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RESEARCH ARTICLE

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Two prospective, multicenter studies for the identification of biomarker signatures for early detection of pulmonary hypertension (PH): The CIPHER and CIPHER-MRI studies

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Abstract

A blood test identifying patients at increased risk of pulmonary hypertension (PH) could streamline the investigative pathway. The prospective, multicenter CIPHER study aimed to develop a microRNA-based signature for detecting PH in breathless patients and enrolled adults with a high suspicion of PH who had undergone right heart catheterization (RHC). The CIPHER-MRI study was added to assess the performance of this CIPHER signature in a population with low probability of having PH who underwent cardiac

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magnetic resonance imaging (cMRI) instead of RHC. The microRNA signature was developed using a penalized linear regression (LASSO) model. Data were modeled both with and without N-terminal pro-brain natriuretic peptide (NT-proBNP). Signature performance was assessed against predefined thresholds (lower 98.7% CI bound of ≥0.73 for sensitivity and ≥0.53 for specificity, based on a meta-analysis of echocardiographic data), using RHC as the true diagnosis. Overall, 926 CIPHER participants were screened and 888 were included in the analysis. Of 688 RHC-confirmed PH cases, approximately 40% were already receiving PH treatment. Fifty microRNA (from 311 investigated) were algorithmically selected to be included in the signature. Sensitivity [97.5% CI] of the signature was 0.85 [0.80–0.89] for microRNA-alone and 0.90 [0.86–0.93] for microRNA+NT-proBNP, and the corresponding specificities were 0.33 [0.24–0.44] and 0.28 [0.20–0.39]. Of 80 CIPHER-MRI participants with evaluable data, 7 were considered PH-positive by cMRI whereas 52 were considered PH-positive by the microRNA signature. Due to low specificity, the CIPHER miRNA-based signature for PH (either with or without NT-proBNP in model) did not meet the prespecified diagnostic threshold for the primary analysis.

KEYWORDS

chronic thromboembolic pulmonary hypertension, diagnostic, microRNA, pulmonary arterial hypertension, screening

INTRODUCTION

Pulmonary hypertension (PH) is a progressive and life-threatening condition that often goes undiagnosed until symptoms are severe.[1–4] The reasons for this diagnostic delay include the nonspecific nature of symptoms (e.g., dyspnea), the rarity of certain PH Groups (e.g., pulmonary arterial hypertension [PAH; Group 1] and chronic thromboembolic pulmonary hypertension [CTEPH; Group 4]), the limitations of investigative tests for PH,[5–9] and the requirement for invasive confirmation of diagnosis.[10,11] PH is defined as a mean pulmonary artery pressure (mPAP) > 20 mmHg and must be diagnosed using right heart catheterization (RHC).[10,11] To avoid unnecessary RHC, all patients with a suspicion of PH should have a transthoracic echocardiogram (TTE) to determine the probability of PH using peak tricuspid regurgitation velocity (TRV) as a surrogate for mPAP.[10–12] However, echocardiograms are not always readily accessible,[13] TRV is not measurable in 15%–45% of patients,[6,8,9,14,15] and echocardiograms do not reliably lead to detection of PH even when signs of PH are present.[5,7]

The availability of an effective blood-based diagnostic biomarker for PH has the potential to substantially improve detection of PH by providing a simple test to help triage patients for echocardiogram and/or RHC.[16] N-terminal pro-brain natriuretic peptide (NT-proBNP) is the only blood biomarker routinely used in PH.[10,11] Aside from screening for PAH in systemic sclerosis (SSc),[17] NT-proBNP is mainly used for prognostication in PH, since—as a biomarker for myocardial wall stress—it is not specific to PH and can fail to detect early PH.[10,11] Micro ribonucleic acid (miRNA) are small, stable, noncoding RNA molecules and evidence suggests that they could have a role as a diagnostic biomarker in PH. For example, in vitro studies have identified miRNAs that are involved in the pathogenesis of PH.[18–24] Several studies have identified miRNAs that are differentially expressed in patients with PH (most commonly from healthy controls) that are putative biomarkers,[25,26] and a recent study found that two miRNAs were able to distinguish patients with PAH from both disease and healthy controls.[27] Moreover, miRNA expression can predict the severity of PAH and CTEPH.[19,23]

The aim of the CIPHER study (NCT04193046) and, a second study, CIPHER-MRI (NCT04480723) was to identify and develop biomarker signatures for PH from circulating miRNAs. If effective, such biomarker signatures would help identify patients who should undergo RHC to confirm or rule out a diagnosis of PH.

METHODS

Study design

CIPHER was a prospective, multicenter study in participants with a suspicion of PH that was intended to identify and develop blood biomarker signatures for PH. The CIPHER-MRI study, also prospective and multicenter, was designed to assess the performance of the signatures derived in CIPHER in a patient population with low suspicion of PH. CIPHER was conducted in 44 sites across nine countries (Belgium, France, Germany, Netherlands, Poland, Spain, Ukraine, UK, and USA), enrolling patients between 23 December 2019 and 20 December 2021 (last-patient-last-visit 18 February 2022). CIPHER-MRI was conducted at eight sites across the UK and Germany, and enrolled patients between January 20, 2021 and May 10, 2022 (last-patient-last-visit May 18, 2022). The sites were mostly PH centers.

Selection and adjudication of participants

The CIPHER study population included prevalent (previously diagnosed within 18 months) and incident (newly diagnosed) participants, of at least 18 years of age, who had undergone RHC. Participants were categorized according to the following subgroups: prevalent non-PH (RHC within 6 months), incident non-PH (RHC within 6 weeks), incident PH (RHC within 6 weeks), or prevalent PH with or without PAH therapy (RHC within 18 months). Patients from all five PH Groups were enrolled (Group 1, PAH; Group 2, PH associated with left heart disease; Group 3, PH associated with lung disease; Group 4, PH associated with pulmonary artery obstructions; Group 5, PH with unclear and/or multifactorial mechanisms).[10,11] These PH Groups were defined as described in Supporting Information: Table S1. Any cases that were not classified or had a classification that did not conform to the study definitions were submitted to a disease classification adjudication committee comprised of authors L. H, D. G. K, A. L, B. A. M, I. R. P, S. R., M. T, M. R. W, and K. M. C. The committee reviewed the patient profile and could request supplemental information where available (such as RHC tracings, X-ray reports, and pulmonary function test results) to determine the correct World Health Organization (WHO) PH Group. During the adjudication process, it emerged that it was not possible to assign every case to a WHO PH Group, and these patients were classified as "unclear PH Group." No changes to the clinical management of the patient were suggested to the principal investigator.

The CIPHER-MRI study enrolled adult participants who had undergone TTE assessment to investigate a suspicion of PH and were considered to have a low or intermediate probability of PH on TTE and who were not referred for RHC based on clinical opinion. Full eligibility criteria are shown in Supporting Information: Tables S2 (CIPHER) and S3 (CIPHER-MRI).

