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The Pyruvate Kinase (PK) to Hexokinase Enzyme Activity Ratio and PK-R Protein Level in the Diagnosis and Phenotype of PK Deficiency

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ABSTRACT

Diagnosis of pyruvate kinase deficiency (PKD), the most common cause of hereditary nonspherocytic hemolytic anemia, remains challenging in routine practice and no biomarkers for clinical severity have been characterized. This prospective study enrolled 41 patients with molecularly-confirmed PKD from 9 North American centers to evaluate the diagnostic sensitivity of pyruvate kinase (PK) enzyme activity and PK:hexokinase (HK) enzyme activity ratio and evaluate the pyruvate kinase-red cell (PK-R) protein level and erythrocyte metabolites as biomarkers for clinical severity. In this population not transfused for ≥90 days before sampling, the diagnostic sensitivity of PK enzyme assay was 90% (95% CI, 77%-97%) whereas the PK:HK ratio sensitivity was 98% (95% CI, 87%-100%). There was no correlation between PK enzyme activity and clinical severity. Transfusion requirements correlated with normalized erythrocyte ATP levels (r=0.527, *P*=0.0016) and PK-R protein levels (r=-0.527, *P*=0.0028). PK-R protein levels were significantly higher in never transfused (median=40.1%, range=9.8-73.9%) versus ever transfused (median=7.7%, range=0.4-15.1%) patients (*P*=0.0014). The PK:HK ratio had excellent sensitivity for PK diagnosis, superior to *PKLR* exon sequencing. Given that the number of *PKLR* variants and genotype combinations limits prognostication based on molecular findings, PK-R protein level may be a useful prognostic biomarker of disease severity and merits further study.

INTRODUCTION

Pyruvate kinase deficiency (PKD) is the most common cause of chronic hereditary non-spherocytic hemolytic anemia and results from mutations in the *PKLR* gene. Over 300 distinct pathogenic mutations have been documented (Pissard*, et al* 2006, Warang*, et al* 2013). Prevalence estimates of PKD vary widely between 1:20,000 and 1:300,000 in Caucasian populations (Beutler and Gelbart 2000, Carey*, et al* 2000), likely because the diagnosis of PKD remains challenging in routine practice (Al-Samkari*, et al* 2020). While the pyruvate kinase (PK) enzyme assay is typically employed as an initial diagnostic screen in patients with unidentified Coombs-negative hemolytic anemia, PK enzyme levels may be falsely normal in patients receiving red cell transfusion within 90 days, in those with profound reticulocytosis (as PK enzyme activity is red cell age-dependent), or in patients with *PKLR* mutations resulting in unusual biochemical properties (Bianchi*, et al* 2019, Grace*, et al* 2015). Molecular testing

similarly presents challenges: up to 20% of affected patients have new *PKLR* variants, up to 10% of patients have variants not detected through routine exon sequencing, and molecular testing may be cost-prohibitive (Grace*, et al* 2018). After the diagnosis is established, there are no known biomarkers of clinical severity which could be utilized for determining prognosis or monitoring. While PK enzyme activity does not have a relation with *PKLR* mutation severity (Grace*, et al* 2018), it remains unresolved whether phenotypic severity has any relation to enzyme activity in PKD. Given the current diagnostic limitations and the broad phenotypic spectrum of disease, alternative diagnostic strategies and biomarkers of clinical severity are needed.

Therefore, we aimed to describe the correlation of PK enzyme activity, erythrocyte PK (PK-R) protein level, and erythrocyte metabolites [adenosine triphosphate (ATP), 2,3-diphosphoglycerate (2,3-DPG)] with clinical phenotype as well as estimate the sensitivity of PK enzyme activity and the pyruvate kinase:hexokinase (PK:HK) ratio to diagnose PKD in a cohort of patients with genetically-confirmed PKD. Because hexokinase (HK) is also red cell age-dependent, the use of the PK:HK ratio may correctly diagnose patients with normal PK enzyme activity in the setting of profound reticulocytosis, a common finding in PKD. This ratio has previously been demonstrated to correctly diagnose PK deficiency in a few select patients (Bianchi*, et al* 2019). In this study, we had the opportunity to establish the validity of this observation in a large number of patients with molecularly-confirmed PKD.

