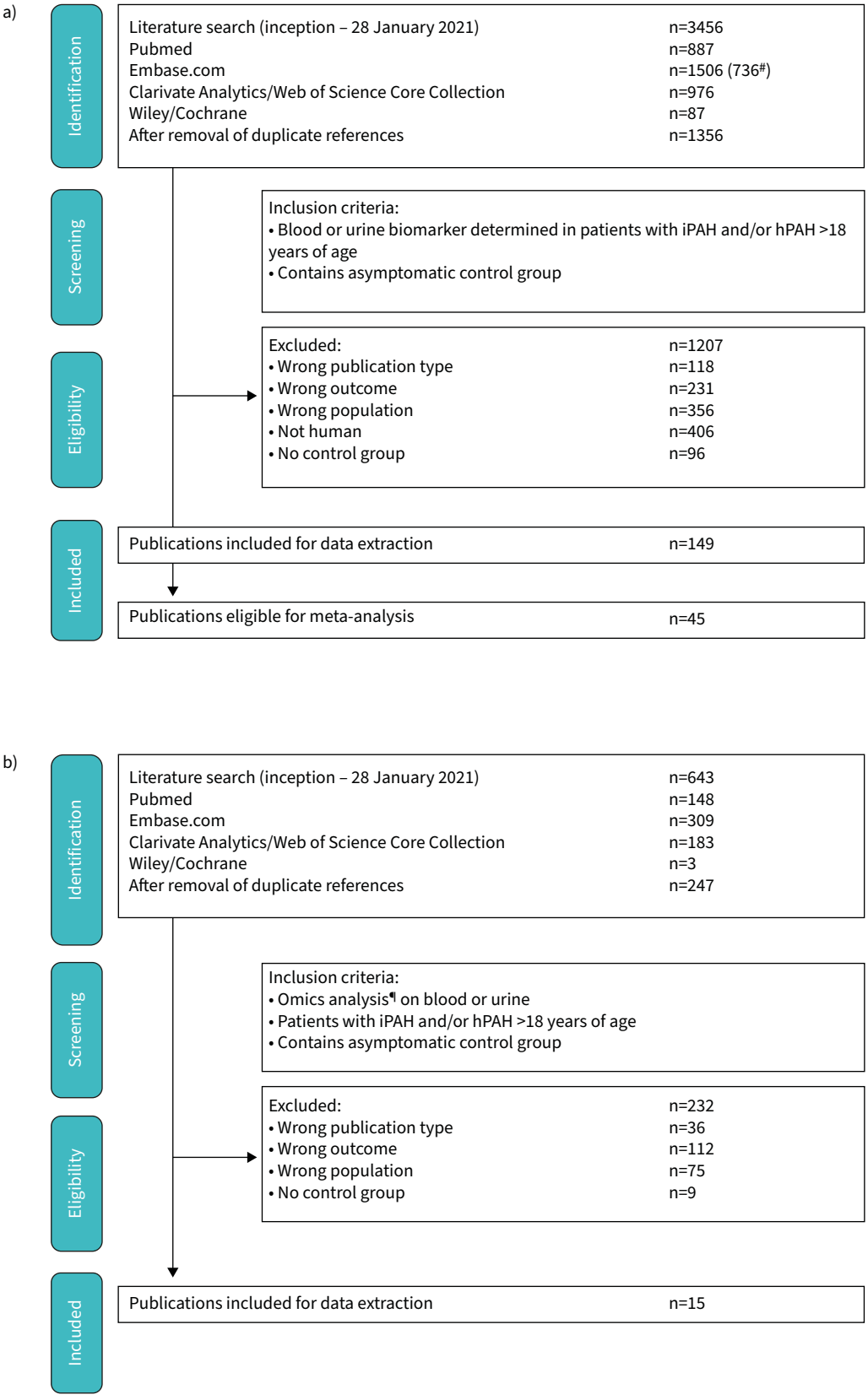

FIGURE 1 Flow chart visualising identification of publications, inclusion and exclusion criteria, and selection of publications eligible for meta-analysis. a) Biomarker search; b) omics search. #: excluding conference abstracts; ¶: transcriptomics, proteomics, metabolomics, glycomics and lipidomics. iPAH: idiopathic pulmonary arterial hypertension; hPAH: hereditary pulmonary arterial hypertension.

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TABLE 1 Summary of 26 meta-analyses

