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Prognostic Value of Novel Hematologic Biomarkers in Patients with Pulmonary Arterial Hypertension

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ABSTRACT

Objective: Pulmonary vascular remodeling and inflammation play a major role in pulmonary arterial hypertension (PAH). Novel hematologic biomarkers have recently been recognized as a risk predictor for cardiovascular, oncologic, and inflammatory diseases. We aimed to investigate the association of hematologic biomarkers with mortality in PAH patients.

Materials and Methods: Fourty-five patients diagnosed with PAH and 45 healthy volunteers were evaluated retrospectively. Concurrent data included clinical, echocardiographic, hemodynamic and hematologic variables. The study population was divided into subgroups based on admission neutrophil to lymphocyte ratio (NLR), neutrophil to monocyte ratio (NMR), platelet to lymphocyte ratio (PLR) values.

Results: The median NMR and NLR levels were lower in healthy subjects than in PAH patients (7.7 (7-8.8) vs 9.2 (6.5-11.6); p= 0.03 and 1.9 (1.4-2.9) vs 2.6 (1.9-3.3); p= 0.04) respectively). The estimated mean survival duration was longer in patients with low NMR levels (93 (95% CI, 86-100) vs. 67 (95% CI, 45-88) months (p=0.006) respectively). NMR independently predicted poor outcome and improved the power of the other prognostic markers (OR 1.4 (95% CI, 1-1.8) p= 0.04); (AUC= 0.91; p< 0.0001).

Conclusions: NMR levels alone or combined with other prognostic factors may predict mortality in patients with PAH.

Keywords: Chronic thromboembolic pulmonary hypertension, Neutrophil to lymphocyte ratio, Neutrophil to monocyte ratio, Pulmonary arterial hypertension, PAH, IPAH

INTRODUCTION

Pulmonary arterial hypertension (PAH) is a devastating disease characterized by increased pulmonary vascular resistance due to vasoconstriction, and pulmonary vascular remodeling, often accompanied by a poor outcome due to right heart failure (1,2). Inflammation is important for the development and progression of PAH (1). As a marker of inflammation and tissue remodeling, elevated hematologic biomarkers have been recently found to have association with disease severity and adverse outcomes in several diseases including cardiovascular and neoplastic diseases (3,4).

Predictive prognostic markers of PAH warrant investigation because some reports have indicated that better treatment results can be achieved by starting affirmative therapies before the PAH begins to worsen (2,5). Uric acid, brain natriuretic peptide, heart rate, 6-minute-walk distance (6MWD), and echocardiographic predictors such as pericardial effusion were indicated to correlation with the prognosis of Idiopathic PAH (5-8). But prognosis assessment in PAH is difficult and no guidance was provided on which parameters were the most important or which values to be used as thresholds.

Until now, the value of hematologic parameters in PAH prognosis has not been reported. Thus, the present study aimed to investigate the potential prognostic role of hematologic biomarkers individually and by combining them with different parameters to enable more robust prognostic information in PAH patients.

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MATERIAL AND METHODS

Data from consecutive patients with PAH (IPAH and chronic thromboembolic pulmonary hypertension (CTEPH)) who were referred to the Department of Cardiology, Türkiye Yüksek İhtisas Training and Research Hospital, Ankara, Turkey, have been prospectively collected in a dedicated database since 2006. Data from a contemporary group of healthy volunteers were also consecutively collected. Healthy controls were individuals with no history of pulmonary or cardiac disease or symptoms.

In this retrospective study, all patients with clinically defined PAH i.e. mean pulmonary arterial pressure (MPAB) ≥25 mmHg at rest and pulmonary artery wedge pressure ≤15 mmHg as measured by right heart catheterization, were included (9). PAH was classified as being associated with chronic thromboembolic pulmonary hypertension and idiopathic as described in recent guidelines (9). PAH patients other than these two etiologic groups including PAH patients associated with congenital heart disease and patients with concomitant left heart disease defined by a left ventricular ejection fraction ≤45% or a pulmonary wedge pressure ≥15 mmHg were excluded from the study. Also, patients with known hematologic diseases and active inflammation were excluded. Date of diagnosis of PAH was established as the date of the first confirmatory right heart catheterization performed in our institution.

Upon referral to our Centre, all patients underwent a complete assessment, including clinical history, physical examination, venous blood samples, echocardiography, lung function test, arterial blood gases, ventilation/perfusion lung scan, 6-minute walking distance (6MWD) under standardized conditions, right heart catheterization and laboratory testing, including serological tests for autoimmune diseases and hematologic parameters (10). Left heart catheterization or computed tomography of the lungs was performed in all patients with suspected left heart or respiratory diseases and when clinically indicated. All tests were performed in our clinic and set as the baseline assessment. These data were collected in a dedicated database along with the time between diagnoses of PAH and subsequent follow-up period. Following referral to our Centre, all of the patients were uniformly treated according to the current guidelines and proposed treatment with algorithms-approved PAH-specific drugs (9). Data from 1 January 2006 to 28 February 2015 were analyzed. The study was conducted in accordance with the Helsinki Declaration after being approved by the ethics committee of our hospital.

Blood Sample Analysis

A complete blood count analysis was performed using the peripheral venous blood samples taken upon admission. The blood samples were collected in a calcium EDTA (Ethylenediaminetetra- acetic acid) tube, and blood counts were evaluated using an auto-analyzer. Some hematologic biomarkers were calculated from the whole blood cell counts. NLR was calculated as the ratio of neutrophils to lymphocytes, PLR was calculated as the ratio of platelets to lymphocytes, and NMR was calculated as the ratio of neutrophils to monocytes. In addition, other routine laboratory findings and serologic tests were examined using the electronic database.

Transthoracic Echocardiography

Comprehensive TTEs were performed to all participants (Philips HD11XE and Envisor HD; Phillips USA, Andover, MA). We acquired images from standard echocardiographic views in accordance with the recommendations of the American Society of Echocardiography (11). Pulmonary artery systolic and mean pressure were derived as the sum of the tricuspid regurgitant gradient and pulmonary regurgitant gradients obtained from continuous wave Doppler and the right atrial pressure as estimated from the inferior vena cava, respectively (11). A great deal of data pertaining to right ventricle tricuspid annular plane systolic excursion measurements was also assessed.