METHODS

A subset of 9 North American sites from the PKD Natural History Study (NHS) (NCT02053480) (Grace*, et al* 2018) participated in a sub-study, in which blood samples were collected at a single time-point from all enrolled patients. All patients had two confirmed *PKLR* mutations and no red cell transfusions for ≥90 days prior to the blood sample. Erythrocyte PK and HK enzyme activity testing were performed using standard biochemical assays (Beutler 1984) at the Stanford Red Blood Cell Special Studies Laboratory (reference range for PK activity, 3.2-6.5 EU/gm Hb and for HK activity, 0.14-0.37 EU/gm Hb). Baseline erythrocyte PK-R protein was quantitated by antibody-based capture and detection using an electrochemiluminescence immunoassay (Meso Scale

Discovery, Rockville, MD) and the signal normalized to a control sample without PKD. Erythrocyte ATP and 2,3-DPG concentrations (μg/ml) in blood were analyzed using qualified tandem mass spectrometry methods and then normalized for individual hemoglobin (Hb) values [ATP and 2,3-DPG concentrations were converted to g/dL and divided by Hb (g/dL)]. Spearman correlation coefficients were calculated to estimate the correlation between continuous variables, and the Wilcoxon rank-sum test was used to assess the association of continuous and binary variables.

RESULTS AND DISCUSSION

Of the 255 patients in the PKD NHS, 41 patients participated in this sub-study. The median age was 25.3 years (range 1.4-60.4) and 80% of patients were splenectomized (Table). Mean measured PK enzyme activity level was 1.1 EU/gm Hb; 37/41 of patients had low PK activity, corresponding to a diagnostic sensitivity of 90% [95% confidence interval (CI), 77%-97%]. Normal PK activity was observed in 4 patients, 3 of whom had high HK activity and a low PK/HK ratio (reference normal mean PK/HK ratio 15.6, range: 8.7-22.5). In total, 40/41 patients had a low PK/HK ratio, corresponding to a sensitivity for PKD diagnosis of 98% (95% CI, 87%-100%). The single patient not accurately diagnosed with this method had a PK enzyme activity of 4.6 EU/gm Hb and a PK/HK ratio of 9.2, which is at the low end of the normal range. There were no correlations between PK enzyme activity and clinical severity indicators, including post-splenectomy hemoglobin (r=0.109, *P*=0.56), total number of lifetime transfusions (r=-0.107, *P*=0.54), transfusion status (ever transfused vs. never transfused; *P*=0.30), or splenectomy status (splenectomized vs. not splenectomized; *P*=0.41).

Normalized ATP levels strongly positively correlated with normalized 2,3-DPG levels (r=0.93, *P*=<0.0001, Figure 1A), moderately positively correlated with total lifetime transfusions (r=0.527, *P*=0.0016, Figure 1B) and percent reticulocyte count (r=0.581, *P*=0.0003, Figure 1C), and were higher in ever transfused (median=0.0022, range=0.0011-0.0029) compared with never transfused patients (median=0.0012, range=0.0008-0.0020), (*P*=0.0037).

PK-R protein percentage was moderately inversely correlated with the total number of transfusions prior to enrollment (r=-0.527, *P*=0.0028, Figure 1D) and was higher in never transfused (median=40.1%, range=9.8-73.9%) compared with ever transfused patients (median=7.7%, range=0.4%-15.1%), (*P*=0.0014, Figure 1E). The PK-R protein level in the Amish population, which shares a common homozygous R479H genotype, was relatively consistent among patients as compared to the wide variation of PK-R protein levels of non-Amish patients with differing genotypes (Table). In addition, PK-R protein levels were numerically higher in patients with two missense *PKLR* variants (median=7.7%) as compared with those with at least one drastic variant (median=2.0%), although this difference was not statistically significant (p=0.36).