Marker	Studies n	Participants n	Mean difference	St. mean difference	Overall effect (p-value)	Tau^2	I^2 (%)	Heterogeneity (p-value)	Forest plot
Haematological markers									
RDW %	4	427	1.83 (1.39–2.26)	0.98 (0.61–2.17)	<0.00001	0.07	51	0.11	Figure 2a
PDW %	3	245	1.42 (0.16–2.67)	0.81 (0.50–1.12)	<0.00001	0.02	19	0.29	Figure S2a
MPV fL	5	361	0.95 (0.76–1.13)	1.0 (0.81–1.25)	<0.00001	0.00	0	0.68	Figure S2b
	6#	395	0.66 (0.24–1.09)	0.72 (0.24–1.19)	0.003	0.27	78	0.0003	
Thrombocytes (×10^9 L^{-1})	7	334	−23.9 (−38.6– −9.2)	−0.38 (−0.62– −0.15)	0.001	0.01	5	0.39	Figure S2c
Hb g dL^{-1}	9	400	−0.59 (−1.23–0.06)	−0.18 (−0.43–0.07)	0.15	0.04	29	0.19	Figure S2d
Hct %	5	229	−1.07 (−3.91–1.76)	−0.21 (−0.76–0.34)	0.46	0.29	74	0.004	Figure S2e
Leukocytes (×10^9 L^{-1})	7	294	−0.23 (−0.70,0.24)	−0.10 (−0.41–0.21)	0.52	0.07	39	0.13	Figure S2f
Metabolic markers									
LDL-c mg dL^{-1}	6	3035	−15.82 (−26.18– −5.46)	−0.44 (−0.65– −0.22)	<0.00001	0.03	46	0.10	Figure 2b
Total cholesterol mg dL^{-1}	4	408	−17.70 (−24.15– −11.26)	−0.52 (−0.73– −0.32)	<0.00001	0.00	67	0.67	Figure S3a
TG mg dL^{-1}	4	198	−32.56 (−54.17– −10.94)	−0.52 (−0.87– −0.17)	0.004	0.04	34	0.21	Figure S3b
Glucose (fasted) mg dL^{-1}	3	103	24.06 (0.54–7.58)	0.48 (0.08–0.87)	0.02	0.00	0	0.85	Figure S3c
HDL-c mg dL^{-1}	6	577	−6.15 (−2.11–14.40)	−0.53 (−1.20–0.15)	0.13	0.63	91	<0.00001	Figure S3d
Coagulation markers									
D-dimer ng mL^{-1}	3	142	245.99 (148.55–343.43)	0.69 (0.27–1.11)	0.001	0.04	27	0.26	Figure 2c
Fibrinogen mg dL^{-1}	4	227	73.75 (−2.58–150.08)	0.84 (−0.14–1.81)	0.09	0.88	90	<0.00001	Figure S4
Inflammatory markers									
IL-6 pg mL^{-1}	5	389	5.01 (2.06–7.96)	0.64 (0.28–0.99)	0.0005	0.08	47	0.11	Figure 2d
CRP mg L^{-1}	8	387	0.74 (0.13–1,6)	0.25 (0.04–0.47)	0.02	0.02	0	0.98	Figure S5a
	9#	493	0.13 (0.10–0.17)	0.77 (−0.08–1.61)	0.08	1.57	94	<0.00001	
sVCAM-1 ng mL^{-1}	3	150	626.72 (29.38–1224.07)	1.03 (0.53–1.52)	<0.00001	0.08	40	0.19	Figure S5b
CXCL-10 pg mL^{-1}	3	171	99.77 (54.53–145.01)	0.82 (0.49–1.16)	<0.00001	0.00	0	0.46	Figure S5c
TIMP-1 ng mL^{-1}	3	224	15.58 (−2.56–33.72)	0.40 (0.13–0.67)	0.003	0.00	0	0.54	Figure S5d
	4#	329	40.15 (1.02–79.29)	0.67 (0.14–1.21)	0.01	0.24	82	0.0009	
sP-selectin ng mL^{-1}	4	180	0.52 (−11.10–12.14)	−0.04 (0.35–0.28)	0.82	0.00	0	0.72	Figure S5e
Cardiac markers									
NT-proBNP pg mL^{-1}	10	1152	1684 (1035–2330)	1.13 (0.93–1.33)	<0.00001	0.03	30	0.17	Figure 2e
	11#	1258	1004 (787–1221)	1.37 (0.96–1.79)	<0.00001	0.39	85	<0.00001	
Renal markers									
UA mg dL^{-1}	5	441	1.77 (1.06–2.48)	0.89 (0.58–1.12)	<0.00001	0.06	51	0.09	Figure 2f
	6#	531	1.52 (0.77–2.27)	0.81 (0.53–1.09)	<0.00001	0.09	59	0.03	
BUN mg dL^{-1}	5	891	1.76 (0.51–3.01)	0.43 (0.29–0.56)	<0.00001	0.00	0	0.48	Figure S6a
Creatinine mg dL^{-1}	10	475	0.03 (−0.04–0.10)	0.13 (−0.08–0.34)	0.23	0.02	20	0.26	Figure S6b
eGFR mL min^{-1}/1.73 m^2	4	180	1.70 (5.98–9.37)	0.09 (−0.32–0.49)	0.67	0.08	47	0.13	Figure S6c
Hepatic markers									
ALT U L^{-1}	3	115	3.57 (−4.18–11.31)	0.18 (−0.56–0.92)	0.37	0.30	71	0.03	Figure S7

Per biomarker the number of studies included, total sample size, mean difference, and the standardised difference between iPAH and non-PH control with 95% confidence interval, p-value of the difference, and heterogeneity of the result (I^2, Tau^2 and p-heterogeneity) are shown. St. mean difference; standardised mean difference; RDW: red cell distribution width; PDW: platelet distribution width; MPV: mean platelet volume; Hb: haemoglobin; Hct: haematocrit; TG: triglycerides; LDL-c: low density lipoprotein; HDL-c: high density lipoprotein; IL-6: interleukin-6; CRP: c-reactive protein; sVCAM-1: circulating vascular cell adhesion molecule-1; CXCL-10: C-X-C motif chemokine ligand-10; TIMP-1: tissue inhibitors of metalloproteinases-1; NT-proBNP: N-terminal prohormone of brain natriuretic peptide; UA: uric acid; BUN: blood urea nitrogen; eGFR: estimated glomerular filtration rate: ALT: alanine transaminase. #: publication excluded due to heterogeneity.

