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Kynurenine–PARP-1 Link Mediated by MicroRNA 210 May Be Dysregulated in Pulmonary Hypertension

ORIGINAL INVESTIGATION

ABSTRACT

Background: Dysregulation of microRNAs is associated with pulmonary hypertension. The present study aimed to determine the alterations in microRNA and microRNA expressions and their role in signaling pathways and investigate the relationship with serum levels of apelin, kynurenine, and endocan in pulmonary hypertension.

Methods: The study design was prospective and single-centered. The study included 32 consecutive treatment-naive patients with precapillary pulmonary hypertension and 55 age and sex-matched healthy controls. All subjects underwent right heart catheterization. mRNA expressions of hypoxia-inducible factor-1 alpha, hypoxia-inducible factor-2 alpha, signal transducer and activator of transcription-3, fibroblast growth factor-2, fibroblast growth factor receptor-1, and poly-ADP-ribose polymerase-1 and microRNA expressions of miRNA-210, miRNA-130a, miRNA-424, miRNA-204, and miRNA-223 were determined by RT-PCR. Concentrations of kynurenine, apelin, and endocan were analyzed by ELISA.

Results: mRNA expressions of hypoxia-inducible factor-2 alpha, signal transducer and activator of transcription-33, and FGF-2 were increased; miRNA-210 and miRNA-130a were increased; miRNA-223 and miRNA-204 were decreased in pulmonary hypertension. Apelin and kynurenine concentrations were decreased in pulmonary hypertension. There were positive correlations: hypoxia-inducible factor-2 alpha-miRNA-424, Apelin-miRNA-424, kynurenine-miRNA-210, signal transducer and activator of transcription-3-PVR, miRNA-210-right atrial pressure, and kynurenine-right atrial pressure. There were negative correlations: poly-ADP-ribose polymerase-1-miRNA-210 and poly-ADP-ribose polymerase-1-right atrial pressure. On multiple logistic regression analyses, miRNA-130a and Apelin were independent risk factors for PH.

Conclusions: We report a novel relationship between the kynurenine and poly-ADP-ribose polymerase-1 signaling pathways that could be mediated by miRNA-210. We also report a connection between the Apelin and hypoxia-inducible factor-2 alpha signaling pathways that could be mediated by miRNA-424. Reduced levels of Apelin and elevated levels of miRNA-130a are associated with pulmonary hypertension. We also find that elevated levels of signal transducer and activator of transcription-3, miRNA-210, and kynurenine and reduced levels of poly-ADP-ribose polymerase-1 correlate with more severe hemodynamics.

Keywords: MicroRNA, kynurenine, poly-ADP-ribose polymerase-1, pulmonary hypertension

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INTRODUCTION

Despite significant advancements in the treatment strategies in the past years, morbidity and mortality remain high for pulmonary hypertension (PH). Pulmonary hypertension is a complex disease with remodeling in pulmonary arterial endothelial cells (PAEC), pulmonary arterial smooth muscle cells (PASMC), and adventitial fibroblasts. This is evidenced by a series of events in the pulmonary vascular system that lead to hyperproliferation, vasoconstriction, and smooth muscle hypertrophy.[1] The current PH medications do not target the elusive upstream molecular origins of PH. Discoveries have identified the dysregulation of microRNAs (miRNAs) linked to the hyperproliferative phenotypes of those cells. MicroRNA is composed of small, RNA molecules that cannot code by themselves

but affect gene expression. Approximately 5500 varieties have been identified in the human genome. Excessive vascular proliferation was observed in patients with decreased miRNA-204 expression after signal transducer and activator of transcription-3 (STAT-3) activation in pulmonary arterial hypertension (PAH).[2] It has been demonstrated that miRNAs-424 and -503 were downregulated after increased fibroblast growth factor receptor-1 (FGFR-1) expression in PAEC, which then resulted in increased proliferation in PAEC and PASMC.[2] Increased poly-ADP-ribose polymerase-1 (PARP-1) was associated with DNA damage and decreased miRNA-223.[2] It has been observed that endothelial dysfunction was related to the Apelin (APLN)–APJ axis in PAH.[3] Increased plasma levels of kynurenine have been found in PAH.[4] Endocane levels were increased in rats with monocrotaline (MCT)-induced PAH.[5] We aimed to evaluate the most commonly studied miRNAs in PH together with their transcriptional factors. We also studied related proteins and their role in signaling pathways and correlated their levels with PH severity.