Trial procedures

Blood samples were taken at the time of study enrollment and the analysis of NT-proBNP and miRNAs was performed at a central laboratory (details in Supporting Information: Appendix 1). All participants had a TTE within 6 weeks from enrollment. TTEs were performed as per routine clinical practice. Participants also had either an RHC (in CIPHER) or a cardiac magnetic resonance imaging (cMRI) (in CIPHER-MRI) to determine their status (PH or non-PH). All TTEs and cMRIs were centrally read in a blinded manner. TTE results were classified as low, intermediate, or high probability of PH per the 2015 European Society of Cardiology/European Respiratory Society (ESC/ERS) guideline recommendations. cMRI categorization of participants into PH and non-PH was performed as described previously[28–30] and as outlined in Supporting Information: Appendix 2. The results of the cMRI were provided to the treating physician, and if they independently decided to perform an RHC, the RHC results were collected and were to be used as the ground truth.

Study objectives and outcome measures

The overall aim of the CIPHER study was to develop a miRNA-based signature for detecting PH in patients with unexplained shortness of breath. The CIPHER-MRI study was added to address the bias in the CIPHER study (which enrolled a higher risk population) and assess the performance of the signature developed in the CIPHER study in a population with low probability of PH by TTE. CIPHER-MRI could therefore help investigate whether the CIPHER biomarker signature could detect PH earlier than the current investigative tools (i.e., in patients at risk of PH but with a low probability of PH by TTE).

Performance of the miRNA signature in distinguishing PH from non-PH was assessed by measuring sensitivity, specificity, positive predictive values (PPVs), negative predictive values (NPVs), accuracy, and area under the receiver operating characteristic curve (AUC).

In CIPHER, the performance of the miRNA signature was evaluated using RHC as the confirmation for PH diagnosis. The diagnostic performance of TTE was also

explored and an informal comparison of the performance of TTE to that of the miRNA signature was performed.

The primary objectives of CIPHER-MRI were to (i) estimate and compare the percentage of participants (considered clinically as not having PH) who were PH-positive according to the CIPHER miRNA signature versus cMRI and (ii) estimate the performance of the CIPHER signature(s) in the CIPHER-MRI population. As CIPHER-MRI participants did not undergo RHC, cMRI was used to classify participants as PH or non-PH, based on a previously published MRI model.[28–30]

The incidence of all treatment-emergent adverse events (TEAEs) in CIPHER and CIPHER-MRI was reported only in relationship to the study procedures, TTE, cMRI, and blood draws.

Statistical methods

For the CIPHER analysis of TTE performance in distinguishing PH from non-PH, CIPHER participants were classified as PH-positive or PH-negative using (i) TRV > 2.4 m/s and (ii) the full 2015 ESC/ERS guideline recommendations for TTE assessment of PH (i.e., TRV > 2.8 m/s and other echocardiographic signs of PH).[2,11] For the calculation of performance using TRV > 2.4 m/s, participants who were missing peak TRV measurements were included and assumed to be PH-negative. For estimating the performance of the full ESC/ERS guidelines, participants with an intermediate or high probability of PH were considered PH-positive, and those with low probability, PH-negative. Participants who could not be classified using these guidelines were excluded from the analysis.

miRNA data were log-transformed before analysis. The training/test ratio in CIPHER was 1:1. The miRNA biomarker signature was trained using the first half of the study data (training set) to allow a prespecified reassessment of sample size halfway through the study using only the training set. After careful statistical review of the performance of several machine learning models, penalized linear regression (LASSO) was used to identify a miRNA biomarker signature. Training was performed using a nested 10-fold cross-validation with 10 random repeats. Data were modeled both with and without NT-proBNP.

Diagnostic performance was estimated by applying the miRNA biomarker signature to the second half of the study data (test set) in CIPHER and, separately, to the entire data set of CIPHER-MRI. In both studies, the signature was considered to have acceptable precision if the lower one-sided 98.7% confidence interval for sensitivity was ≥0.73 AND the lower one-sided 98.7% confidence interval for specificity was ≥0.53. The thresholds for sensitivity and specificity were obtained from a meta-analysis of echocardiogram performance.[31] Additional statistical details may be found in Supporting Information: Appendix 1.

Sample size calculation

The sample size for the number of PH and non-PH participants (n_{PH} and $n_{non\text{-}PH}$) needed to meet the minimally acceptable sensitivity and specificity criteria of 0.73 and 0.53, respectively, was derived for a joint 95% confidence region for true positive fraction (sensitivity) and false positive fraction (1 – specificity) made up of the cross-product of two one-sided, 98.7% Wilson score confidence intervals based on an asymptotic method[32] and supplanted via simulation. Simulation sample size calculations showed that $n_{PH} = 291$ and $n_{non\text{-}PH} = 97$ yielded 91% power to detect acceptable diagnostic performance. The sample size was doubled to $2^*(291 + 97) = 776$ participants to estimate diagnostic performance in the second half of the study. To ensure sufficient numbers of non-PH participants, the target sample size was subsequently increased to ~900.

Regarding CIPHER-MRI sample size: assuming a minimum of 80 study participants, an 80% two-sided Wilson confidence interval for the true proportion of participants that were biomarker-positive according to the CIPHER miRNA signature assured that the width of the 80% confidence interval was no greater than 0.14.

RESULTS

Patient populations

The CIPHER study enrolled patients who had undergone RHC. Of a total of 926 participants screened, 888 met eligibility criteria and had an evaluable biomarker sample (evaluable set) (Figure 1). Median (range) age was 63 (19; 95) years and 61% of participants were female. Of 688 participants with RHC-confirmed PH, 346 (50%) were prevalent cases (diagnosed up to 18 months before enrollment), 279 (40%) were receiving PAH-specific medications, and 65% were in WHO funcional class (FC) III/IV. The most common PH Group was PAH (41%), followed by CTEPH (24%) and PH due to left heart disease (20%). Baseline characteristics of the training set ($n = 441$) and the test set ($n = 447$) are shown in Tables 1 and 2.

