bjh short report

The pyruvate kinase (PK) to hexokinase enzyme activity ratio and erythrocyte PK protein level in the diagnosis and phenotype of PK deficiency

Hanny Al-Samkari,[1] Kathryn Addonizio,[2] Bertil Glader,[3] D. Holmes Morton,[4,5] Satheesh Chonat,[6,7] Alexis A. Thompson,[8] Kevin H. M. Kuo,[9] Yaddanapudi Ravindranath,[10] Heng Wang,[11] Jennifer A. Rothman,[12] Janet L. Kwiatkowski,[13] Charles Kung,[14] Penelope A. Kosinski,[14] Hasan Al-Sayegh,[2] Wendy B. London[2] and Rachael F. Grace[2]

[1]*Division of Hematology, Massachusetts General Hospital, Harvard Medical School,* [2]*Dana-Farber/Boston Children's Cancer and Blood Disorders Center, Harvard Medical School, Boston, MA,* [3]*Lucile Packard Children's Hospital, Stanford University, Palo Alto, CA,* [4]*Central Pennsylvania Clinic for Special Children & Adults, Belleville, PA,* [5]*Lancaster General Hospital, Lancaster, PA,* [6]*Department of Pediatrics, Emory University School of Medicine, Atlanta, GA,* [7]*Aflac Cancer and Blood Disorders Center, Children's Healthcare of Atlanta, Atlanta, GA,* [8]*Ann & Robert H. Lurie Children's Hospital of Chicago, Chicago, IL, USA,* [9]*University of Toronto, University Health Network, Toronto, ON, Canada,* [10]*Children's Hospital of Michigan,Wayne State University School of Medicine, Detroit, MI,* [11]*DDC Clinic for Special Needs Children, Middlefield, OH,* [12]*Duke University Medical Center, Durham, NC,* [13]*Children's Hospital of Pennsylvania and Perelman School of Medicine of the University of Pennsylvania, Philadelphia, PA, and* [14]*Agios Pharmaceuticals, Cambridge, MA, USA*

Correspondence: Hanny Al-Samkari, Division of Hematology, Massachusetts General Hospital, Zero Emerson Place Suite 118 Office 112, Boston, MA 02114, USA.
E-mail: hal-samkari@mgh.harvard.edu

Summary

Diagnosis of pyruvate kinase deficiency (PKD), the most common cause of hereditary non-spherocytic haemolytic anaemia, remains challenging in routine practice and no biomarkers for clinical severity have been characterised. This prospective study enrolled 41 patients with molecularly confirmed PKD from nine North American centres to evaluate the diagnostic sensitivity of pyruvate kinase (PK) enzyme activity and PK:hexokinase (HK) enzyme activity ratio, and evaluate the erythrocyte PK (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 the PK enzyme assay was 90% [95% confidence interval (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 normalised 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 the never transfused [median (range) 40·1 (9·8–73·9)%] *versus* ever transfused [median (range) 7·7 (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.

Keywords: pyruvate kinase, pyruvate kinase deficiency, hexokinase, enzyme assay, pyruvate kinase protein.

First published online 28 May 2020
doi: 10.1111/bjh.16724

Pyruvate kinase deficiency (PKD) is the most common cause of chronic hereditary non-spherocytic haemolytic anaemia and results from mutations in the *PKLR* gene. Over 300 distinct pathogenic mutations have been documented.[1,2] Prevalence estimates of PKD vary widely between 1:20 000 and 1:300 000 in Caucasian populations,[3,4] likely because the diagnosis of PKD remains challenging in routine practice.[5] While the pyruvate kinase (PK) enzyme assay is typically employed as an initial diagnostic screen in patients with unidentified Coombs-negative haemolytic anaemia, 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.[6,7] 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.[8] After the diagnosis is established, there are no known biomarkers of clinical severity that could be utilised for determining prognosis or monitoring. While PK enzyme activity does not have a relation with *PKLR* mutation severity,[8] 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 the disease, alternative diagnostic strategies and biomarkers of clinical severity are needed.