METHODS

Study Design, Inclusion, and Exclusion Criteria

The study design was prospective and single-centered. Thirty-two treatment-naive patients aged 18-80 years with precapillary pulmonary hypertension (group 1 PH) and chronic thromboembolic pulmonary hypertension (CTEPH) from a cardiology out-patient clinic were included in the study between January 1, 2018, and June 17, 2019. The control group, which comprised 55 age- and sex- matched healthy participants who presented to a medical out-patient clinic for routine annual physicals, was chosen in a consecutive manner without exclusion criteria. Right heart catheterization was performed in all cases. Individuals with a known history of left heart disease (low-moderate-normal EF heart failure, severe mitral and aortic valve diseases, a history of cardiomyopathy), lung diseases, advanced renal failure (eGFR <15 mL/min), advanced liver failure, malignancy, multiple risk factors for LV diastolic dysfunction, PH due to pulmonary artery obstructions, and PH with unclear and multifactorial mechanisms were excluded from the study.

HIGHLIGHT

- This study demonstrates relationships between the kynurenine and poly-ADP-ribose polymerase-1 signaling pathways, Apelin and hypoxia-inducible factor-2 alpha signaling pathways, and associations of reduced levels of Apelin and elevated levels of miRNA-130a with pulmonary hypertension. Furthermore, this study demonstrates that elevated levels of signal transducer and activator of transcription-3, miRNA-210, and kynurenine and reduced levels of poly-ADP-ribose polymerase-1 correlate with more severe hemodynamics. These findings would stimulate researchers to further study those signaling pathways in pulmonary hypertension.

Samples and Measurements

RNA was isolated from nucleated blood elements obtained from blood samples. cDNA synthesis was performed for both mRNA and miRNA. mRNA expressions of hypoxia-inducible factor (HIF)-1α, HIF-2α, STAT-3, fibroblast growth factor-2 (FGF-2), FGFR-1, and PARP-1 and miRNA expressions of miRNA-210, miRNA-130a, miRNA-424, miRNA-204, and miRNA-223 were determined by RT-PCR. Total RNA extraction with Trizol was performed for RNA isolation from the samples. RNA isolation from nucleated blood cells for gene expression evaluation was performed with Trizol Reagant according to the manufacturer's kit protocol. The concentration and purity of the isolated RNA were carried out with the aid of the Nanodrop apparatus. The obtained samples were prepared for cDNA synthesis of miRNA for RT-PCR analysis.

For mRNA expression analysis, cDNA synthesis from isolated RNAs was performed according to the manufacturer's protocol using the Transcriptor High Fidelity cDNA synthesis kit (CatNo: 05 081 955 001, Roche, Germany) with oligo d (T) primer and Reversible Transcriptase enzyme (RT). miRNA cDNA Synthesis Kit with Poly (A) Polymerase Tailing (abm) from the patient group and control group was performed. The resulting cDNAs were used to determine targeted mRNA and miRNA expression levels by reacting with specific primers. Blood samples were collected from the control and patient groups centrifuged at 2500 rpm for 10 minutes to obtain serum. The serum was maintained at −20°C until the completion of the study samples. Serum APLN, endocane, and kynurenine levels were determined by using ELISA kit protocols. Human APLN Elabscience commercial kit was used for APLN, while Bioassay Technology Laboratory assay kits were used for kynurenine and endocane.