than three publications or as median with IQR are summarised in supplementary tables S4 and S5. Selected biomarkers are shown in figure 2 (see Materials and methods). These include RDW, LDL-c, d-dimer, IL-6, NT-proBNP and UA. Forest plots for PDW, MPV, thrombocytes, total cholesterol, triglycerides, fasting glucose, CRP, sVCAM-1, CXCL-10, TIMP1 and BUN are provided in the supplementary material (supplementary figures S2–7, supplementary table S6).

Evaluation of publication bias

Egger's regression analysis revealed a significant association ($p<0.10$) between effect size and standard error for MPV and thrombocytes. After correction for possible publication bias by Duval and Tweedie's trim and fill, the mean difference between PAH and control groups remained significant. The fail-safe test indicated that a minimum of five publications were required to bring the differences to a clinically trivial value, defined as a standardised mean difference of <−0.25 or 0.25. This suggests that the chance that the observed difference relies on publication bias is small (supplementary table S7). Funnel plots of all meta-analyses are given in supplementary figure S8a–z.

Haematological markers: RDW

All five publications on RDW were eligible for meta-analysis. RDW was determined in treatment-naïve iPAH [24] and PAH [25] patients, and in PAH patients receiving vasodilatory treatment [11, 26]. As a reference, asymptomatic controls [11, 24–26] and patients suspected of PH [26] or common disease controls [24] were included (figure 2a). Meta-analysis confirmed a positive mean difference of 1.67% (95% CI 1.45–1.89, $p<0.00001$) between PAH and non-PH control (table 1). For RDW no sensitivity, specificity or diagnostic accuracy could be extracted from the original data.

A rise in RDW is predictive for the presence of PH in patients with acute pulmonary embolism [27] or systemic sclerosis [26, 28]. RDW was positively associated with pulmonary artery pressure [11, 24], right atrial pressure [24], pulmonary vascular resistance [24], BNP [26] and NT-proBNP [29], and inversely with 6- min walk distance (6MWD) [24, 26, 29]. Remarkably, in one study RDW performed better than NT-proBNP and IL-6 as prognostic markers in PAH patients [27].

Other markers in the haematological domain are summarised in table 1. PDW was increased with a mean difference of 1.42% (95% CI 0.16–2.67, $p<0.00001$, supplementary figure S2a), as well as MPV (0.95 fL (95% CI 0.76–1.13, $p<0.00001$; supplementary figure S2b), while thrombocyte count was decreased by a mean of -23.9×10^9 cells L^{-1} (95% CI −38.6– −9.2, p=0.001); supplementary figure S2c). Eligible for meta-analysis but without significant differences were haemoglobin, haematocrit and leukocytes (supplementary figure S2d–f).

Metabolic markers: LDL-c

LDL-c was reported in six publications eligible for meta-analysis and determined in patients with PAH receiving vasodilatory treatment. Asymptomatic controls [11, 30–32] or patients with cardiovascular disease or patients suspected of PH [8] were included as reference (figure 2b). All measurements were performed in blood obtained after >8 h of fasting. LDL-c was lower in patients with PAH, with a mean difference of −15.82 mg dL^{-1} (95% CI −26.18– −5.46, $p<0.00001$) (table 1). For LDL-c no sensitivity, specificity or diagnostic accuracy could be extracted from the original data. Decreased insulin sensitivity and altered lipid metabolism in iPAH are a possible consequence of chronic inflammation, malnourishment and alterations in liver function [33, 34]. LDL-c was not related to haemodynamic parameters, NT-proBNP, 6MWD or body mass index. LDL-c was negatively associated with 3-year survival in PAH (hazard ratio 0.18 mmol L^{-1} (95% CI 0.07–0.47), $p<0.01$, corrected for statin use) [30]. A similar relationship has been described in chronic heart failure [35, 36].

A lower LDL-c in patients with PAH was accompanied by a lower mean total cholesterol of −17.70 mg dL^{-1} (95% CI −24.15– −11.26, $p<0.00001$; supplementary figure S3a) and lower mean triglycerides of −32.56 mg dL^{-1} (95% CI −54.17– −10.94, p=0.004; supplementary figure S3b). Despite the availability of six publications, no significant difference was found in meta-analyses for high-density lipoprotein (HDL-c) (mean difference −6.15 mg dL^{-1}, 95% CI −2.11–14.40, p=0.13; supplementary figure S3d) or fasting glucose (supplementary figure S3c).