Follow-Up Assessment and Identification of Survival Predictors

During the study period, all participating patients were interviewed at a control visit and quarterly thereafter in our clinic to evaluate symptoms, World Health Organization functional classification, current medication, and any potential worsening cardiopulmonary events that might have occurred since last observation. Pulmonary endarterectomy therapy was planned for none of our chronic thromboembolic pulmonary hypertension (CTEPH) patients. Follow-up was censored at the date of the outcome event and treating physicians or relatives were asked for the cause and circumstances of death. Echocardiographic parameters, laboratory parameters, and 6MWD were analyzed for their predictive value on survival.

Besides, the study population was divided into subgroups based on PLR, NLR, NMR values. Effects of these biomarkers on outcome were studied by constructing a receiver–operating characteristic (ROC) curve. High risk groups were defined as follows according to their cut-off values: For PLR >134, for NLR >2.2, for NMR >9.2. Tricuspid Annular Plane Systolic Excursion (TAPSE), 6-minute walking distance (6MWD), mean pulmonary artery pressure (MPAP) and pericardial effusion were considered as prognostic factors for PAH (5-7). All-cause mortality was the primary outcome. A further stratification model was generated according to NLR, PLR and NMR levels and the presence of pericardial effusion.

Statistical Analyses

We used the Kolmogorov-Smirnov test to assess the normality of numeric variables. We made comparisons between 2 groups by Mann-Whitney U test and we presented descriptive statistics for subgroup comparisons, including median and interquartile range. To analyze categorical data, we used a $\chi 2$ test (or Fisher's exact test if any expected cell count was <5), and we presented descriptive statistics as number and percentages. Although the primary objective of our study was the comparison among PAH subgroups according to their hematologic biomarkers; We also performed descriptive statistical analysis comparing the baseline laboratory and demographic characteristics of PAH patients, with that of the healthy population. A P-value ≤0.05 was considered statistically significant. Statistical tests were two-sided.

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To evaluate the correlations between hematologic biomarkers and the currently known PAH associated prognostic indicators (TAPSE, 6MWD and MPAP), we used the Spearman's ρ correlation analysis. Furthermore, we used the ROC curve to determine the cut-off point and the area under the curve (AUC) of significant biomarkers. For clinical convenience, we displayed the cut-off values of those; neutrophil to monocyte ratio (NMR), neutrophil to lymphocyte ratio (NLR) and Platelet to lymphocyte ratio (PLR); as a pre-specified dichotomous variables in order to determine the prognostic significance (that was done to facilitate a meaningful clinical interpretation of the results). In addition, the study tested the statistical significance of the difference between the areas under the ROC curves using the method proposed by Hanley and McNeil (12). When indicated, we also reported analyses performed using those variables as a continuous variable.

For the survival analysis, all causes of mortality were included in the Kaplan–Meier analysis. No patient died of non-cardiopulmonary causes, and no patients were lost to follow-up till the end of our study. Kaplan–Meier survival curves were assessed and compared with the log-rank test according to the clinical subgroups. The date of the first confirmatory right heart catheterization establishing the presence of idiopathic PAH or CTEPH was considered to be the baseline from which survival was measured. Cox proportional-hazards models, both univariate and adjusted for adopted prognostic factors including patient age at first confirmatory right heart catheterization, were performed as additional analyses. Also, we looked for the collinearities among covariates to analyze appropriate Cox models and all variables in cox-regression analysis were normally distributed as shown by Kolmogorov–Smirnov tests.

Model and score derivation

To develop a mortality risk score, we assigned points to predictor variables proportional to the size of their regression coefficients in the model. For the variables above the categorized levels, we constructed two risk score models derived from hematologic biomarkers according to the presence of pericardial effusion. We appointed 2 points for pericardial effusion, 2 points for NMR, 1 point for NLR, and 1 point for PLR, and data was analyzed according to the cumulative risk scores (SCORE and PE-SCORE). Also we preselected TAPSE, 6MWD and MPAP as currently known indicators of PAH related adverse events (6-8). ROC curve was used to determine the optimized cut-off points and to compare the AUC of our risk models. High risk groups were defined according to following cut-off values: For SCORE ≥3 points and for PE-SCORE ≥5 points.

Comparison of the predictive performance of models for mortality

Logistic Regression with a forward stepwise variable selection was used to predict the probability of death in PAH. By using the currently known prognostic factors and our proposed hematologic scoring variables, we created different models for the probability of mortality. The c-statistic, a measure of the area under the ROC curve (which tests the hypothesis that these models performed significantly better than chance (indicated by a c-statistic ≥0.5)) was used to quantify the predictive validity and discriminatory capacity of our proposed models (13). In addition to model discriminatory ability (the ROC curve analysis), model calibration of each adjusted model was tested by the Hosmer-Lemeshow goodness-of-fit test. Explanatory power was tested using the pseudo- R^2 statistic according to the ‘‘Nagelkerke R^2’’ to assess the degree to which the model explained the variance of the binary outcome.

Internal validation group

We used a split-sample approach to develop and internally validate our mortality risk score. As a subsidiary analysis, we also ran the same analyses in a validation group that had the two thirds of our study group (IPAH patients). All analyses were performed with IBM SPSS 14 (SPSS Statistics version 14, IBM Corp).

RESULTS

Demographic, clinical, hematologic and echocardiographic characteristics of PAH patients on admission.

A total of 45 patients with PAH were included. Median age was 49 (32-58) years, 29 patients (64%) were females, 30 had IPAH (67%), and 15 (33%) had CTEPH. CTEPH patients were older, more likely to have pericardial effusion, higher CRP and RDW levels, and shorter 6MWD. Functional class was similar in PAH subgroups and didn’t alter the effect of our hematologic biomarkers or derived models on mortality.

Of the 45 PAH patients, 18 (40%) had PLR >134, 27 (60%) had NLR >2.2 and 21 (47%) had NMR >9.2. Seventy-eight (n=14) and eighty-one (n=17) percent of PAH patients who had PLR and NMR values above the categorized points in sequence; also had NLR values above the cut-off point of 2.2 (p=0.04 and p=0.007). While PAH patients with PLR >134 levels had median NLR values higher than the ones with PLR ≤134 levels; no difference observed in terms of median NMR values between PLR subgroups (3.3(2.3-5.2) vs 2.1(1.8-2.9) p=0.009 and 9.3(7.2-11.6) vs 8.6(6.3-9.9) p=0.55 respectively). In patients with high NLR levels, 6MWD was shorter, NMR, PLR values were higher, and pericardial effusion was more common.

The median age of controls was 49 (30-59) years and 30 subjects (67%) were females. Although no difference was observed between the median WBC and platelet counts; the groups were not similar in terms of median MCV, RDW, and MPV levels. Median values of NMR and NLR were lower in controls than that of the overall PAH patients (7.7 (7-8.8) vs 9.2 (6.5-11.6) p=0.03 and 1.9 (1.4-2.9) vs 2.6 (1.9-3.3) p=0.04 respectively). Demographic, clinical, echocardiographic, functional, and hematologic characteristics of the study patients are shown in Table 1 and 2.