This study complied with the principles outlined in the Declaration of Helsinki. The study protocol was approved by the Medical Ethics Committee (protocol no. 60116787-020/86982). All participants provided informed consent. Patients were not involved in the design, conduct, reporting, or dissemination plans of our research.

Patient and Public Involvement

Patients were not directly involved in this research.

Statistical Analyses

The sample size was calculated assuming a large effect size of 0.7 between the groups. The resulting sample size was 26 patients for each group. Eighty percent of power would be obtained with 95% confidence. After the study completion, we calculated the effect size considering the number of PH patients (n = 32) and healthy controls (n = 55). The effect size was 1.2 in APLN difference between the groups, and we reached 99.2% power with 95% confidence. All statistical analyses were performed using SPSS 25.0 software. Kolmogorov Smirnov and Shapiro Wilk tests were used for the determination of normal distribution. Continuous variables were defined by the mean ± standard deviation, and categorical variables were determined by number and percent. When parametric test assumptions were provided, the independent samples *t*-test was used to compare independent

group differences. When the parametric test assumptions were not provided, the Mann–Whitney *U*-test was used to compare independent group differences. Besides, the relationships between continuous variables were analyzed by Spearman correlation analysis, and the differences between categorical variables were analyzed by Chi-square analysis. Univariate and multiple logistic regression analysis models were used for determining the risk factors. $P < .05$ was considered statistically significant.

RESULTS

Comparison of basic clinical features, laboratory parameters, and echocardiographic measurements are presented in Table 1. There were no significant differences between the groups in terms of age and gender. mRNA expressions of HIF-2α, STAT-3, and FGF-2 were increased; miRNA-210 and miRNA-130a were increased; miRNA-223 and miRNA-204 were decreased in PH. Apelin and kynurenine concentrations were decreased in PH. There were positive correlations: HIF-2α-miRNA-424: r = 0.474, P = .011; APLN-miRNA-424: r = 0.385, P = .030; kynurenine–miRNA-210: r = 0.551, P = .004; STAT-3-pulmonary vascular resistance (PVR): r = 0.478, P = .006; miRNA-210–right atrial pressure (RAP): r = 0.536, P = .07; kynurenine–RAP: r = 0.409, P = .022. There were negative correlations: PARP-1-miRNA-210: r = (–)0.561, P = .007; PARP-1-RAP: r = (–)0.424, P = .27 (Figures 1 and 2). On multiple logistic regression analyses, miRNA-130a (O.R.= 1.257, P = .016) and APLN (O.R.= 0.223, P = .004) were independent risk factors for PH.

DISCUSSION

The main findings of this study were (1) APLN, and miRNA-130a were associated with PH; (2) altered levels of miRNA-210, kynurenine, and PARP-1 correlate with more severe hemodynamics; and (3) there might be a novel relationship between the kynurenine and PARP-1 signaling pathways that could be mediated by miRNA-210. These findings might expand the current literature as miRNAs have been recognized as promising biomarkers and therapeutic options for many cardiovascular diseases.[6,7]

Recent discoveries have identified the dysregulation of miRNAs in PH. Jin et al[8] showed that miRNA-210 levels increased within 48 h in human PASMC culture exposed to hypoxia. They showed that elevation of miRNA-210 due to hypoxia was associated with a decrease in an iron–sulfur complex in mitochondria during acute hypoxic stress. Similarly, Gou et al[9] demonstrated increased levels of 5 miRNAs out of 265 miRNAs, and that miRNA-210 was the dominant miRNA in human PASMCs. In keeping with those studies, we also found that miRNA-210 expression level was significantly higher in the patient group than in controls. Further studies may target the inhibition of miRNA-210 to reverse the pathophysiological changes. Another study by Meloche et al[10] found that PARP-1 release was associated with DNA damage. They showed that HIF-1α was increased by PARP-1 activation and consequently observed decreased proliferation and apoptosis in PASMC. Simultaneously, both *in vitro* and two large *in vivo* studies with the PARP-1 inhibitors they were able to reverse changes in PAH.[10] We demonstrated that kynurenine was significantly decreased in patients and a moderate positive relationship was found between kynurenine and miRNA-210. Therefore, we think that the kynurenine and PARP-1 signal might be related to miRNA-210. Further studies may focus on PARP-1 signaling and kynurenine pathway to inhibit miRNA-210 to halt the progression of the disease.