In CIPHER, 142 (16%) participants with PH were considered by two independent adjudicators due to difficulty in determining their PH subtype from clinical

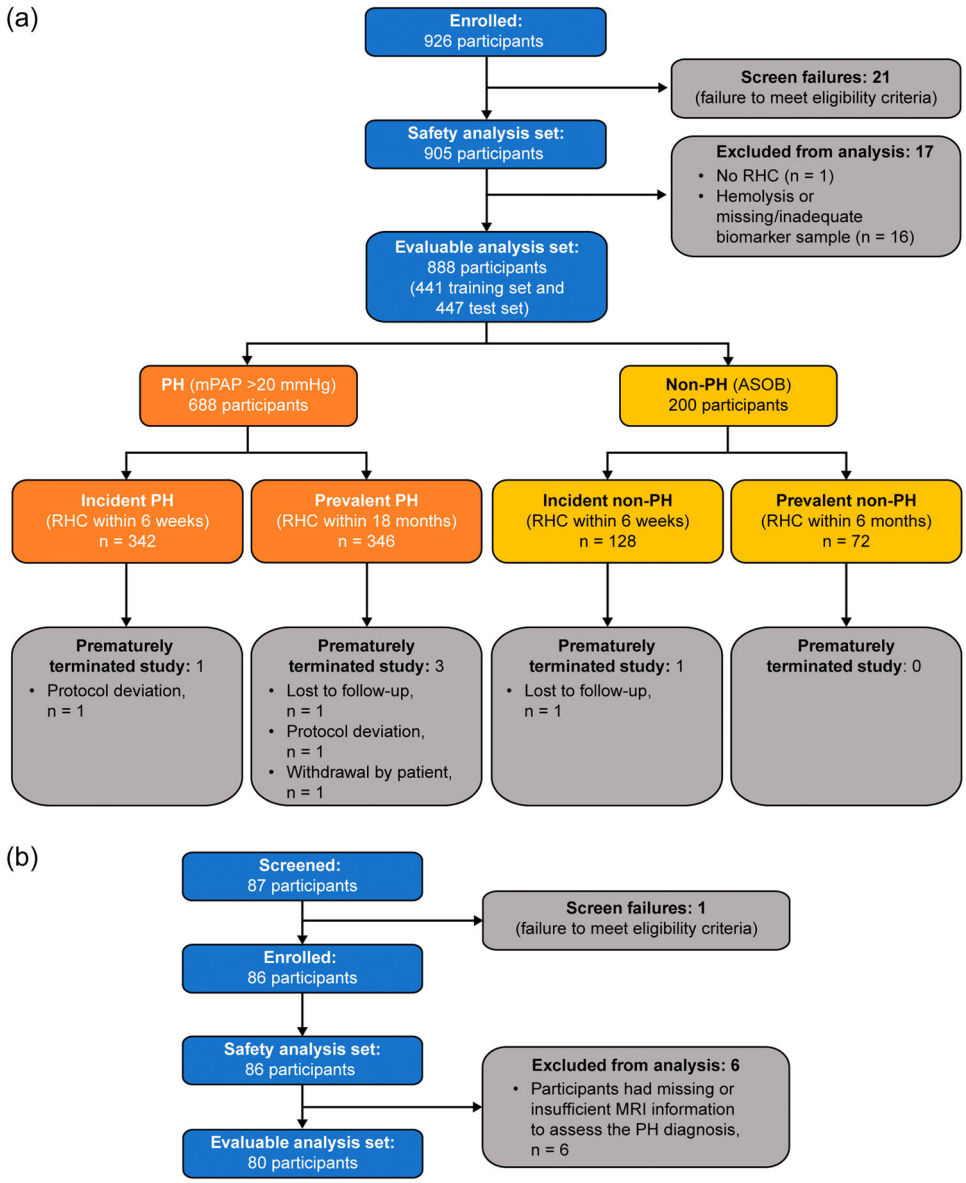

FIGURE 1 Patient flow diagram for (a) CIPHER and (b) CIPHER-MRI. With the exception of one patient (who did not have an RHC), all patients who prematurely discontinued the CIPHER study were included in the evaluable analysis set. ASOB, atypical shortness of breath; mPAP, mean pulmonary arterial pressure; MRI, magnetic resonance imaging; PH, pulmonary hypertension; RHC, right heart catheterization.

data per protocol. Of these, 72 patients were sent to a third reviewer, with 11 of these 72 participants requiring a consensus meeting among the whole panel. PH etiology could not be determined in 27 participants.

The CIPHER-MRI study enrolled patients who had low or intermediate probability of PH by TTE and therefore did not undergo RHC. Eighty-seven participants were screened, and 80 participants were included in the evaluable set. The percentage of patients who were considered by the treating physician as either low or intermediate probability of PH compared with the percentage of patients who were classified as such after central reading of TTEs is shown in Supporting Information: Table S5. Baseline characteristics of the safety analysis set ($N = 86$) are shown in Supporting Information: Table S6: median (range) age was 60 (20;81) years, 63% of participants were female, and 26% were in WHO FC III/IV.

CIPHER: Performance of biomarker signature

A total of 311 miRNAs were included in the statistical analyses after accounting for missing data and hemolysis

TABLE 1 Baseline demographics and characteristics of CIPHER participants (training set).

Characteristic	PH ($N = 350$)	Non-PH ($N = 91$)
Age (years)	64.3 (14.47)	56.7 (14.39)
Female	207 (59.1%)	58 (63.7%)
Race		
American Indian or Alaska Native	0	0
Asian	2 (0.6%)	1 (1.1%)
Black or African American	20 (5.7%)	6 (6.6%)
Native Hawaiian or other Pacific Islander	0	0
White	321 (91.7%)	84 (92.3%)
Not reported	3 (0.9%)	0
Multiple	4 (1.1%)	0
BMI (kg/m^2)	$N = 347$, 29.5 (7.42)	$N = 90$, 29.6 (8.47)
TRV (m/s)		
N	319	65
Mean (SD)	3.3 (0.73)	2.4 (0.49)
Median	3.3	2.5
Range	(1; 5)	(1; 4)
Echocardiographic probability of PH as estimated by PI		
N	347	91
Low risk of PH	43 (12.4%)	67 (73.6%)
Intermediate risk of PH	84 (24.2%)	17 (18.7%)
High risk of PH	220 (63.4%)	7 (7.7%)
Echocardiographic probability of PH as estimated by central reading[a]		
N	281	36
Low risk of PH	10 (3.6%)	16 (44.4%)
Intermediate risk of PH	75 (26.7%)	17 (47.2%)
High risk of PH	196 (69.8%)	3 (8.3%)
Not applicable	64	54
Hemodynamics		
mPAP (mmHg)	40.4 (11.58)	15.8 (2.71)
mRAP (mmHg)	$N = 348$, 9.5 (6.10)	4.8 (2.84)
PCWP/PAWP (mmHg)	$N = 345$, 12.8 (6.48)	8.5 (3.56)
LVEDP (mmHg)	$N = 34$, 16.0 (7.48)	$N = 6$, 9.3 (5.28)
CO (L/min)	$N = 348$, 4.9 (1.87)	$N = 89$, 5.2 (1.44)
PVR (dyn s/cm^5)	$N = 346$, 538.2 (372.52)	$N = 89$, 119.4 (56.84)
NT-proBNP, plasma sample (pmol/L)	$N = 337$, 160.7 (258.25)	$N = 90$, 33.7 (72.55)
PH Group		
N	333	0
Group 1	138 (41.4%)	–
Group 2	79 (23.7%)	–
Group 3	30 (9.0%)	–
Group 4	68 (20.4%)	–
Group 5	18 (5.4%)	–
WHO Functional Class		
N	348	21
I	17 (4.9%)	4 (19.0%)
II	99 (28.4%)	5 (23.8%)
III	210 (60.3%)	12 (57.1%)
IV	22 (6.3%)	0

Note: Categorical data are presented as *n* (%) and continuous data are presented as mean (SD) unless otherwise stated. *N* numbers are as stated in column headings ($N = 350$ for PH and $N = 91$ for non-PH), unless otherwise stated. Group 1 PH, pulmonary arterial hypertension; Group 2 PH, PH associated with left heart disease; Group 3 PH, PH associated with lung diseases and/or hypoxia; Group 4 PH, PH associated with pulmonary artery obstructions; Group 5 PH, PH with unclear and/or multifactorial mechanisms.