Therefore, in the present study 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 PK:hexokinase (PK:HK) ratio to diagnose PKD in a cohort of patients with genetically confirmed PKD. Because 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 shown to correctly diagnose PKD in a few select patients.[6] In the present study, we had the opportunity to establish the validity of this observation in a large number of patients with molecularly confirmed PKD.

Patients and methods

A subset of nine North American sites from the PKD Natural History Study (ClinicalTrials.gov Identifier: NCT02053480)[8] 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[9] at the Stanford Red Blood Cell Special Studies Laboratory [reference range for PK activity, 3·2–6·5 eu/g haemoglobin (Hb) and for HK activity, 0·14–0·37 eu/g Hb]. Baseline erythrocyte PK-R protein was quantified by antibody-based capture and detection using an electrochemiluminescence immunoassay (Meso Scale Discovery, Rockville, MD, USA) and the signal normalised to a control sample without PKD. Erythrocyte ATP and 2,3-DPG concentrations (μg/ml) in blood were analysed using qualified tandem mass spectrometry methods and then normalised for individual Hb values [ATP and 2,3-DPG concentrations were converted to g/l and divided by Hb (g/l)]. 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 Natural History Study, 41 patients participated in this sub-study. The median (range) age was 25·3 (1·4–60·4) years and 80% of the patients were splenectomised (Table I). The mean measured PK enzyme activity level was 1·1 eu/g 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 four patients, three of whom had high HK activity and a low PK:HK ratio [reference normal mean (range) PK:HK ratio 15·6 (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/g 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 Hb ($r = 0{\cdot}109$, $P = 0{\cdot}56$), total number of lifetime transfusions ($r = -0{\cdot}107$, $P = 0{\cdot}54$), transfusion status (ever transfused vs. never transfused; $P = 0{\cdot}30$), or splenectomy status (splenectomised vs. not splenectomised; $P = 0{\cdot}41$).

Normalised ATP levels strongly positively correlated with normalised 2,3-DPG levels ($r = 0{\cdot}93$, $P < 0{\cdot}0001$, Fig 1A), moderately positively correlated with total lifetime transfusions ($r = 0{\cdot}527$, $P = 0{\cdot}0016$, Fig 1B) and percentage reticulocyte count ($r = 0{\cdot}581$, $P = 0{\cdot}0003$, Fig 1C), and were higher in ever transfused [median (range) 0·0022 (0·0011–0·0029)] compared with never transfused patients [median (range) 0·0012 (0·0008–0·0020)] ($P = 0{\cdot}0037$).

The PK-R protein percentage was moderately inversely correlated with the total number of transfusions prior to enrollment ($r = -0{\cdot}527$, $P = 0{\cdot}0028$, Fig 1D) and was higher in never transfused [median (range) 40·1 (9·8–73·9)%] compared with ever transfused patients [median (range) 7·7 (0·4–15·1)%] ($P = 0{\cdot}0014$, Fig 1E). The PK-R protein level in the Amish population, which shares a common homozygous R479H genotype, was relatively consistent amongst patients as compared to the wide variation of PK-R protein levels of non-Amish patients with differing genotypes

Table I. Patients' characteristics and results of erythrocyte metabolite and PK enzyme assays.

Patient characteristics (N = 41)	Value
Age at enrollment, years, median (range)	25·3 (1·4–60·4)
Female, *n* (%)	21 (51)
Amish, *n* (%)	21 (51)
Splenectomy, *n* (%)	33 (80)
Total lifetime RBC transfusions, *n*, median (range) (n = 34)	17 (0–149)
Red cell metabolite levels	**Result**
2,3-DPG level, μg/ml, median (range) (n = 39)	796 (573–995)
Normalised 2,3-DPG level, median (range) (n = 38)	0·0083 (0·0059–0·0126)
ATP level, μg/ml, median (range) (n = 39)	179 (78–233)
Normalised ATP level, median (range) (n = 38)	0·0021 (0·0008–0·0029)
PK-R protein levels	**Result**
PK-R protein percentage*, 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/g Hb, median (range)	1·1 (0·2–4·6)
HK activity, eu/g Hb, median (range)	0·7 (0·3–1·4)
PK activity (category), *n* (%)	
Low (<3·2 eu/g Hb)	37 (90)
Normal (3·2–6·5 eu/g Hb)	4 (10)
High (>6·5 eu/g Hb)	0 (0)
PK:HK ratio†, median (range)	1·88 (0·30–9·20)
Low PK/HK ratio, *n* (%)	40 (98)

