Distinct plasma gradients of microRNA-204 in the pulmonary circulation of patients suffering from WHO Groups I and II pulmonary hypertension

Leonard E. Estephan[1,*], Michael V. Genuardi[1,2,*] ⓘ, Chad M. Kosanovich[2], Michael G. Risbano[1,3], Yingze Zhang[3], Nancy Petro[3], Annie Watson[1], Yassmin Al Aaraj[1], John C. Sembrat[1,3], Mauricio Rojas[3], Dmitry A. Goncharov[1,3], Marc A. Simon[1,3], Elena A. Goncharova[1,3], Anjali Vaidya[4], Akaya Smith[5], Jeremy Mazurek[6], Yuchi Han[6] and Stephen Y. Chan[1,2]

[1]Center for Pulmonary Vascular Biology and Medicine, Pittsburgh Heart, Lung, Blood, and Vascular Medicine Institute, Division of Cardiology, Department of Medicine, University of Pittsburgh School of Medicine and University of Pittsburgh Medical Center, Pittsburgh, PA, USA; [2]Division of Cardiology, University of Pittsburgh School of Medicine and University of Pittsburgh Medical Center, Pittsburgh, PA, USA; [3]Division of Pulmonary Allergy and Critical Care Medicine, University of Pittsburgh School of Medicine and University of Pittsburgh Medical Center, Pittsburgh, PA, USA; [4]Division of Cardiovascular Medicine, Temple University Health Systems, Philadelphia, PA, USA; [5]Division of Pulmonary Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA; [6]Division of Cardiovascular Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

Abstract

Pulmonary hypertension (PH), a heterogeneous vascular disease, consists of subtypes with overlapping clinical phenotypes. MicroRNAs, small non-coding RNAs that negatively regulate gene expression, have emerged as regulators of PH pathogenesis. The muscle-specific micro RNA (miR)-204 is known to be depleted in diseased pulmonary artery smooth muscle cells (PASMCs), furthering proliferation and promoting PH. Alterations of circulating plasma miR-204 across the trans-pulmonary vascular bed might provide mechanistic insights into the observed intracellular depletion and may help distinguish PH subtypes. MiR-204 levels were quantified at sequential pulmonary vasculature sites in 91 patients with World Health Organization (WHO) Group I pulmonary arterial hypertension (PAH) (n = 47), Group II PH (n = 22), or no PH (n = 22). Blood from the right atrium/superior vena cava, pulmonary artery, and pulmonary capillary wedge was collected. Peripheral blood mononuclear cells (PBMCs) were isolated (n = 5/group). Excretion of miR-204 by PAH-PASMCs was also quantified in vitro. In Group I patients only, miR-204 concentration increased sequentially along the pulmonary vasculature (log fold-change slope = 0.22 [95% CI = 0.06–0.37], P = 0.008). PBMCs revealed insignificant miR-204 variations among PH groups (P = 0.12). Cultured PAH-PAMSCs displayed a decrease of intracellular miR-204 (P = 0.0004), and a converse increase of extracellular miR-204 (P = 0.0018) versus control. The stepwise elevation of circulating miR-204 across the pulmonary vasculature in Group I, but not Group II, PH indicates differences in muscle-specific pathobiology between subtypes. Considering the known importance of miR-204 in PH, these findings may suggest pathologic excretion of miR-204 in Group I PAH by PASMCs, thereby accounting for decreased intracellular miR-204 concentration.

Keywords

Introduction

Pulmonary hypertension (PH) is a deadly and morbid vascular disease, classified by the World Health Organization

*Equal contributors.

Corresponding author:
Stephen Y. Chan, Center for Pulmonary Vascular Biology and Medicine, Pittsburgh Heart, Lung, Blood, and Vascular Medicine Institute, Division of Cardiology, Department of Medicine, University of Pittsburgh School of Medicine and University of Pittsburgh Medical Center, 200 Lothrop Street, BST1704.2, Pittsburgh, PA 15261, USA.
Email: chansy@pitt.edu

(WHO) into subtypes based on histopathologic, hemodynamic, and clinical features,[1] and subject to distinct management strategies.[2,3] Group I pulmonary arterial hypertension (PAH) is now defined by a mean pulmonary arterial pressure (mPAP) > 20 mmHg, a pulmonary arterial wedge pressure (PAWP) ≤ 15 mmHg, and a pulmonary vascular resistance (PVR) > 3 Wood units (WU), accompanied by specific clinical criteria. Notably, however, previous hemodynamic criteria for PAH were set at mPAP ≥ 25 mmHg[4] and have been the basis for most clinical studies of this disease thus far. Extensive vascular inflammation, a hallmark of this PH subtype, is consistent with several underlying causes of PAH, including, but not limited to, a range of connective tissue diseases and HIV infection.[5] Group I PAH disease progression often includes the upregulation of highly inflammatory cytokines and chemokines paired with elevated activity of perivascular inflammatory classes of immune cells.[6] These phenotypes contribute to poor outcomes in Group I disease such as aberrant vascular remodeling and lesion development.[7] While traditionally associated with dismal prognosis, pulmonary vasodilator therapy has been effective in improving long-term morbidity and other outcomes in PAH.[8]

In contrast to Group I disease, Group II PH is driven by left heart disease[9,10] but is similarly associated with poor outcomes.[11] In general, Group II disease has not been shown to be responsive to pulmonary vasodilator therapy; treatment with agents proved to be effective in Group I PAH may be potentially harmful.[12] Group II PH is defined by elevated mPAP with PAWP ≥ 15 mmHg and left heart disease,[9,10] but there are contexts where the hemodynamic profiles of Group II PH may mimic decompensated Group I PAH, such as when both the PVR and PAWP are both elevated.[13] Despite showing such similarities, Group I and Group II PH may nonetheless be driven by distinct molecular mechanisms. Study of the markers of disease unique to Group I and II patients may aid in the description and further elucidation of disease pathophysiology.

Recently, several microRNAs (miRNAs) have emerged as molecules with direct pathogenic importance in the development and severity of PH.[14] Intracellularly, miRNAs negatively regulate gene expression across the transcriptome via engagement of specific nucleic acid sequences, leading to transcript degradation or inhibition of protein translation. Dynamic alterations to their expression have been found to carry pervasive pathogenic consequences in PH and may be of diagnostic or prognostic value.[15] As expressed in stable biomolecules as either extracellular microparticles[16,17] or RNA–protein complexes,[18] plasma-circulating miRNAs from diseased vascular tissue may reflect disease state and also may be active endocrine or paracrine mediators in PH pathogenesis.[18,19] MiR-204 is a muscle-specific miRNA that is highly active in cardiomyocytes and vascular muscle cells. MiR-204 has been found to be downregulated in diseased pulmonary arterial smooth muscle cells (PASMCs) in PAH and is linked to signaling molecules crucial to PH including PPAR-γ, TGF-β1, STAT3, SHP2, BRD4, NFAT, and HIF-1α.[20–25] Mir-204 may also mediate the protective action of mesenchymal stromal cell-derived exosomes[26] and altered levels correlate with more angiogenic and proliferative disease[27] in both the pulmonary and coronary circulation.[28] Paradoxically, however, miR-204 appears to circulate at high levels in patients with PH, as a recent investigation found increased plasma miR-204 in a heterogeneous population of patients with PH encompassing multiple WHO groups.[29]

We aimed to describe the levels of plasma miR-204 in a population of patients with well-characterized PH to assess and compare plasma miR-204 levels among patients with WHO Group I PAH, Group II PH, and no PH. Additionally, we sought to assess the dynamic profile of miR-204 across the pulmonary vascular bed and compare this profile among PH groups. As a comparison molecule, we chose another circulating muscle-specific miRNA, miR-208, which has been previously reported to be elevated in acute myocardial infarction, heart failure, viral myocarditis, and atrial fibrillation.[30] Unlike miR-204, however, intracellular miR-208 has been implicated in right ventricular (RV) rather than vascular smooth muscle dysfunction in PH.[31] Furthermore, levels of circulating miR-208 have not been shown to be elevated in PH compared to non-PH patients except in the buffy coat of patients with severe cases of disease (mPAP ≥ 40 mmHg).[29]

Methods

Patient population

All patients with known or suspected PH and referred for right heart catheterization (RHC) during their routine clinical care during 2014–2018 at two designated PH Comprehensive Care Centers—the University of Pittsburgh Medical Center (UPMC) and the Hospital of the University of Pennsylvania (HUP)—were eligible for inclusion. Procedures indicated for either diagnostic or disease monitoring purposes were included. Patients were prospectively enrolled by study staff before RHCs were performed.