There are two important conclusions from this study. The first is that the PK:HK ratio appears to have excellent sensitivity for the diagnosis of PKD. With a sensitivity of 98% in this study population of non-transfused patients, this ratio was more sensitive than PK activity in isolation and even more sensitive than *PKLR* exon sequencing to diagnose PKD (Grace*, et al* 2018). Therefore, based on this data, the PK:HK ratio may be considered a standard component of the PKD diagnostic evaluation. Individuals who are heterozygous carriers of a *PKLR* variant will also often have low PK activity; thus, genetic testing has an important role as a confirmatory test (Bianchi*, et al* 2019). In addition, genetic testing will often be required to establish the diagnosis in patients with recent transfusions as well as those with suspected PKD and a normal PK enzyme activity and PK:HK ratio. The second major conclusion is that erythrocyte PK enzyme activity does not correlate with markers of clinical severity in PKD, but that erythrocyte PK-R protein and ATP levels do, in particular the need for transfusions.

While a positive correlation between erythrocyte ATP level and 2,3-DPG level and a negative correlation between erythrocyte ATP level and disease severity seem counterintuitive, these findings may be due to the higher reticulocyte count noted in patients with more robust hemolysis and lower hemoglobin levels (Figure 1C). Reticulocytes have higher ATP on a per-cell basis than mature erythrocytes (Mentzer*, et al* 1971), and higher reticulocyte counts in patients with severe disease are likely indicative of the elevated erythropoietic drive necessary to compensate for more severe hemolysis. PK-R protein levels appear to correlate with transfusion needs. Given that

the number of *PKLR* variants and genotype combinations limits predicting outcomes based on molecular findings, erythrocyte PK-R protein levels may represent a better marker of disease severity. Additional studies to further elucidate the prognostic value of erythrocyte PK-R protein in patients could help to confirm whether this is a useful future biomarker for clinicians managing patients with PKD.

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Table. Patient characteristics and results of erythrocyte metabolite and PK enzyme assays. RBC, red blood cell; DPG, diphosphoglycerate; ATP, adenosine triphosphate; PK-R, pyruvate kinase-red cell; PK, pyruvate kinase; HK, hexokinase; EU, enzyme unit; Hb, hemoglobin.