Abbreviations: BMI, body mass index; CO, cardiac output; LVEDP, left ventricle end diastolic pressure; mPAP, mean pulmonary arterial pressure; mRAP, mean right atrial pressure; NT-proBNP, N-terminal pro-brain natriuretic peptide; PAWP, pulmonary artery wedge pressure; PCWP, pulmonary capillary wedge pressure; PH, pulmonary hypertension; PI, principal investigator; PVR, pulmonary vascular resistance; SD, standard deviation; TRV, tricuspid regurgitation velocity; WHO, World Health Organization.

[a]Central reading is considered primary reading to be used in analyses.

(details in the Supporting Information, Appendix). Of these 311 miRNAs, approximately 50 miRNAs were identified using the LASSO regression model and included in the miRNA biomarker signature. The sensitivity of the miRNA biomarker signature with miRNA alone in the model was 0.85 [97.5% CI: 0.80–0.89] and the specificity was 0.33 [97.5% CI: 0.24–0.44] (Figure 2, Table 4). The sensitivity and specificity of the miRNA biomarker signature including NT-proBNP in the model with miRNA

TABLE 2 Baseline demographics and characteristics of CIPHER participants (test set).

Characteristic	PH ($N = 338$)	Non-PH ($N = 109$)
Age (years)	61.7 (14.57)	57.8 (14.66)
Female	208 (61.5%)	66 (60.6%)
Race		
N	335	106
American Indian or Alaska Native	3 (0.9%)	0
Asian	6 (1.8%)	0
Black or African American	31 (9.3%)	4 (3.8%)
Native Hawaiian or other Pacific Islander	1 (0.3%)	0
White	281 (83.9%)	91 (85.8%)
Not reported	13 (3.9%)	10 (9.4%)
Multiple	0	1 (0.9%)
BMI (kg/m^2)	$N = 335$, 29.4 (6.98)	$N = 106$, 27.7 (6.80)
TRV (m/s)		
N	288	81
Mean (SD)	3.3 (0.75)	2.4 (0.49)
Median	3.3	2.4
Range	(1; 6)	(1; 4)
Echocardiographic probability of PH as estimated by PI		
N	333	108
Low risk of PH	58 (17.4%)	87 (80.6%)
Intermediate risk of PH	69 (20.7%)	15 (13.9%)
High risk of PH	206 (61.9%)	6 (5.6%)
Echocardiographic probability of PH as estimated by central reading[a]		
N	265	40
Low risk of PH	13 (4.9%)	17 (42.5%)
Intermediate risk of PH	77 (29.1%)	21 (52.5%)
High risk of PH	175 (66.0%)	2 (5.0%)
Not applicable	65	67
Hemodynamics		
mPAP (mmHg)	$N = 337$, 40.5 (13.15)	15.9 (2.91)
mRAP (mmHg)	$N = 334$, 9.3 (5.24)	$N = 108$, 4.6 (2.96)
PAWP (mmHg)	$N = 330$, 12.5 (6.42)	$N = 108$, 8.5 (3.13)
LVEDP (mmHg)	$N = 42$, 11.4 (4.75)	$N = 15$, 12.9 (6.27)
CO (L/min)	$N = 331$, 4.8 (1.72)	$N = 106$, 5.6 (1.45)
PVR ($dyn\ s/cm^5$)	$N = 333$, 568.8 (415.97)	$N = 104$, 116.0 (53.70)
NT-proBNP in plasma sample (pmol/L)	$N = 328$, 174.9 (265.14)	$N = 103$, 22.8 (30.20)
PH Group		
N	284	0
Group 1	113 (39.8%)	–
Group 2	44 (15.5%)	–
Group 3	37 (13.0%)	–
Group 4	79 (27.8%)	–
Group 5	11 (3.9%)	–
WHO Functional Class		
N	326	15
I	12 (3.7%)	2 (13.3%)
II	101 (31.0%)	7 (46.7%)
III	194 (59.5%)	6 (40.0%)
IV	19 (5.8%)	0

Note: Categorical data are presented as *n* (%) and continuous data are presented as mean (SD) unless otherwise stated. *N* numbers are as stated in column headings ($N = 338$ for PH and $N = 109$ for non-PH), unless otherwise stated. Group 1 PH, pulmonary arterial hypertension; Group 2 PH, PH associated with left heart disease; Group 3 PH, PH associated with lung diseases and/or hypoxia; Group 4 PH, PH associated with pulmonary artery obstructions; Group 5 PH, PH with unclear and/or multifactorial mechanisms.

Abbreviations: BMI, body mass index; CO, cardiac output; LVEDP, left ventricle end diastolic pressure; mPAP, mean pulmonary arterial pressure; mRAP, mean right atrial pressure; NT-proBNP, N-terminal pro-brain natriuretic peptide; PAWP, pulmonary artery wedge pressure; PH, pulmonary hypertension; PI, principal investigator; PVR, pulmonary vascular resistance; SD, standard deviation; TRV, tricuspid regurgitation velocity; WHO, World Health Organization.

