



Molecular heterogeneity of pyruvate kinase deficiency

Paola Bianchi and Elisa Fermo

Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico Milano, UOC Ematologia, UOS Fisiopatologia delle Anemie, Milan, Italy

Haematologica 2020
Volume 105(9):2218-2228

ABSTRACT

Red cell pyruvate kinase (PK) deficiency is the most common glycolytic defect associated with congenital non-spherocytic hemolytic anemia. The disease, transmitted as an autosomal recessive trait, is caused by mutations in the *PKLR* gene and is characterized by molecular and clinical heterogeneity; anemia ranges from mild or fully compensated hemolysis to life-threatening forms necessitating neonatal exchange transfusions and/or subsequent regular transfusion support; complications include gallstones, pulmonary hypertension, extramedullary hematopoiesis and iron overload. Since identification of the first pathogenic variants responsible for PK deficiency in 1991, more than 300 different variants have been reported, and the study of molecular mechanisms and the existence of genotype-phenotype correlations have been investigated in-depth. In recent years, during which progress in genetic analysis, next-generation sequencing technologies and personalized medicine have opened up important landscapes for diagnosis and study of molecular mechanisms of congenital hemolytic anemias, genotyping has become a prerequisite for accessing new treatments and for evaluating disease state and progression. This review examines the extensive molecular heterogeneity of PK deficiency, focusing on the diagnostic impact of genotypes and new acquisitions on pathogenic non-canonical variants. The recent progress and the weakness in understanding the genotype-phenotype correlation, and its practical usefulness in light of new therapeutic opportunities for PK deficiency are also discussed.

Correspondence:

PAOLA BIANCHI,
paola.bianchi@policlinico.mi.it

Received: May 7, 2020.

Accepted: July 3, 2020.

Pre-published: July 23, 2020.

doi:10.3324/haematol.2019.241141

©2020 Ferrata Storti Foundation

Material published in *Haematologica* is covered by copyright. All rights are reserved to the Ferrata Storti Foundation. Use of published material is allowed under the following terms and conditions:

<https://creativecommons.org/licenses/by-nc/4.0/legalcode>.

Copies of published material are allowed for personal or internal use. Sharing published material for non-commercial purposes is subject to the following conditions:

<https://creativecommons.org/licenses/by-nc/4.0/legalcode>,

sect. 3. Reproducing and sharing published material for commercial purposes is not allowed without permission in writing from the publisher.



Pyruvate kinase enzyme

Pyruvate kinase (PK) is an allosterically regulated glycolytic enzyme that catalyzes the irreversible conversion of phosphoenolpyruvate to pyruvate, with the synthesis of one molecule of ATP. Since mature red blood cells totally depend on the ATP generated by glycolysis for maintaining cell integrity and function, PK plays a crucial role in erythrocyte metabolism; insufficient energy production may impair red blood cell homeostasis, leading to premature removal of PK-deficient erythrocytes from the circulation by the spleen.^{1,2} A secondary decrease in PK activity has been observed in the presence of reduced red cell membrane surface (as in hereditary spherocytosis³) or in acquired hematologic conditions (e.g., acute myeloid leukemias, or myelodysplastic syndromes),^{4,5} suggesting a functional relationship between structural membrane integrity and PK activity, and a wider involvement of glycolytic enzymes in cell control.^{4,5}

The three-dimensional structures of a number of prokaryotic and eukaryotic PK have been solved to a high resolution, showing that in almost all organisms, functional PK is a homotetramer of approximately 200-240 kDa.⁶⁻⁹ Each subunit contains four domains, namely a small N-terminal helical domain (residues 1-84); an A domain with (β/α)₈ barrel topology (residues 85-159 and 263-431); a β-stranded B domain (residues 160-262), inserted between helix α₃ and strand β of the A domain, and a C domain with α+β topology (residues 432-574)^{10,11} (Figure 1A, B). The active site is located between the A and B domains, whereas the C domain contains the binding site for fructose 1,6 bisphosphate.¹² Subunit interactions at the interfaces between the A domains and the C domains, as well as A/B and A/C interdomain interactions within one subunit are considered to be key determinants of the allosteric response of the enzyme. PK is quite a stable protein, and can last

the entire lifespan of erythrocytes.¹³ Pathological mutations causing PK deficiency can be localized in any of the protein domains, with major clusters in specific regions, such as the interface between the A and C domains, the A/A' intersubunit interface, the hydrophobic core of the A domain, and the fructose 1,6 bisphosphate-binding site^{6,10,14,15} (Figure 1C).

Several human PK mutants have been produced as recombinant forms and biochemically characterized^{10,11,13,16,17} showing that amino acid substitutions can affect thermostability, catalytic efficiency, and response to the allosteric effector.

Diagnosis of pyruvate kinase deficiency

The diagnostic workup for PK deficiency is based on the patient's personal and family medical history and clinical examination, and on several laboratory investigations, including the spectrophotometric assay of red blood cell PK activity.¹⁸ Molecular analysis of the *PKLR* gene is necessary to confirm the diagnosis, and overcomes the limitations of the enzymatic test, which may give false positive results in the case of heterozygous carriers, or false negative results in the case of recent transfusion, or an increased number of reticulocytes. Therefore, recently

published guidelines and recommendations conclude that enzyme analyses and DNA studies are complementary techniques for diagnosing PK deficiency.¹⁹

With the advent of next-generation sequencing (NGS) techniques, the *PKLR* gene is usually included in panels designed for diagnosing hereditary hemolytic anemias,²⁰⁻²⁴ allowing detection of an increasing number of cases, thus reducing misdiagnosis, and highlighting the extreme phenotypic variability of PK deficiency²⁵⁻²⁷ (Table 1).

Gene and variants

The *PKLR* gene, located on chromosome 1q21, consists of 12 exons and is approximately 9.5 kb in size.³¹ The gene encodes for the liver (L) and erythrocyte (R) isoforms of the enzyme according to tissue-specific promoters;^{31,32} ten exons are shared by the two isoforms, while exons 1 and 2 are specifically transcribed to the PK-R and PK-L mRNA, respectively. The cDNA encoding PK-R is 2060 bp long and codes for 574 amino acids (Figure 1A). In the R-type promoter region, two CAC boxes and four GATA motifs are located within 270 bp from the translational initiation codon; the proximal 120 bp region has basal promoter activity and the region from -120 to -270 works as a powerful enhancer in erythroid cells.³¹