The study protocol was approved separately by the Institutional Review Boards (IRBs) from each respective institution (IRB No. PRO11070366, University of Pittsburgh; IRB No. 818660, University of Pennsylvania). Informed consent was universally obtained and all study procedures conformed to the ethical standards of the Declaration of Helsinki.

Right heart catheterization

A pulmonary artery (PA) catheter (Edwards, Irvine, CA, USA) was advanced into the central venous system (superior vena cava [SVC]), right atrium (RA), right ventricle (RV), and PA by experienced operators in individuals at

rest in the supine position after appropriate catheter calibration and zeroing.[32] Pressure waveforms from the RA, PA, and pulmonary capillary wedge (PCW) positions were recorded in duplicate at end-expiration using the Xper Cardio Physiomonitoring System at UPMC (Philips, Melborne, FL, USA) and Horizon Cardiology Cardiovascular Information System at HUP (McKesson, San Francisco, CA, USA). Cardiac output (CO) was calculated by the Fick method using main PA and peripheral oxyhemoglobin saturations.

Blood sampling

Blood samples were drawn from our 91-patient cohort at the RA/SVC, PA, and the PCW positions during RHC. Samples were transferred into BD Vacutainer® tubes (BD, Franklin Lakes, NJ, USA), treated with standard anticoagulant ethylenediaminetetraacetic acid (EDTA), and subsequently spun at 2800 RCF in a Medilite Centrifuge (Thermo Scientific, Waltham, MA, USA) for 10 min to initiate plasma separation. Aliquots of plasma (350 μL) were stored at –80°C until future RNA extraction.

From a separate patient cohort (n = 15) referred for RHC, peripheral blood mononuclear cells (PBMCs) were isolated following blood collection using a Ficoll density gradient (Corning, New York, USA), as previously described.[33] Isolated cells were stored in freezing media comprising 10% dimethyl sulfoxide (DMSO), 20% fetal bovine serum (FBS), and 70% Dulbecco's Modified Eagle Medium (DMEM). The mixtures were promptly frozen in liquid nitrogen (LN$_2$) before RNA extraction.[33] These patients were designated as either Group I PAH (n = 5), Group II PH (n = 5), or no PH (n = 5), and were not included in the original cohort due to a later date of enrollment and sample collection.

Cultured human PASMCs

Primary distal PASMCs from patients with PAH and patients with non-diseased lungs were provided by the University of Pittsburgh Vascular Medicine Institute Cell Processing Core in accordance with procedures approved by University of Pittsburgh IRB and CORID. Cell isolation, characterization, and maintenance were performed as previously described.[34,35] Briefly, pre-capillary pulmonary arteries (< 1.5 mm of outer diameter) were dissected out and adherent lung parenchymal tissue was removed using a microscissor and scalpel. The arteries were minced into 1 mm^2 blocks and plated on tissue culture plates with a small drop of Lonza SmBM smooth muscle cell basal media supplemented with an SmGm-2 growth media kit (Lonza Group, Basel, Switzerland). On the following day, a full volume of culture media was added to the plates and left undisturbed for 3–5 days. The media was then subsequently changed every other day until the cells reached confluence. Isolated PASMCs were characterized by immunocytochemical analysis of alpha-smooth muscle actin (α-SMA),

SM22alpha, smooth muscle myosin heavy chains (Cell Signaling Technology, Danvers, MA, USA), and cell morphology.

Primary PASMCs (passage 3–6) from one non-diseased control patient (40-year-old woman) and one scleroderma-associated PAH (Scl-PAH) patient (54-year-old woman on intravenous prostacyclin) were experimentally utilized in this study. Approximately four months before the harvest of lung tissue, invasive hemodynamic study confirmed a mPAP of 53 mmHg and PVR of 4.4 WU in the Scl-PAH patient. PASMC cell stocks from each patient were grown in Falcon Brand T-75 flasks (Corning, NY, USA) using SmGM-2 growth media. Scl-PAH (n = 3) and control (n = 3) cells were then plated in triplicate on Falcon Brand flat-bottom six-well plates (Corning, NY, USA) at a seeding density of 200,000 cells/well using SmBM smooth muscle cell basal media with 5% FBS and Pen Strep antibiotics (10,000 U/mL) (Thermo Fisher Scientific, Waltham, MA, USA). After 48 h of spreading time, plating media was removed, and 1.5 mL of SmBM smooth muscle cell basal media with 1% FBS and Pen Strep antibiotics was added to each well. After an additional 48 h, 150 μL of conditioned media was added to 800 μL of Qiazol lysis buffer (Qiagen, Hilden, Germany). The entirety of cells per well was lysed with a separate 800 μL of Qiazol lysis buffer. Both media and cells were frozen in lysis buffer at –80°C for subsequent RNA extraction. For cell culture data, three independent experiments were performed in triplicate.

RNA extraction

All RNA samples derived from plasma and PBMC samples were confirmed to have spectrophotometric absorption far below 0.3 over a range of 410–430 nm (414 nm, absorbance peak of free hemoglobin) using a Synergy HTX Multi-Mode Microplate Reader (Biotek, Winooski, VT, USA), a previously described cutoff to rule out substantial hemolysis.[36] Aliquots of plasma and PBMCs were thawed on ice and centrifuged at 4°C (Centrifuge 5427 R, Eppendorf, Hamburg, Germany) at 12,500 rpm for 15 min before batch processing. Resulting supernatant volumes of 150 μL were collected for RNA extraction as described previously.[24,37] Importantly, to ensure the accuracy and precision of this protocol, we compared the quantitative results of this single spike-in miRNA protocol to other recently described operating procedures[38] utilizing a mixture of two separate spike-in control miRNAs as well as an independent spike-in control c-miRNA during complementary DNA (cDNA) synthesis. Over 10 separate and randomly selected plasma samples, quantitative fold-change of candidate miRNAs did not significantly differ when comparing calculations derived from single versus multiple spike-in protocols. As such, we elected to utilize a single spike-in control miRNA protocol (cel-67 mimic, Thermo Fisher Scientific, Waltham, MA, USA) in order to remain consistent with our prior reported findings of circulating miRNAs.[39–41]

After thawing, cultured PASMCs and their respective media underwent a similar extraction procedure for miRNA quantification. For extracellular miRNA expression, a single spike-in control miRNA (cel-67 mimic, Thermo Fisher Scientific, Waltham, MA, USA) was used in media as a normalization factor. For cellular miRNA expression values in both PASMCs and PBMCs, the small nucleolar RNA, C/D box 48 (RNU 48) (Thermo Fisher Scientific, Waltham, MA, USA) was used as a housekeeping gene.

