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Raised erythrocyte creatine in patients with pulmonary arterial hypertension — Evidence for subclinical hemolysis

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Summary
Background: Pulmonary arterial hypertension (PAH) has been associated with hemolytic conditions such as sickle cell disease but the possible role of hemolysis in the pathogenesis or pathophysiology of other forms of PAH has not been studied. Erythrocyte lifespan is the gold-standard test of hemolysis and may be measured by assaying erythrocyte creatine (EC) levels. EC decreases as the erythrocyte ages, so patients with hemolysis have high EC levels.
Methods: We measured EC and other parameters of hemolysis in patients with idiopathic and connective tissue associated PAH and normal controls.
Results: In patients with PAH ($n = 40$), EC levels were higher than in controls $n = 30$ (patients EC 1.72 mcmol/g HgB 95%CI[1.51, 1.96], controls EC 1.05 mcmol/g HgB [0.93, 1.19], $p < 0.0001$). High levels of EC correlated with worse 6 min walk ($r = -0.42$, $p < 0.0001$) and worse functional class ($p = 0.002$). Other indirect indices of hemolysis (total lactate dehydrogenase, red cell distribution width) were also increased in patients with PAH relative to controls.
Conclusions: There is evidence of subclinical hemolysis in patients with PAH, and higher levels of hemolysis are associated with poorer exercise capacity.

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Introduction

Pulmonary arterial hypertension (PAH) is a catastrophic disease affecting mostly young people.[1] The term PAH refers to a specific hemodynamic phenotype of pulmonary hypertension (PH, defined as mean pulmonary artery pressure >25 mmHg) with low left-sided cardiac pressures and high pulmonary vascular resistance. The diagnosis given to an individual patient is one of the subdivisions of the PAH class — idiopathic PAH, hereditary PAH, PAH associated with medical conditions such as connective tissue diseases, congenital heart disease etc.[1] The pathogenesis of PAH is complex and incompletely understood although inflammation and dysfunction of growth factor regulation are central to the process.[1]

Over the last decade there has been increasing interest in hemolysis as a potential cause of pulmonary hypertension. Free hemoglobin, when released from the erythrocyte acts as a 'sump' for nitric oxide (NO), and promotes endothelial dysfunction with thrombosis, inflammation, vasoconstriction and smooth muscle proliferation in the capillaries — the hyperhemolysis paradigm.[2] Similar pathophysiological changes are also seen in PAH.[3] In a study of hemoglobin—diaspirin solution given to patients after cardiac surgery as a blood-product alternative, a dose dependent rise in pulmonary artery pressure was observed.[4] Pulmonary hypertension has been reported in almost all chronic hemolytic diseases, such as the thalassemias and sickle cell anemia.[5,6] In the most recent PH consensus document, hemolysis has been accepted as an 'associated condition' PAH subclass, although many patients with hemolysis have PH associated with left heart disease or anemia-induced high cardiac output.[1,7] There are case reports of PAH-associated micro-angiopathic hemolysis.[8,9] A recent work also described an increase in red cell distribution width (RDW) in PAH patients, a parameter which may increase in hemolytic anemia.[10]

There are many laboratory parameters of hemolysis. The gold-standard test is to measure red-blood-cell (RBC) lifespan or half-life, which is reduced in hemolytic diseases.[11] Hemoglobin, hematocrit, reticulocyte counts and biochemical parameters (LDH, bilirubin, haptoglobin) are also useful, although these parameters may be affected by non-hemolytic factors such as bone marrow function, inflammation and liver disease.[11] RBC lifespan is measured by radio-labeling RBCs, re-injecting the cells into the patient and observing elimination of the radio-isotope over time.[12] These techniques are not commonly available outside of research laboratories. An alternative is to assay Erythrocyte Creatine (EC) levels, since RBC creatine levels decrease as the cell ages.[13] EC levels correlate well with the gold-standard 51-Cr labeling technique.[14] The clinical utility of EC has been established in a cohort of patients with hemolytic anemia and hemolysis caused by mechanical heart valves.[13,15]

We hypothesized that hemolysis might be present in PAH and this study hypothesis was that EC levels will be higher in PAH patients than in controls.

Methods

The study was approved by the Institutional Research Board of Rabin Medical Center and all subjects gave written informed consent to participate.

Participants

We enrolled patients during routine follow-up in the PH clinic at our center. Inclusion criteria for the PAH group were based on the standard hemodynamic criteria (Mean PAP >25 mmHg, PCWP ≤15 mmHg and PVR >3 Wood Units). Patients with idiopathic, hereditary or connective tissue disease associated PAH were eligible. Healthy control subjects were invited to participate, typically drawn from hospital staff or family members accompanying patients to the clinic. Exclusion criteria were known hemolytic disease, glucose-6-phosphatase deficiency, blood transfusion within 3 months or clinically evident blood loss within 3 months of study enrollment. Physiological and demographic data were extracted from the medical record.