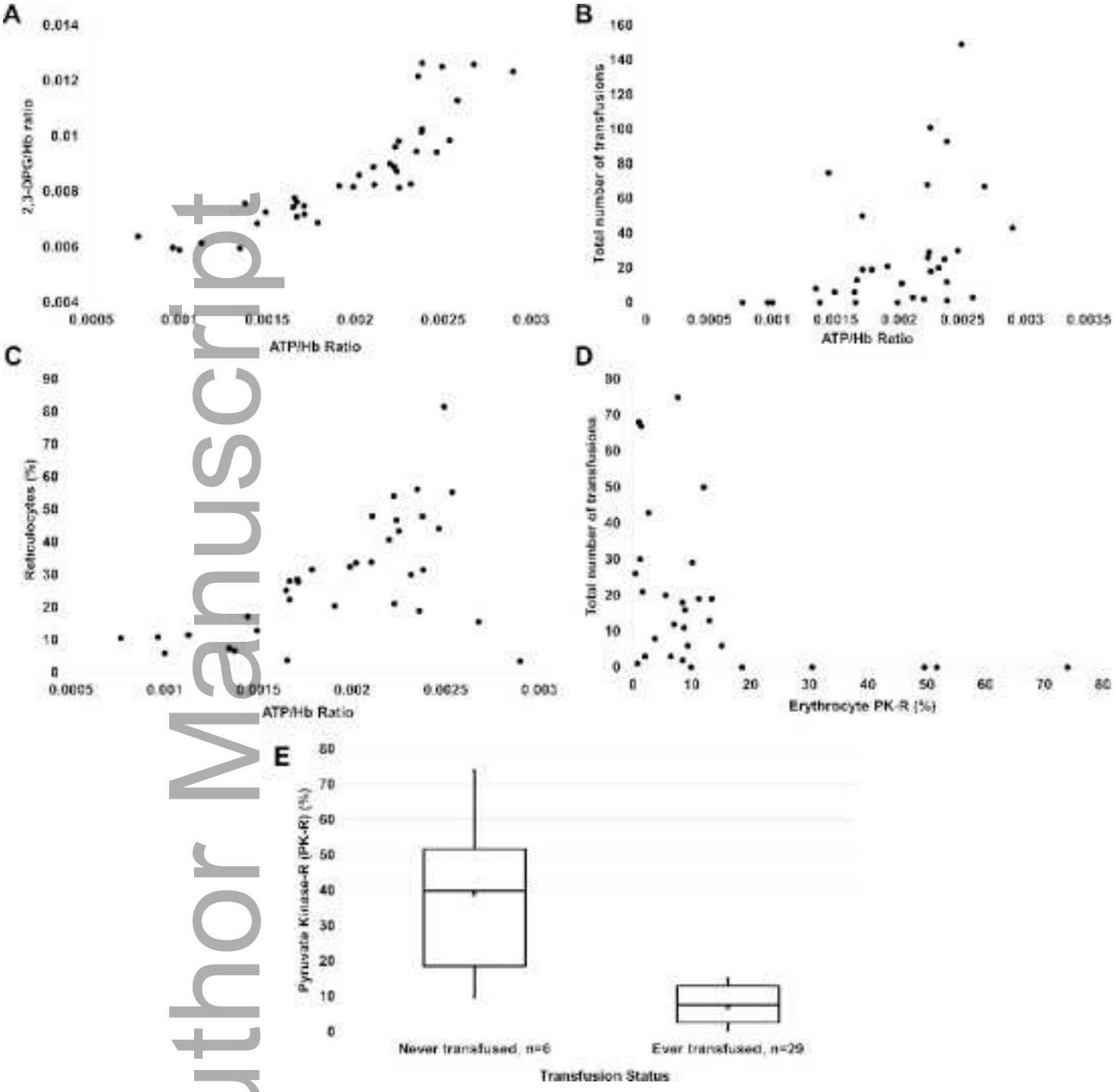
Patient Characteristics (N=41)	**Value**
Median age at enrollment (years, range)	25.3 (1.4-60.4)
Female	21 (51%)
Amish	21 (51%)
Splenectomy	33 (80%)
Total lifetime RBC transfusions, median (range) (N=34)	17 (0-149)
Red Cell Metabolite Levels	**Result**
2,3-DPG (µg/ml) level, median (range) (N=39)	796 (573-995)
Normalized 2,3-DPG level, median (range) (N=38)	0.0083 (0.0059-0.0126)
ATP (µg/ml) level, median (range) (N=39)	179 (78-233)
Normalized ATP level, median (range) (N=38)	0.0021 (0.0008-0.0029)
PK-R Protein Levels	**Result**
PK-R protein percentage[a], median (range) (N=37)	8.70 (0.41-73.95)
Non-Amish, median (range) (N=16)	3.20 (0.41-73.95)
Missense/Missense, n=7	7.66 (0.41-73.95)
Missense/Non-missense, n=8	2.03 (0.72-51.71)
Non-missense/Non-missense, n=1	2.02 (-)
Amish (range), median (N=21)	9.06 (4.53-15.62)
Enzyme Activity (N=41)	**Result**
PK activity (EU/gm Hb), median (range)	1.1 (0.2-4.6)
HK activity (EU/gm Hb), median (range)	0.7 (0.3-1.4)
PK activity (category)	

Low (<3.2 EU/gm Hb)	37 (90%)
Normal (3.2-6.5 EU/gm Hb)	4 (10%)
High (>6.5 EU/gm Hb)	0 (0%)
PK:HK ratio[b] median (range)	1.88 (0.30-9.20)
Low PK/HK ratio	40 (98%)

[a]Normalized to hemoglobin concentration and expressed as the percentage of protein present in a healthy subject without PKD.

[b]Normal range for PK:HK ratio is 8.7-22.5.

Figure 1. Relation of erythrocyte metabolites with one another or markers of clinical severity in PKD. (A) Erythrocyte ATP versus erythrocyte 2,3-DPG (N=38, r=-0.93; *P* <0.0001). (B) Erythrocyte ATP versus total number of lifetime RBC transfusions (N=33, r=0.527, *P* =0.0016). (C) Erythrocyte ATP versus percent reticulocyte count (N=35, r=0.581, *P*=0.0003). (D) Erythrocyte PK-R protein versus total number of lifetime RBC transfusions (N=30, r=-0.527, *P*=0.0028). (E) Erythrocyte PK-R protein level in never transfused (N=6) versus ever transfused (i.e., received at least one red cell transfusion over the lifetime, N=29) patients (*P*=0.0014).