Coagulation markers: D-dimer

From the available markers representing coagulation pathways, meta-analyses could be performed for fibrinogen and d-dimer levels. D-dimer was studied in treatment-naïve iPAH patients [37] and in PAH patients receiving vasodilatory treatment [8, 38], and results were compared to asymptomatic controls (figure 2c). Meta-analysis revealed a significantly higher d-dimer level in patients with PAH compared to

a) **Haematological**

RDW, %		PAH		Control		Standardised mean difference		Risk of bias
Study	*Type*	*Mean±SD*	*n*	*Mean±SD*	*n*	*Weight*	*Mean (95% CI)*	P I R T
Petrauskas, L.A. et al. (2019)	PAH	15.9±2.8	181	14.2±1.1	101	28%	0.72 (0.47–0.98)	- + + ?
Decker, I. et al. (2011)	**iPAH**	**14.9±2.1**	**30**	**13.2±1.0**	**12**	**10%**	**0.88 (0.18–1.58)**	+ ? + ?
Yildiz, A. et al. (2013)	PAH	17.3±2.2	25	15.6±0.8	22	12%	0.98 (0.37–1.59)	- + + ?
Yaylali, Y.T. et al. (2019)	PAH	16.3±2.6	21	13.4±1.2	35	12%	1.55 (0.93–2.17)	- ? + +
Total effect PAH	p<0.00001		257		170	100%	0.98 (0.61–1.34)	
Mean difference (95% CI)	1.83% (1.39–2.26)							

b) **Metabolic**

LDL-c, mg·dL^{-1}		PAH		Control		Standardised mean difference		Risk of bias
Study	*Type*	*Mean±SD*	*n*	*Mean±SD*	*n*	*Weight*	*Mean (95% CI)*	P I R T
Jasiewicz, M. et al. (2014)	PAH	105±37	26	140±36	30	11%	−0.95 (−1.50– −0.39)	- ? + ?
Kopec, G. et al. (2017)	**iPAH**	**101±31**	**140**	**124±46**	**2431**	**33%**	**−0.51 (−0.68– −0.34)**	- + + ?
Varol, E. et al. (2011)	PAH	96±31	22	111±34	25	10%	−0.45 (−1.04–0.13)	+ ? + ?
Wang, G.F. et al. (2020)	**iPAH**	**79±27**	**177**	**84±23**	**103**	**27%**	**−0.19 (−0.44–0.05)**	- ? + ?
Yaoita, N. et al. (2014)	PAH	96±26	19	100±31	15	9%	−0.12 (−0.76–0.52)	? ? + +
Yildiz, A. et al. (2013)	PAH	98±20	25	144±35	22	10%	−0.53 (−1.07–0.01)	? ? + ?
Total effect iPAH	**p=0.03**		**317**		**2534**	**60%**	**−0.37 (−0.68– −0.06)**	
Total effect PAH	p<0.0001		409		2626	100%	−0.43 (−0.64– −0.23)	
Mean difference (95% CI)	−15.82 mg·dL^{-1} (−26.18– −5.46)							

c) **Coagulation**

D-dimer, ng·mL^{-1}		PAH		Control		Standardised mean difference		Risk of bias
Study	*Type*	*Mean±SD*	*n*	*Mean±SD*	*n*	*Weight*	*Mean (95% CI)*	P I R T
Yaoita, N. et al. (2014)	PAH	1630±328	19	880±500	15	29%	0.30 (−0.39–0.98)	? ? + ?
Vrigkou, E. et al. (2019)	PAH	657±547	30	355±175	10	26%	0.61 (−0.12–1.34)	- ? + +
Can, M.M., et al. (2010)	PAH	420±310	34	190±90	34	45%	1.00 (0.49–1.50)	- ? + ?
Total effect PAH	p=0.001		83		59	100%	0.68 (0.27–1.11)	
Mean difference (95% CI)	246 ng·mL^{-1} (149–343)							

d) **Inflammatory**

IL-6, pg·mL^{-1}		PAH		Control		Standardised mean difference		Risk of bias
Study	*Type*	*Mean±SD*	*n*	*Mean±SD*	*n*	*Weight*	*Mean (95% CI)*	P I R T
Soon, E. et al. (2010)	**iPAH**	**18.9±49.8**	**70**	**5.7±1.8**	**21**	**24%**	**0.30 (−0.19–0.79)**	+ - + ?
Rhodes, C.J. et al. (2011)	**iPAH**	**8.6±18.7**	**139**	**1.4±1.4**	**40**	**31%**	**0.43 (0.08–0.79)**	+ - + ?
Prins, K.W. et al. (2018)	PAH	15.1±21	40	2.5±5.5	10	16%	0.65 (−0.06–1.36)	? ? + +
Heresi, G.A. et al. (2017)	**iPAH**	**4.5±3.6**	**14**	**2.4±1.7**	**14**	**14%**	**0.73 (−0.04–1.50)**	? - + ?
Itoh, T. et al. (2006)	**iPAH**	**2.6±1.6**	**28**	**0.6±0.4**	**13**	**15%**	**1.47 (0.73–2.20)**	- ? + ?
Total effect iPAH	**p=0.004**		**251**		**88**	**84%**	**0.65 (0.21–1.09)**	
Total effect PAH	p=0.0005		291		98	100%	0.64 (0.28–0.99)	
Mean difference (95% CI)	5.01 pg·mL^{-1} (2.06–7.96)							