RNA quantification

Levels of plasma miR-204 and miR-208 were quantified using reverse transcription-quantitative (real time) polymerase chain reaction (RT-PCR) in all sample types. Reverse transcription of circulating miRNAs was performed using a TaqMan MicroRNA Assay (Thermo Fisher Scientific, Waltham, MA, USA) to generate cDNA, which was then amplified using Taqman probes, primer sets, and an Applied Biosystems QuantStudio 6 Flex Real-Time PCR System. Fold change of miRNAs was calculated by formulas previously described,[24] specifically for plasma samples as well as for intracellular miRNA levels in PASMCs and PBMCs. In order to account for any differences in cellular content among comparators when calculating extracellular miRNA analysis in culture media, fold change quantifications were normalized to the relative level of total cellular RNA in each sample (designated as "normalized fold change").

PH and WHO Group ascertainment

Presence or absence of PH for all patients with PH was adjudicated by third-party PH specialists, based on all patient history and diagnostic information available, including hemodynamic measurements obtained from the qualifying RHC. In this study, the more hemodynamically stringent criteria were used for defining Group I PAH (as the more recent classification scheme was released after this study had completed enrollment), i.e. mPAP > 25 mmHg, PAWP ≤ 15 mmHg, and PVR > 3 WU. Diagnostic criteria of the Group II PH subset included both mPAP > 25 mmHg and PAWP ≥ 15 mmHg accompanied by left heart disease.[9,10] Subsequent PH classification was based on these standardized criteria specified by WHO guidelines.[4] Results of miRNA assays were not available to physicians making PH group determinations.

Statistical analysis

Baseline and hemodynamic characteristics within groups were expressed as mean ± SD for continuous variables and compared between the three patient groups (Group I, Group II, and no PH) using ANOVA with post-hoc pairwise comparisons using the Tukey method. Categorical parameters were compared among groups using χ^2 tests. After

Shapiro–Wilk tests of normality, cellular and extracellular miRNA analyses were performed using unpaired parametric t-tests and were expressed as mean ± SEM. PBMC miRNA values from each of the patient groups (Group I, Group II, and no PH) were compared using ANOVA combined with Dunn's multiple comparisons testing.

Multiple comparative analyses were performed on pulmonary vascular bed plasma. First, mean miRNA expression levels were compared across PH subgroups and were represented as fold change from the mean non-diseased miRNA expression value for each vascular region. Spearman's correlation coefficients were calculated between the concentration of miR-204 at each of the three catheter positions (RA/SVC, PA, and PCW) and hemodynamic parameters. At each of the three catheter positions, the expression level of miR-204 was evaluated among patients with Group I, Group II, and no PH using Wilcox rank-sum tests to make pair-wise comparisons. To describe the dynamic change in miR-204 concentration across the pulmonary vascular bed, the ratio of miR-204 concentration at each position to concentration at the RA position for each patient was plotted.

We designed a linear mixed-effects interaction model to test whether the log mirR-204 concentrations changed differently when measured sequentially from RA to PA to PCW in patients with Group I versus Group II PH. In this model, sample source was included as an ordered categorical fixed effect and patient number was included as a random intercept effect. To test whether log miR-204 concentrations changed differently across the pulmonary vascular bed, a PH group × sample source interaction term was included. Additional adjustment was made for hospital (UPMC versus HUP). A second set of models tested the slope of the miR-204 concentrations over the vascular bed in the cohort stratified by PH group (Group I, Group II, no PH). Again, patient number was included as a random effect and adjustment was made for hospital of origin. In all analyses, complementary models were created for miR-208 for comparison. Confidence intervals for fixed effects were obtained by the bootstrap method based on 1000 simulations.

We examined whether slope of miR-204 over the pulmonary vascular bed has sensitivity and specificity for Group I PAH by creating receiver operating characteristic curves for the gradient between miR-204 levels at the PA or PCW (whichever was higher) and RA level.

All comparisons were two sided, with alpha = 0.05. Statistical analyses were performed using Prism version 7.0 c (GraphPad Software, La Jolla, CA, USA) and R version 3.4.0 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Demographic characteristics by PH group are presented in Table 1. Patients in Group II were substantially older

(approximately 10 years on average; $P = 0.008$) than patients in Group I, while race and gender were fairly similar among the three groups. Hemodynamic parameters differed substantially among groups (Table 2), although pairwise comparison of Group I and Group II patients showed similar mPAP (43.5 vs. 41.7 mmHg, respectively; $P = 0.8$). PVR varied significantly among patients with Group I, Group II, and no PH (6.4 vs. 3.7 vs. 1.5 WU, respectively; $P < 0.001$). Variations in creatinine clearance were also noted. However, despite the hemodynamic and creatine clearance differences, patients in all three groups had similar cardiac output and cardiac index.

The expression levels of miR-204 at the three anatomical sites did not differ between groups (Fig. 1). Notably, miR-204 was found to trend upwards sequentially from the RA/SVC to PA to wedge (PCW) positions within Group I PAH patients, but such stepwise increase was not observed in Group II PH patients (Fig. 2a, 2c). We modeled log miR-204 fold-change as a function of anatomic source as well as source-PH group (Group I vs. Group II) interaction, adjusting for hospital and a patient-level random effect. We found significant interaction between source and PH group ($P = 0.006$), indicating that there is a difference in miR-204 gradient over the pulmonary vasculature comparing Group I and Group II. We tested whether miR-204 gradients were non-zero by stratifying by PH group and modeling log fold-change as a function of anatomic source, again adjusting for hospital and patient-level random effects. We found that in Group I patients, miR-204 concentration increased from RA → PA → PCW (log fold change slope = 0.22 [95% CI = 0.06–0.37], $P = 0.008$), while this was not true in Group II patients (log concentration slope −0.13 [95% CI = −0.26–0.01], $P = 0.06$) or patients without PH (slope 0.02 [95% CI = −0.21–0.24], $P = 0.9$). In contrast, there was no difference in miR-208 fold-change gradient in Group I compared to Group II PH (interaction $P = 0.2$) using the same model (Fig. 2b, 2d).

We sought to determine if quantification of miR-204 expression at these anatomic locations correlated with hemodynamic parameters. We report Spearman correlations between location-restricted miR-204 expression levels and

Table 1. Patient demographics by PH classification.

	Total	No PH	Group I	Group II	P value
n	91	22	47	22	
Age (years) (±SD)	58.5 ± 13.4	57.3 ± 12.1	55.5 ± 13.9	66.0 ± 10.8	**0.008**
Sex					0.81
Female	58 (64)	15 (68)	30 (64)	13 (59)	
Male	33 (36)	7 (32)	17 (36)	9 (41)	
Race					0.88
White	75 (82)	18 (82)	39 (83)	18 (82)	
Black	11 (12)	3 (14)	5 (11)	3 (14)	
Other/unknown	5 (6)	1 (4)	3 (6)	1 (4)	

Values are presented as (n(%)) unless otherwise specified.
PH, pulmonary hypertension. P values ≤ 0.05 are bolded.