Kim et al[11] evaluated the relationship between APLN and FGF-2 and found a decreased APLN signal and increased FGF2 and FGFR1 in PAEC. Increased proliferation in PAEC and PASMC was observed due to decreased APLN and decreased miRNA-424 and miRNA-503. With the restoration of

Table 1. Baseline Clinical, Laboratory, and Hemodynamic Characteristics of Subjects (n = 87)

	Controls (n = 55)	Pulmonary Hypertension (n = 32)	*P*
Age (years)	60 ± 11	60 ± 12	.137
Gender, F (%)	33 (60)	22 (68)	.414
Etiology of PAH, n (%)			
Idiopathic PAH	_	5 (16)	
APAH–CHD	_	4 (12)	
APAH–CTD	_	8 (25)	
CTEPH	_	15 (47)	
6 MWD (m)	_	358.86 ± 129.07	
2	_	17	
WHO FC 3	_	13	
4	_	2	
NT pro-BNP (pg/mL)		1014.71 ± 1893.24	
STAT-3 (ct)	23.1 ± 2.9	24.45 ± 2.6	.034
FGF-2 (ct)	29.5 ± 2.2	30.6 ± 1.6	.031
HIF-2α(ct)	25.4 ± 7.9	29.4 ± 6.7	.005
miR-223 (ct)	23.9 ± 7.0	20.2 ± 6.1	.033
miR-210 (ct)	22.9 ± 6.0	25.4 ± 3.7	.018
miR-130a (ct)	22.3 ± 4.8	27.5 ± 4.4	.000
miR-204 (ct)	24.0 ± 8.7	21.4 ± 6.0	.031
Apelin (ng/mL)	2.55±0.81	1.69 ± 0.61	.000
Kynurenin (ng/mL)	2.29±2.04	1.86 ± 2.15	.015
Pulmonary hemodynamics			
Mean RAP (mm Hg)	_	8.68 ± 5.07	
Mean PVR (WU)	_	6.77 ± 4.06	
Fick Cardiac Index (L/min/m^2)	_	2.77 ± 1.0	
mVO_2 (%)	_	64.02 ± 9.5	

Values are given as percentages or means SD. CTEPH, chronic thromboembolic pulmonary hypertension; CT, cycle threshold; *FGF2*, fibroblast growth factor-2; HIF-2α, hypoxia-inducible factor-2 alpha; mVO_2, Mixed Venous Oxygen Saturation; miR, MicroRNA; NT pro-BNP, N-terminal pro-brain natriuretic peptide; PAH, pulmonary arterial hypertension; APAH–CHD PAH associated with congenital heart disease; APAH–CTD, PAH Associated With Connective Tissue Disease; PVR, pulmonary vascular resistance; RAP, right atrial pressure; STAT-3, Signal Transducer and Activator of Transcription-3; 6 MWD, six-minute walk distance; WHO FC, World Health Organization functional class.