All of our patients were treated with approved PAH-specific medications. Treatment of patients at the end of the follow-up according to the PAH subgroups is shown in supplementary table 1. A larger number of patients with PAH after diagnosis were treated with endothelin receptor antagonists. In the overall population, 20 (44%) patients were treated with combination therapy. Treatment modality had no significant effect on mortality regardless of being whether mono-therapy or combination treatment.

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Table 1: Demographic, clinical and echocardiographic characteristics of the study patients.

	Control N=45	Overall PAH n=45	*p-value	IPAH n=30	CTEPH n=15	**p-value
Age, (years)	49 (30-59)	49 (32-58)	0.94	40 (28-50)	58 (53-68)	**0.001**
Sex n, (%)						
Female	30 (67)	29 (64)	0,8	19 (63)	10 (67)	0.8
Follow-up duration (months)		26 (14-56)		27 (14-56)	26 (20-63)	0.57
Hemoglobin (mg/dL)	13.8 (13.2-15.9)	13.6 (12.3-15.3)	0.82	14.1 (12.4-15.7)	12.6 (11.9-13.7)	0.06
Platelet (10^3/mm^3)	222 (204-251)	204 (175-276)	0.35	203 (161-269)	248 (190-298)	0.06
White blood cells (x10^9/L)	7 (6.5-8.3)	7.6 (6.6-8.9)	0.28	7.8 (6.1-9.2)	7.4 (6.7-7.9)	0.92
MCV (fl)	90 (83-92)	85 (78-91)	**0.04**	86 (81-92)	83 (74-88)	0.14
MPV (fl)	8.8 (8.2-9.6)	9.2 (8.7-10.2)	**0.02**	9.2 (8.6-10.4)	9.2 (8.7-9.6)	0.77
RDW (%)	13.6 (13-15)	16.5 (14.1-17.4)	**0.001**	15 (13.9-17.3)	17.2 (15.9-18.7)	**0.03**
NLR	1.9 (1.4-2.9)	2.6 (1.9- 3.3)	**0.04**	2.7 (1.9-3.9)	2.3 (1.9-3.1)	0.59
PLR	97 (86-123)	106 (83-157)	0.12	104 (71-164)	141 (89-153)	0.39
NMR	7.7 (7-8.8)	9.2 (6.5-11.6)	**0.03**	9.4 (6.9-11.9)	8.4 (5.6-9.5)	0.09
Creatinine (mg/dL)		0.9 (0.7-1)		0.8 (0.7-0.9)	1 (0.8-1.1)	0.05
CRP (mg/L)		5.7 (3.4-11.4)		4 (2.9-7.3)	8.6 (5.9-14.9)	**0.02**
Iron (µg/dL)		61 (40-68)		62 (39-68)	60 (43-69)	0.82
Ferritin (ng/mL)		25.3 (13.3-38.4)		26.6 (13.3-46.1)	21.4 (14.0-28.8)	0.16
mPAP (mmHg)		55 (45-65)		52 (45-66)	55 (45-65)	0.9
TAPSE (mm)		13 (12-15)		13 (12-15)	12 (11-13)	0.4
6MWD (meters)		290 (220-340)		335 (240-380)	280 (130-310)	**0.03**
Functional Class n, (%)						
Class II		21 (47)		17 (57)	4 (27)	**0.03**
Class III		18 (40)		8 (27)	10 (67)	
Class IV		6 (13)		5 (16)	1 (6)	
Pericardial effusion n, (%)		22 (49)		11 (37)	11 (73)	**0.02**
PLR ≤134 n, (%)		27 (60)		20 (67)	7 (47)	0.19
PLR >134 n, (%)		18 (40)		10 (33)	8 (53)	
NLR ≤2.2 n, (%)		18 (40)		12 (40)	6 (40)	1.00
NLR >2.2 n, (%)		27 (60)		18 (60)	9 (60)	
NMR ≤9.2 n, (%)		24 (53)		14 (47)	10 (67)	0.2
NMR >9.2 n, (%)		21 (47)		16 (53)	5 (33)	
Medication n, (%)						
Mono-therapy		25 (56)		16 (53)	9 (60)	0.67
Combination treatment		20 (44)		14 (47)	6 (40)	
Death n, (%)		9 (20)		4 (13)	5 (33)	0.13

MPV, mean platelet volume; MCV, mean corpuscular volume; RDW, red blood cell distribution width; NLR, neutrophil to lymphocyte ratio; NMR, neutrophil to monocyte ratio; PLR, platelet to lymphocyte ratio; CRP, C-reactive protein; mPAP, mean pulmonary arterial pressure; TAPSE, tricuspid annular plane systolic excursion; 6MWD, six-minute walk distance; IPAH, idiopathic pulmonary arterial hypertension; CTEPH, chronic thromboembolic pulmonary hypertension. * Comparisons between controls and PAH **Comparisons between IPAH and CTEPH subgroups.

Correlations between hematologic biomarkers and PAH associated prognostic indicators

After we investigated the correlations of hematological parameters with each other, we did the same analysis with PAH associated prognostic indicators in our study group. In addition to the identified correlation between RDW and PLR, MPV showed mild correlation with NMR (rs =0.36, p=0.01and rs =0.30, p= 0.04 respectively). Both CRP and age showed no correlation with prognostic factors. Similar to the relationships, found between TAPSE, 6MWD and our risk score (PE-SCORE) (rs =0.44, p=0.003) and (rs = -0.60, p<0.0001), TAPSE and 6MWD were negatively associated with increased NLR (rs = -0.37, p=0.01 and rs = -0.40, p= 0.007 respectively). There was a lack of association between NMR, PLR and the PAH associated prognostic indicators.

Follow-Up, survival analyses, and prognostic factors

In the overall observation period of 26 (14-56) months, 9 cardio-pulmonary deaths occurred in patients with PAH and estimated mean survival time was 85 (95% CI, 72-99) months. In the overall population, Five-, 9-, 11-, 12- and 40-month survival rates were 98%, 95%, 93%, 86%, 80% and 74% respectively (Figure 1A).

Estimated mean survival times according to categorized biomarker levels were: for NMR, 93 (95% CI, 86-100) vs. 67 (95% CI, 45-88) months (p=0.006); for NLR 103 (95% CI, 92-113) vs. 67 (95% CI, 50-84) months (p=0.047); for PLR 100 (95% CI, 90-110) vs. 62 (95% CI, 42-82) months (p=0.024) respectively.