e) **Cardiac**

NT-proBNP, pg·mL^{-1}		PAH		Control		Standardised mean difference		Risk of bias
Study	*Type*	*Mean±SD*	*n*	*Mean±SD*	*n*	*Weight*	*Mean (95% CI)*	P I R T
Hennigs, J.K. et al. (2014)	PAH	3106±4661	52	240±173	11	8%	**0.66 (0.00–1.33)**	? + + +
Calvier, L. et al. (2016)	**iPAH**	**1575±1863**	**41**	**250±141**	**8**	**5%**	**0.76 (−0.02–1.53)**	? ? ? ?
Fares, W.H. et al. (2012)	PAH	7420±6682	25	3601±1230	33	10%	0.84 (0.30–1.39)	? ? + ?
Renard, S. et al. (2013)	**iPAH**	**1917±2497**	**49**	**335±331**	**50**	**15%**	**0.87 (0.46–1.29)**	? ? + ?
Fijalkowska, A. et al. (2006)	**iPAH**	**2562±2713**	**36**	**46±24**	**9**	**5%**	**1.01 (0.25–1.77)**	+ ? + +
Lu, G.H. et al. (2020)	**iPAH**	**409±126**	**338**	**285±74**	**338**	**30%**	**1.20 (1.04–1.37)**	+ ? + +
Zhu, T. et al. (2019)	**iPAH**	**2142±1920**	**27**	**87±63**	**20**	**8%**	**1.38 (0.74–2.03)**	? + + ?
Andreassen, A.K. et al. (2006)	**iPAH**	**2875±2402**	**17**	**59±50**	**10**	**5%**	**1.42 (0.54–2.30)**	+ ? + +
Wang, K.Y. et al. (2015)	**iPAH**	**934±891**	**21**	**60±65**	**27**	**8%**	**1.46 (0.81–2.10)**	? + + ?
Yang, D. et al. (2013)	**iPAH**	**1579±778**	**20**	**465±11**	**20**	**6%**	**1.99 (1.22–2.76)**	? ? + +
Total effect iPAH	**p<0.00001**		**549**		**482**	**52%**	**1.20 (1.00–1.41)**	
Total effect PAH	p<0.00001		626		526	100%	1.13 (0.93–1.33)	
Mean difference (95% CI)	1684 pg·mL^{-1} (1035–2330)							

* Malhotra, R et al. (2013), excluded (method)

f) **Renal**

Uric acid, mg·dL^{-1}		PAH		Control		Standardised mean difference		Risk of bias
Study	*Type*	*Mean±SD*	*n*	*Mean±SD*	*n*	*Weight*	*Mean (95% CI)*	P I R T
Suzuki, S. et al. (2016)	PAH	6.3±1.7	18	6.0±1.8	30	17%	0.17 (−0.4–0.75)	? ? ? +
Jasiewicz, M. et al. (2014)	PAH	6.3±2.4	26	4.5±1.5	24	17%	0.85 (0.27–1.43)	+ ? + ?
Jiang, X. et al. (2008)	**iPAH**	**6.3±2.0**	**78**	**4.5±1.4**	**98**	**28%**	**1.03 (0.71–1.34)**	+ ? + ?
Zhu, T. et al. (2019)	**iPAH**	**7.6±2.4**	**27**	**5.3±1.4**	**20**	**15%**	**1.10 (0.48–1.73)**	? + + +
Nagaya, N. et al. (1999)	**iPAH**	**7.5±2.5**	**90**	**4.9±1.2**	**30**	**23%**	**1.15 (0.71–1.59)**	+ ? + +
Total effect iPAH	**p<0.00001**		**195**		**148**	**66%**	**1.07 (0.84–1.31)**	
Total effect PAH	p<0.00001		239		2202	100%	0.89 (0.58–1.21)	
Mean difference (95% CI)	1.77 pg·dL^{-1} (1.06–2.48)							

* Yaoita, N et al. (2014), excluded (control group severe CVD)

FIGURE 2 Forest plots of selected biomarkers. a) The haemtological biomarker RDW: red cell distribution width; b) the metabolic biomarker LDL-c: low density lipid-cholesterol; c) the coagulation biomarker d-dimer; d) the inflammatory biomarker IL-6: interleukin-6; e) the cardiac biomarker NT-proBNP: N-terminal prohormone of brain natriuretic peptide; f) the renal biomarker UA: uric acid; CVD: cardiovascular disease. Risk of bias (QUADAS-2) – P: patient inclusion; I: index test (biomarker); R: reference standard (diagnosis); T: flow and timing. Publications in bold type represent biomarker levels of idiopathic pulmonary arterial hypertension (iPAH) and/or hereditary pulmonary arterial hypertension (hPAH) uniquely.

asymptomatic controls, with a mean difference of 245.99 ng mL^{-1} (95% CI 148.55–343.43, p=0.001, table 1), in contrast to fibrinogen (73.75 ng mL^{-1}, 95% CI −2.58–150.08, p=0.09); supplementary figure S4), all consistent with the hypothesis that hypercoagulability and *in situ* thrombosis may contribute to disease pathobiology in PAH [39].