Sample analysis

After giving informed consent, venous blood samples were drawn from each patient. Complete blood count and full chemistry panel were performed routinely in the hospital laboratory (Chemistry - Olympus AU2700, Olympus, Melville NY; Hematology-Advia 120, Siemens, Deerfield, IL). The EC was performed as per the technique described in detail elsewhere.[13] In brief, plasma and buffy coat are aspirated from the blood sample after centrifugation. A small sample of mixed packed RBCs is then hemolyzed in a four-fold volume of 1 g/L saponin solution over 20 min at room temperature. Aliquots of hemolyzed RBCs were then frozen at −80 °C until analysis. Hemosylate was deproteinized with 0.15 mol/L $Ba(OH)_2$ and 0.15 mol/L $ZnSO_4$. Supernatant was obtained by centrifugation and filtration. Creatine concentration in the supernatant was measured with the enzymatic assay method involving creatine amidinohydrolase, sarcosine oxidase and peroxidase.[13] Measured data were expressed as micromole creatine per gram of hemoglobin (mcmol/g Hb). Aliquots of serum were collected from centrifuged clotted-blood and stored at −80 °C for LDH isoenzyme assay (Hydragel 150 assay, Sebia, Norcross, GA).

Statistical analysis

Subjects were grouped for analysis according to their enrollment diagnosis. Data were tabulated as Mean [95% Confidence Intervals] (CI). Parameters found to be significantly right-skewed were log transformed before analysis, with mean/CI reported as anti-logs.[16] Differences between groups were tested with Student's *t*-test, analysis of variance (ANOVA), the Wilcoxon Ranked Sums test or the Chi-Squared test as appropriate. The relationship between parameters of interest was modeled by linear modeling or general linear modeling, as required by the type of data analyzed. We performed a series of pre-planned post-hoc analyses to evaluate the relationship between EC and traditional hemolytic parameters, PAH sub-diagnoses, use of PAH-specific medication disease severity. All analyses were performed on R statistical software version 2.11.1.[17]

Table 1 Participants in the study — demographic and laboratory results.

	PAH	Normals	*p* value
Age	52 [47, 56]	41 [31, 45]	0.01
Sex (M/F)	11/29	14/16	0.10*
PAH Subdiagnosis	Idiopathic 24		
	Connective Tissue 16		
PAH treatment	Pre-treatment 23		
	PDE5-I 7		
	ERA 11		
	Prostanoid 10		
	Monotherapy 8		
	Combination 9		
Hemoglobin (g/dl)	13.3 [12.7, 14.0]	14.0 [13.5, 14.4]	0.15
RDW (%)	15.2 [14.6, 15.9]	12.8 [12.6, 13.0]	<0.0001†
Erythrocyte creatine (mcmol/g Hb)	1.72 [1.51, 1.96]	1.05 [0.93, 1.19]	<0.0001
LDH total (units/l)	494 [454, 539]	348 [323, 374]	<0.0001
LDH-1%	25.2 [23.5, 27.0]	27.3 [25.6, 29.2]	0.10
AST (units/l)	24 [21, 28]	21 [19, 23]	0.12
ALT (units/l)	22 [18, 26]	18 [15, 22]	0.15
Total Bilirubin (mg/dl)	0.65 [0.55, 0.75]	0.56 [0.47, 0.66]	0.22
Unconjugated Bilirubin (mg/dl)	0.46 [0.37, 0.57]	0.45 [0.38, 0.54]	0.89
Haptoglobin (mg/dl)	111 [93, 134]	97 [84, 112]	0.24
NT-proBNP (pg/ml)	461 [291, 732]	Not measured	—

All data are presented as mean [95% confidence interval] except for count data. All *p* values represent the result of a *t*-test except where marked: *Chi-Squared, †Wilcoxon Rank Sum test.
Abbreviations: AST, Aspartate Aminotransferase; ALT, Alanine aminotransferase; LDH, Lactate Dehydrogenase; RDW, Red cell distribution width; BNP, Brain natriuretic peptide; PAH, Pulmonary Arterial Hypertension; PDE5-I, Phosphodiesterase inhibitor; ERA, Endothelin Receptor Anatagonist.

Results

Seventy subjects were enrolled (Table 1). Patients with PAH were older than controls ($p = 0.01$). Patients with PAH had significantly higher levels of erythrocyte creatine and LDH, Fig. 1. RDW was also significantly higher in PAH compared to controls.