In patients with IPAH, estimated mean survival time was 82 (95% CI, 69-96) months and five-, 9-,11-,12- , 24- and 40-month survival rates were 96% (95% CI, 89–100%), 93% (95% CI, 83– 97%), 93% (95% CI, 83– 97%), 89% (95% CI, 79–96%), and 80% (95% CI, 70–90%) respectively. Also in patients with CTEPH, 5-, 9-, 11-, 12- , 24- and 40-month survival rates were 100%, 100%, 93% (95% CI, 91– 100%), 80% (95% CI, 70– 91%), 65% (95% CI, 40– 89%) and 65% (95% CI, 40– 89%) respectively (p= 0.22) (Figure 1C).

As significant associations of hematologic biomarkers with unfavorable outcome observed, we analyzed predictive ability of each biomarker and pairwise comparisons of their predictive abilities were investigated. NMR, NLR and PLR had significantly better predictive ability compared with TAPSE, 6MWD and MPAP (all p<0.05), whilst there were no significant differences among the first three biomarkers (Table 3). While NMR had the highest sensitivity (89%), PLR was the most specific (72%) for prediction of mortality, with appropriate cut-off values.

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Table 2. Baseline characteristics of the patient subgroups based on neutrophil to monocyte ratio levels.

	Overall N=45	NMR ≤9.2 n=24	NMR >9.2 n=21	p-value
Age, (years)	49 (32-58)	50 (36-64)	41 (31-52)	0.12
Sex n, (%)				
Female	29 (64)	15 (62)	14 (67)	0.77
Type n, (%)				
CTEPH	15 (33)	10 (42)	5 (24)	0.20
IPAH	30 (67)	14 (58)	16 (76)	
Follow-up duration (months)	26 (14-56)	31 (15-70)	24 (12-51)	0.39
Hemoglobin (mg/dL)	13.6 (12.3-15.3)	12.6 (11.8-15.3)	13.6 (12.6-15.2)	0.41
Platelet (10^3/mm^3)	204 (175-276)	207 (182-286)	204 (173-269)	0.60
White blood cells (x10^9/L)	7.6 (6.6-8.9)	6.9 (6.3-8.5)	7.8 (7.2-10.1)	0.19
RDW (%)	16.5 (14.1-17.4)	15.9 (14-18)	16.7 (14.6-17.3)	0.99
MCV (fl)	85 (78-91)	86 (72-91)	85 (82-91)	0.41
MPV (fl)	9.2 (8.7-10.2)	9.2 (8.7-9.8)	9.5 (8.7-10.4)	0.41
NLR	2.6 (1.9- 3.3)	1.9 (1.5-2.8)	2.9 (2.4-4.5)	**0.001**
PLR	106 (83-157)	102 (81-155)	128 (92-157)	0.5
NMR	9.2 (6.5-11.6)	6.6 (5.5-8.3)	11.6 (9.6-12.4)	**<0.001**
Creatinine (mg/dL)	0.9 (0.7-1)	0.9 (0.7-1.1)	0.9 (0.7-1)	0.50
CRP (mg/L)	6 (3-11)	6 (3-13)	5 (4-9)	0.72
Iron (µg/dL)	61 (40-68)	62 (37-94)	52 (42-67)	0.59
Ferritin (ng/mL)	25 (13-38)	23 (13-29)	32 (15-43)	0.19
mPAP (mmHg)	55 (45-65)	60 (45-65)	55 (45-65)	0.61
TAPSE (mm)	13 (12-15)	13 (12-15)	12 (11-14)	0.62
6MWD (meters)	290 (220-340)	295 (200-380)	290 (220-340)	0.29
Functional Class n, (%)				
Class II	21 (47)	12 (50)	9 (43)	
Class III	18 (40)	11 (46)	7 (33)	0,15
Class IV	6 (13)	1 (4)	5 (24)	
Pericardial effusion n, (%)	22 (49)	10 (41)	12 (57)	0.30
PLR ≤134 n, (%)	27 (60)	15 (62)	12 (57)	0.71
PLR >134 n, (%)	18 (40)	9 (37)	9 (43)	
NLR ≤2.2 n, (%)	18 (40)	14 (58)	4 (19)	**0.007**
NLR >2.2 n, (%)	27 (60)	10 (42)	17 (81)	
SCORE	2 (1-3)	1 (0-1.5)	3 (3-4)	**<0.001**
PE-SCORE	3 (1-4)	1.5 (0-3)	5 (3-6)	**<0.001**
Medication n, (%)				
Mono-therapy	25 (56)	12 (50)	13 (62)	0.42
Combination treatment	20 (44)	12 (50)	8 (38)	
Death n, (%)	9 (20)	1 (4)	8 (38)	**0.007**

CTEPH, chronic thromboembolic pulmonary hypertension; IPAH, idiopathic pulmonary arterial hypertension; RDW, red blood cell distribution width; MCV, mean corpuscular volume; MPV, mean platelet volume; NLR, neutrophil to lymphocyte ratio; NMR, neutrophil to monocyte ratio; PLR, platelet to lymphocyte ratio; CRP, C-reactive protein; mPAP, mean pulmonary arterial pressure; TAPSE, tricuspid annular plane systolic excursion; 6MWD, six-minute walk distance; PE-SCORE, pulmonary emboli SCORE.