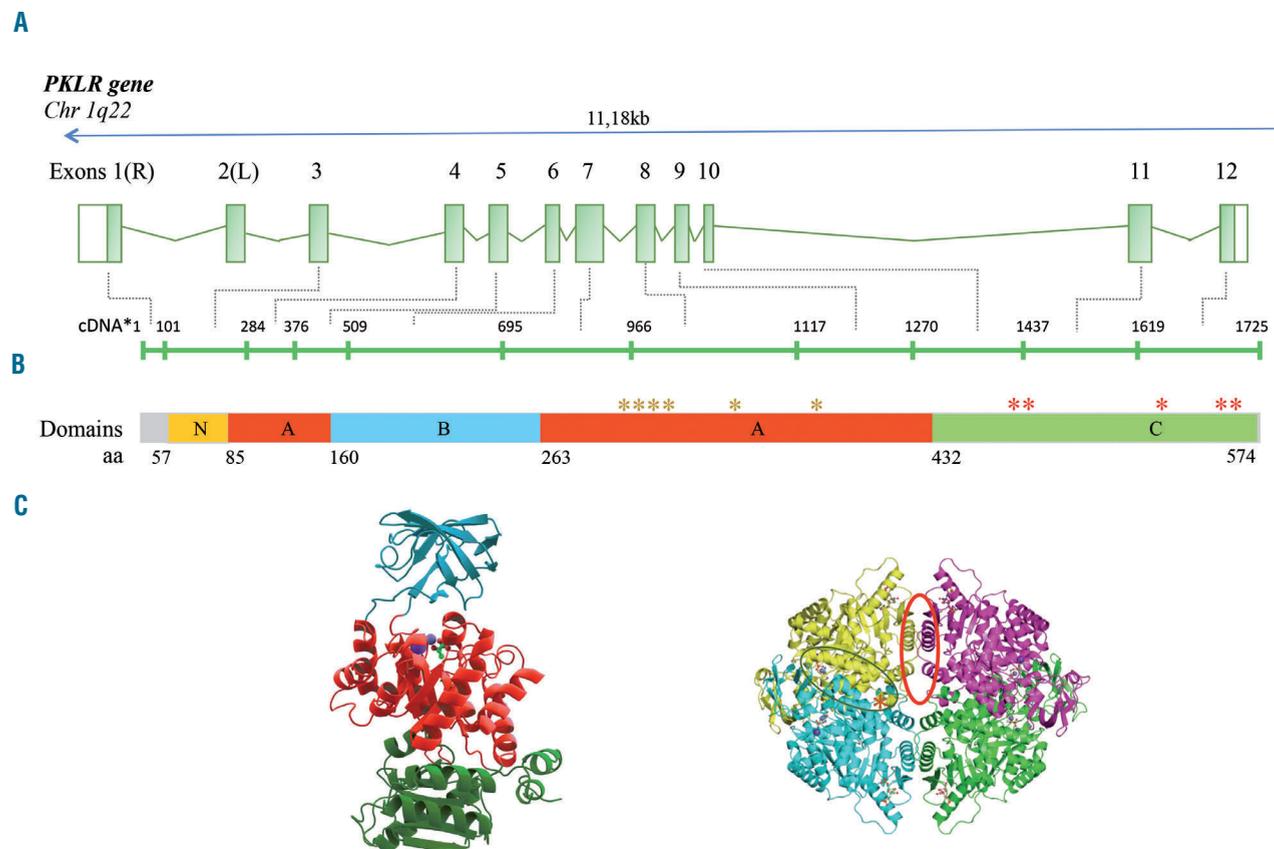


Figure 1. *PKLR* gene and red cell pyruvate kinase structure. (A) The *PKLR* gene, its chromosomal localization, extension and intron/exon organization. Numbering and mutations are usually reported in the literature using the RPK cDNA sequence of the *PKLR* gene, with the A of the initiation ATG being assigned number +1 (Transcript refseq ID NM_000298.5). (B) Structural domains of the human PK-R monomer, the N-terminal domain is reported in yellow, A-domain in red, B-domain in light blue and C-domain in green. The corresponding amino acids are reported below. *Represents the localization of residues directly involved in the allosteric site and catalytic center (yellow) and in the fructose 1,6 bisphosphate (FBP) activator (red). (C) Ribbon representation of the human erythrocyte pyruvate kinase monomer (left) in complex with the substrate and the allosteric activator fructose-1,6-diphosphate (red and purple) and tetramer based on the crystal structure described by Valentini et al.¹⁰ Circles indicate the A'A' and the A/C subunit interfaces.

The number of known pathogenic variants is continuously increasing. In a recent inventory, Canu *et al.*³³ reported 260 mutations in the *PKLR* gene; the Human Genome Mutation Database (HGMD) reports 290 pathogenic variants (update March 2020); a detailed inventory of *PKLR* variants is also available in the *PKLR* Leiden Open Variation Database (<https://databases.lovd.nl/shared/genes/PKLR>), including a more specific data collection (e.g., congress presentations and unpublished results).

The HGMD does not yet include the results obtained in a single analysis of 257 patients with PK deficiency enrolled in the Pyruvate Kinase Deficiency Natural History Study (PKD NHS), a multicenter, international study; 127 different pathogenic variants were detected, comprising 84 missense and 43 non-missense variants (including 20 stop-gain variants, 11 affecting splicing, 5 large deletions, 4 in-frame indels, and 3 promoter variants).^{34,35} A similar distribution is observed by stratifying variants reported by the HGMD according to the type of mutations (Figure 2).

A list of the more commonly detected mutations and variants with geographical distribution and ethnic background is reported in Table 2; similar information for the rarer/unique variants is available in mutation databases. Molecular analysis of the *PKLR* gene by Sanger sequencing usually covers the entire coding region, flanking intronic sequences and the erythroid-specific promoter. NGS analysis allows more extensive sequencing than the Sanger method (generally including entire coding and intronic flanking regions, promoter, 3' upstream, and 5' downstream regions) and can give information on the presence of large indels. Other techniques (e.g., multiplex ligation-dependent probe amplification or assays of copy number variations, comparative genomic hybridization arrays or digital polymerase chain reaction) can also be used to this latter purpose. Variants are usually reported in the literature using the RPK cDNA sequence of the *PKLR* gene, with the A of the initiation ATG being assigned number +1 (Transcript refseq ID NM_000298.5). American College of Medical Genetics and Genomics (ACMG) guidelines should be followed to interpret and assess sequence variants.⁴²

Promoter and enhancer variants

Only a few pathogenic variants have been identified in the promoter region, mostly clustering at two functionally important sequences, such as the consensus binding motif for GATA-1 at nucleotides c.-69 to -74,^{43,45} and a regulatory element (PKR-RE1) whose core CTCTG extends from nucleotides c.-87 to -83.^{46,47} The variant c.-72A>G, located in the GATA-1 motif, was found to be associated with low mRNA expression, and to be responsible for severe anemia when present in the homozygous state.^{15,45} Other variants have been reported with uncertain pathogenic significance: the variant c.-109C>T described by Pissard *et al.*,⁴⁸ while not directly modifying any known binding site for a transacting factor, was found to be located within a region displaying basic promoter activity, very close to the region described as an erythroid enhancer⁴⁹ (Figure 3).

At the moment a clear disease-causing association of variants located in the enhancer region is not well established. Some reported variants in this regions, such as c.-148C>T,⁴⁹ or deletion reported at nucleotides c.-249delA or c.-248delT, do not seem to affect the expression of the gene, thus are considered non-pathogenic.⁴⁶

Coding region variants

The large majority of pathogenic variants are located in the coding region. Mutations are distributed throughout the *PKLR* gene and affect all exons (Figure 4). Most of them (about 66%) are missense mutations (Figure 2). Not every mutation detected by DNA sequencing can be immediately classified as a disease-causing variant, and should be considered 'variants of unknown clinical significance' until their pathogenic nature is confirmed by functional analysis such as PK enzymatic assays, western blotting, reverse transcriptase polymerase chain reaction analysis, or gene reporter assays.⁵⁰ This is especially important when patients' samples are not accompanied by complete clinical and laboratory information.

Most variants in PK deficiency affect residues critical to the structure and/or function of the enzyme. However, it is not possible to establish a direct relationship between the severity of a pathogenic variant and its position; most patients in fact are compound heterozygous for two

Table 1. Recent studies performed by next-generation sequencing technologies in patients with hemolytic anemias.

Reference	Method	N. of genes analyzed	N. of cases studied with CHA	PKD diagnosis	New diagnosis and number and type of mismatched diagnoses
15	t-NGS	35	36	2	2 new PKD
28	t-NGS	55	43	8	8 new PKD
29	WES	n.a.	4	4	4 new PKD
30	t-NGS	76	21 ^a	6	3 new PKD 2 CDA → PKD 1 DBA → PKD
27	t-NGS	76	21 ^b	6	4 new PKD 2 CDA → PKD
25	t-NGS	34 and 71	74 ^c	7	7 CDA → PKD
23	t-NGS	33	57	3	2 new PKD 1 CDA → PKD

Number of genes included in the panel, number of cases analyzed in each study and cases diagnosed with pyruvate kinase deficiency are shown. Next-generation sequencing analysis allowed modification of a previous diagnosis; the number and the type of mismatched diagnosis is reported in the last column. ^aAll transfusion-dependent patients. ^bNo diagnosis despite extensive laboratory investigations. ^cSuspected diagnosis of congenital dyserythropoietic anemia. CHA: chronic hemolytic anemias; PKD: pyruvate kinase deficiency; t-NGS: targeted next-generation sequencing; WES: whole-exome sequencing; n.a.: not available; CDA: congenital dyserythropoietic anemia; DBA: Diamond-Blackfan anemia.

mutations and it is therefore difficult to determine the severity of any one individual variant in critical regions of the gene.

Splice site variants

Splice site variants have been reported in all exon/intron boundary sequences. Most of these variants affect the ± 1 or ± 2 nucleotides of the donor/acceptor sequences, and are consequently considered to have a drastic effect on splicing, causing unstable and degraded mRNA.

Care should be taken in the interpretation of more internal variants that require functional analysis before

defining their pathogenicity; this is the case of rare *PKLR* variants such as c.1269+5G>A,⁴⁸ c.507-20C>A¹⁴, c.100+10G>A and c.375+10G>T,⁴⁸ considered by authors to affect the splicing only basing on *in silico* analysis.