Table 2. Hemodynamic parameters and laboratory findings by pulmonary hypertension (PH) classification.

|	Total	No PH	Group I	Group II	P value
n	91	22	47	22	
RA (mmHg)	9.3 ± 7.8	7.3 ± 5.1	7.7 ± 4.7	14.9 ± 12.3	<**0.001**
Systolic PA (mmHg)	61.0 ± 26.4	29.8 ± 6.3	72.4 ± 21.1	67.6 ± 24.7	<**0.001**
Diastolic PA (mmHg)	23.1 ± 10.2	13.0 ± 4.7	26.8 ± 8.7	25.3 ± 10.8	<**0.001**
mPA (mmHg)	37.2 ± 15.5	19.2 ± 5.0	43.5 ± 12.9	41.7 ± 13.8	<**0.001**
PCWP (mmHg)	13.2 ± 6.9	11.0 ± 5.6	10.7 ± 5.1	20.8 ± 5.9	<**0.001**
PVR (WU)	4.5 ± 3.2	1.5 ± 0.7	6.4 ± 3.2	3.7 ± 1.6	<**0.001**
Peripheral SaO$_2$ (%)	96.0 ± 2.8	96.7 ± 2.2	95.8 ± 3.0	96.0 ± 2.7	0.51
Cardiac output (L/min)	5.8 ± 1.9	6.0 ± 1.5	5.8 ± 2.3	5.6 ± 1.3	0.77
Cardiac index (L/min/m^2)	2.9 ± 1.0	3.1 ± 0.9	3.0 ± 1.1	2.7 ± 0.6	0.35
Creatinine, plasma (mg/dL)	1.2 ± 0.9	0.9 ± 0.3	1.2 ± 0.9	1.5 ± 1.2	**0.05**
Creatinine clearance* (mL/min/1.73 m^2)	72.1 ± 28.4	84.5 ± 21.6	74.6 ± 29.1	54.3 ± 24.9	<**0.001**
Sodium, plasma (mmol/L)	137.6 ± 4.1	138.7 ± 3.0	137.2 ± 4.6	137.2 ± 3.9	0.33
Potassium, plasma (mEq/L)	4.0 ± 0.5	4.1 ± 0.4	4.0 ± 0.5	4.1 ± 0.5	0.35
Blood urea nitrogen (mg/dL)	21.6 ± 14.3	15.1 ± 5.2	21.3 ± 13.7	28.8 ± 18.3	**0.005**
Hemoglobin, plasma (g/dL)	13.0 ± 1.8	13.7 ± 1.7	12.9 ± 1.9	12.4 ± 1.6	0.07

Values are mean ± SD. P values ≤ 0.05 are bolded.
Pressures are end-expiratory means unless otherwise noted.
*Calculated from serum creatinine and anthropometrics by the CKD-Epi equation.[54]
RA, right atrium; PA, pulmonary artery; PCWP, pulmonary capillary wedge pressure; PVR, pulmonary vascular resistance. P values ≤ 0.05 are bolded.
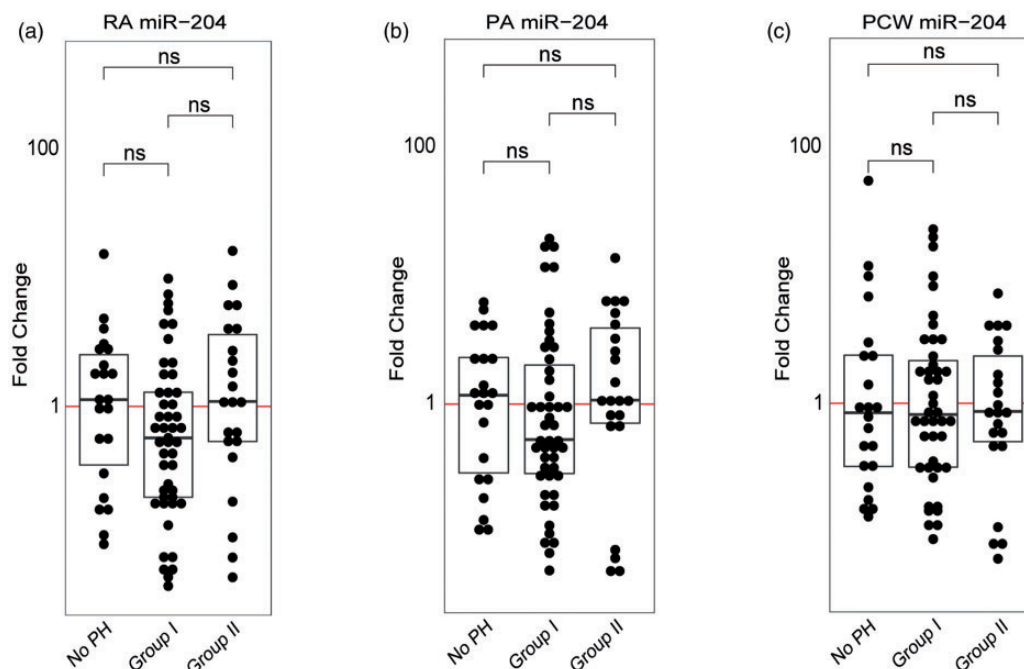
Fig. 1. Comparison of miR-204 concentration by anatomical site across the pulmonary circulation of Groups I and II PH patients. Using RT-qPCR, miR-204 expression was quantified from plasma derived from the (a) RA/SVC, (b) PA, and (c) PCWP. At the RA and PA locations, median miR-204 concentration was higher in Group II vs. Group I PAH patients, although there was a high degree of variability and a pair-wise comparison was not statistically significant. Overlaid boxes show median along with 1st and 3rd quartiles. At each anatomic site, Wilcox rank-sum tests were used to make pair-wise comparisons between PH groups.

clinical parameters in Table 3. Overall, the strength of the correlations were modest; however, clear negative correlations were observed between cardiac output and index and miR-204 levels at all anatomic sites.

To further characterize the relation between PH group and miR-204 gradient in the pulmonary vasculature, we examined the receiver operating characteristics (ROC) of miR-204 gradient to detect the presence of Group I PAH (Fig. 3a). The area under the curve was 0.72, suggesting modest test characteristics. In contrast, miR-208 gradients across the pulmonary vasculature showed poor discriminatory ability for Group I disease (Fig. 3b).

In a comparison of both cellular and extracellular miR-204 from PAH patient-derived PASMCS (PAH-PASMCs derived from a 53-year-old female scleroderma patient on intravenous prostacyclin with mPAP 53 mmHg and PVR 4.4 WU) versus control (PASMCs derived from a 40-year-old woman) in vitro, we examined whether the miR-204 progressively increasing expression gradient observed in pulmonary vascular bed plasma could be consistent with disease-relevant PASMC release. Intracellular miR-204 levels were decreased in PAH-PASMCs compared with control ($P = 0.0004$) (Fig. 4a). Conversely, extracellular miR-204 levels were normalized to total cellular RNA content in culture in order to account for any differences in cellular content among comparators and were found to be increased in PAH-PASMC cultures as compared with control ($P = 0.0018$) (Fig. 4b). No significant differences in

expression profiles of either the cells or media were evident for miR-208 (Fig. 4c, 4d).

Finally, to determine if the differences in the plasma miR-204 gradient correlated with miR-204 levels in blood-borne cells, we quantified miR-204 expression levels in isolated PBMCs from Group I and II PH patients compared with control. No significant differences were observed among groups for miR-204 or miR-208 (Fig. 4e, 4f).

Discussion

In this study, we quantified the levels of circulating miRNA, miR-204, across the pulmonary vascular bed in patients with well-phenotyped PH subtypes. We found that miR-204, previously described as being downregulated in PASMCs of PAH patients,[20] specifically increased in concentration across the pulmonary vascular bed in patients with Group I PAH. Our in vitro studies of diseased PASMCs suggest that cellular excretion of miR-204 is a plausible mechanism for this finding. The stepwise increase in concentration was not seen for Group II PH patients. Considering the known importance of this miRNA in PH,[20–23] its tissue specificity, and its unique upward elevation across the pulmonary circulation, such findings implicate differences in muscle-specific pathobiology between Group I and II PH subtypes.