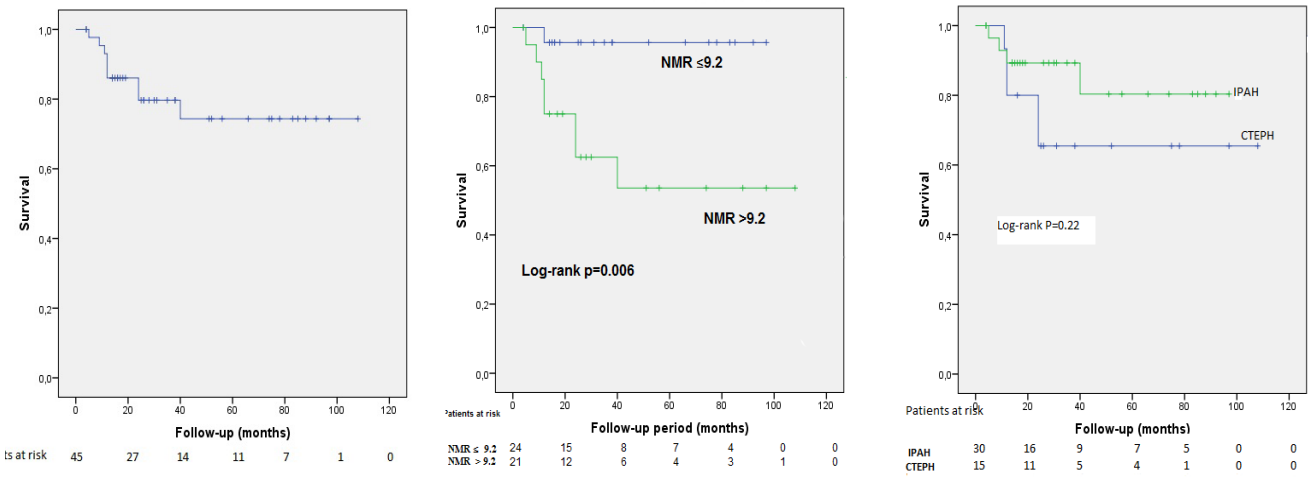

Figure-1. Kaplan-Meier cumulative survival curves of the patients with pulmonary arterial hypertension A) in all patients B and C) in subgroups. IPAH indicates idiopathic pulmonary arterial hypertension and CTEPH indicates chronic thromboembolic pulmonary hypertension. NMR, neutrophil to monocyte ratio.

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Table-3. Comparison of the predictive ability of prognostic factors and models in patients with PAH.

	AUC (95% CI)	p-value
NMR	0.77 (0.63-0.91)	0.01
NLR	0.72 (0.53-0.92)	0.04
PLR	0.76 (0.59-0.94)	0.02
TAPSE	0.30 (0.14-0.46)	0.07
6MWD	0.32 (0.14-0.49)	0.09
mPAP	0.56 (0.37-0.76)	0.56
PE-SCORE+RF	0.91 (0.81-1.00)	<0.0001
PE-SCORE	0.90 (0.79-1.00)	<0.0001

NMR, neutrophil to monocyte ratio; NLR, neutrophil to lymphocyte ratio; PLR, platelet to lymphocyte ratio; TAPSE, tricuspid annular plane systolic excursion; 6MWD, six-minute walk distance; mPAP, mean pulmonary arterial pressure. PE-SCORE, Pulmonary emboli SCORE. RF, model derived from TAPSE, 6MWD and MPAP; SCORE, model derived from NMR, NLR and PLR; PE-SCORE, model derived from pericardial effusion, NMR, NLR and PLR; AUC, area under curve; CI, confidence interval.

In PAH patients with NMR above the categorized level, 5-, 9-,11-,12- , 24- and 40-month survival rates were 95% (95% CI, 95–100%), 90% (95% CI, 78–96%), 85% (95% CI, 69–92%), 75% (95% CI, 55–86%), 62% (95% CI, 40–78%), 54% (95% CI, 32–63%) respectively (p= 0.006) (Figure 1B). While In IPAH patients with NMR above the categorized level 5-, 9-,12-, and 40-month survival rates were 93% (95% CI, 86–100%), 87% (95% CI, 78–95%), 80% (95% CI, 70–93%), 67% (95% CI, 50–82%), CTEPH group with NMR above the categorized level had 80%(95% CI, 65–100%), 60% (95% CI, 38–82%), and 20% (95% CI, 5–39%) 11-,12- and 24-month survival rates respectively. In IPAH patients with lower NMR, all patients survived and only one death was observed in CTEPH patients with similar categorized levels.

Cox regression analyses confirmed the difference across our clinical subgroups of PAH in terms of categorized hematologic biomarkers. In the univariate analysis NLR, PLR, NMR, MPAP and pericardial effusion were found as the significant predictive variables (Supplementary table 2). Of the available individual clinical characteristics, NMR was the only significant prognostic factor after adjusting for appropriate confounding factors (6MWD, MPAP) by cox regression analysis (OR 1.4 (95% CI, 1-1.8) P=0.04). Than we performed the analysis considering only two (NMR and pericardial effusion) variables and found an independent association between NMR and mortality (OR 1.1 (95% CI, 1-1.2) p=0.04). Also pericardial effusion tended to be an independent significant factor for prognosis (OR 6.3 (95%CI 0.76-53) p=0.08). When the multivariate analysis performed considering pericardial effusion and optimized cut-off levels of NMR and PLR, only categorized NMR level was independently associated with mortality (OR 7.9, 95%CI (1.1-65) p= 0.04).(PLR: OR 3.2 (95%CI 0.6-17) p=0.18; pericardial effusion: OR 3.9 (95%CI 0.4-38) p=0.24). The percentage of death in our patients according to the categorized NMR level and the presence of pericardial effusion is shown in figure 2.

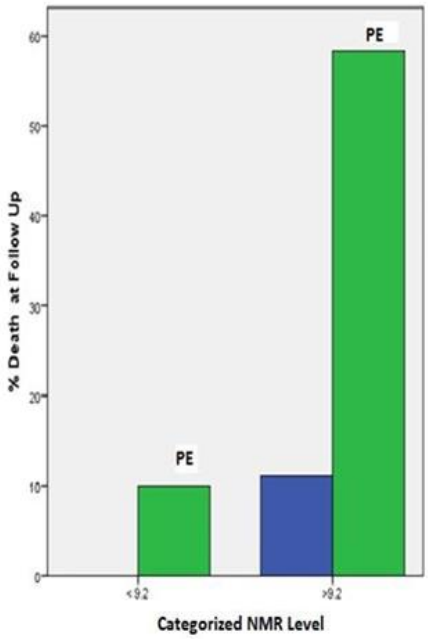

Figure 2: The mortality rates during follow-up period according to categorized neutrophil to monocyte ratio (NMR) level in PAH patients with and without pericardial effusion (PE).

Risk stratification models and risk score performance to predict events

In our group, we developed mortality risk scores by using the regression coefficients of the predictive variables (NMR, NLR, PLR and pericardial effusion), which were detected as 2.6, 1.4, 1.5, 2.5 respectively. The predictive ability and the explained degree of mortality for the two risk score models derived from our variables; with and without pericardial effusion, were 91%, 0.54 and 89%, 0.46 respectively. Patients who died had higher risk scores than those who survived (supplemantary table 2), and the two risk scores had the significantly better predictive ability for cardio-pulmonary death than the score derived from known prognostic indicators. Both scores were significantly associated with mortality on the univariate analyses (SCORE: OR 3.2, 95% CI 1.4-6.9 p=0.005; PE-SCORE: OR 2.3, 95% CI 1.4–4 p=0.002). When multivariable analysis was performed, including both scores with the model (RF) derived from known prognostic indicators separately, associations with unfavorable outcomes continued to be significant (SCORE: OR 4.8, 95%CI 1.4-15 p=0.008; PE-SCORE: OR 3.2, 95%CI 1.4–7.1 p=0.004). The crude associations of our risk scores with unfavorable outcomes are shown in Figure 3. Within the PE-SCORE, the percentage of hematologic biomarkers above the categorized levels are shown in figure 4.