It is worth noting that some missense mutations in the coding region may also affect splicing, in particular when located in the first/last nucleotides of the exons, e.g., c.507G>A,¹⁷ c.694G>T,^{48,51} c.1269G>C,⁵² or the c.1436G>A variant (p.R479H),³⁸ located in the last nucleotide of exon 10, typically but not exclusively found in the Amish community. The deleterious effect of these variants should always be considered in genetic counseling, or in evaluat-

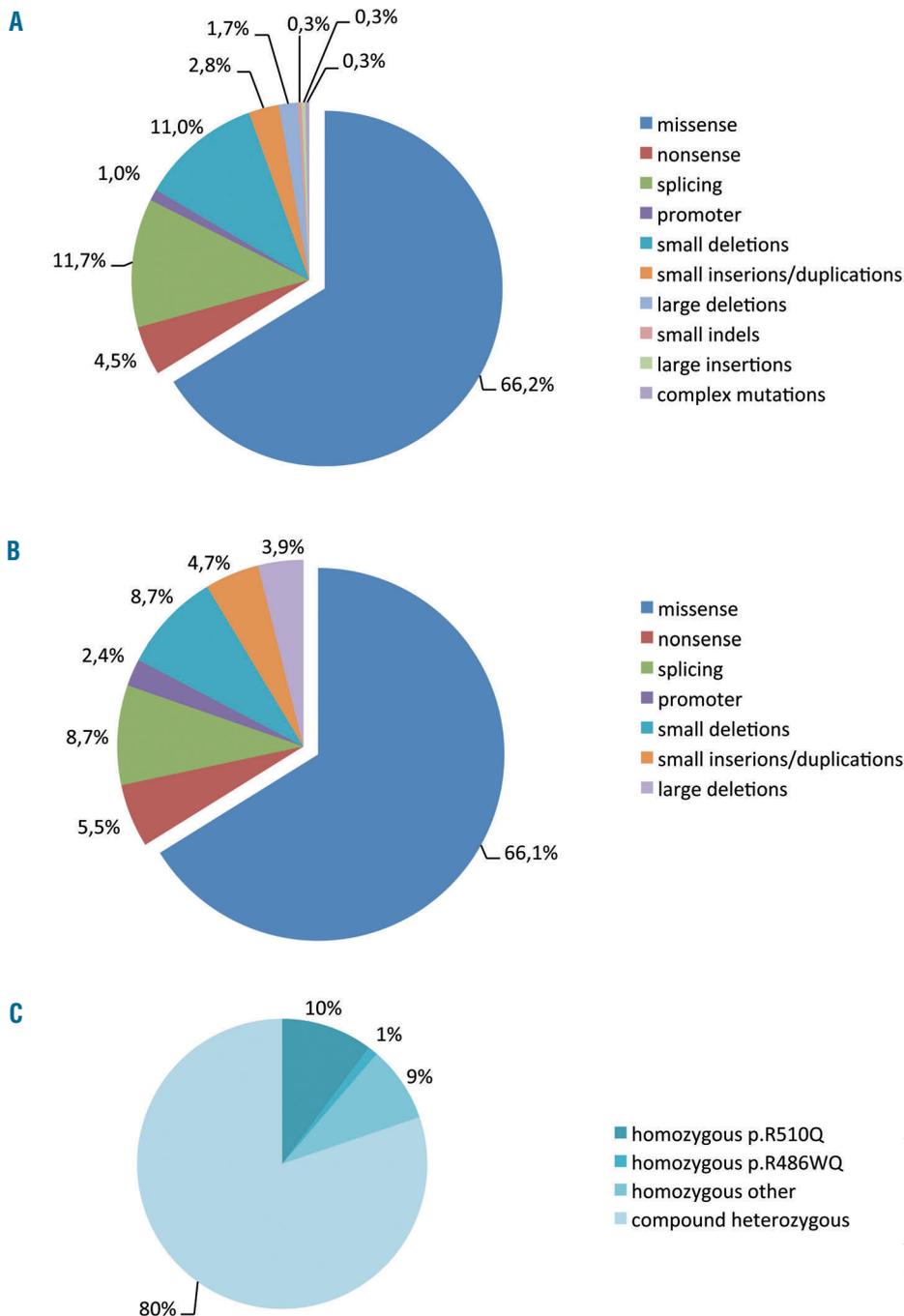


Figure 2. Type of *PKLR* pathogenic variants. (A) The type of *PKLR* pathogenic variants (n=290) reported in the Human Genome Mutation Database (March 2020). (B) The type of *PKLR* mutations (n=127) reported in a series of 257 patients with pyruvate kinase deficiency.³⁵ (C) Genotypes in a series of 177 unrelated patients with pyruvate kinase deficiency.³⁵

ing the impact of genotype in clinical trials (see the section “From genotype to new therapies”).

Deep intronic variants

An increasing number of pathogenic deep intronic mutations has been described across different disease conditions,⁵³ and these mutations have been considered to justify the number of PK-deficient patients in whom it is not possible to find molecular defects. Among the 278 participants initially enrolled in the PKD NHS, 21 were considered ineligible for the study because of the inability to demonstrate two pathogenic variants even after excluding the large deletion analyzed by long-range polymerase chain reaction.^{34,35} This aspect has important implications, obviously from the diagnostic point of view, but also with regards to the possibility to access new specific therapies, as further discussed in the next paragraphs.^{26,54} Laboratory

confirmation of deep intronic variants is often difficult and may require specific testing such as loss of heterozygosity by analyzing an allele-specific cDNA, or the more complex minigene construct approach.⁵⁵

In a recent study a deep intronic mutation (c.283+109C>T in intron 2) was detected by whole exome sequencing in compound heterozygosity with the missense mutation p.G332S, and was considered responsible for creating an alternative spliceosome by *in silico* analysis; rapid mRNA degradation was confirmed by the observation of loss of heterozygosity of the p.G332S variant at the cDNA level.⁵⁶

In another study, 13 PK-deficient related individuals with one or no pathogenic variants identified in the *PKLR* gene were analyzed by whole exome sequencing or whole genome sequencing. Five patients had an alternative diagnosis with mutations in *GATA1*, *KIF23*, and

Table 2. Most common mutations of the *PKLR* gene, ethnic distribution and allelic frequency.

Mutation	Effect	Exon	Mutation type	Geographical distribution/ethnicity	rs	Allelic frequency (gnomAD Exome)	Ref.
c.1529G>A	p.R510Q	11	Missense	Nothern EU, USA	rs113403872	0.000358	34
c.1456C>T	p.R486W	11	Missense	Southern EU, India	rs116100695	0.00305	34,36
c.1468C>T	p.R490W	11	Missense	Japan	rs200133000	0.000127	37
c.721G>T	p.E241*	7	Nonsense	Caucasian	rs201953584	0.0000485	16
c.994G>A	p.G332S	8	Missense	Caucasian	rs773626254	0.0000557	16
c.992 A>G	p.D331G	8	Missense	India	rs1443439423	0.00000398	36

Pathogenic variants with strong ethnic association

c.1436G>A	p.R479H/abnormal splicing	10	Missense/splicing	Pennsylvania Amish, Indian	rs118204085	0.000127	36,38
c.829G>A	p.E277K	7	Missense	African	rs147689373	0.000694	39
c.1437-518_1618+440del	1149 bp deletion	i10-i11	Large deletion	Roma community	na	na	40
c.283+1914_1434del	5006 bp deletion	i3-10	Large deletion	Vietnamese	na	na	17,41



Figure 3. DNA sequence of the erythroid-specific *PKLR* promoter region. Conserved sequences between human and rat PKR promoter are underlined, the black arrow indicates the PK-R transcriptional start site. Yellow boxes indicate the GATA-1 motif, the green boxes identify the CAC/Sp1 motif and the blue box identifies the PKR-RE regulatory element. Colored arrows indicate motif direction. Mutated nucleotides reported in the literature associated with pyruvate kinase deficiency are indicated in red and reported in more detail in the box.

Promoter Mutations (ref)	
-72 A>G (44,45)	-83G>C (46)
-73G>C (43,46)	-109C>T (48)
c.-88_-84delTCTCT (47)	

PIEZO1 genes, whereas in five other cases, whole genome sequencing identified different intronic variants, all predicted to perturb normal mRNA processing and confirmed by minigene assays.⁵⁵

Large insertions/deletions

Large indels are rare, possibly due to the technical difficulties in identifying them. The most frequent is the deletion of the 1149 bp characteristic of the Roma community, which leads to skipping of exon 11.⁴⁰ A very large deletion of 5006 bp that results (at the cDNA level) in the loss of exons 4 to 11 (c.283+1914_c.1434del5006) has been described in patients of Vietnamese origin.^{17,41} A large homozygous insertion of 367 bp (c.939_940ins367) containing an Alu element (AluYb9)⁵⁷ was identified in two unrelated children with severe transfusion-dependent hemolytic anemia, from the Middle East. Other variants have been reported although the exact cut-off point has not been identified (3 of the 5 different large deletions identified within the PKD NHS).³⁵

As for deep intronic variants, the search for large indels may add to the costs of analysis and require techniques not always available; however, it should always be considered when one mutation or no mutations at all are detected in a patient with clinical and biochemical diagnosis of PK deficiency. The eventuality of a large deletion at the heterozygous level should always be taken into account in patients carrying homozygous pathogenic variants, in whom the allelic transmission has not been confirmed through analysis of the parents.