The feasibility of using circulating miRNA concentration gradients across a vascular bed to make inferences about
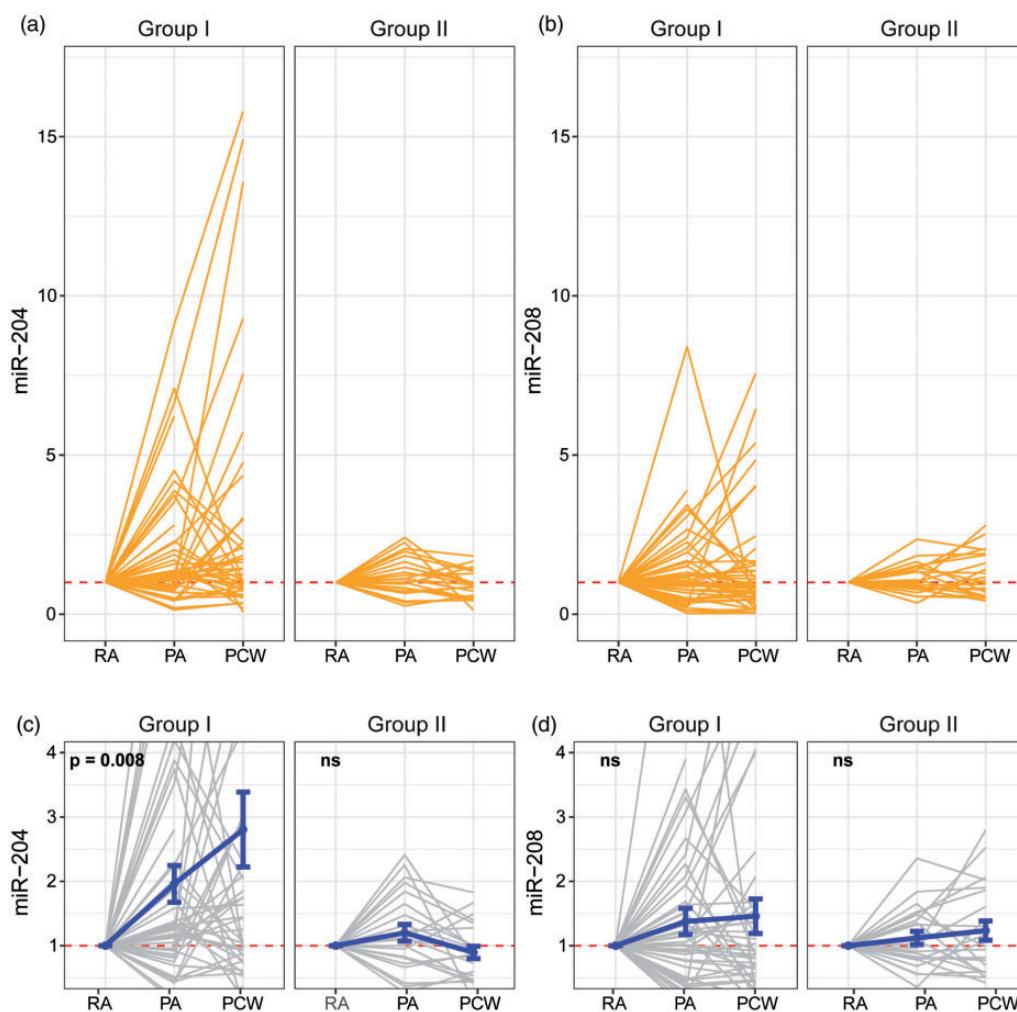
Fig. 2. Relative concentration of miRNAs across the right ventricular-pulmonary artery vascular bed in Group I and II PH patients. Anatomical site fold change values were computed for levels of circulating (a) miR-204 and (b) miR-208 for patients classified as Group I and II PH using RT-PCR, expressed as a ratio normalized to the RA concentration for each patient. Each individual line is a unique patient. (c, d) Group averages, with standard error, are shown for each disease group and miRNA. Circulating miR-204 displayed a significant upward trend across the serial anatomic positions in Group I PAH, but not Group II PH patients. No similar trend was observed for c-miR-208 in either PH group. *P* values represent testing against the null hypothesis of a zero slope using models described in the text.

vascular tissue miRNA release has been previously demonstrated in prior investigations in the coronary circulation.[42] In our study, elevated plasma levels of miR-204 in the pulmonary capillary bed and pulmonary veins (as drawn from the PCW position) compared to RA concentrations (Fig. 2) correlate with prior observations that miR-204 is decreased intracellularly in PASMCs in Group I PAH.[20] Such reciprocal directions of intracellular and extracellular miRNAs are not without precedent, as aerobically exercising skeletal muscle tissue has been shown to release specific miRNAs to allow for a more efficient reduction of miRNA activity in the intracellular space of source tissue.[37] Similarly, our findings offer the foundation for a tempting molecular explanation, whereby a net balance favoring release of miR-204 and increase of this miRNA across the transpulmonary vascular gradient in Group I PAH may be instrumental in facilitating the known pathogenic decrease of intracellular miR-204 in

pulmonary vascular smooth muscle cells in PAH.[20] To explain the relatively low levels of miR-204 observed in our study at the RA/SVC position, specific but as-of-yet unknown molecular controls of degradation, clearance, or uptake of circulating miR-204 may be at play as well.

Conversely, given the substantial stress on left ventricular function in most forms of Group II PH,[12] one could speculate that more robust release of miR-204 from the strained left ventricle could also help to maintain miR-204 levels throughout the circulation. In turn, this may generate a less favorable gradient for pulmonary vascular muscle release in Group II PH. However, it is notable that the average miR-204 pulmonary gradient was flat not only in Group II PH patients but also in persons without PH (Fig. 2), thus favoring the notion that a specific pathobiology of miR-204 unique to Group I PAH pulmonary vasculature may be a more prominent driver of the difference among PH

Table 3. Spearman correlation coefficients for miR-204 concentrations from the right atrium, pulmonary artery, and the pulmonary capillary wedge position vs. hemodynamic and echocardiographic parameters.

	Right atrium		Pulmonary artery		Pulmonary capillary wedge	
	ρ	P value	ρ	P value	ρ	P value
RA (mmHg)	0.0126	0.91	0.0289	0.79	−0.0526	0.63
Systolic PA (mmHg)	**−0.2835**	**0.007**	**−0.2525**	**0.02**	−0.1883	0.08
Diastolic PA (mmHg)	−0.1684	0.11	−0.1188	0.26	−0.0828	0.45
mPA (mmHg)	**−0.2596**	**0.01**	**−0.2156**	**0.04**	−0.1746	0.11
PCWP (mmHg)	0.0856	0.42	0.0797	0.45	−0.0281	0.80
PVR (WU)	−0.1932	0.07	−0.115	0.28	−0.0266	0.81
Cardiac output (L/min)	**−0.2525**	**0.02**	**−0.299**	**0.005**	**−0.3509**	**0.001**
Cardiac index (L/min/m^2)	**−0.2662**	**0.02**	**−0.2893**	**0.008**	**−0.3272**	**0.003**
RV size*	−0.2012	0.06	−0.1898	0.08	−0.0990	0.37
RV function*	−0.1077	0.31	−0.0584	0.58	−0.0064	0.95
RA size*	−0.0913	0.40	−0.1376	0.20	−0.0876	0.43

*Right ventricular (RV) and right atrial (RA) parameters were based on echocardiographic assessment and treated as an ordered categorical variable with possible values in ascending order of normal, mildly abnormal, moderately abnormal, or severely abnormal.