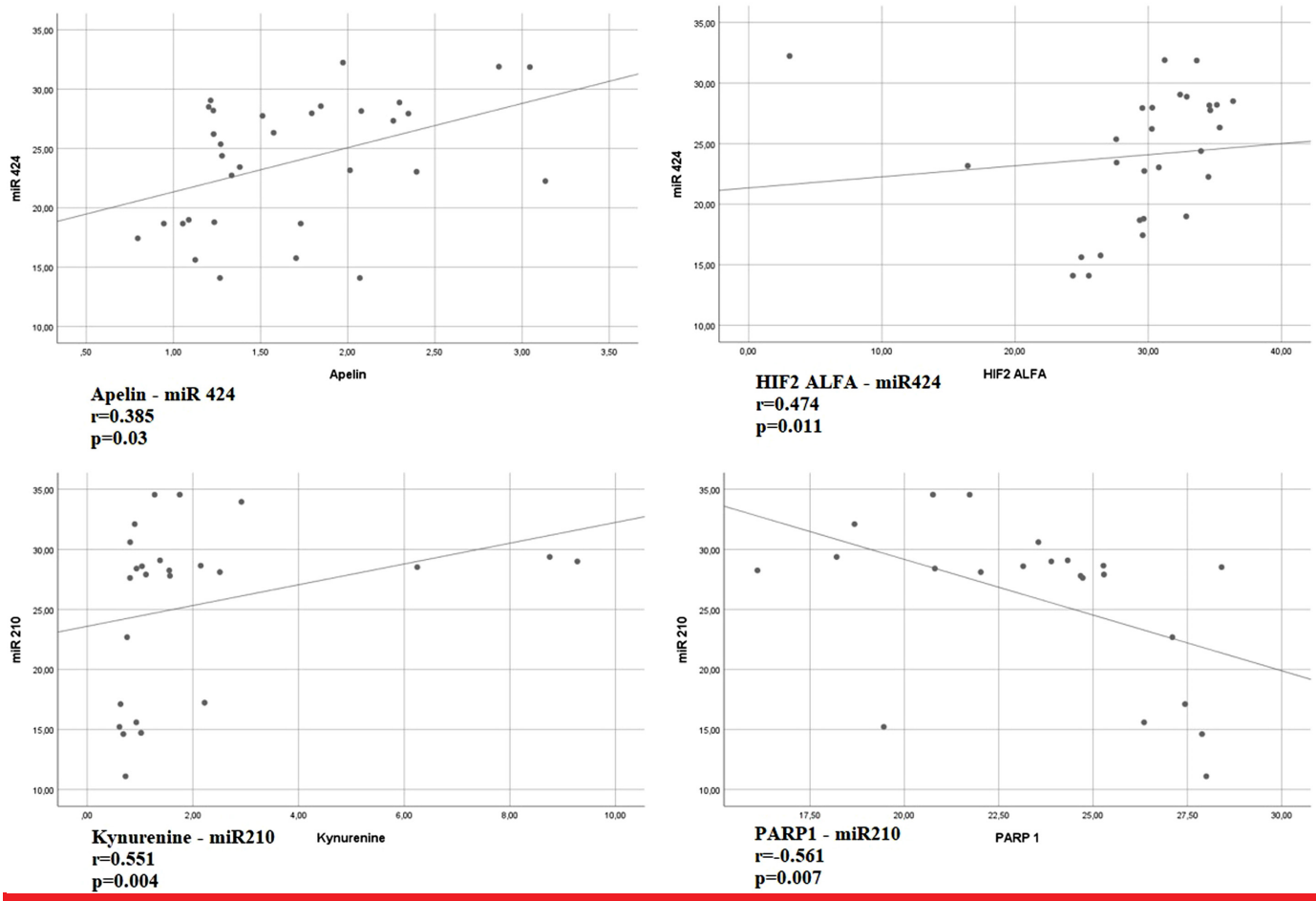

Figure 1. Correlation plots for levels of Apelin and miR-424, hypoxia-inducible factor-2 alpha (HIF-2α) mRNA and miR-424, kynurenine and miR-210, and poly-ADP-ribose polymerase-1 (PARP-1) mRNA and miR-210.