[a]Central reading is considered primary reading to be used in analyses.

were 0.90 [97.5% CI: 0.86–0.93] and 0.28 [97.5% CI: 0.20–0.39], respectively. Neither analysis (with or without NT-proBNP in the model) met the prespecified diagnostic threshold, which required both a lower 98.7% CI bound of 0.73 or higher for sensitivity and a lower 98.7% CI bound of 0.53 or higher for specificity (Figure 2, Table 4). This result is based on the per protocol strategy, which

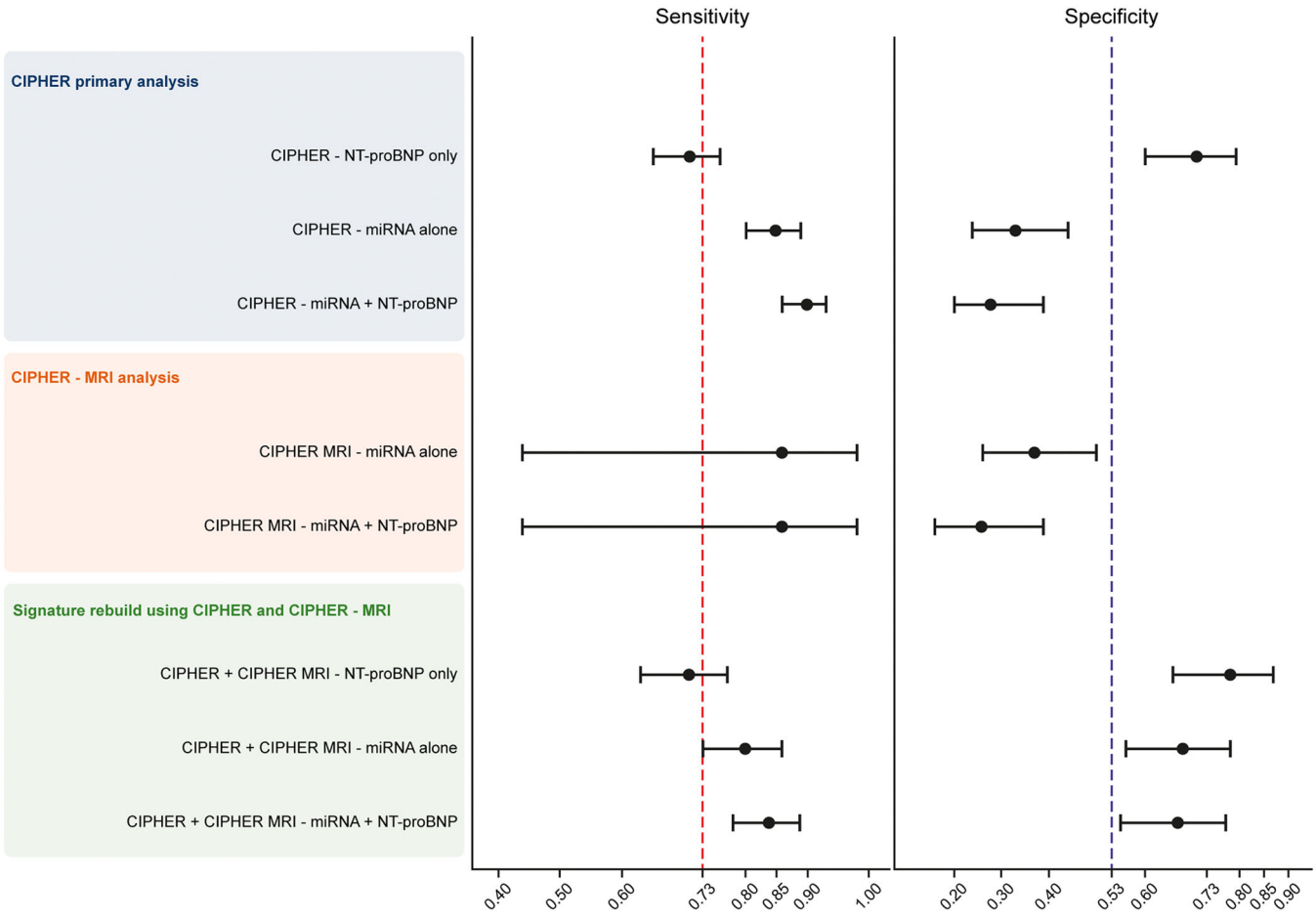

FIGURE 2 Sensitivity and specificity of biomarker signatures in plasma compared with prespecified criteria for sensitivity and specificity. CIPHER primary analysis: biomarker signatures tested on second half of CIPHER study data. CIPHER-MRI primary analysis: CIPHER biomarker signatures tested on all evaluable CIPHER-MRI data. Post-hoc signature rebuild: new biomarker signatures tested on pooled, resplit CIPHER and CIPHER-MRI data. Biomarker signatures are either miRNA-alone (only miRNA included in model), NT-proBNP-alone (only NT-proBNP included in model) or miRNA + NT-proBNP (miRNA and NT-proBNP both included in model). Red dashed lines show the hypothesis threshold for specificity and sensitivity at 0.53 and 0.73, respectively. 97.5% confidence intervals applied. miRNA, micro ribonucleic acid; MRI, magnetic resonance imaging; NT-proBNP, N-terminal-pro B-type natriuretic peptide.

prespecified that the biomarker signature be finalized on the first available 50% of the CIPHER data, with the final estimation of the signature performance reported on the second half of the CIPHER data.

CIPHER: Performance of TTE results

Of the participants included in the test set ($n = 447$), TRV measurements were missing for 78 participants (17%). Assuming these 78 participants were PH-negative, TRV > 2.4 m/s showed a sensitivity of 0.77 [97.5% CI: 0.71–0.82] and a specificity of 0.66 [97.5% CI: 0.55–0.75] (Table 3).

The full ESC/ERS TTE algorithm (i.e., peak TRV plus other echocardiographic signs of PH) could not be applied in 142 participants (32%) due to missing information. Therefore, the probability of PH was assessed in the remaining 305 participants from the test set. The observed sensitivity and specificity were 0.95 [97.5% CI: 0.91–0.97] and 0.43 [97.5% CI: 0.27–0.60] when the result of "intermediate probability of PH" is combined with the result of "high probability of PH" (Table 3).

CIPHER-MRI: Performance of biomarker signature

None of the CIPHER-MRI participants underwent RHC, and participants were classified as PH or non-PH according to cMRI. Seven (9%) CIPHER-MRI participants were considered PH-positive by cMRI, six of whom were considered PH-positive by the miRNA biomarker. Of the remaining 73 participants considered

TABLE 3 Performance of TTE assessment of PH probability (test set, N = 447).

(A) TRV			
	Diagnosis definition		
	TTE TRV result	**Final interpretation**	**Count**
RHC result			
Positive	TRV > 2.4 m/s	TP	260
	TRV ≤ 2.4 m/s	FN	28
	No TRV estimated	FN	50
Negative	TRV > 2.4 m/s	FP	37
	TRV ≤ 2.4 m/s	TN	44
	No TRV estimated	TN	28
Sensitivity (97.5% CI)	0.77 (0.71,0.82)		
Specificity (97.5% CI)	0.66 (0.55,0.75)		
PPV (97.5% CI)	0.88 (0.83,0.91)		
NPV (97.5% CI)	0.48 (0.39,0.57)		
(B) Full 2015 ESC/ERS guideline recommendations for TTE assessment of PH probability			
	Diagnosis definition		
	TTE probability	**Final interpretation**	**Count**
RHC result			
Positive	High	TP	175
	Intermediate	TP	77
	Low	FN	13
Negative	High	FP	2
	Intermediate	FP	21
	Low	TN	17
Sensitivity (97.5% CI)	0.95 (0.91,0.97)		
Specificity (97.5% CI)	0.43 (0.27,0.60)		
PPV (97.5% CI)	0.92 (0.87,0.95)		
NPV (97.5% CI)	0.57 (0.37,0.75)		

Note: Calculating using a two-sided Wilson score confidence interval for one sample proportion without correction for continuity.