Inherited pyruvate kinase hyperactivity

Inherited hyperactivity of red blood cell PK (OMIM 102900) has been reported in only three families with apparent asymptomatic conditions with different etiologies.⁵⁸⁻⁶⁰ This rare condition was attributed in the past to a heterozygous mutation in the *PKLR* gene⁶¹ or to the persistent expression of the fetal isozyme PK-M2.⁵⁹ More recently, there was a report of a family characterized solely by the increased expression of a kinetically normal PK-R, in the absence of mutations in *PKLR* coding and regulatory regions as well as variations in *PKLR* copy number, and exclusion of co-segregation with the *PKLR* locus;

in this case, the authors postulated that the causative mutation resides in a novel, unidentified locus, responsible for upregulating *PKLR* gene expression.⁶⁰

Other genes associated with pyruvate kinase deficiency

KLF1 is a transcription factor involved in terminal erythroid differentiation, and regulates many of the genes implicated in red cell enzyme deficiencies, including *PKLR*. Decreased PK activity in the absence of *PKLR* mutations has been reported in patients who were compound heterozygotes for *KLF1* variants, possibly leading to misdiagnosis of these cases with PK deficiency;⁶² these patients displayed severe, transfusion-dependent neonatal anemia with a broad spectrum of red cell morphological abnormalities and a remarkable persistence of fetal and embryonic globin synthesis.

Geographical distribution of *PKLR* variants

PK deficiency has a worldwide geographical distribution. A careful literature review established that the prevalence of clinically-diagnosed PK deficiency was likely between 3.2 and 8.5 cases per million in Western populations, while the prevalence of diagnosed and undiagnosed PK deficiency could possibly be as high as 51 cases per million.⁶³⁻⁶⁵ The causes of this variability can be explained by the rarity of the disorder, the high percentage of undiagnosed cases and the absence of disease registries and specific population studies.⁶⁵

There is a high frequency of PK deficiency in Middle East and sub-Saharan Africa populations, possibly due to selective pressure from malaria;^{39,66} some reports have, in fact, suggested that PK deficiency provides protection against infection and replication of *P. falciparum* in murine models and in *ex vivo* experiments with red cells from PK-deficient patients.⁶⁷⁻⁶⁹ Most of the molecular variants reported are private, and it is thus difficult to define a geographical distribution. Despite this, the most frequent mutations in PK deficiency are distributed with a strong ethnic and regional background; in addition, some mutations have a high frequency in specific populations as a result of a founder effect (Table 2).

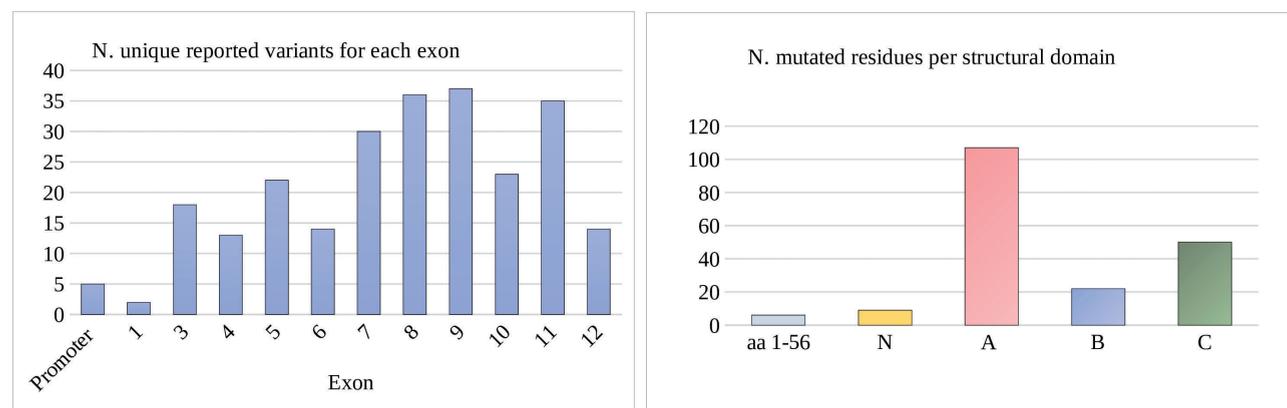


Figure 4. Distribution of variants along the *PKLR* gene and pyruvate kinase structural domains. Distribution of unique pathogenic variants reported in the Human Genome Mutation Database along exons (left side), and distribution of affected residues in the four different structural domains (right side). aa: amino acid; N: N-terminal domain.

Mutations and clinical phenotype: the genotype-phenotype correlation

The broad spectrum of clinical presentations reported in PK deficiency reflects the extensive molecular heterogeneity,^{16,26,34} and the search for a correlation between the genotype and the phenotype has been the matter of study for many years.

The genotype-phenotype correlation has been investigated in clinical studies and by *in vitro* production and characterization of recombinant mutant proteins of the human enzyme,^{10,13,16,70} showing that patients with severe phenotypes more commonly carry nonsense mutations or missense pathogenic variants affecting the active site or stability of the PK protein.^{15,16,36,71}

A recent analysis evaluated the genotype-phenotype correlation in the PKD NHS.³⁵ In addition to the volume of patients/data collected (257 patients, 177 of them unrelated), analysis of this cohort had the great advantage of homogeneous data collection. Mutation types were classified according to previous approaches as missense (M) or non-missense (NM) (including nonsense, frameshift, splicing mutations, large deletions, in-frame indels, and promoter variants); patients with NM/NM mutations were found to have a more severe phenotype, with lower hemoglobin levels after splenectomy, a higher number of transfusions throughout their lifetime, a higher rate of iron overload, and a higher rate of splenectomy, when compared with patients with M/M or M/NM *PKLR* mutations. This categorization has some obvious limitations; in fact, although it is easy to predict the effects of a nonsense variant, because, independently of its nature, it results in protein degradation, predicting the effect of missense variants is more complex, and must take into account the effects on functional properties and the stability of the mutated protein. Studies on the biochemical characterization of recombinant mutant PK enzymes have actually warned against predictions of the effects of missense mutations simply based on the location and the nature of the replaced residues; as an example, the two most frequently reported mutations p.R486W and p.R510Q both affect arginine residues located at the A/C interface, but result in substantially different effects. The p.R486W substitution leads to an enzyme with moderately altered kinetic parameters, but does not affect protein stability, whereas the p.R510Q replacement is likely to disrupt a local network of hydrogen bonds and ultimately results in protein instability and altered allosteric responsiveness to ATP inhibition.^{10,13}

The structural architecture of the PK molecule contributes greatly to the heterogeneity of biochemical properties of the abnormal variants; in fact, the majority of patients with PK deficiency are compound heterozygous for two missense mutations, and may therefore have several different combinations of tetramers, each with distinct kinetic, allosteric and structural properties.

In addition, it is known that in patients with identical genotypes other genetic or environmental factors may affect the phenotype. This has been observed in a large number of patients homozygous for the p.R510Q mutant reported in three studies.^{16,34,72} In all series, variability in the severity of the disease and the well-being of the patients was observed, even within the same family. Patients displayed a wide range of hemoglobin levels

(4.9-12.2 g/dL¹⁶ and 6.7-11.5 g/dL³⁴) with a broad spectrum of ages at diagnosis (0-56 and 0-47 years), but similar rates of splenectomy (44% and 37%).

Phenotypic variability within the same family has been confirmed by the analysis of 88 siblings from 38 families:³⁵ with intraclass correlations ranging from 0.4-0.61; about the same degree of similarity has been found either within or between sibling clusters for hemoglobin, total bilirubin, splenectomy, and cholecystectomy.³⁵

Finally, PK-deficient patients usually tolerate anemia well, so the decision to transfuse or treat a patient is based on how the patient feels rather than on an arbitrary hemoglobin threshold.¹⁶ This is in part justified by the increased 2,3-diphosphoglycerate level typically found in these patients; as an important regulator of the oxygen affinity of hemoglobin, 2,3-diphosphoglycerate may enhance oxygen delivery.⁷⁴ Al-Samkari *et al.*²⁶ reported an illustrative case: despite continued severity of anemia after splenectomy, a PK-deficient patient did not require blood transfusion, maintaining a normal social life into adulthood. Quality of life assessments, including the Functional Assessment of Chronic Illness Therapy Fatigue subscale [FACIT-F, final score of 48 (score range 0-52)] and the Functional Assessment of Cancer Therapy [FACT-G, score of 96 (score range 0-104)], confirmed the patient's good quality of life.