PA, pulmonary artery; PCWP, pulmonary capillary wedge pressure; PVR, pulmonary vascular resistance. P values ≤ 0.05 are bolded.
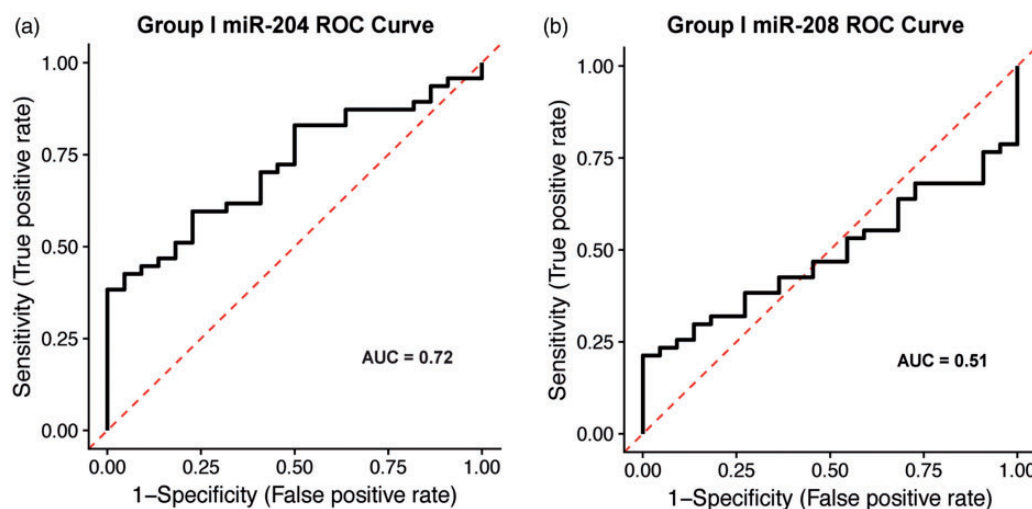

Fig. 3. Receiver operating characteristic curves. The maximum fold change value from PA or PCW to RA/SVC was calculated and its discriminatory ability to detect Group I vs. Group II disease was tested. MiR-204 gradient (a) showed modest discriminatory characteristics; conversely, miR-208 gradient (b) did not distinguish Group I from II disease.

subtypes. Future studies that can differentiate the release versus degradation and uptake of miR-204 will be instrumental in defining the key molecular aspects of this model. In contrast to miR-204, miR-208, although influential in stages of RV pathology in PH,[31] did not show the same differential response to Group I versus Group II disease. The underlying molecular mechanism for this observation remains unclear, but the explanation may rest in the tissue specificity of miRNA release kinetics from diseased PASMCs versus cardiomyocyte tissue.

Although we propose that the primary contributor to this finding of disease mediated miR-204 excretion in Group I

PAH appear to be PASMCs (Fig. 4), we do not fully discount the potential release of miR-204 by other cell types across the vascular bed. While our data indicate that dynamic alterations of miR-204 do not appear robustly in blood-borne immune and inflammatory cells (Fig. 4e, 4f), it is plausible that contributing slightly to this plasma expression gradient could include nearby pulmonary artery endothelial cells (PAECs) that undergo apoptosis and dysfunction throughout the progression of PAH.[43] Despite miR-204 being regarded as a muscle-specific miRNA, prior studies demonstrate that expression of this molecule in cultured human PAECs occurs at detectable levels, albeit at
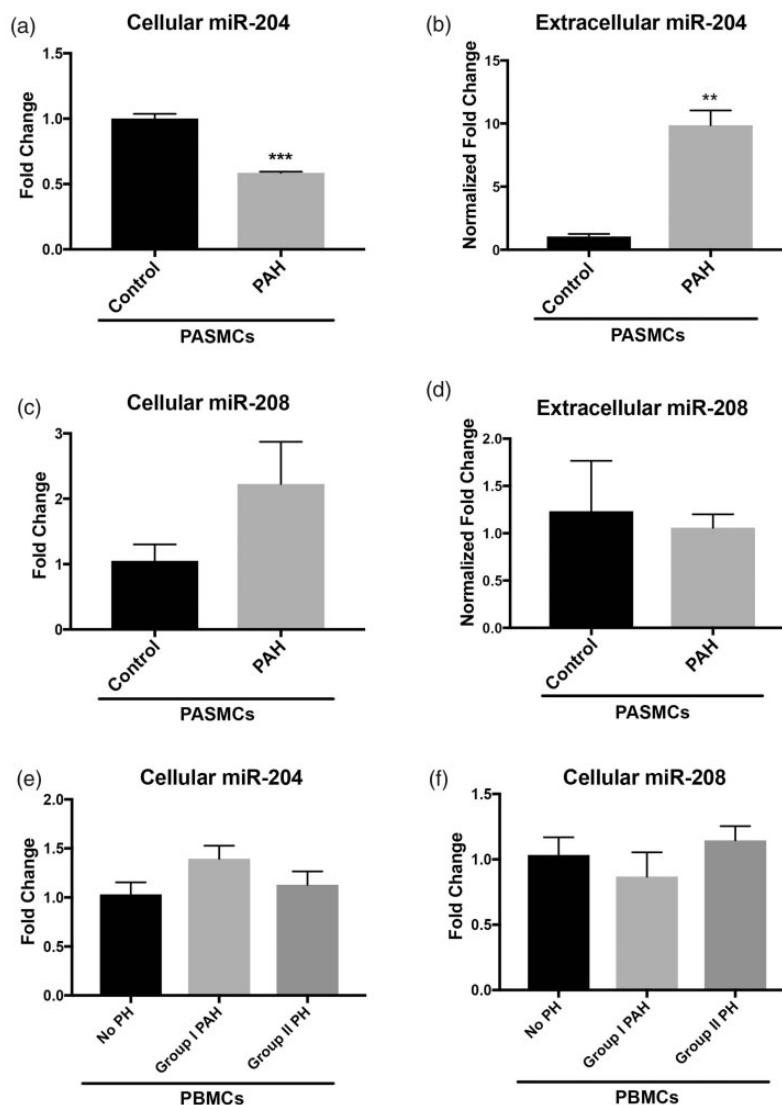
Fig. 4. Comparison of miRNA levels in PAH patient-derived pulmonary artery smooth muscle cells and peripheral blood mononuclear cells. From both diseased (n = 3) and control (n = 3) PASMC cultures, by RT-qPCR, intracellular (a) and extracellular (b) miR-204 levels as well as intracellular (c) and extracellular (d) miR-208 levels were quantified. Intracellular miR-204 levels were decreased in PAH-PASMCs compared with control. Conversely, extracellular miR-204 levels were found to be increased in PAH-PASMC cultures as compared with control. No significant differences in expression profiles of either the cells or media were evident for miR-208. Mean miRNA levels in control cells (no PH) were normalized to fold change of 1, to which relevant samples were compared. Notably, in panels (b, d), extracellular levels were normalized to total intracellular RNA in cultured cells in order to account for any differences in cellular content among comparators. In peripheral blood mononuclear cells (PBMC), intracellular content of (E) miR-204 and (F) miR-208 did not display significant variations in patients with Group I (n = 5) PH, Group II (n = 5) PH, and no PH (n = 5). Data represent mean ± SEM (*P < 0.05, **P < 0.01, ***P < 0.001).

approximately seven times less than in cultured human PASMCs.[20] Therefore, although its presence in this cell type appears minimal, some miR-204 excretion from human PAECs, and potentially other cell types, could be possible.