Inflammatory markers: IL-6

From 10 publications reporting on IL-6, five were eligible for meta-analysis. All studies detected elevated levels of circulating IL-6 in treatment-naïve iPAH [40], or iPAH receiving vasodilatory treatment [27, 41–45] and naïve PAH [46, 47] or PAH patients receiving treatment [48]. Findings were compared to asymptomatic controls (figure 2d). A significant rise in IL-6 levels was observed in PAH compared to non-PH controls (mean difference 5.01 (95% CI 2.06–7.96) pg mL^{-1}, p=0.0005) (table 1).

Il-6 levels were negatively associated with the number of circulating endothelial progenitor cells [41] and were elevated in parallel to several interleukins [44], as well as CXCL-10 [42], monocyte chemoattractive protein-1 (MCP-1) [47, 48], tumour necrosis factor-α (TNF-α) [40, 46–48], placental growth factor (PlGF) [40], soluble vascular endothelial growth factor (VEGF) receptor-1 (sVEGFR-1) [40], VEGF-A [40], VEGF-D [40] and markers related to thrombogenesis [45]. IL-6 was negatively associated with right ventricular function [47] and 6MWD [27, 40], while positively to World Health Organization (WHO) functional class [27], NT-proBNP [27, 40] and mean right atrial pressure [40]. IL-6 levels were predictive for all-cause mortality [27, 44] in PAH. No data on diagnostic accuracy, including ROC and AUC were available for meta-analysis.

Eight publications detected a subtle elevation in CRP levels in PAH [11, 41, 49–57] (supplementary figure S5a), (mean difference 0.74 mg L^{-1}, 95% CI 0.13–1.6, p=0.02) (table 1). However, since only one study was predicted to bring the difference to a clinically insignificant value, the risk of bias is significant. The study of Wang *et al.* [50] yielded an AUC of 0.51 (p=0.899) with a 85% specificity but low (39%) sensitivity [50], when using a diagnostic cut-off of 2.7 mg L^{-1} CRP, indicating diagnostic accuracy is low in an external validation cohort consisting of iPAH and asymptomatic controls. CRP is commonly attributed to other cardiovascular or inflammatory disease [58], and these data indicate that an elevated CRP lacks the specificity required for detection of PAH among non-PH controls.

Other inflammatory markers that were eligible for meta-analysis and significantly increased in patients with iPAH compared to non-PH controls included: sVCAM-1 (mean difference of 626.72 ng mL^{-1}, 95% CI 29.38–1224.07, p=0.003; supplementary figure S5b), CXCL-10 (mean difference 99.77 pg mL^{-1}, 95% CI 54.53–145.01, p<0.00001; supplementary figure S5c) and TIMP-1 (mean difference of 15.58 ng mL^{-1}, 95% CI −2.56–33.72, p=0.003; supplementary figure S5d). No significant difference was observed for sP-selectin (supplementary figure S5e). From these markers no sensitivity, specificity or diagnostic accuracy could be extracted from the original data.

Cardiac markers: NT-proBNP

11 publications reporting on NT-proBNP met the inclusion criteria, 10 of which were eligible for meta-analysis. NT-proBNP was measured in treatment-naïve iPAH patients [32, 59–63], as well as in iPAH [50–52, 62–64] and PAH patients receiving vasodilatory treatment [55, 56, 65, 66]. Data were compared to asymptomatic controls [32, 57, 59–63, 66] or subjects suspected of PH [55, 56, 65] (figure 2e). The overall mean difference was 1684 pg mL^{-1}, (95% CI 1035–2330, p<0.00001) (table 1).

Wang *et al.* [50] determined the diagnostic accuracy of NT-proBNP in patients with iPAH among asymptomatic controls employing a cut-off >89.25 pg mL^{-1} (AUC 0.87, p<0.0001) with a sensitivity of 89% and specificity of 78%. Similarly, Malhotra *et al.* [52] detected PAH patients receiving vasodilatory treatment among asymptomatic controls with an AUC of 0.714. However, with a specificity of 78% [50], NT-proBNP is not suitable for identifying PAH amongst patients with left heart disease.

NT-proBNP was positively associated with markers of disease severity, including right ventricular function, including pulmonary vascular resistance [60, 65], right atrial pressure [60], right ventricular dimensions [59, 61, 66] and exercise tolerance (WHO functional class [51, 60, 65]). NT-proBNP was inversely related to 6MWD [51, 65], cardiac index [60, 65] and mixed venous oxygen concentration [60, 65]. In addition, NT-proBNP decreased significantly after initiation of treatment, in line with decreased pulmonary vascular resistance and is predictive of survival [59, 60, 65]. NT-proBNP was not dependent on the location of blood draw or pulmonary capillary wedge procedure [55].

Renal markers: uric acid

Six publications reporting on UA levels were included in this review, five of which were eligible for meta-analysis. UA levels were measured in treatment-naïve iPAH patients [32, 62, 67], iPAH patients receiving treatment [8, 68] and PAH patients on treatment [54, 69], and compared to asymptomatic controls [8, 32, 54, 62, 67–69] (figure 2f). Meta-analyses detected a significantly higher UA level in PAH compared to control with a mean difference of 1.77 mg dL^{-1} (95% CI 1.06–2.48, p<0.00001) (table 1).