Epigenetic factors and co-inheritance

Other causes of variability of clinical expression in PK deficiency could depend on possible individual differences in metabolic or proteolytic activity, which may modulate the basic effect of the mutations on ineffective erythropoiesis^{74,75} or differences in splenic function, and on the ability to compensate for the enzyme deficiency by overexpressing isozymes or using alternative pathways.¹⁶ In addition, other factors, such as genetic background, concomitant functional polymorphisms of other enzymes, post-translational or epigenetic modifications, and co-inheritance of other diseases may greatly contribute to the phenotypic heterogeneity and complications.

Patients with PK deficiency usually develop secondary iron overload with a multifactorial pathogenesis, involving chronic hemolysis, ineffective erythropoiesis, and transfusion therapy;⁷⁶⁻⁷⁸ *HFE* mutations p.C282Y and p.H63D have been proposed as additional risk factors.^{79,80} Similarly, the co-inheritance of the *UGT1A1* TA promoter polymorphism may contribute to the occurrence of gallstones, which are detected with increased frequency after the first decade of life in PK-deficient patients.^{81,82}

The concomitance of PK deficiency and other hereditary anemias, such as glucose-6-phosphate dehydrogenase deficiency, hemoglobinopathies, and red blood cell membrane defects, has been reported on rare occasions, with variable contributions of the different diseases to the severity of hemolysis, and should always be considered when interpreting clinical severity.^{82,83} The number of these reports has grown in recent years due to the increased use of NGS technologies, allowing identification of multiple disease-associated variants in patients affected by congenital hemolytic anemias and complex patterns of inheritance.^{25,84,30}

Heterozygosity for a mutation in the *PKLR* gene may

accompany other red cell diseases, confounding the hematologic pattern and sometimes making the diagnosis challenging; likewise, concomitant causes of anemia may explain some patients with decreased PK activity and only one mutation detected upon molecular analysis. In a series of 56 French patients diagnosed with PK deficiency by enzymatic assay and submitted to a molecular diagnosis, 17 cases were reported to carry a heterozygote PK mutation; in three of them, an association with other defects was found, namely a membrane defect, a hexokinase deficiency and a glucose-6-phosphate dehydrogenase deficiency.⁴⁸ In these cases, complete hematologic investigation and molecular characterization of the involved genes are needed to clarify the correct diagnosis, also in view of therapeutic approaches and genetic counseling.^{85,86}

Co-inheritance of heterozygous HbS and PK deficiency (either in the homozygous or heterozygous state)⁸⁷⁻⁸⁹ may induce sickling and worsening of phenotype. In the reported cases, the increase of intraerythrocytic 2,3-diphosphoglycerate concentration induced by the PK deficiency resulted in a decreased oxygen affinity which favored sickling.

The possible contribution of a heterozygous PK deficiency to modifying the clinical expression of a membrane defect or other congenital anemias is still debated. Some authors excluded a synergetic effect between carriership for PK deficiency in patients with concomitant hereditary spherocytosis,^{85,90} while others reported that the co-inheritance of heterozygous PK deficiency was associated with an aggravation of the phenotype in two families, one affected by hereditary spherocytosis⁹¹ and the other by congenital dyserythropoietic anemia associated with a *GATA1* mutation.⁹²

From genotype to new therapies

The treatment of PK deficiency is based on supportive measures, including blood transfusions, splenectomy, and managing complications. The only curative treatment is hematopoietic stem cell transplantation (HSCT); however, due the risk of graft-versus-host disease, this should be considered only in severe cases or when it represents the only realistic therapeutic option.

New therapeutic options that range from a small molecule PK activator to gene therapy are being developed, and may change the way of treating PK deficiency in the future. In this context, a confirmed diagnosis is crucial to have access to these new therapies, and consequently genotyping is becoming a need for most patients; moreover, it may influence the outcome of the treatment and must therefore be taken into account when directing the patient to possible therapies.

Hematopoietic stem cell transplantation

van Straaten *et al.* recently evaluated the indications, procedures employed and outcomes of HSCT in the series of all the patients with PK deficiency treated between 1996 and 2015 (16 patients from Europe and Asia, no patients resulted as being treated in the USA in that period).⁹³ Two additional cases were recently reported.^{94,95}

The analysis of the genotypes of the treated patients showed a great heterogeneity, and surprisingly, no prevalence of nonsense pathogenic variants. Despite this, as reported in Table 3, most of the missense variants in this series affected amino acid residues that participate directly in the allosteric and catalytic binding site of the enzyme, supporting the observation of genotype-phenotype analysis. As expected, no correlation was observed

Table 3. Patients who have undergone hematopoietic stem cell transplantation and their genotype.

Pt	Sex	Country	Genotype	Mutation effect	Splenectomy	Age at HCST	Year	Outcome	Structure ref	
Pt 1	M	Asia	Unknown	Unknown	Unknown	No	5 y	1996	Alive	
Pt 2	F	EU	p.[E241*;R532W]	Nonsense	Missense ¹	Yes	15	2002	Deceased	10,11
Pt 3	F	Asia	p.[K348N;R359H]	Missense ²	Missense ²	No	1 y 7 mo	2009	Alive	47
Pt 4	F	EU	p.[E241*;R488Q]	Nonsense	Missense	No	3 y	2009	Alive	
Pt 5	M	Asia	p.[R40Q;D339N]	Missense	Missense ³	No	2 y 6 mo	2009	Alive	10
Pt 6	F	EU	p.[M377fs;M377fs]	Nonsense	Nonsense	Yes	17 y	2010	Deceased	
Pt 7	F	EU	p.[G165V;R510Q]	Missense	Missense ⁴	Yes	39 y	2011	Deceased	16
Pt 8	F	EU	p.[G511E;E538*]	Missense	Nonsense	Yes	7 y	2013	Alive	
Pt 9	M	EU	p.[I494T;R559*]	Missense	Nonsense	No	6 y	2013	Deceased	
Pt 10	M	Asia	p.[V283A;I314T]	Missense	Missense ³	No	1 y 6 mo	2013	Alive	10
Pt 11	M	EU	p.[K541fs;K541fs]	Nonsense	Nonsense	Yes	10 y	2014	Deceased	
Pt 12	M	Asia	p.[D221Y;I314T]	Missense ³	Missense ³	No	9 y	2014	Alive	10,17
Pt 13	M	Asia	p.[V283A;V283A]	Missense	Missense	No	1 y 6 mo	2015	Alive	
Pt 14	M	EU	p.[D331Q;D339H]	Missense ⁵	Missense ³	Yes	41 y	2015	Alive	10,14
Pt 15	M	Asia	c.[1270-3C>A];p.[G540*]	Nonsense	Nonsense	Yes	11 y	Unknown	Alive	
Pt 16	F	Asia	c.[1270-3C>A];p.[G540*]	Nonsense	Nonsense	No	8 y	Unknown	Alive	
Ref 94	F	China	p.[I314T;I314T]	Missense ³	Missense ³	No	Unknown	Unknown	Alive	
Ref 95	M	Japan	p.[Pro145Hisfs;Pro145Hisfs]	Nonsense	Nonsense	Yes	32 y	Unknown	Alive	

Missense variants falling in "strategic" functional amino acid residues or associated with documented thermo-unstable variants are reported in bold. ¹Directly involved in the fructose 1,6 biphosphate activator. ²Directly involved in the substrate and cation binding sites. ³Residues directly involved in the allosteric site and catalytic center. ⁴Highly unstable. ⁵Proximity of the substrate-binding site.

between the type of mutations and the outcome of the treatment.

Allosteric activator (AG-348)

AG-348 is an allosteric activator of PK-R that binds in a pocket at the dimer-dimer interface, distinct from the allosteric activator fructose 1,6 bisphosphate binding domain, inducing the active R-state conformation of the PK-R tetramer.

Preclinical studies showed that AG-348 enhanced activity *in vitro* in wild-type PK and in a broad spectrum of *PKLR* mutations; this finding was consistent with the known binding site for AG-348, which is distinct from the areas of the most common *PKLR* mutations.¹¹ Data from phase I and phase II studies demonstrated that the glycolytic pathway is activated upon treatment with AG-348, and that 54% of PK-deficient subjects experienced a rise in hemoglobin, all of whom had at least one missense mutation.^{54,96} It has therefore been hypothesized that a minimal level of full-length PK protein is required for enzyme activation, excluding patients carrying two nonsense variants from the potential benefits of the treatment.⁵⁴ In an AG-348 clinical trial, evaluating p.R479H as a splicing variant other than a simple amino acid substitution caused an increase of the percentage of patients with a hemoglobin response from 48% to 54%.⁵⁴

A more recent study investigated the effect of *ex vivo* treatment with AG-348 on enzyme activity, thermostability, protein levels and ATP in PK-deficient red cells from 15 patients with different genotypes, including the most frequently reported variants in Caucasian p.R486W and p.R510Q;⁹⁷ the overall results showed a mean 1.8-fold increase in PK activity and a 1.5-fold increase in ATP levels. Protein analyses suggested that a sufficient level of protein is required for cells to respond to AG-348 treatment, as previously reported.⁵⁴ Interestingly, the thermostability of PK was also found to be significantly improved upon *ex vivo* treatment with AG-348, but with a high variability in response among the different genotypes; this was particularly evident in PK patients carrying the common mutation p.R510Q, which is known to affect catalytic activity only slightly, but to be highly unstable.¹³ Overall, these data demonstrated that the clinical utility of AG-348 in PK-deficient patients is influenced by the type of mutations, and that variability in the response can also be increased by the compound heterozygosity that is present in most patients. Prospective studies in patients across a broader range of genotypes and disease severity are required to identify patients who can benefit most from the treatment.