RBC, red blood cell; DPG, diphosphoglycerate; ATP, adenosine triphosphate; PK-R, pyruvate kinase-red cell; PK, pyruvate kinase; HK, hexokinase; eu, enzyme unit; Hb, haemoglobin.
*Normalised to Hb concentration and expressed as the percentage of protein present in a healthy subject without PKD.
†Normal range for PK:HK ratio is 8·7–22·5.

(Table I). 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 the present 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.[8] Therefore, based on these 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.[6] 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 haemolysis and lower Hb levels (Fig 1C). Reticulocytes have higher ATP on a per-cell basis than mature erythrocytes,[10] and higher reticulocyte counts in patients with severe disease are likely indicative of the elevated erythropoietic drive necessary to compensate for more severe haemolysis. 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.

Acknowledgements

H. Al-Samkari is the recipient of the National Hemophilia Foundation-Shire Clinical Fellowship Award, the Harvard KL2/Catalyst Medical Research Investigator Training Award, and the American Society of Hematology Scholar Award.

Sources of funding

This study was supported by funding from Agios Pharmaceuticals.

Author contributions

H. Al-Samkari wrote the first draft of the manuscript and contributed to data analysis, creation of tables and figures, critical revision of the manuscript, and final approval; K. Addonizio contributed to data collection, data analysis, creation of tables and figures, critical revision of the manuscript, and final approval; B. Glader provided red cell enzyme data and contributed to data collection, critical revision of the