Post-hoc analysis of erythrocyte creatine

We correlated EC with other accepted measurements of hemolysis with linear regression. EC correlated significantly with RDW ($r = 0.57$, $p < 0.0001$), LDH ($r = 0.49$, $p < 0.0001$) and Hb ($r = -0.40$, $p < 0.0001$), but not with haptoglobin, AST and bilirubin. In a subgroup analysis, EC was highest in PAH-connective tissue disease (2.06 95%CI [1.68, 2.53]), followed by idiopathic PAH (1.54 95%CI[1.32, 1.86]) and then normals (1.05 95%CI[0.93, 1.19]); ANOVA $p < 0.0001$, Tukey test $p \leq 0.05$ for all comparisons. EC levels were not different between groups of patients taking or not taking PAH-specific medications, when analyzed as treated vs untreated (*t*-test, $p = 0.44$) or when grouped by class of PAH medication (ANOVA, $p = 0.54$).

In the PAH subject group, we analyzed the interaction between EC and disease severity. We found a significant negative correlation between Erythrocyte Creatine and 6 min walk distance ($r = -0.42$, $p < 0.0001$), Fig. 2. There were also significantly higher EC levels in patients with worse NYHA functional class: NYHA I-II (1.38 95%CI[1.19, 1.61]) or NYHA III-IV (2.04 95%CI[1.71, 2.44]) $p = 0.002$, Fig. 3. There was no significant correlation between EC and NT-proBNP ($r = 0.18$, $p = 0.27$). No significant correlations were found between echocardiographically estimated SPAP or the presence of right ventricular dysfunction. Over 36 months of follow-up, 13/40 PAH subjects died. There was no significant difference in EC between the survivors (1.75 95%CI[1.51, 2.03]) and non-survivors (1.71 95%CI[1.29, 2.26]) $p = 0.97$.

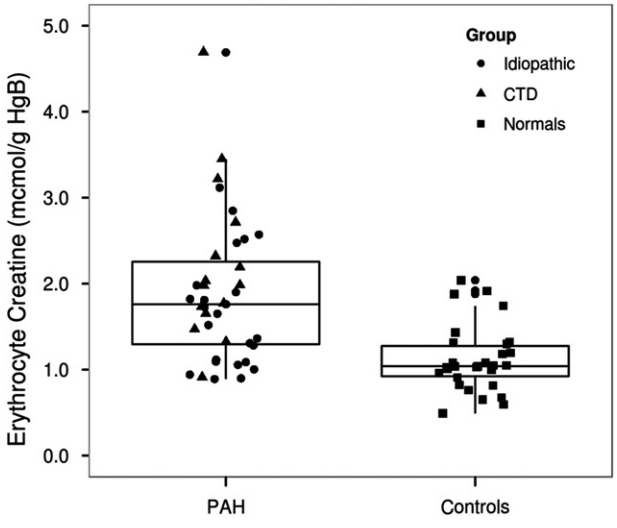

Figure 1 Erythrocyte Creatine levels in the different diagnostic groups. PAH patients are grouped together for analysis.

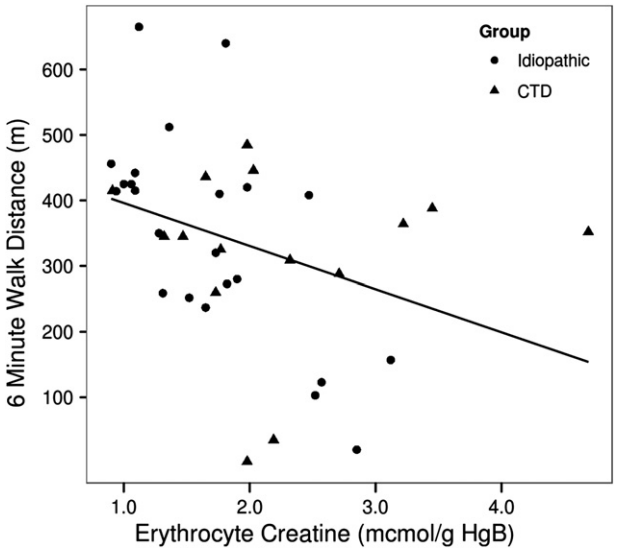

Figure 2 Correlation between Erythrocyte Creatine levels and 6 min walk. PAH patients were analyzed together as a group.

Discussion

We analyzed hemolytic parameters in 40 patients with PAH and 30 healthy controls. We demonstrated that RBC lifespan (as assessed by Erythrocyte Creatine EC) is shorter in patients with PAH, with highest values seen in patients with PAH associated with connective tissue disease. This indicates that subclinical hemolysis is present in patients with PAH.