Gene therapy

Ex vivo gene therapy for hematologic genetic disorders is becoming a reality in clinical practice. This gene therapy strategy is based on an autologous transplant in which the infused cells are genetically corrected *ex vivo*. There are several ongoing clinical trials on the use of gene therapy for rare anemias (β -thalassemia, sickle cell disease, Fanconi anemia) and hereditary metabolic diseases.^{98,99} Preclinical studies focused on the treatment of PK deficiency by gene therapy have been successfully performed.¹⁰⁰⁻¹⁰² Autologous cells corrected with a lentiviral vector carrying a codon optimized version of the wild-type cDNA sequence of the PK-R gene have been demonstrated to be able to compensate the disease phenotype in a murine model, without any adverse effect related to the procedure.¹⁰² This procedure has been designated as an orphan drug by the European Medicines Agency (EU/3/14/1330; <https://goo.gl/T4N6mO>) and by the U.S. Food and Drug Administration (DRU-2016-5168).¹⁰³

An open-label, phase 1 gene therapy study consisting of autologous hematopoietic stem and progenitor cells transduced *ex vivo* with a lentiviral vector encoding for the PK enzyme has been approved and recently opened. PK-deficient patients with confirmed genotype and severe, transfusion-dependent anemia despite splenectomy may be eligible for enrollment (www.clinicaltrial.gov NCT04105166).

Conclusions

One of the clear advantages of NGS technologies is the availability of molecular testing for rare diseases in many laboratories, resulting in increased awareness of rare congenital conditions, in the dramatically increased number of molecular variants, in the reduced time of diagnosis and number of misdiagnoses. However, the huge amount of data obtained should be interpreted in the light of knowledge of the pathogenic basis of diseases and always supported by functional studies: on top of the molecular lesion itself, the effect of mutations on the expression and functionality of the protein is known for only a few variants. In addition, the study of compensatory effects of other metabolic pathways and cellular involvement (e.g., membrane channel activities, membrane stability) in response to energy depletion will offer new insights into the interpretation of the effect of *PKLR* mutations and phenotype.

References

- Nathan DG, Oski FA, Miller DR, et al. Life-span and organ sequestration of the red cells in pyruvate kinase deficiency. *N Engl J Med*. 1968;278(2):73-81.
- Aisaki K, Aizawa S, Fujii H, et al. Glycolytic inhibition by mutation of pyruvate kinase gene increases oxidative stress and causes apoptosis of a pyruvate kinase deficient cell line. *Exp Hematol*. 2007;35(8):1190-1200.
- Andres O, Loewecke F, Morbach H, et al. Hereditary spherocytosis is associated with decreased pyruvate kinase activity due to impaired structural integrity of the red blood cell membrane. *Br J Haematol*. 2019;187(3):386-395.
- Boivin P, Galand C, Hakim J, et al. Acquired red cell pyruvate kinase deficiency in leukemias and related disorders. *Enzyme*. 1975;19(5-6):294-299.
- Lin G, Xie Y, Liang X, Wu X, et al. Study on red cell enzymes and isoenzymes in patients with leukemia and myelodysplastic syndromes. *Zhonghua Xue Ye Xue Za Zhi*. 1997;18(7):350-353.
- Mattevi A, Valentini G, Rizzi M, et al. Crystal structure of Escherichia coli pyruvate kinase type I: molecular basis of the allosteric transition. *Structure*. 1995;3:729-741.
- Muirhead H, Clayden DA, Barford D, et al. The structure of cat muscle pyruvate kinase. *EMBO J*. 1986;5:475-481.
- Larsen TM, Laughlin LT, Holden HM, et al. Structure of rabbit muscle pyruvate kinase complexed with Mn²⁺, K⁺, and pyruvate. *Biochemistry*. 1994;33(20):6301-6309.
- Valentini G, Chiarelli LR, Fortin R, Speranza ML, Galizzi A, Mattevi A. The allosteric regulation of pyruvate kinase. *J Biol Chem*.