The correlation coefficients in Table 3 do not offer consistent evidence that circulating miR-204 increases with disease severity. Indeed, we found that lower cardiac output (which may be evidence of more advanced disease) is associated with higher miR-204 levels, albeit the correlation was

modest. However, we also found that there is a subtle negative correlation between PA pressure and circulating miR-204 concentration. The reasons for these seemingly contradictory relations are unclear. It is possible that the hypothesized miR-204 excretion by PASMCs is accelerated early in disease process but slows as disease moves into advanced stage, as evidenced by very high PA pressure. It is also possible that miR-204 is affected by treatment with PH-specific medications, more likely to be used in advanced cases. Finally, impaired clearance might explain the

association between low cardiac output and higher serum concentrations. These hypotheses offer avenues for further study.

We did not have sufficient power to test whether miR-204 levels or miR-204 pulmonary gradients were different within Group I for patients with more versus less advanced disease. This is a limitation of our study and could be the basis for future investigations. It also should be acknowledged that in contradistinction to previous work showing high levels of circulating miR-204 in the PA of patients with elevated PAP compared to patients with normal PAP,[29] we found similar overall concentrations of miR-204 in patients with no PH, Group I, and Group II disease. There may be several possible explanations for this discrepancy, including the fact that the previously studied cohort included a heterogeneous PH population and defined the presence or absence of disease on the basis of PAP alone.

Beyond the molecular details, these differences of miR-204 profiles suggest the applicability of this circulating miRNA as a potential tool for PH group differentiation. While hemodynamic stratification of Group II PH patients may be helpful,[44] there still exists substantial confusion in distinguishing Groups I and II PH, thereby affecting not only clinical management but also appropriate stratification of patients for clinical trial design in this large but heterogeneous patient population. Other blood-borne biomarkers for Group II PH have been proposed, such as endothelin-1,[45] but without adequate specificity. Only a few circulating miRNAs[46] have been reported in this context to date, but unlike miR-204 in this study, no others have been quantified across the trans-pulmonary vascular gradient and thus with direct mechanistic links to miRNA release. How miR-204 gradients in the pulmonary circulation might be responsive to various disease conditions as reflected through non-invasive imaging[47–50] or exercise testing[51–53] remains to be seen and may be an intriguing avenue for investigation, particularly if such analytic combinations could improve the modest receiver operator characteristics of miR-204 gradients alone (Fig. 3).

We note several limitations in our study. Further work is necessary to expand upon the relatively small sample size and contained geographic and demographic focus in this study; however, we note that despite the moderate sample size, patients in this study all have PH phenotypes well described by clinical specialists across two PH referral centers. Our sample was not large enough, however, to analyze gradient variation by disease severity strata in Groups I and II separately. In our investigation of downstream miR-204 expression in peripheral blood by analyzing PBMCs, we had to obtain samples from patients outside of our main cohort of study, as these same samples from our original patients were not available. Additionally, we were also unable to obtain matching peripheral venous plasma from our main cohort of patients, and thus we could not rule out the possibility of distinct miR-204 profiles of WHO PH subgroups in the periphery. Therefore, future studies including a larger

variety of samples coming from the same cohort of individuals would likely provide a fuller explanation of miRNA expression relationships occurring in different vascular regions. Further, typical technical challenges drawing samples from a truly occlusive PCW position may contribute to variability in that measurement. Insight into circulating miRNA expression profiles that differ based on the extent of disease could merit studies in the future. Lastly, our study was focused on Group I and II subsets of PH, but inclusion of patients qualifying for the remainder of WHO classifications may uncover novel findings differentiating these subtypes further.

In conclusion, we report a unique and stepwise elevation of circulating miR-204 levels along a gradient though the pulmonary circulation in patients with Group I PAH, but not in patients with Group II PH. We also report an extension of reported models of depleted miR-204 in diseased PASMCs of Group I PAH, by demonstrating the release of this molecule into the media of cultured disease cells. This finding could also partly explain the establishment of the sequential increase of expression across the pulmonary vascular bed that we associate with Group I disease. Considering the known importance of miR-204 in PH, this gradient offers a molecular foundation for discerning specific pathobiologies between these PH subtypes, with potential implications for designing future diagnostic strategies to better differentiate these clinical groups.

Conflict of interest

Funding

ORCID iD

Michael V. Genuardi ⓘ http://orcid.org/0000-0002-8008-7526

References

1. Simonneau G, Montani D, Celermajer DS, et al. Haemodynamic definitions and updated clinical classification of pulmonary hypertension. *Eur Respir J* 2019; 53: pii: 1801913.
2. Galie N, Humbert M, Vachiery JL, et al. 2015 ESC/ERS Guidelines for the Diagnosis and Treatment of Pulmonary Hypertension. *Rev Esp Cardiol (Engl Ed)* 2016; 69: 177.
3. Galie N, Humbert M, Vachiery JL, et al. 2015 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension: The Joint Task Force for the Diagnosis and Treatment of Pulmonary Hypertension of the European Society of

Cardiology (ESC) and the European Respiratory Society (ERS): Endorsed by: Association for European Paediatric and Congenital Cardiology (AEPC), International Society for Heart and Lung Transplantation (ISHLT). *Eur Heart J* 2016; 37: 67–119.

4. Simonneau G, Gatzoulis MA, Adatia I, et al. Updated clinical classification of pulmonary hypertension. *J Am Coll Cardiol* 2013; 62: D34–41.

5. Price LC, Wort SJ, Perros F, et al. Inflammation in pulmonary arterial hypertension. *Chest* 2012; 141: 210–221.

6. Groth A, Vrugt B, Brock M, et al. Inflammatory cytokines in pulmonary hypertension. *Respir Res* 2014; 15: 47.

7. Rabinovitch M, Guignabert C, Humbert M, et al. Inflammation and immunity in the pathogenesis of pulmonary arterial hypertension. *Circ Res* 2014; 115: 165–175.

8. Farber HW, Miller DP, Poms AD, et al. Five-year outcomes of patients enrolled in the REVEAL Registry. *Chest* 2015; 148: 1043–1054.

9. Bossone E, D'Andrea A, D'Alto M, et al. Echocardiography in pulmonary arterial hypertension: from diagnosis to prognosis. *J Am Soc Echocardiogr* 2013; 26: 1–14.

10. Hoeper MM, Bogaard HJ, Condliffe R, et al. Definitions and diagnosis of pulmonary hypertension. *J Am Coll Cardiol* 2013; 62: D42–50.

11. Vanderpool RR, Saul M, Nouraie M, et al. Association between hemodynamic markers of pulmonary hypertension and outcomes in heart failure with preserved ejection fraction. *JAMA Cardiol* 2018; 3: 298–306.

12. Berthelot E, Bailly MT, Hatimi SE, et al. Pulmonary hypertension due to left heart disease. *Arch Cardiovasc Dis* 2017; 110: 420–431.

13. Vachiery JL, Adir Y, Barbera JA, et al. Pulmonary hypertension due to left heart diseases. *J Am Coll Cardiol* 2013; 62: D100–108.

14. Min PK and Chan SY. The biology of circulating microRNAs in cardiovascular disease. *Eur J Clin Invest* 2015; 45: 860–874.

15. Chun HJ, Bonnet S and Chan SY. Translating microRNA biology in pulmonary hypertension: it will take more than "miR" words. *Am J Respir Crit Care Med* 2016; 195: 167–178.

16. Chen J, Hu C and Pan P. Extracellular vesicle MicroRNA transfer in lung diseases. *Front Physiol* 2017; 8: 1028.

17. Creemers EE, Tijsen AJ and Pinto YM. Circulating microRNAs: novel biomarkers and extracellular communicators in cardiovascular disease? *Circ Res* 2012; 110: 483–495.

18. Hale A, Lee C, Annis S, et al. An Argonaute 2 switch regulates circulating miR-210 to coordinate hypoxic adaptation across cells. *Biochim Biophys Acta* 2014; 1843: 2528–2542.