Estimated mean survival times according to categorized risk levels for our models were (for PE-SCORE) 102 (95% CI, 94-110) months vs. 37 (95% CI, 17-58) months and (for SCORE) 104 (95% CI, 97-111) vs. 55 (95% CI, 34-75) months (Figure 5A and 5B).

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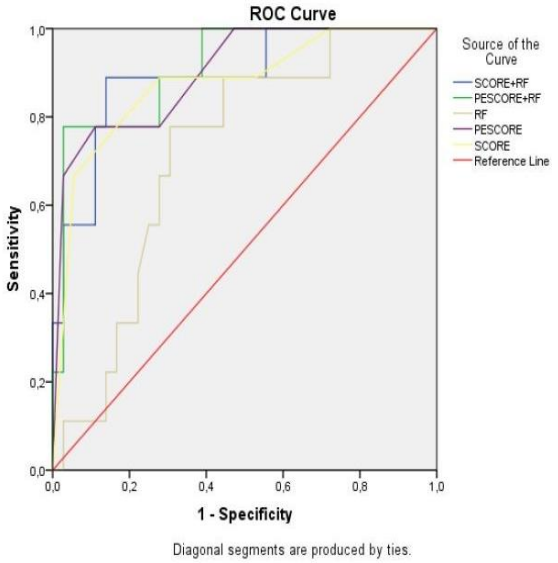

	AUC (95% CI)	P-value	Nagelkerke R Square*	Predictive probability %
PESCORE+RF	0.91 (0.81-1.00)	<0.0001	0.58	91
PESCORE	0.90 (0.79-1.00)	<0.0001	0.54	91
SCORE + RF	0.89 (0.77-1.00)	<0.0001	0.53	89
SCORE	0.87 (0.74-1.00)	0.001	0.46	89
RF	0.72 (0.55-0.88)	0.04	0.14	78

Figure-3. Comparison of ROC curves of our proposed risk stratification models individually and in combination with other models in predicting mortality in patients with PAH. RF, model derived from TAPSE, 6MWD and MPAP; SCORE, model derived from neutrophil to monocyte ratio (NMR), neutrophil to lymphocyte ratio (NLR) and Platelet to lymphocyte ratio (PLR); PE-SCORE, model derived from pericardial effusion, NMR, NLR and PLR; AUC, area under curve; CI, confidence interval. *By using the pseudo- R^2 statistic according to the ‘‘Nagelkerke R^2’’, explanatory power of our models was tested to assess the degree to which the model explained the variance of the mortality.

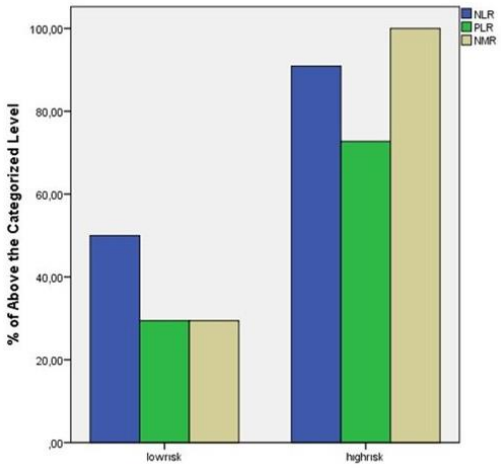

Figure 4: The percentage of hematologic biomarkers above the categorized level according to combined risk stratification score with presence of pericardial effusion (PE-SCORE). NMR, neutrophil to monocyte ratio; NLR, neutrophil to lymphocyte ratio and PLR, platelet to lymphocyte ratio.

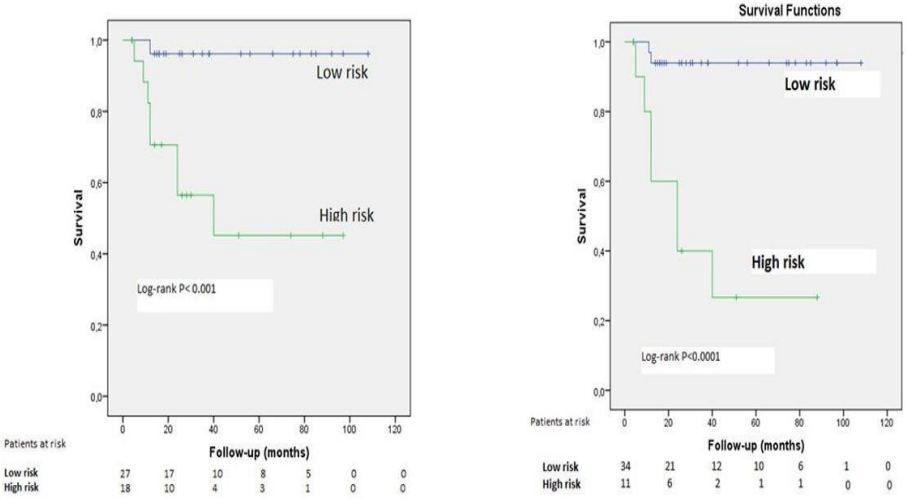

Figure 5: Kaplan-Meier cumulative survival curves of patients with pulmonary arterial hypertension A) according to combined risk stratification score without the presence of pericardial effusion (SCORE) B) according to combined risk stratification score with presence of pericardial effusion (PE-SCORE)

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Internal validation group

Analyses of the IPAH patients (n= 30) yielded essentially identical results. Patients with unfavorable outcomes had higher values of risk scores, compared with patients who survived (median SCORE of 2 (1-3) vs. 4 (4-4) and median PE-SCORE of 2 (0-3) vs. 6 (6-6) respectively, all p=0.002). The predictive ability for the two risk score models derived from our variables; with and without pericardial effusion, were 97% and 93% respectively. The model derived from known prognostic indicators, both risk scores and combined scores were compared for cardio-pulmonary death by ROC curve analysis. For combined scores AUC was 0.98 (95%CI 0.94-1) p=0.002, for PE-SCORE 0.98 (95%CI 0.93-1) p=0.002, for SCORE 0.96 (95%CI 0.89-1) p=0.003 and for known prognostic indicator score 0.77 (95%CI 0.59-0.96) p=0.08 respectively. Other analyses with the biomarkers and combined scores also yielded essentially identical results as shown for our group (data not shown).