Abbreviations: CI, confidence interval; ERS, European Respiratory Society; ESC, European Society of Cardiology; FN, false negative; FP, false positive; NPV, negative predictive value; PH, pulmonary hypertension; PPV, positive predictive value; RHC, right heart catheterization; TN, true negative; TP, true positive; TRV, tricuspid regurgitation velocity; TTE, transthoracic echocardiogram.

non-PH by cMRI, 46 (63%) were PH-positive by the CIPHER miRNA biomarker signature. The performance of the signature in the CIPHER-MRI population is summarized in Figure 2 and Table 4.

Post-hoc analysis of combined CIPHER and CIPHER-MRI datasets

As the nonrandomized, temporal 50/50 split between the training and the test set used in CIPHER could have created temporal bias and an imbalance in the participants' baseline characteristics, the signature was redeveloped (retrained) in an exploratory, post-hoc analysis, pooling data from both CIPHER and CIPHER-MRI. The signature was rebuilt using similar modeling techniques (LASSO with a nested 10-fold cross-validation with 10 random repeats). These combined data were randomly split 70/30 (new train/new test), so that the new signature was built using 70% of the evaluable data: 684 participants were in the new training set and 284 were in the new test set. These adjustments resulted in improved specificity

TABLE 4 Performance of biomarker signatures in plasma samples.

Biomarker signature	AUC	Accuracy	Sensitivity [97.5% CI]	Specificity [97.5% CI]	PPV [97.5% CI]	NPV [97.5% CI]
CIPHER primary analysis (N = 447)						
NT-proBNP	0.78	0.71	0.71 [0.65; 0.76]	0.71 [0.60; 0.79]	0.88 [0.83; 0.92]	0.44 [0.36; 0.52]
miRNA	0.69	0.72	0.85 [0.80; 0.89]	0.33 [0.24; 0.44]	0.80 [0.75; 0.84]	0.42 [0.31; 0.54]
miRNA + NT-proBNP	0.72	0.75	0.90 [0.86; 0.93]	0.28 [0.20; 0.39]	0.80 [0.75; 0.84]	0.48 [0.35; 0.62]
CIPHER-MRI analysis (N = 80)						
NT-proBNP	NR	NR	0 [0; 0.42]	0.81 [0.69; 0.89]	NR	NR
miRNA	0.62	0.41	0.86 [0.44; 0.98]	0.37 [0.26; 0.5]	0.12 [0.05; 0.25]	0.96 [0.79; 0.99]
miRNA + NT-proBNP	0.61	0.31	0.86 [0.44; 0.98]	0.26 [0.16; 0.39]	0.10 [0.04; 0.22]	0.95 [0.73; 0.99]
Post-hoc signature rebuild using combined, resplit CIPHER and CIPHER-MRI data (N = 284)						
NT-proBNP	NR	NR	0.71 [0.63; 0.77]	0.78 [0.66; 0.87]	NR	NR
miRNA	0.80	0.77	0.80 [0.73; 0.86]	0.68 [0.56; 0.78]	0.86 [0.80; 0.91]	0.58 [0.47; 0.69]
miRNA + NT-proBNP	0.82	0.79	0.84 [0.78; 0.89]	0.67 [0.55; 0.77]	0.86 [0.80; 0.91]	0.63 [0.51; 0.74]

Abbreviations: AUC, area under the receiver operating characteristic curve; CI, confidence interval; miRNA, micro ribonucleic acid; MRI, magnetic resonance imaging; NPV, negative predictive value; NR, not reported; NT-proBNP, N-terminal pro-brain natriuretic peptide; PPV, positive predictive value.

(to 0.68 [97.5% CI: 0.56–0.78]) while maintaining the sensitivity (0.80 [97.5% CI: 0.73–0.86]), achieving the prespecified analysis performance (Figure 2, Table 4). While the improved signature did not outperform TTE overall (Table 3), it did have a slightly better sensitivity than that of NT-proBNP alone in the same pooled post-hoc data set: sensitivity of NT-proBNP was 0.71 [97.5%: 0.63–0.77] and specificity was 0.78 [97.5% CI: 0.66–0.87].

CIPHER safety results

Safety results are summarized in Supporting Information: Table S7. Of the 905 participants in the safety analysis set, 9 (1.3%) participants had adverse events (AEs) (mild 5 [0.7%], moderate 2 [0.3%], and severe 2 [0.3%]) in the PH Group and 2 (1.0%) participants had mild AEs in the non-PH Group. There were no SAEs related to the mandated procedure or AEs leading to permanent discontinuation of the study. There were no deaths reported in the study.

CIPHER-MRI safety results

Of 86 participants in the safety analysis set, 1 (1.4%) participant had a TEAE (solitary fibrous tumor in the incident, non-PH Group) (Supporting Information: Table S8). There were no serious AEs related to the mandated procedure, AEs leading to permanent discontinuation of the study, or AEs associated with COVID-19.

DISCUSSION

A diagnostic biomarker test should be trained and tested in large cohorts of treatment-naïve patients who have a confirmed diagnosis and are representative of the patients for whom the test is intended. This is challenging for PH given that the treatable subgroups of PH are rare and that a diagnosis must be confirmed invasively by RHC, usually in a specialist center. In CIPHER, the largest study of its kind in PH, approximately 50 miRNAs were algorithmically selected to be included in a miRNA biomarker signature. The sensitivity and specificity of the miRNA signature alone were 0.85 and 0.33, respectively, in the held-out test set (338 PH and 109 non-PH participants). Inclusion of NT-proBNP in the miRNA biomarker signature improved the sensitivity to 0.90 at the cost of a slight decrease in specificity, which was 0.28. Neither analysis of the miRNA signature (with or without inclusion of NT-proBNP) met the prespecified minimum requirements for specificity (lower 97.5% confidence interval bound of 0.53).

We also tested TTE detection of PH and found that using TRV > 2.4 m/s alone achieved a reasonable balance of sensitivity (0.77) and specificity (0.66), whereas applying the full 2015 ESC/ERS guidelines was more sensitive (0.95) but less specific (0.43) than TRV alone. However, 78 participants (17%) in the TRV-only analysis had no TRV measurement and were assumed to be PH-negative, while 142 (32%) could not be classified by TTE probability of PH per the full ESC/ERS guidelines and were excluded from that analysis. Acknowledging the limitation of the missing data, TTE performed reasonably well at determining PH probability.