miRNA-424 and miRNA-503, FGF2 and FGFR1 levels fell and pathological changes regressed.[11] Yang et al[12] demonstrated that the APLN receptor agonist named "MM07" significantly reduced right ventricular pressure and hypertrophy in MCT-induced PH. MM07 also reduced muscle development in the pulmonary arteries. MM07 was shown to increase the phosphorylation and release of eNOS. It then promoted proliferation in human pulmonary artery cells and reduced apoptosis. These data showed that APLN receptor agonists could be used in PAH by correcting the pharmacokinetic profiles.[12] We found that miRNA-424 and APLN were significantly lower in the patient group. We observed decreased levels of miRNA-424 similar to the study by Kim et al.[11] However, they reported that their PAH-CHD patients had significantly higher miR-424(322) levels than controls. We had lower number of PAH-CHD patients in our cohort.[13] We also found a positive and statistically significant relationship between PARP-1 and APLN. Therefore, APLN may be associated with DNA damage. Further studies may examine the APLN pathway to observe the alterations in the muscle development in the pulmonary arteries.

Bertero et al[14] found that miRNA130/301 directly affected vasomotor tone via peroxisome proliferator-activated receptor as well as enhancing vascular proliferation by providing interaction between cells. Moreover, they showed that EDN1 was upregulated by miRNA130/301 to cause STAT-3 activation, which in turn caused vasoconstriction in PASMC. Therefore, the miRNA130/301 family was found to play an essential role in the pathogenesis of PH.[14] Similarly, we demonstrated that miRNA-130a expression level was significantly increased in the patient group, and miRNA-130a and APLN were independent risk factors for PH. This suggests that miRNA-130a and APLN might have a role in the pathogenesis of PH.

We demonstrated altered levels of miR130a, miR210, Apelin, kynurenine, and PARP-1. Those alterations could contribute to the understanding of the pathogenesis of PAH. Further studies are needed to confirm our findings. MiRNAs may be used as diagnostic and prognostic biomarkers as well as therapeutic targets. These developments are promising for PAH. Alterations in measured miRNA levels have been demonstrated in various clinical studies. This could be due to many factors.[15] While these may be related to the amount of the sample and the way it is taken, they are also affected by the additional diseases of the sampled person and the drugs he uses. Therefore, it is important to standardize these variables.[16] In studies intended for use as therapeutic targets, some miRNA-based drugs are currently in clinical trials,