19. Deng L, Blanco FJ, Stevens H, et al. MicroRNA-143 activation regulates smooth muscle and endothelial cell crosstalk in pulmonary arterial hypertension. *Circ Res* 2015; 117: 870–883.

20. Courboulin A, Paulin R, Giguere NJ, et al. Role for miR-204 in human pulmonary arterial hypertension. *J Exp Med* 2011; 208: 535–548.

21. Meloche J, Potus F, Vaillancourt M, et al. Bromodomain-containing protein 4: the epigenetic origin of pulmonary arterial hypertension. *Circ Res* 2015; 117: 525–535.

22. Potus F, Graydon C, Provencher S, et al. Vascular remodeling process in pulmonary arterial hypertension, with focus on miR-204 and miR-126 (2013 Grover Conference series). *Pulm Circ* 2014; 4: 175–184.

23. Zhou G, Chen T and Raj JU. MicroRNAs in pulmonary arterial hypertension. *Am J Respir Cell Mol Biol* 2015; 52: 139–151.

24. Bertero T, Lu Y, Annis S, et al. Systems-level regulation of microRNA networks by miR-130/301 promotes pulmonary hypertension. *J Clin Invest* 2014; 124: 3514–3528.

25. Yu H, Xu M, Dong Y, et al. 1,25(OH)2D3 attenuates pulmonary arterial hypertension via microRNA-204 mediated Tgfbr2/Smad signaling. *Exp Cell Res* 2018; 362: 311–323.

26. Lee C, Mitsialis SA, Aslam M, et al. Exosomes mediate the cytoprotective action of mesenchymal stromal cells on hypoxia-induced pulmonary hypertension. *Circulation* 2012; 126: 2601–2611.

27. Bockmeyer CL, Maegel L, Janciauskiene S, et al. Plexiform vasculopathy of severe pulmonary arterial hypertension and microRNA expression. *J Heart Lung Transplant* 2012; 31: 764–772.

28. Meloche J, Lampron MC, Nadeau V, et al. Implication of inflammation and epigenetic readers in coronary artery remodeling in patients with pulmonary arterial hypertension. *Arterioscler Thromb Vasc Biol* 2017; 37: 1513–1523.

29. Wei C, Henderson H, Spradley C, et al. Circulating miRNAs as potential marker for pulmonary hypertension. *PloS One* 2013; 8: e64396.

30. Corsten MF, Dennert R, Jochems S, et al. Circulating MicroRNA-208b and MicroRNA-499 reflect myocardial damage in cardiovascular disease. *Circ Cardiovasc Genet* 2010; 3: 499–506.

31. Paulin R, Sutendra G, Gurtu V, et al. A miR-208-Mef2 axis drives the decompensation of right ventricular function in pulmonary hypertension. *Circ Res* 2015; 116: 56–69.

32. Kovacs G, Avian A, Olschewski A and Olschewski H. Zero reference level for right heart catheterisation. *Eur Respir J* 2013; 42: 1586–1594.

33. Florentin J, Coppin E, Vasamsetti SB, et al. Inflammatory macrophage expansion in pulmonary hypertension depends upon mobilization of blood-borne monocytes. *J Immunol* 2018; 200: 3612–3625.

34. Goncharov DA, Kudryashova TV, Ziai H, et al. Mammalian target of rapamycin complex 2 (mTORC2) coordinates pulmonary artery smooth muscle cell metabolism, proliferation, and survival in pulmonary arterial hypertension. *Circulation* 2014; 129: 864–874.

35. Kudryashova TV, Goncharov DA, Pena A, et al. HIPPO-integrin-linked kinase cross-talk controls self-sustaining proliferation and survival in pulmonary hypertension. *Am J Respir Crit Care Med* 2016; 194: 866–877.

36. Shah JS, Soon PS and Marsh DJ. Comparison of methodologies to detect low levels of hemolysis in serum for accurate assessment of serum microRNAs. *PloS One* 2016; 11: e0153200.

37. Ramos AE, Lo C, Estephan L, et al. Specific circulating microRNAs display dose-dependent responses to variable intensity and duration of endurance exercise. *Am J Physiol Heart Circ Physiol* 2018; 315: H273–283.

38. Nielsen S, Akerstrom T, Rinnov A, et al. The miRNA plasma signature in response to acute aerobic exercise and endurance training. *PloS One* 2014; 9: e87308.

39. Baggish AL, Hale A, Weiner RB, et al. Dynamic regulation of circulating microRNA during acute exhaustive exercise and sustained aerobic exercise training. *J Physiol* 2011; 589: 3983–3994.

40. Baggish AL, Park J, Min PK, et al. Rapid upregulation and clearance of distinct circulating microRNAs after prolonged aerobic exercise. *J Appl Physiol (1985)* 2014; 116: 522–531.

41. Min PK, Park J, Isaacs S, et al. Influence of statins on distinct circulating microRNAs during prolonged aerobic exercise. *J Appl Physiol (1985)* 2016; 120: 711–720.

42. De Rosa S, Fichtlscherer S, Lehmann R, et al. Transcoronary concentration gradients of circulating microRNAs. *Circulation* 2011; 124: 1936–1944.

43. Jurasz P, Courtman D, Babaie S, et al. Role of apoptosis in pulmonary hypertension: from experimental models to clinical trials. *Pharmacol Ther* 2010; 126: 1–8.

44. Fang JC, DeMarco T, Givertz MM, et al. World Health Organization Pulmonary Hypertension group 2: pulmonary hypertension due to left heart disease in the adult–a summary statement from the Pulmonary Hypertension Council of the International Society for Heart and Lung Transplantation. *J Heart Lung Transplant* 2012; 31: 913–933.

45. Giaid A, Yanagisawa M, Langleben D, et al. Expression of endothelin-1 in the lungs of patients with pulmonary hypertension. *N Engl J Med* 1993; 328: 1732–1739.

46. Jin P, Gu W, Lai Y, et al. The circulating microRNA-206 level predicts the severity of pulmonary hypertension in patients with left heart diseases. *Cell Physiol Biochem* 2017; 41: 2150–2160.

47. Freed BH, Collins JD, Francois CJ, et al. MR and CT imaging for the evaluation of pulmonary hypertension. *JACC Cardiovasc Imaging* 2016; 9: 715–732.

48. Johns CS, Kiely DG and Swift AJ. Novel imaging techniques in pulmonary hypertension. *Curr Opin Cardiol* 2018; 33: 587–593.

49. Rajiah P. The evolving role of MRI in pulmonary hypertension evaluation: a noninvasive approach from diagnosis to follow-up. *Radiology* 2018; 289: 69–70.

50. Rengier F, Melzig C, Derlin T, et al. Advanced imaging in pulmonary hypertension: emerging techniques and applications. *Int J Cardiovasc Imaging* 2018; Epub ahead of print.

51. Dumitrescu D, Nagel C, Kovacs G, et al. Cardiopulmonary exercise testing for detecting pulmonary arterial hypertension in systemic sclerosis. *Heart* 2017; 103: 774–782.

52. Farina S, Correale M, Bruno N, et al. The role of cardiopulmonary exercise tests in pulmonary arterial hypertension. *Eur Respir Rev* 2018; 27: 170134.

53. Zhao Q, Liu Z, Ma X, et al. [Value of cardiopulmonary exercise testing in comparing cardio-pulmonary function among patients with three kinds of pulmonary artery hypertension]. *Zhonghua Yi Xue Za Zhi* 2015; 95: 3598–3601.

54. Levey AS, Stevens LA, Schmid CH, et al. A new equation to estimate glomerular filtration rate. *Ann Intern Med* 2009; 150: 604–612.