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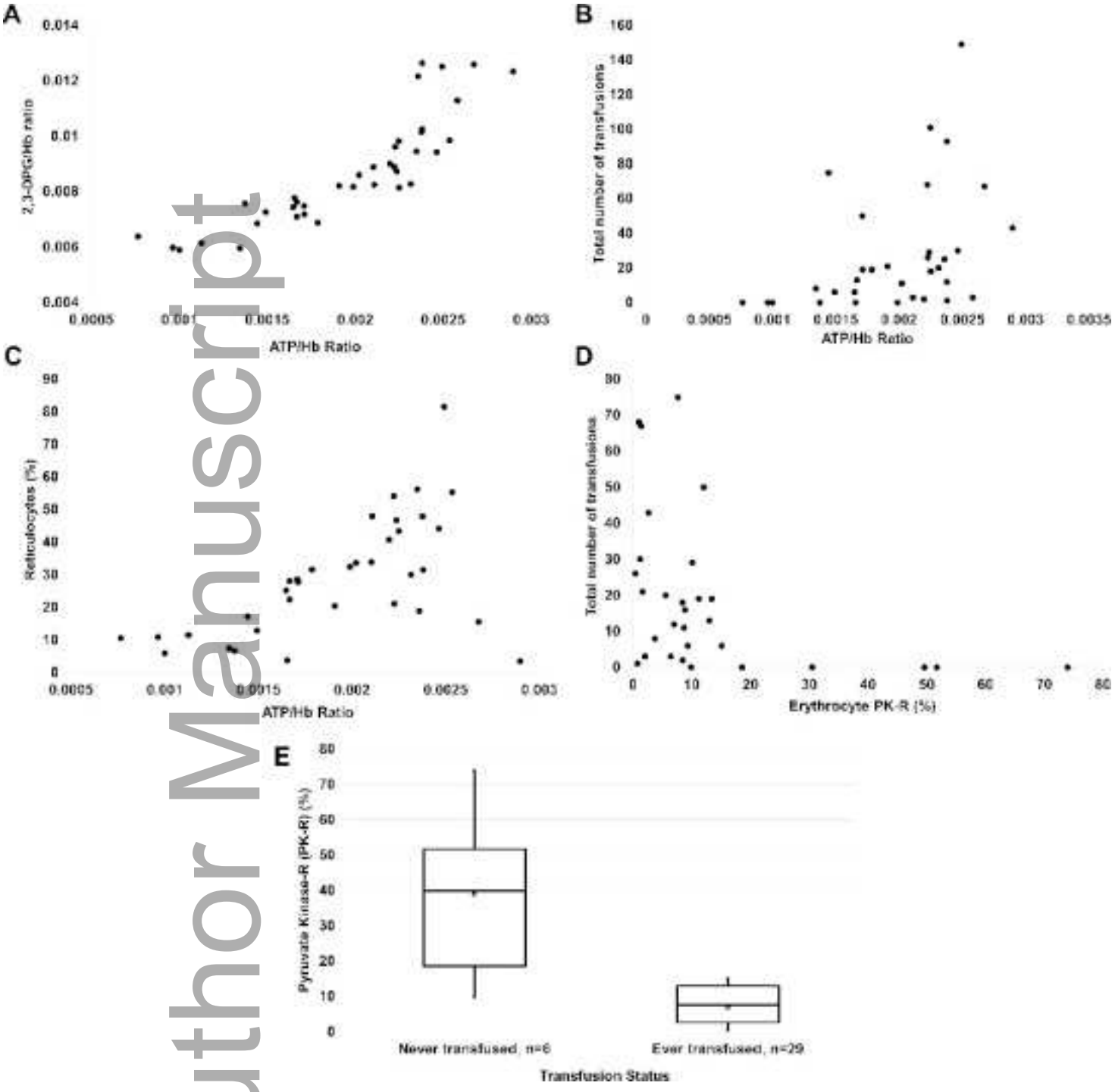

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