As it is not ethical to perform RHC in cases where it is not indicated, CIPHER-MRI participants did not undergo RHC. The finding that 46 (63%) of 73 participants who were considered non-PH by cMRI were PH-positive by the CIPHER miRNA biomarker could suggest that the miRNA signature is able to detect PH at an earlier stage than current investigative tools. However, although cMRI is a good surrogate for RHC, it is not a diagnostic test and, therefore, the true sensitivity of the signature in these patients is not known. In addition, 9 (11%) patients had SSc (Supporting Information: Table S5) and the impact of this on the signature is not known.

These results are based on a miRNA signature built with data from the first half of the CIPHER study (441 participants) and tested on data from the second half of the study (447 participants), and, separately, to CIPHER-MRI participants. Due to the limiting nature of this type of analysis, the signature was redeveloped in an exploratory, post-hoc analysis, combining CIPHER and CIPHER-MRI data randomly split 70/30 into train/test. This exploratory analysis was done to address concerns of a temporal bias and potential imbalance amongst the baseline characteristics across the training and test sets. For example, most sites first enrolled prevalent PH patients that were already in their database before enrolling more incident patients and more non-PH patients. In addition, some sites predominantly enrolled in the first half of the study while other sites contributed more in the second half, some sites predominantly enrolled non-PH patients and some sites enrolled more patients than others, with the number of patients contributed by each site varying widely from 1 to 127. This exploratory analysis resulted in improved specificity while maintaining the sensitivity achieved with the prespecified analysis, and successfully met the prespecified criteria. While the improved signature did not outperform TTE overall, it did appear to have better sensitivity compared with NT-proBNP. Further studies are needed to confirm whether a miRNA signature could be a more effective blood-based diagnostic biomarker than NT-proBNP for PH.

Although the CIPHER study was only powered to assess the performance of the miRNA signature in distinguishing broad PH from non-PH, PH is a large collection of highly heterogeneous diseases with completely different etiologies, defined only by one common hemodynamic feature (mPAP >20 mmHg).[10,11] Elevated mPAP eventually leads to right heart strain, which can be detected by measuring NT-proBNP levels.[10,11] While NT-proBNP is a useful component of the diagnostic work-up in PH, it is not specific to PH, and it can miss early or "borderline" PH. In the CIPHER data set (high suspicion of PH), NT-proBNP performed well (AUC 0.78). However, NT-proBNP may have been used in the screening process for the enrollment of CIPHER cohort, which may have introduced a positive bias for the performance of NT-proBNP in CIPHER. As NT-proBNP is not able to distinguish between the drivers of elevated right heart strain,[10,11] an effective miRNA signature could offer greater sensitivity, as suggested by the post-hoc findings from the combined CIPHER and CIPHER-MRI data.

To the best of our knowledge, the CIPHER study is the largest prospective diagnostic biomarker study in PH to date, and specifically the largest attempt to identify biomarkers that can discriminate patients with PH from the general symptomatic referral population and best reflect the potential clinical utility of the biomarkers. There are several key study limitations. First, 77% of the study population had PH and half of those had prevalent disease (diagnosed up to 18 months before enrollment) and almost two-thirds (of those with WHO FC available) had WHO FC III/IV symptoms. This study population was chosen because it would require a much larger sample size to develop a diagnostic test for PH in a patient cohort that is representative of the general population of patients with unexplained breathlessness, because a confirmed diagnosis is required and PH is diagnosed invasively by RHC. Additional studies are needed to assess the performance of the signature in newly diagnosed, treatment-naïve patients. Second, the CIPHER study is only powered to examine the performance of the miRNA signature in detecting broad PH versus non-PH, thus the CIPHER signature cannot be used to distinguish treatable subgroups of PH. Similar to the first point, it would be challenging to prospectively enroll the numbers of RHC-confirmed patients with treatable forms of PH required to perform such a study. Third, the CIPHER study is also limited by the temporal split of study data into training and test sets. The study team was able to address this temporal bias post-hoc, and the signature was redeveloped in an exploratory, post-hoc analysis, combining CIPHER and CIPHER-MRI data randomly split 70/30 into train/test to rebuild the signature. These adjustments resulted in improved specificity and maintained the sensitivity achieved with the primary analysis and met the prespecified criteria for sensitivity and specificity. While the miRNA signature still did not outperform TTE, it was more sensitive than NT-proBNP in this analysis.

MiRNA sampling could be a safe and effective way to support a minimally invasive, easily administered suite of diagnostic tests that may or may not include TTE. In the future, studies should consider having a larger derivation cohort sample size, or more focus on specific PH subgroups. These approaches would address several

limitations of the CIPHER study and allow for further subanalyses, including the ability to distinguish between different types or drivers of PH. Another approach would be to utilize the combined CIPHER and CIPHER-MRI cohorts as a validation cohort for future biomarker signature work in PH. For example, this cohort could be used to validate retrospective studies performed in local or national biobanks. Additional data or analyses that maximize the utility of the CIPHER cohorts may lend additional support for the use of a miRNA signature in the diagnostic work-up of PH. Future work should include the development of miRNA signatures to distinguish PH subpopulations either by clinical or molecular classification. For example, a biomarker that can distinguish between different PH subgroups would be clinically useful, given that (i) 139 CIPHER participants required adjudication to determine their specific PH Group in this study and that (ii) a PH Group could not be assigned in 27 cases. Distinguishing SSc-associated PH from SSc without PH would also allow us to identify treatable patients with poor prognosis.

CONCLUSION

The prospective, multicenter CIPHER study was designed to build a miRNA signature for PH; however, the signature did not meet the prespecified minimum requirements for specificity. Additional analyses that minimize these limitations, such as utilizing the combined CIPHER study data with external datasets to serve as a validation cohort—particularly for PH subclassification group signatures—are required to further investigate the utility of a miRNA signature in the diagnostic work-up of PH.

AUTHOR CONTRIBUTIONS

Debbie Quinn acts as a guarantor for this manuscript and, as such, accepts full responsibility for the work. All authors were involved in the conception of the study design. Yiu-Lian Fong proposed and initiated the study concept with scientific rationale, contributed to data interpretation and manuscript preparation and review. Allan Lawrie, Kelly Chin, David G. Kiely, Bradley A. Maron, Stephan Rosenkranz, Mark Toshner, Martin R. Wilkins, Luke Howard, and Ioana R. Preston collected the study data. Cheng He, Li Zhou, and Lihan Zhou were responsible for sample analysis. Cynthia Gargano was responsible for the statistical data analysis. All authors contributed to the interpretation of findings, critically reviewing the paper for intellectual content, and approved the final version of the manuscript for submission.