UA levels in PAH patients were positively associated with right ventricular volume [68], pulmonary vascular resistance [67, 68] and WHO functional class [67, 68], and negatively correlated with cardiac output [67, 68] and mixed venous saturation [68]. UA decreased significantly after initiation of vasodilatory treatment, proportional to the decrease in pulmonary vascular resistance [67, 68]. UA is an independent predictor of 3-year mortality in iPAH [67] and heart failure [70].

BUN was the second renal marker that was analysed. We observed a significant increase of 1.76 mg dL^{-1} (95% CI 0.51–3.01, p<0.0001; supplementary figure S6a). Creatinine and estimated glomerular filtration rate were eligible but not significantly altered (supplementary figure S6b–c).

Hepatic markers

In three individual studies reporting on alanine aminotransferase (ALT) in treatment-naïve iPAH patients [62], iPAH patients receiving vasodilatory treatment [49] and treatment-naïve PAH patients [37], no significant difference was observed in our meta-analysis (supplementary figure S7). No other hepatic marker was eligible for meta-analysis.

Omics studies

The omics search strategy generated a total of 643 articles: 148 in PubMed, 309 in Embase.com, 183 in Clarivate Analytics/Web of Science Core Collection and three in Wiley/Cochrane Library. After removal of duplicates, 247 remained (represented in figure 1b). We identified 15 publications that analysed metabolomic [71–80] and proteomic profiles [81–85] in iPAH and PAH patients in plasma [71–76, 78, 79, 81, 83, 85, 86] and serum [77, 80, 84, 85, 87]. 14 studies compared signatures to asymptomatic controls, while two studies used common disease controls [72, 74]. Liquid and gas chromatography coupled with mass spectrometry or multiplex assays were the most frequently used methods to detect altered metabolites, proteins or antigens. Targeting component analysis was performed employing a variety of statistical tests (supplementary table S1). Metabolomic studies mainly described glycolytic shift and increased fatty-acid metabolism in patients with PAH, implicating an enhanced glycolytic catabolic state [71, 72–74, 76–79], which Rhodes *et al*. [72], and He *et al*. [75] validated in independent cohorts. Proteomic studies describe induced growth factors [82], including erythropoietin [85], hepatic growth factor [82], and inflammatory or immune-response pathways, including complement C4a [81] and several interleukins [85]. Outcomes are summarised in supplementary table S8.

Discussion

Biomarkers may contribute to early noninvasive detection and monitoring of disease. To our knowledge, this is the first systematic review with meta-analyses to evaluate the performance of diagnostic blood markers in patients with group 1 PAH. In this meta-analysis, we identified RDW, LDL-c, d-dimer, NT-proBNP, IL-6 and UA as biomarkers with the largest observed difference and sample size. Plasma NT-proBNP levels showed the largest difference between PAH and non-PH controls. Although it has a high sensitivity for PAH, NT-proBNP lacks specificity to distinguish PAH from other heart diseases. For other biomarkers, including IL-6, RDW, LDL-c, d-dimer and UA, insufficient data were available for meta-analysis of diagnostic accuracy. Owing to the lack of clinical validation, none of the newly proposed biomarkers could equal the sensitivity and specificity of NT-proBNP for detection of PAH.

Performance of current biomarkers in PAH diagnosis

Clinical adoption and implementation of new biomarkers is subject to strict performance metrics and involves: 1) an evidence-based relation between a biomarker and disease; 2) statistical quantification of the

predictive strength of biomarker level for the presence of disease, by using calculation of clinical sensitivity and specificity or evaluating ROC curves in diagnostic studies; and 3) availability of multiple independent data sources with sufficient sample sizes and power. When considering the first criterion, the current meta-analysis demonstrates that for various biomarkers a consistent and reproducible relation between PAH and biomarker levels can be found. By using a predefined search and selection strategy 26 biomarkers showed differential expression between the PAH and control population, reflecting the various pathophysiological processes (domains) that contribute to PAH. The number of biomarkers identified in this review is limited by the requirement of a minimum of three publications reporting on a given biomarker to perform a meta-analysis. This approach visualises biomarkers that have consistently been shown to relate to PAH (*i.e.* in at least three studies) but may ignore promising biomarkers that have not been reproduced in other studies. Markers included in less than three studies or expressed as medians were rendered unsuitable for meta-analysis and are depicted in supplementary tables S4 and S5. These markers include serotonin (5-Ht), asymmetric dimethylarginine (ADMA), angiopoietin-1 (Ang-1), BNP, endostatin, endothelin-1 (ET-1), galectin-3 (Gal-3), hepatocyte growth factor (HGF), high mobility group box 1 (HMGB-1), IL-8, MCP-1, matrix metalloproteinase-2 and -8 (MMP-2, -8), sodium (Na), PlGF, stem cell factor (SCF), sF-selectin, superoxide dismutase (SOD), sVCAM, transforming growth factor-β1 (TGF-β1), angiopoietin-1 receptor-2 (Tie-2), TIMP-4, VEGF and TNF-α (supplementary table S4), and caveolin-1 (CAV-1), HbA1c, IL-12, potassium (K), mean corpuscular volume (MCV), nitric oxide (NO), osteopoietin (OPN), provirus integration site for moloney murine leukaemia virus kinase (Pim-1), selenoprotein-P (Se-p), FGF-2, endoglin (Eng), kynurine (KYN), osteoprotegerin (OPG), N-terminal propeptide of type III procollagen (PIIINP), soluble fms-like tyrosine kinase 1 (sFLT), tissue factor pathway inhibitor (TFPI), thrombomodulin, tryptophan (TRP) and VEGFR1 (supplementary table S5). More studies focusing on these markers would clarify the relation between these markers and PAH.