- 2000;275(24):18145-18152.
10. Valentini G, Chiarelli LR, Fortin R, et al. Structure and function of human erythrocyte pyruvate kinase. Molecular basis of nonspherocytic hemolytic anemia. *J Biol Chem.* 2002;277(26):23807-23814.
 11. Kung C, Hixon J, Kosinski PA, et al. AG-348 enhances pyruvate kinase activity in red blood cells from patients with pyruvate kinase deficiency. *Blood.* 2017;130(11):1347-1356.
 12. Jurica MS, Mesecar A, Heath PJ, et al. The allosteric regulation of pyruvate kinase by fructose-1,6-bisphosphate. *Structure.* 1998; 6:195-210.
 13. Wang C, Chiarelli LR, Bianchi P, et al. Human erythrocyte pyruvate kinase: characterization of the recombinant enzyme and a mutant form (R510Q) causing nonspherocytic hemolytic anemia. *Blood.* 2001;98(10): 3113-3120.
 14. Kedar P, Hamada T, Warang P, et al. Spectrum of novel mutations in the human PKLR gene in pyruvate kinase-deficient Indian patients with heterogeneous clinical phenotypes. *Clin Genet.* 2009;75(2):157-162.
 15. Svidnicki M, Santos A, Fernandez JAA, et al. Novel mutations associated with pyruvate kinase deficiency in Brazil. *Rev Bras Hematol Hemoter.* 2018;40(1):5-11.
 16. Zanella A, Fermo E, Bianchi P, et al. Pyruvate kinase deficiency: the genotype-phenotype association. *Blood Rev.* 2007;21(4):217-231.
 17. Fermo E, Bianchi P, Chiarelli LR, et al. Red cell pyruvate kinase deficiency: 17 new mutations of the PK-LR gene. *Br J Haematol.* 2005;129(6):839-846.
 18. Beutler E. *Red Cell Metabolism: A Manual of Biochemical Methods.* New York: Grune & Stratton, Inc.; 1984
 19. Bianchi P, Fermo E, Glader B, et al; with the endorsement of EuroBloodNet, the European Reference Network in Rare Hematological Diseases. Addressing the diagnostic gaps in pyruvate kinase deficiency: consensus recommendations on the diagnosis of pyruvate kinase deficiency. *Am J Hematol.* 2019;94(1):149-161.
 20. Agarwal AM, Nussenzweig RH, Reading NS, et al. Clinical utility of next-generation sequencing in the diagnosis of hereditary haemolytic anaemias. *Br J Haematol.* 2016;174(5):806-814.
 21. Del Orbe Barreto R, Arrizabalaga B, De la Hoz AB, et al. Detection of new pathogenic mutations in patients with congenital haemolytic anaemia using next-generation sequencing. *Int J Lab Hematol.* 2016;38(6):629-638.
 22. Iwasaki T, Yamamoto T, Muramatsu H, et al. Clinical impact of captured-based targeted sequencing in diagnosis of congenital hemolytic anaemia. *Rinsho Ketsueki.* 2016;57:1489
 23. Roy NB, Wilson EA, Henderson S. A novel 33-gene targeted resequencing panel provides accurate, clinical-grade diagnosis and improves patient management for rare inherited anaemias. *Br J Haematol.* 2016;175(2):318-330.
 24. Fermo E, Vercellati C, Marcello AP, et al. Use of next generation sequencing panel to clarify undiagnosed cases of hereditary hemolytic anaemias. *Blood.* 2017;130 (Supplement 1):3480.
 25. Russo R, Andolfo I, Manna F, Gambale A, et al. Multi-gene panel testing improves diagnosis and management of patients with hereditary anaemias. *Am J Hematol.* 2018;93(5):672-682.
 26. Al-Samkari H, van Beers EJ, Kuo KHM, et al. The variable manifestations of disease in pyruvate kinase deficiency and their management. *Haematologica.* 2020;105(9):2229-2239.
 27. Shefer Averbuch N, Steinberg-Shemer O, Dgany O, et al. Targeted next generation sequencing for the diagnosis of patients with rare congenital anaemias. *Eur J Haematol.* 2018;101(3):297-304.
 28. Jamwal M, Aggarwal A, Palodhi A, et al. Next-generation sequencing-based diagnosis of unexplained inherited hemolytic anaemias reveals wide genetic and phenotypic heterogeneity. *J Mol Diagn.* 2020;22(4):579-590.
 29. Qin L, Nie Y, Chen L, et al. Novel PLKR mutations in four families with pyruvate kinase deficiency. *Int J Lab Hematol.* 2020;42(2):e84-e87.
 30. Kedar PS, Harigae H, Ito E, et al. Study of pathophysiology and molecular characterization of congenital anemia in India using targeted next-generation sequencing approach. *Int J Hematol.* 2019;110(5):618-626.
 31. Kanno H, Fujii H, Miwa S. Structural analysis of human pyruvate kinase L-gene and identification of the promoter activity in erythroid cells. *Biochem Biophys Res Commun.* 1992;188(2):516-523.
 32. Noguchi T, Tamada K, Inoue H, et al. The L- and R-type isozymes of rat pyruvate kinase are produced from a single gene by use of different promoters. *J Biol Chem.* 1987;262(29):14366-14371.
 33. Canu G, De Bonis M, Minucci A, Capoluongo E. Red blood cell PK deficiency: an update of PK-LR gene mutation database. *Blood Cells Mol Dis.* 2016;57:100-109.
 34. Grace RF, Bianchi P, van Beers EJ, et al. Clinical spectrum of pyruvate kinase deficiency: data from the Pyruvate Kinase Deficiency Natural History Study. *Blood.* 2018;131(20):2183-2192.
 35. Bianchi P, Fermo E, Lezon-Geyda K, et al. Genotype-phenotype correlation and molecular heterogeneity in pyruvate kinase deficiency. *Am J Hematol.* 2020;95(5):472-482.
 36. Warang P, Kedar P, Ghosh K et al. Molecular and clinical heterogeneity in pyruvate kinase deficiency in India. *Blood Cells Mol Dis.* 2013;51(3):133-137.
 37. Zanella A, Bianchi P. Red cell pyruvate kinase deficiency: from genetics to clinical manifestations. *Baillieres Best Pract Res Clin Haematol.* 2000;13(1):57-81.
 38. Kanno H, Ballas SK, Miwa S, et al. Molecular abnormality of erythrocyte pyruvate kinase deficiency in the Amish. *Blood.* 1994;83(8):2311-2316.
 39. Machado P, Manco L, Gomes C, et al. Pyruvate kinase deficiency in sub-Saharan Africa: identification of a highly frequent missense mutation (G829A;Glu277Lys) and association with malaria. *PLoS One.* 2012;7:e47071.
 40. Baronciani L, Beutler E. Molecular study of pyruvate kinase deficient patients with hereditary nonspherocytic hemolytic anemia. *J Clin Invest.* 1995;95(4):1702-1709.
 41. Costa C, Albuissou J, Le TH, et al. Severe hemolytic anemia in a Vietnamese family, associated with novel mutations in the gene encoding for pyruvate kinase. *Haematologica.* 2005;90(1):25-30.
 42. Richards S, Aziz N, Bale S, et al. ACMG Laboratory Quality Assurance Committee. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med.* 2015;17(5):405-424.
 43. Marcello AP, Vercellati C, Fermo E, et al. A case of congenital red cell pyruvate kinase deficiency associated with hereditary stomatocytosis. *Blood Cells Mol Dis.* 2008;41(3): 261-262.
 44. Coutinho R, Bento C, Almeida H, et al. Complex inheritance of chronic haemolytic anaemia. *Br J Haematol.* 2009;144(4):615-616.
 45. Manco L, Ribeiro ML, Maximo V, et al. A new PKLR gene mutation in the R-type promoter region affects the gene transcription causing pyruvate kinase deficiency. *Br J Haematol.* 2000;110(4):993-997.
 46. van Wijk R, van Solinge WW, Nerlov C, et al. Disruption of a novel regulatory element in the erythroid-specific promoter of the human PKLR gene causes severe pyruvate kinase deficiency. *Blood.* 2003;101(4):1596-1602.
 47. Kager L, Minkov M, Zeithofer P, et al. Two novel missense mutations and a 5bp deletion in the erythroid-specific promoter of the PKLR gene in two unrelated patients with pyruvate kinase deficient transfusion-dependent chronic nonspherocytic hemolytic anemia. *Pediatr Blood Cancer.* 2016;63(5):914-916.
 48. Pissard S, Max-Audit I, Skopinski L, et al. Pyruvate kinase deficiency in France: a 3-year study reveals 27 new mutations. *Br J Haematol.* 2006;133(6):683-689.
 49. de Vooght KM, van Wijk R, van Wesel AC, et al. Characterization of the -148C>T promoter polymorphism in PKLR. *Haematologica.* 2008;93(9):1407-1408.
 50. Gallagher PG, Glader B. Diagnosis of pyruvate kinase deficiency. *Pediatr Blood Cancer.* 2016;63(5):771-772.
 51. Titapiwatanakun R, Hoyer JD, Crain K, et al. Relative red blood cell enzyme levels as a clue to the diagnosis of pyruvate kinase deficiency. *Pediatr Blood Cancer.* 2008;51(6): 819-821.
 52. Zanella A, Bianchi P, Baronciani L, et al. Molecular characterization of PK-LR gene in pyruvate kinase-deficient Italian patients. *Blood.* 1997;89(10):3847-3852.
 53. Gallagher PG, Maksimova Y, Lezon-Geyda K, et al. Aberrant splicing contributes to severe α -spectrin-linked congenital hemolytic anemia. *J Clin Invest.* 2019;129 (7):2878-2887.
 54. Grace RF, Rose C, Layton M, et al. Safety and efficacy of mitapivat in pyruvate kinase deficiency. *N Engl J Med.* 2019;381(10):933-944.
 55. Lezon-Geyda K, Rose MJ, McNaull MA, et al. PKLR intron splicing-associated mutations and alternate diagnoses are common in pyruvate kinase deficient patients with single or no PKLR coding mutations. *Blood.* 2018;132 (Suppl 1):3607.
 56. Bagla S, Bhambhani K, Gadgeel M, et al. Compound heterozygosity in PKLR gene for a previously unrecognized intronic polymorphism and a rare missense mutation as a novel cause of severe pyruvate kinase deficiency. *Haematologica.* 2019;104(9):e428-e431.
 57. Lesmana H, Dyer L, Li X, et al. Alu element insertion in PKLR gene as a novel cause of pyruvate kinase deficiency in Middle Eastern patients. *Hum Mutat.* 2018;39(3): 389-393.
 58. Zurcher C, Loos JA, Prins HK. Hereditary high ATP content of human erythrocytes.