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CONFLICT OF INTEREST STATEMENT

Luke Howard has served as a member of the CIPHER steering committee for Janssen pharmaceutical companies of Johnson & Johnson, Gossamer Bio, and Lung Biotechnology; has received consulting fees from Altavant; has received research grants from Janssen pharmaceutical companies of Johnson & Johnson; has received speaker fees from Bayer PLC, Janssen pharmaceutical companies of Johnson & Johnson, and Merck; has received support for attending meetings and/or travel from Janssen pharmaceutical companies of Johnson & Johnson; has been a member of an advisory board for Acceleron, Janssen pharmaceutical companies of Johnson & Johnson, and Merck; and is a shareholder in iOWNA and Circular. David G. Kiely has served as a member of the CIPHER steering committee for Janssen pharmaceutical companies of Johnson & Johnson and his institution has received support from the National Institute of Health Research Sheffield Biomedical Research Centre as part of the support for the present manuscript. He has received consulting fees and honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events from Janssen pharmaceutical companies of Johnson & Johnson, Ferrer, Altavant, MSD, United Therapeutics, Gossamer, and Liquidia; has received support for attending meetings and/or travel from Janssen pharmaceutical companies of Johnson & Johnson, Ferrer, MSD and United Therapeutics; has participated on a Data Safety Monitoring Board of Advisory Board for Janssen pharmaceutical companies of Johnson & Johnson and MSD; is a member of the Clinical Reference Group for Specialized Respiratory Medicine (NHS England, unpaid); and is the lead of the UK National Audit of Pulmonary Hypertension (paid). David G. Kiely's institution has received grants or contracts from Janssen pharmaceutical companies of Johnson & Johnson, National Institute of Health Research Sheffield Biomedical Research Centre and Ferrer. Allan Lawrie has served has received payment for serving as a steering committee member for Janssen pharmaceutical companies of Johnson & Johnson (as part of the support for the present manuscript), has received support for attending meetings and/or travel

from Janssen pharmaceutical companies of Johnson & Johnson. He receives funding from the British Heart Foundation through a Senior Basic Science Research Fellowship (FS/18/52/33808), the Imperial British Heart Foundation Imperial Centre for Research Excellence (RE/18/4/34215), Alexion Pharmaceuticals, and Apple Inc (Investigator Awards). Bradley A. Maron has served as a member of the CIPHER steering committee for Janssen pharmaceutical companies of Johnson & Johnson; and has received consultancy fees from Actelion Pharmaceuticals, Tenax, and Regeneron and grants from Deerfield, Boston Biomedical Innovation Center and the Cardiovascular Medical Research Education Foundation, He has two patents pending and one patent issued relevant to the submitted work. He has served as PI or co-PI on various projects: 5R01HL139613-03: PI on NIH R01 award focusing on molecular mechanisms that regulate vascular fibrosis in PAH ($1,748,134); NIH R01HL163960: Co-PI on NIH R01 award using network medicine to prognosticate patients with PH ($286,861); U54HL119145 and Boston Biomedical Innovation Center (BBIC): PI on NIH-funded project to develop an antibody therapeutic for CTEPH ($341,589); Brigham IGNITE award: PI on project to develop an antibody therapeutic for CTEPH ($50,000); NIH R01HL153502: PI on NIH-funded project to clarify the mechanisms regulating NEDD9-SMAD3 interactions in thrombotic vascular disease ($864,664); NIH R01HL155096-01: PI on NIH-funded project to clarify individualize the pathophenotype of patients with PH ($809,353). Ioana R. Preston has served as a member of the CIPHER steering committee for Janssen pharmaceutical companies of Johnson & Johnson, Merck, Liquidia; she has received consulting fees and honoraria for lectures, presentations, manuscript writing or educational events from Janssen pharmaceutical companies of Johnson & Johnson, Altavant, Gossamer, and United Therapeutics; has received support for attending meetings and/or travel from Janssen pharmaceutical companies of Johnson & Johnson, Merck, and United Therapeutics; Ioana Preston's institution has received grants or contracts from Janssen pharmaceutical companies of Johnson & Johnson, Merck, United Therapeutics, Respira, Bellerophon. Stephan Rosenkranz has served as a steering committee member for Janssen pharmaceutical companies of Johnson & Johnson (as part of the support for the present manuscript), has received remunerations for lectures and/or consultancy from Abbott, Acceleron, Actelion, Aerovate, Altavant, AOP, AstraZeneca, Bayer, Boehringer-Ingelheim, Edwards, Ferrer, Gossamer, Janssen, Lilly, MSD, United Therapeutics, Vifor. His institution has received research grants from Actelion, AstraZeneca, Bayer, Janssen pharmaceutical companies of Johnson & Johnson, and Lempo. Mark Toshner has served as a steering committee member for Janssen pharmaceutical companies of Johnson & Johnson (as part of the support for the present manuscript), has received support for attending meetings and/or travel from Janssen pharmaceutical companies of Johnson & Johnson & GSK and has been a member of an advisory board for MorphogenIX. Martin R Wilkins has served as a member of the CIPHER steering committee for Janssen pharmaceutical companies of Johnson & Johnson and his institution received clinical research facility and Biomedical Research Centre infrastructure support from the National Institute of Health Research Sheffield Biomedical Research Centre as part of the support for the present manuscript. Martin R. Wilkins has received consulting fees from MorphogenIX, VIVUS, Janssen pharmaceutical companies of Johnson & Johnson, Kindaset, Chiesi, Aerami and BenevolentAI and has patents planned, issued and/or pending with Imperial Innovations (patent submitted for prognostic protein model and diagnostic miRNA model and patent for ZIP12 as a drug target); has participated in an adjudication committee for three clinical trials for Acceleron and in a study safety committee for GSK. Martin R. Wilkins institute has received grants or contracts from the British Heart Foundation (RE/18/4/34215 center support). Yiu-Lian Fong was an employee of Janssen Pharmaceuticals Inc. at the time of study, and owns shares of stock/stock options in Johnson & Johnson. Cynthia Gargano is an employee of Janssen Pharmaceuticals Inc. and owns shares of stock/stock options in Johnson & Johnson. Debbie Quinn, Dimitri Stamatiadis, and Xavier Gitton are employees of Actelion Pharmaceuticals Ltd, a Janssen pharmaceutical company of Johnson & Johnson, and own shares of stock/stock options in Johnson & Johnson. Kelly M Chin has received payment for work on steering, advisory, or adjudication committee work with Arena, Bayer, Gossamer Bio, Janssen Pharmaceuticals of Johnson & Johnson, and Merck, payment for consulting work with Altavant, and her institution has received research support for clinical studies overseen by her from Altavant, Gossamer Bio, Janssen pharmaceutical companies of Johnson & Johnson, Merck, Pfizer, and United Therapeutics. Cheng He, Li Zhou, and Lihan Zhou are employees of MiRXES Lab and received support from Janssen Pharmaceuticals of Johnson & Johnson during the conduct of this study.

ETHICS STATEMENT

The protocol and other study documentation for each study were reviewed and approved by the relevant Institutional Review Board/Independent Ethics Committee for each site before the studies were initiated

(Supporting Information: Table S4) and each patient gave written informed consent.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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