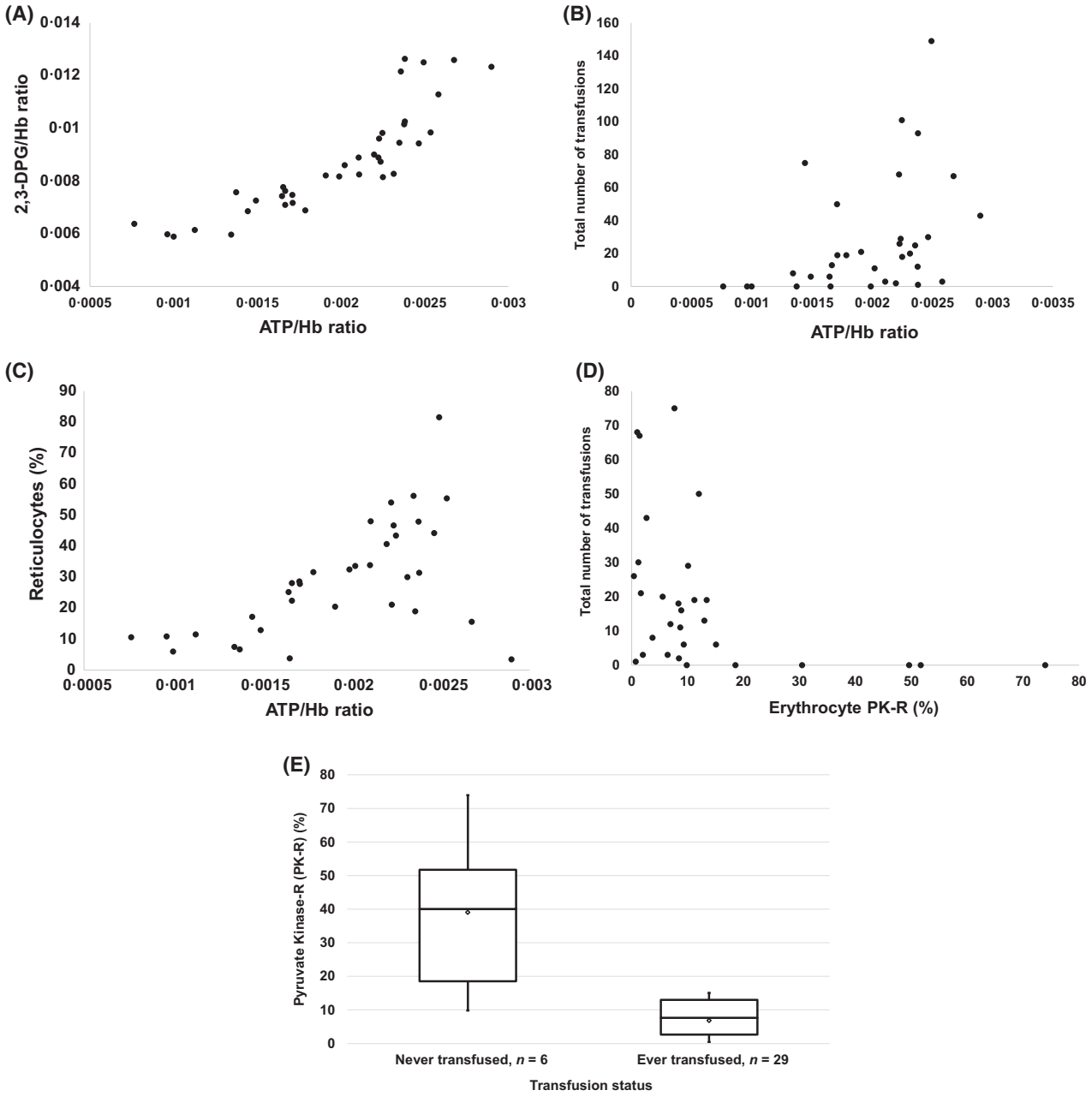

Fig 1. Relation of erythrocyte metabolites with one another or markers of clinical severity in PKD. (A) Erythrocyte ATP *versus* erythrocyte 2,3-DPG ($N = 38$, $r = -0{\cdot}93$; $P < 0{\cdot}0001$). (B) Erythrocyte ATP *versus* total number of lifetime RBC transfusions ($N = 33$, $r = 0{\cdot}527$, $P = 0{\cdot}0016$). (C) Erythrocyte ATP *versus* percentage reticulocyte count ($N = 35$, $r = 0{\cdot}581$, $P = 0{\cdot}0003$). (D) Erythrocyte PK-R protein *versus* total number of lifetime red blood cell (RBC) transfusions ($N = 30$, $r = -0{\cdot}527$, $P = 0{\cdot}0028$). (E) Erythrocyte PK-R protein level in never transfused ($N = 6$) *versus* ever transfused (i.e., received at least one RBC transfusion over the lifetime, $N = 29$) patients ($P = 0{\cdot}0014$).

manuscript, and final approval; D. Morton contributed to data collection, critical revision of the manuscript, and final approval; S. Chonat contributed to data collection, critical revision of the manuscript, and final approval; A. Thompson contributed to data collection, critical revision of the manuscript, and final approval; K. Kuo contributed to data collection, critical revision of the manuscript, and final approval; Y. Ravindranath contributed to data collection, critical revision of the manuscript, and final approval; H. Weng contributed to data collection, critical revision of the manuscript, and final approval; J. Rothman contributed to data collection, critical revision of the manuscript, and final approval; J. Kwiatkowski contributed to data collection, critical revision of the manuscript, and final approval; C. Kung contributed to critical revision of the manuscript and final approval; P. Kosinski contributed to data analysis, critical

revision of the manuscript and final approval; H. Al-Sayegh contributed to data analysis, critical revision of the manuscript, and final approval; W. London contributed to data analysis, critical revision of the manuscript, and final approval; R. Grace contributed to study design, data collection, data analysis, creation of tables and figures, critical revision of the manuscript, and final approval.