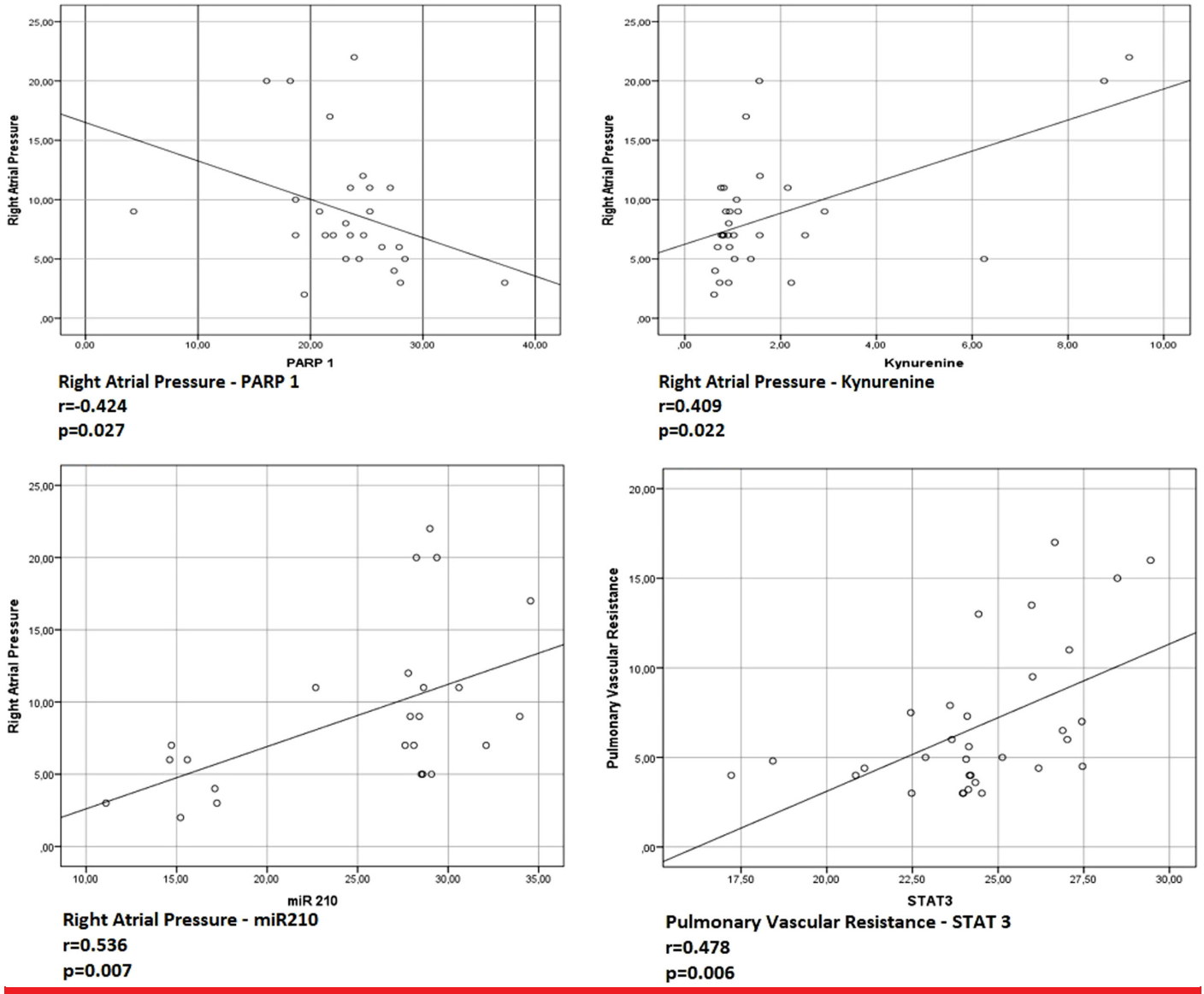

Figure 2. Correlation plots for levels of poly-ADP-ribose polymerase-1 (PARP-1) mRNA and right atrial pressure (RAP), kynurenine and RAP, miR-210 and RAP, signal transducer and activator of transcription-3, and pulmonary vascular resistance.

although large animal studies and phase I and/or II clinical trials are lacking. Such trials would guide its use as a diagnostic and prognostic marker based on miRNA and its use as a therapeutic target, and this may alter poor long-term outcomes of these patients.[15]

Study Limitations

We studied plasma samples of the patients rather than tissue changes at the cellular level. This is a single-center study with a small number of heterogenous group of patients. Therefore, our findings need to be confirmed in larger studies with more homogenous group of patients.

CONCLUSIONS

We report a novel relationship between the kynurenine and PARP-1 signaling pathways that could be mediated by miRNA-210. We also report a connection between the APLN and HIF-2α signaling pathways that could be mediated by miRNA-424. Reduced levels of APLN and elevated levels of miRNA-130a are associated with PH. We also found that higher levels of STAT-3, miRNA-210, and kynurenine and lower levels of PARP-1 correlate with more severe hemodynamics. These findings support the development of novel therapeutic strategies targeting the augmentation of APLN and PARP-1 signaling, as well as inhibition of kynurenine, miRNA-210, miRNA-130a, and HIF-2α signaling. Furthermore, it provides important insight into the conduct of future clinical studies of pathobiology in PH patients.

Data Availability Statement: All data relevant to the study are included in the article.

Ethics Committee Approval: Ethical committee approval was received from the Ethics Committee of Pamukkale University (protocol no. 60116787-020/86982).

Informed Consent: All participants provided informed consent. Patients were not involved in the design, conduct, reporting, or dissemination plans of our research.

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