- Folia Haematol Int Mag Klin Morphol Blutforsch. 1965;83(4):366-376.
59. Max-Audit I, Rosa R, Marie J. Pyruvate kinase hyperactivity genetically determined: metabolic consequences and molecular characterization. *Blood*. 1980;56(5):902-909.
 60. van Oirschot BA, Francois JJ, van Solinge WW, et al. Novel type of red blood cell pyruvate kinase hyperactivity predicts a remote regulatory locus involved in PKLR gene expression. *Am J Hematol*. 2014;89(4):380-384.
 61. Beutler E, Westwood B, van Zwieten R, et al. G→T transition at cDNA nt 110 (K37Q) in the PKLR (pyruvate kinase) gene is the molecular basis of a case of hereditary increase of red blood cell ATP. *Hum Mutat*. 1997;9(3):282-285.
 62. Viprakasit V, Ekwatanakit S, Riolueang S, et al. Mutations in Kruppel-like factor 1 cause transfusion-dependent hemolytic anemia and persistence of embryonic globin gene expression. *Blood*. 2014;123(10):1586-1595.
 63. Beutler E, Gelbart T. Estimating the prevalence of pyruvate kinase deficiency from the gene frequency in the general white population. *Blood*. 2000;95(11):3585-3588.
 64. Carey PJ, Chandler J, Hendrick A, et al. Prevalence of pyruvate kinase deficiency in northern European population in the north of England. Northern Region Haematologists Group. *Blood*. 2000;96(12):4005-4006.
 65. Secrest MH, Storm M, Carrington C, et al. Prevalence of pyruvate kinase deficiency: a systematic literature review. *Eur J Haematol*. 2020 Apr 12. [Epub ahead of print]
 66. van Bruggen R, Gualtieri C, Iliescu A, et al. Modulation of malaria phenotypes by pyruvate kinase (PKLR) variants in a Thai population. *PLoS One*. 2015;10:e0144555.
 67. Min-Oo G, Fortin A, Tam MF, et al. Pyruvate kinase deficiency in mice protects against malaria. *Nat Genet*. 2003;35(4):357-362.
 68. Ayi K, Min-Oo G, Serghides L, et al. Pyruvate kinase deficiency and malaria. *New Eng J Med*. 2008;358(17):1805-1810.
 69. Qidwai T, Jamal F, Singh S. Exploring putative molecular mechanisms of human pyruvate kinase enzyme deficiency and its role in resistance against *Plasmodium falciparum* malaria. *Interdiscipl Sci*. 2014;6(2):158-166.
 70. van Wijk R, Huizinga EG, van Wesel AC, et al. Fifteen novel mutations in PKLR associated with pyruvate kinase (PK) deficiency: structural implications of amino acid substitutions in PK. *Hum Mutat*. 2009; 30(3):446-453.
 71. Jaouani M, Manco L, Kalai M, et al. Molecular basis of pyruvate kinase deficiency among Tunisians: description of new mutations affecting coding and noncoding regions in the PKLR gene. *Int J Lab Hematol*. 2017;39(2):223-231.
 72. Christensen RD, Yaish HM, Johnson CB, et al. Six children with pyruvate kinase deficiency from one small town: molecular characterization of the PK-LR gene. *J Pediatr*. 2011;159(4):695-697.
 73. Lakomek M, Winkler H, Pekrun A, et al. Erythrocyte pyruvate kinase deficiency. The influence of physiologically important metabolites on the function of normal and defective enzymes. *Enzyme Protein*. 1994;48(3):149-163.
 74. Finkenstedt A, Bianchi P, Theurl I, et al. Regulation of iron metabolism through GDF15 and hepcidin in pyruvate kinase deficiency. *Br J Haematol*. 2009;144(5):789-793.
 75. Goto T, Ubukawa K, Kobayashi I, et al. ATP produced by anaerobic glycolysis is essential for enucleation of human erythroblasts. *Exp Hematol*. 2019;72:14-26.e1
 76. van Beers EJ, van Straaten S, Morton DH, et al. Prevalence and management of iron overload in pyruvate kinase deficiency: report from the Pyruvate Kinase Deficiency Natural History Study. *Haematologica*. 2019;104(2):e51-e53.
 77. Zanella A, Berzuini A, Colombo MB, et al. Iron status in red cell pyruvate kinase deficiency: study of Italian cases. *Br J Haematol*. 1993;83(3):485-490.
 78. Mojzikova R, Koralkova P, Holub D, et al. Iron status in patients with pyruvate kinase deficiency: neonatal hyperferritinaemia associated with a novel frameshift deletion in the PKLR gene (p.Arg518fs), and low hepcidin to ferritin ratios. *Br J Haematol*. 2014;165(4):556-563.
 79. Zanella A, Bianchi P, Iurlo A, et al. Iron status and HFE genotype in erythrocyte pyruvate kinase deficiency: study of Italian cases. *Blood Cells Mol Dis*. 2001;27(3):653-661.
 80. Rider NL, Strauss KA, Brown K, et al. Erythrocyte pyruvate kinase deficiency in an old-order Amish cohort: longitudinal risk and disease management. *Am J Hematol*. 2011;86(10):827-834.
 81. Grace RF, Zanella A, Neufeld EJ, et al. Erythrocyte pyruvate kinase deficiency: 2015 status report. *Am J Hematol*. 2015;90(9):825-830.
 82. Perseu L, Giagu N, Satta S, et al. Red cell pyruvate kinase deficiency in Southern Sardinia. *Blood Cells Mol Dis*. 2010;45(4):280-283.
 83. Branca R, Costa E, Rocha S, et al. Coexistence of congenital red cell pyruvate kinase and band 3 deficiency. *Clin Lab Haematol*. 2004;26(4):297-300.
 84. Christensen RD, Yaish HM, Nussenzweig RH, Agarwal AM. Siblings with severe pyruvate kinase deficiency and a complex genotype. *Am J Med Genet A*. 2016;170(9):2449-2452.
 85. Vercellati C, Marcello AP, Fermo E, et al. A case of hereditary spherocytosis misdiagnosed as pyruvate kinase deficient hemolytic anemia. *Clin Lab*. 2013;59(3-4):421-424.
 86. Fermo E, Vercellati C, Marcello AP, et al. Hereditary xerocytosis due to mutations in *piezo1* gene associated with heterozygous pyruvate kinase deficiency and beta-thalassemia trait in two unrelated families. *Case Rep Hematol*. 2017;2017:2769570.
 87. Cohen-Solal M, Préhu C, Wajcman H, et al. A new sickle cell disease phenotype associating Hb S trait, severe pyruvate kinase deficiency (PK Conakry), and an alpha2 globin gene variant (Hb Conakry). *Br J Haematol*. 1998;103(4):950-956.
 88. Alli N, Coetzee M, Louw V, et al. Sickle cell disease in a carrier with pyruvate kinase deficiency. *Hematology*. 2008;13(6):369-372.
 89. Manco L, Vagace JM, Relvas L, et al. Chronic haemolytic anaemia because of pyruvate kinase (PK) deficiency in a child heterozygous for haemoglobin S and no clinical features of sickle cell disease. *Eur J Haematol*. 2010;84(1):89-90.
 90. Zarza R, Moscardo M, Alvarez R, et al. Coexistence of hereditary spherocytosis and a new red cell pyruvate kinase variant: PK Mallorca. *Haematologica*. 2000;85(3):227-232.
 91. van Zwieten R, van Oirschot BA, Veldthuis M, et al. Partial pyruvate kinase deficiency aggravates the phenotypic expression of band 3 deficiency in a family with hereditary spherocytosis. *Am J Hematol*. 2015;90(3):E35-E39.
 92. Pereira J, Gonzalez A, Vagace J, et al. Congenital dyserythropoietic anemia associated to a GATA1 mutation aggravated by pyruvate kinase deficiency. *Ann Hematol*. 2016;95(9):1551-1553.
 93. van Straaten S, Bierings M, Bianchi P, et al. Worldwide study of hematopoietic allogeneic stem cell transplantation in pyruvate kinase deficiency. *Haematologica*. 2018;103(2):e82-e86.
 94. He Y, Luo J, Lei Y, et al. A novel PKLR gene mutation identified using advanced molecular techniques. *Pediatr Transplant*. 2018;22(2): e13143.
 95. Shimomura M, Doi T, Nishimura S, et al. Successful allogeneic bone marrow transplantation using immunosuppressive conditioning regimen for a patient with red blood cell transfusion dependent pyruvate kinase deficiency anemia. *Hematol Rep*. 2020;12(1):8305.
 96. Yang H, Merica E, Chen Y, et al. Phase 1 single and multiple-ascending-dose randomized studies of the safety, pharmacokinetics, and pharmacodynamics of AG-348, a first-in-class allosteric activator of pyruvate kinase R, in healthy volunteers. *Clin Pharmacol Drug Dev*. 2019;8(2):246-259.
 97. Rab MAE, van Oirschot BA, Kosinski PA, et al. AG-348 (Mitapivat), an allosteric activator of red blood cell pyruvate kinase, increases enzymatic activity, protein stability, and ATP levels over a broad range of PKLR genotypes. *Haematologica*. 2020 Jan 23. [Epub ahead of print]
 98. Dunbar CE, High KA, Joung JK, et al. Gene therapy comes of age. *Science*. 2018;359(6372):eaan4672.
 99. Magrin E, Miccio A, Cavazzana M. Lentiviral and genome-editing strategies for the treatment of β -hemoglobinopathies. *Blood*. 2019;134(15):1203-1213.
 100. Meza NW, Alonso-Ferrero ME, Navarro S, et al. Rescue of pyruvate kinase deficiency in mice by gene therapy using the human isoenzyme. *Mol Ther*. 2009;17(12):2000-2009.
 101. Meza NW, Quintana-Bustamante O, Puyet A, et al. In vitro and in vivo expression of human erythrocyte pyruvate kinase in erythroid cells: a gene therapy approach. *Hum Gene Ther*. 2007;18(6):502-514.
 102. Garcia-Gomez M, Calabria A, Garcia-Bravo M, et al. Safe and efficient gene therapy for pyruvate kinase deficiency. *Mol Ther*. 2016;24(7):1187-1198.
 103. Quintana-Bustamante O, Fañanas-Baquero S, Orman I, et al. Gene editing of PKLR gene in human hematopoietic progenitors through 5' and 3' UTR modified TALEN mRNA. *PLoS One*. 2019;14(10):e0223775.