DISCUSSION

The present study is the first to investigate the potential prognostic role of hematologic biomarkers in PAH patients. Our results showed that as a marker of inflammation and remodeling, NMR increased in PAH patients compared to healthy controls, independently predicting mortality. Our risk stratification model on admission identified patients with poor outcomes more accurately than other prognostic factors.

A comprehensive assessment of prognosis in PAH patients helps clinicians determine the therapeutic strategy in clinical practice. Although a number of biomarkers (e.g. CRP, uric acid, NT-proBNP) have been reported in association with disease severity and prognosis in PAH, only NT-proBNP is widely used in clinical settings (14). Hematologic biomarkers might provide a promising new marker for estimating the prognosis of patients with PAH as they are readily available worldwide.

Association between inflammatory cell types and immune response to inflammation and vascular remodeling may be relevant to several different aspects of PAH (1). Inflamed tissues due to the deregulated immunity and altered metabolism, are attributable to the recruitment of monocytes and neutrophils, in addition to locally proliferating lymphocyte populations (1). Elevated levels of several cytokines and chemokines correlate with a worse clinical outcome in PAH patients and may serve as biomarkers of disease progression. The fact that inflammation precedes vascular remodeling in experimental PAH suggests that altered immunity is a cause rather than a consequence of vascular disease. Although little attention has been given to the neutrophils in the pathogenesis of PAH, it is evident both in experimental and clinical studies that neutrophil elastase can influence pathogenesis. Enhanced neutrophil elastase has recently been revealed in smooth muscle cells from patients with IPAH (1,15). Neutrophil elastase can trigger immune inflammatory response, and by repressing it both clinical and experimentally, induced disease progression regresses (16). During inflammatory conditions, neutrophil count increases, and the amount of circulating blood monocytes decrease by migration to tissues and differentiation to macrophages. Experimental and certain PAH related diseases are characterized with macrophage infiltration. Even in patients with IPAH, recruitment of lung macrophages is evident (1,17). Activation of macrophages is also closely linked to epigenetic changes that stimulate and induce pro-inflammatory cytokines, proliferation of vascular fibroblasts and altered host metabolism in experimental models of PAH. Changes in metabolic phenotype involving a switch to glycolysis, fatty acid oxidation, and production of reactive oxygen species underlie the abnormal interaction of fibroblasts and macrophages. Also, there is recent evidence for macrophage granulocyte-macrophage colony-stimulating factor (GM-CSF) and leukotriene B4 (LTB4) signaling pathways in PAH development. Reversing the metabolic phenotype by blocking macrophage-derived LTB4 biosynthesis or signal transduction reverses experimental PAH and the pathological features of PAH in terms of macrophage recruitment and activation (18). Besides, higher levels of RDW were shown to be associated with inflammation and systolic pulmonary arterial pressure (19, 20). Recently, Can et all, found that although no difference observed in platelet count, MPV was significantly high in adult patients with IPAH than in healthy control patients (21). They suggested that platelet activation may directly impact the pathogenesis of PAH. In addition to the parallel findings with this study, MPV, RDW, NMR and NLR were also significantly higher in our patients according to normal subjects. CRP level failed to be found as a prognostic marker in our analysis, and also it was not different in our subgroups, possibly due to the exclusion of patients with increased CRP assuming a subclinical infection. Observed significant correlations of our hematologic biomarkers with MPV and RDW suggest them as a marker of underlying inflammation and confirm the interactions of inflammatory cells with each other in PAH. Zheng et all, supported our idea by defining significant associations between elevated MPV levels and IPAH severity though they observed no difference in terms of prognosis (2). By determining individual prognostic values of NMR, NLR, and PLR and independent prognostic values of NMR, our data revealed the reported findings regarding inflammation above.

Consistent with previous findings, the prognostic value of pericardial effusion, a known surrogate marker of survival in PAH patients, was significant with unadjusted analyses but showed a clear trend to be significant after adjustment (8). Also patients with higher NMR levels had higher mortality rates if they had pericardial effusion. Increased functional class, treatment modality, and uric acid level did not predicted poor prognosis for PAH patients. Some established risk factors like 6MWD, and TAPSE failed to reach statistical significance. This discrepancy might be explained by the small study population and few patients who were severely diseased in functional class IV in our study. In ROC analyses, hematologic biomarkers outperformed TAPSE, mPAP, and 6MWD in predicting prognosis. Even after adjusting for these established markers, only NMR independently predicted mortality in multivariate analysis. This finding suggested NMR as a prognostic non-invasive, in-expensive and easily accessible marker complementary to currently used markers irrespective of treatment modality and functional class. Also, by excluding patients with known hematologic diseases and active inflammation, the idea of higher NMR levels being an element of the PAH disease process rather than a result of other comorbid conditions is favored.

In PAH patients, Humbert et all, reported 1-year and 3-year survival rates of 83% and 58%, with a baseline NYHA functional class III and IV rate of 67% and 14% (22). In our study, 12-month and 40 month survival rates were 93% and 74% respectively. Baseline NYHA functional classes and treatment modalities might explain why the survival in our study was relatively better. Although survival rates were not different between CTEPH and IPAH patients, when the outcome had analyzed according to optimized NMR levels, lower survival rates were observed in CTEPH group. Distinct inflammatory profiles and alterations in metabolic function of PAH vascular cells can be the cause of different phenotypic characteristics of different PAH subtypes.

It is likely that identifying new biomarkers with different origins or making combined use of them can provide additional prognostic information for an individual patient; therefore risk stratification models are needed. NMR and pericardial effusion were the dominant risk factors in our models. Our risk scores appeared to provide an improved clinical risk stratification model on which to add such biomarkers with the goal of optimal risk prediction. Testing in additional data sets will assess the broader generalizability of our findings, and in this manner, treatment goals can be identified to reduce functional impairment and prolong life.

This study represented a retrospective single-center experience conducted in a small patient group due to the rarity of the disease. The limited outcome did not allow including many variables in multivariable analyses of mortality. Nonetheless, NMR remained predictive of mortality after individually adjusting for important clinical variables, supporting its robust prognostic value. To minimize referral bias, we started our survival analysis from the first diagnosis of PAH by right heart catheterization. Finally, we did not validate our score performance in an external cohort. Future large studies with long follow-up and diverse population are needed to confirm the clinical relevance of our findings.