Disclosures

Al-Samkari: Consultancy (Agios, Dova, Moderna), Research funding (Agios, Dova, Amgen). Addonizio: None. Glader: Scientific Advisor and Consultancy (Agios), Research Funding (Agios). Morton: None. Chonat: Advisory board (Alexion, Agios). Thompson: Consultancy (Bluebirdbio, Celgene, Novartis), Research Funding (Bluebirdbio, Celgene, Novartis, Baxalta). Kuo: Honoraria (Alexion, Novartis); Consultancy (Agios, Alexion, Bluebirdbio, Celgene, Novartis, Pfizer); Data Safety Monitoring Board Chair (Bioverativ). Ravindranath: Research Funding (Agios). Weng: None. Rothman: Research Funding (Agios, Novartis, Pfizer). Consultancy (Agios, Novartis, Pfizer). Kwiatkowski: Consultancy (Celgene, Bluebirdbio, Imara, Agios). Research Funding (Apopharma, Novartis, Terumo, Bluebirdbio). Kung: Employee (Agios), Shareholder (Agios). Kosinski: Employee (Agios), Shareholder (Agios). Al-Sayegh: None. London: Consultancy (United Therapeutics, ArQule, Inc.). Grace: Scientific Advisor (Agios), Consultancy (Agios, Dova), and Research Funding (Agios, Novartis).

References

1. Pissard S, Max-Audit I, Skopinski L, Vasson A, Vivien P, Bimet C, et al. Pyruvate kinase deficiency in France: a 3-year study reveals 27 new mutations. *Br J Haematol*. 2006;**133**:683–9.
2. Warang P, Kedar P, Ghosh K, Colah R. Molecular and clinical heterogeneity in pyruvate kinase deficiency in India. *Blood Cells Mol Dis*. 2013;**51**:133–7.
3. Beutler E, Gelbart T. Estimating the prevalence of pyruvate kinase deficiency from the gene frequency in the general white population. *Blood*. 2000;**95**:3585–8.
4. Carey PJ, Chandler J, Hendrick A, Reid MM, Saunders PW, Tinegate H, et al. Prevalence of pyruvate kinase deficiency in northern European population in the north of England. Northern Region Haematologists Group. *Blood*. 2000;**96**:4005–6.
5. Al-Samkari H, van Beers EJ, Kuo KH, Barcellini W, Bianchi P, Glenthoj AB, et al. The variable manifestations of disease in pyruvate kinase deficiency and their management. *Haematologica*. 2020 [Epub ahead of print]. DOI: https://doi.org/10.3324/haematol.2019.240846.
6. Bianchi P, Fermo E, Glader B, Kanno H, Agarwal A, Barcellini W, et al. Addressing the diagnostic gaps in pyruvate kinase deficiency: consensus recommendations on the diagnosis of pyruvate kinase deficiency. *Am J Hematol*. 2019;**94**:149–61.
7. Grace RF, Zanella A, Neufeld EJ, Morton DH, Eber S, Yaish H, et al. Erythrocyte pyruvate kinase deficiency: 2015 status report. *Am J Hematol*. 2015;**90**:825–30.
8. Grace RF, Bianchi P, van Beers EJ, Eber SW, Glader B, Yaish HM, et al. Clinical spectrum of pyruvate kinase deficiency: data from the Pyruvate Kinase Deficiency Natural History Study. *Blood*. 2018;**131**:2183–92.
9. Beutler E. Red Cell Metabolism: A Manual of Biochemical Methods. New York: Grune & Stratton Inc.; 1984.
10. Mentzer WC Jr, Baehner RL, Schmidt-Schonbein H, Robinson SH, Nathan DG. Selective reticulocyte destruction in erythrocyte pyruvate kinase deficiency. *J Clin Investig*. 1971;**50**:688–99.