As far as we know, this is the first investigation of hemolysis in PAH patients. We measured an index of RBC survival rather than derived/indirect measures of hemolysis such as LDH. This gives a strong support to our hypothesis, since RBC survival is the gold-standard test of hemolysis.[11] Levels of EC in PAH patients, especially those with connective tissue disease were only slightly lower than patients with recognized hemolysis from mechanical heart valves (2.16 mcmol/g Hb, 95%CI[1.89, 2.43]).[15] Patients with overt hemolytic anemia have the highest reported EC levels (5.77 mcmol/g Hb, 95%CI[4.33, 8.16]).[13] Although we did not measure the gold-standard test of RBC lifespan (51-Cr labeling), EC is an established index of hemolysis which correlates with the gold-standard.[13,14] Another interesting phenomenon was the correlation between shortened RBC survival (high EC) and worse exercise capacity. This is interesting because poor exercise capacity is a known risk factor for mortality, although in this small sample there was no difference in EC between survivors and non-survivors. The PAH cohort included untreated and treated patients, since some (n = 23) were recruited during their diagnostic workup before commencement of therapy. We did not establish any effect of therapy on EC although this finding must be interpreted with caution due to the small sample size. The prognostic significance of EC and the effect of medication on EC can only be established in a larger study.

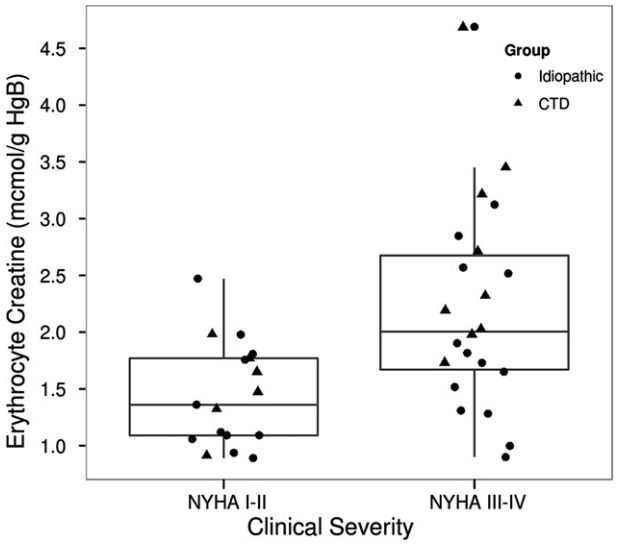

Figure 3 Erythrocyte Creatine levels in the PAH patients according to disease severity. Patients were assigned to two groups - mild disease (NYHA I-II) or severe disease (NYHA III-IV).

It is not possible to determine from the current study whether the hemolysis present in PAH patients represents a cause, effect or epi-phenomenon of the illness. Highest levels of EC were observed in PAH patients with connective tissue disease (scleroderma or lupus in this cohort). Both scleroderma and lupus are associated with hemolysis via micro-angiopathic or auto-immune mechanisms, respectively. Hemolysis in PAH has only been reported in isolated case reports, where patients also had thrombocytopenia.[9,8] We speculate that these patients may have had occult lupus presenting as PAH, since thrombocytopenia is unusual in the presentation of idiopathic PAH. The theory that hemolysis promotes PH by depleting nitric oxide (NO) is derived from study of patients with sickle cell disease, where large amounts of RBC contents are spilled into the plasma during a crisis.[2] Some authors have questioned whether the hyperhemolysis paradigm is sufficient to explain the degree of PH in sickle patients.[11] Given that hemolysis during a sickle crisis is clinically significant compared to the subclinical hemolysis we observed in the PAH patients, it seems unlikely that a hyperhemolysis paradigm is a primary cause of PAH. However, as Hebbel points out, there are many other potentially toxic substances released from a hemolyzed RBC such as arginase and RBC microparticles, and these may also contribute to the development of PH. Intuitively it is more likely that hemolysis-associated loss of NO might exacerbate vasoconstriction in the already sick endothelial bed of the diseased PAH lung.[11] This might explain the correlation between high EC levels and lower 6 min and worse NYHA functional class, and also the finding that PAH patients with high RDW have worse mortality.[10] Further studies are clearly needed to better define the nature of the association between hemolysis and PAH.

Conclusion

To summarize, there is biochemical evidence of subclinical hemolysis in patients with PAH, although the cause and consequences of this process are not yet clear. Increased

hemolysis is associated with worse exercise capacity. We consider this work to be hypothesis-generating.

Authors' contributions

BF conceived the study, recruited patients, performed laboratory analyses, analyzed the data and drafted the manuscript. OT performed the erythrocyte creatine assays, analyzed the data and edited the manuscript for important intellectual content. LAF, MK, YR recruited patients, performed laboratory analyses and edited the manuscript for important intellectual content. MRK conceived the study, recruited patients and edited the manuscript for important intellectual content. All authors approved the final draft for publication.

Conflicts of interest

This study was investigator-initiated and was supported by an unrestricted financial grant from Rafa Pharmaceuticals, Israel for purchase of laboratory equipment and reagents and for inter-laboratory transportation of samples. The funding source had no influence on the study design, implementation, analysis or drafting of the manuscript. None of the authors have any competing interests that could have influenced the conduct or reporting of this study.

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