With regard to the second criterion, while out of 26 meta-analyses, 17 biomarkers were consistently related to the presence of disease, data on ROC curves and calculation of clinical sensitivity and specificity for diagnosis of PAH were only available for NT-proBNP and CRP [50]. Independent validation, preferably in studies including a heterogeneous group of patients and including patients suspected or at risk of developing PAH are needed to clarify diagnostic accuracy, with a focus on providing sensitivity and specificity of a biomarker for disease at relevant and reproducible cut-off values. The latter is an essential step in the identification of biomarkers that may replace invasive diagnostics.

With regard to the third criterion, the drawback of most studies included in this review is a low sample size. The combined sample sizes were largest for NT-proBNP and LDL-c (1152 and 3035, respectively); most other analyses are based on a combined sample size below 450 subjects. Including low sample sizes carries the risk of bias and skewing of data to a selected patient population. This is a general limitation that may be addressed by biobanking, or concurrent analysis of biomarkers in clinical trials. A more systematic approach to biomarker studies may aid authors to increase the number of subjects in biomarker studies

Altogether, our systemic review and meta-analysis reveals a considerable number of biomarkers that were consistently found to be altered in PAH. However, these biomarkers lack the scientific underpinning to replace invasive diagnostics in PAH, either because data on them are lacking or because of a lack of specificity.

Future directions for biomarker development in PAH

Considering the fact that research on single biomarkers has failed to identify a single biomarker with sufficient sensitivity and specificity to foster noninvasive PAH diagnosis, various approaches may be considered to improve noninvasive diagnostics in the future. The first involves combining biomarkers with a strong relation to PAH pathophysiology, which have insufficient diagnostic accuracy on an individual basis, for example, implementing a panel of circulating biomarkers from several domains, weighed by importance to improve biomarker specificity. Based on our meta-analyses, a set of readily available biomarkers may be proposed: a panel including NT-proBNP, IL-6, RDW, UA and LDL-c could potentially be used to score the risk of PAH among clinically similar diseases. A second approach involves combining biomarkers with the strength of noninvasive radiological or haemodynamic measurements. This approach has proven successful in the OPTICS study [88] or DETECT study [89] to exclude iPAH, and in the European Respiratory Society (ERS)/European Respiratory Journal (ERJ) risk criteria and the REVEAL risk stratification [90] to predict outcome in PAH. A third approach may involve unbiased collection of large data sets, including proteomics, transcriptomics and metabolomics, which measure multiple diagnostic biomarkers representative for multiple disease domains in PAH [91]. A PAH-like signature can be used to distinguish iPAH from other diseases. An example is provided by Rhodes *et al.* [92], employing a selection of nine proteins derived from plasma proteomics, which accurately predict disease outcome in iPAH patients. We believe collaborative biobanks and concomitant analysis of biomarkers in

clinical trials and registries are an efficient step forward to improve translation to a clinical setting. External validation cohorts should include patients suspected of PH, and a thoroughly characterised control cohort that contains clinically similar and common diseases.

Strengths and limitations

This review has certain strengths. First, the search strategy of the current study was designed to cover all diagnostic biomarkers research in PAH thus far, resulting in a database on PAH biomarkers of unanticipated size. Second, the meta-analysis was designed to identify biomarkers with consistent performance over several studies. Although this approach may neglect novel, promising biomarkers to a certain extent, the design guarantees identification of biomarkers that were identified in at least three studies, thereby providing surrogate external validation of the biomarker. Third, we focused on easily accessible blood biomarkers thereby potentially bridging the technical gap towards implementation of diagnostic biomarkers in clinical care.

In addition, this meta-analysis has a number of limitations. The major limitation is the lack of validation and calculation of diagnostic accuracy of biomarkers outside their discovery cohort. This renders the reviewing process of sensitivity and specificity for detection of PAH impossible. Second, the meta-analyses were hampered by the limited number of publications addressing iPAH uniquely. Handling iPAH and hPAH patients together as one group, and extracting data of group 1 PAH as second best, meant inclusion of patients with PAH associated with connective tissue disease, congenital heart disease, and drug or toxin use, which may have introduced bias. Next, due to the limited number of studies, we chose not to exclude publications based on QUADAS-2 risk of bias scores, which may have led to inclusion of unreliable data and may have attributed to heterogeneity. However, correction of the most evident sources of bias (treatment status, diagnosis) indicated that bias was negligible.

Conclusion

This study summarises a large number of biomarker studies performed in PAH during the last three decades. Most of the described studies investigated the performance of one single blood biomarker. We conclude that none of these biomarkers have sufficient diagnostic accuracy to replace invasive diagnostics, as all single biomarkers lacked specificity. Using a combination of multiple biomarkers may improve specificity, and this can be achieved by combining a number of routinely available blood tests as well as *via* an unbiased omics approach.

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