CONCLUSION

The present study demonstrates NMR as an independent prognostic factor in patients with PAH. A combination of hematologic biomarkers enabled us to develop a novel risk stratification model for survival.

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Author Contributions: BŞ, NÖŞ, MS, OT: Study design, Literature review, Data collection and processing, Patient therapy, Analysis **BŞ:** Data collection, Writing, Revisions

Ethical approval: All procedures performed in studies involving human participants were in accordance with the institutional and/or national research committee's ethical standards and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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Supplemantary Table 1: Medication of our study patients.

Medical treatment n, (%)	Overall PAH n=45	IPAH n=30	CTEPH n=15	Deaths n=9	Survivors n=36
Prostanoids	4 (9)	1 (3)	3 (20)	1 (11)	3 (8)
ERA	18 (40)	15 (50)	3 (20)	1 (11)	17 (47)
PDE	3 (7)	0 (0)	3 (20)	2 (22)	1 (3)
Prostanoids+ ERA	4 (9)	3 (10)	1 (7)	2 (22)	2 (5)
Prostanoids + PDE	1 (2)	0 (0)	1 (7)	0 (0)	1 (3)
ERA + PDE	8 (18)	4 (13)	4 (26)	1 (11)	7 (20)
Prostanoids + Era + PDE	7 (15)	7 (24)	0 (0)	2 (23)	5 (14)
Mono-therapy	25 (56)	16 (53)	9 (60)	4 (44)	21 (58)
Combination treatment	20 (44)	14 (47)	6 (40)	5 (56)	15 (42)

ERA, Endothelin receptor antagonists; PDE, Phosphodiesterase type-5 inhibitors.

Supplemantary Table 2: Comparison of the baseline characteristics of the fatal cases and survivors.

	Overall n=45	**Deaths N=9**	**Survivors N=36**	**p-value**	**Univariate OR (95% CI)**	**p-value**
Age, (years)	49 (32-58)	50 (32-57)	47 (33-59)	0.9	0.9 (0.9-1)	0.86
Sex n, (%)						
Female	29(64)	7(78)	22(61)	0.45	2.2 (0.4-12.3)	0.35
Follow-up duration (months)	26 (14-56)	12 (11-24)	31 (16-74)	**0.003**	1.0 (0.99-1.15)	0.05
Hemoglobin (mg/dL)	13.6 (12.3-15.3)	12.6 (12.2-13.6)	14 (12.3-15.5)	0.15	1.4 (0.9-2)	0.14
Platelet (10^3/mm^3)	204 (175-276)	256 (204-306)	197 (163-271)	0.06	1.1 (0.9-1.2)	0.08
RDW (%)	16.5 (14.1-17.4)	17.1 (15.1-18.2)	15.9 (14-17.3)	0.33	0.96 (0.75-1.2)	0.75
White blood cells (x10^9/L)	7.6 (6.6-8.9)	7.4 (7.2-8)	7.7 (6.4-9)	0.74	0.9 (0.6-1.2)	0.57
MCV (fl)	85 (78-91)	83 (82-91)	85 (77-91)	0.91	0.98 (0.9-1.1)	0.72
MPV (fl)	9.2 (8.7-10.2)	9.2 (8.6-9.7)	9.2 (8.7-10.2)	0.84	1.2 (0.6- 1.25)	0.51
NLR	2.6 (1.9- 3.3)	3.3 (2.3-6.3)	2.4 (1.8-3.2)	**0.04**	1.3 (1.04-1.58)	**0.02**
PLR	106 (83-157)	157 (135-300)	103 (78-146)	**0.01**	1.0 (1.00-1.01)	**0.02**
NMR	9.2 (6.5-11.6)	10.1 (9.4-11.8)	8.3 (6.1-9.8)	**0.01**	1.1 (1-1.2)	**0.01**
Creatinine (mg/dL)	0.9 (0.7-1)	0.8 (0.7-0.99)	0.9 (0.7-1)	0.5	2.7 (0.7-102)	0.6
CRP (mg/L)	5.7 (3.4-11.4)	5.7 (3.6-6.8)	5.6 (3.3-14)	0.4	0.36 (0.93-1.2)	0.36
Iron (µg/dL)	61 (40-68)	47 (42-65)	61 (39-76)	0.5	1.01 (0.98-1.0)	0.25
Ferritin (ng/mL)	25.3 (13.3-38.4)	38 (22.3-61.6)	24 (13-34)	0.1	0.98 (0.96-1)	0.15
mPAP (mmHg)	55 (45-65)	55 (50-55)	55 (45-65)	0.56	1.02 (1.01-1.03)	**0.01**
TAPSE (mm)	13 (12-15)	12 (11-12)	13 (12-15)	0.06	1.4 (0.9-2.1)	0.08
6MWD (meters)	290 (220-340)	240 (160-320)	300 (240-375)	0.09	1 (0.99-1)	0.10
Functional Class n, (%)						
Class II	21 (47)	2 (22)	19 (53)	0.24	3.6 (0.6-22)	0.28
Class III	18 (40)	5 (56)	13 (36)			
Class IV	6 (13)	2 (22)	4 (11)			
Pericardial effusion n, (%)	22 (49)	8 (89)	14 (39)	**0.01**	12.6 (1.4-111.7)	**0.02**
Type n, (%)						
KTEPH	15 (33)	5 (56)	10 (28)	0.13	0.3 (0.07-1.4)	0.12
IPAH	30 (67)	4 (44)	26 (72)			
PLR ≤134 n, (%)	27 (60)	2 (22)	25 (69)	**0.01**	7.9 (1.4-44.6)	**0.02**
PLR >134 n, (%)	18 (40)	7 (78)	11 (31)			
NLR ≤2.2 n, (%)	18 (40)	1 (11)	17 (47)	0.06	7.1 (0.8-63)	0.08
NLR >2.2 n, (%)	27 (60)	8 (89)	19 (53)			
NMR ≤9.2 n, (%)	24 (53)	1 (11)	23 (64)	**0.007**	10.1 (1.3-80.6)	**0.03**
NMR >9.2 n, (%)	21 (47)	8 (89)	13 (36)			
SCORE	2 (1-3)	4 (3-4)	2 (0-3)	**<0.0001**	3.2 (1.4-6.9)	**0.005**
PE-SCORE	3 (1-4)	6 (5-6)	2 (1-4)	**<0.0001**	2.3 (1.4-4)	**0.002**
Medication						
Mono-therapy	25 (56)	4 (44)	21 (58)	0.48	1.5 (0.4-5.7)	0.52
Combination treatment	20 (44)	5 (56)	15 (42)			