Thursdays Webinars

EuroBloodNet

Recommendations on PKD diagnosis

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Fondazione IRCCSCa' Granda Ospedale Maggiore Policlinico of Milan
Phatophysiology of Anemias Unit
ERN-EuroBloodNet subnetwork Red Blood Cells Disorders
Milan – Italy
8th October 2020

Co-funded by the Health Programme of the European Union

European Reference Network for rare or low prevalence complex diseases
Network Hematological Diseases (ERN EuroBloodNet)

Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico
Sistema Socio Sanitario Regione Lombardia

Conflicts of interest

Consultant at Agios Pharmaceutics

Practical issues before starting

- ✓ **30-35min presentation (30 slides max) + 15 min Q&A session**
- ✓ **Microphones will be muted by host to avoid back noise**
- ✓ **Please, stop your video to improve internet conexion**
- ✓ **Send your questions during the presentation through the chat, they will be gathered and answered after the presentations.**

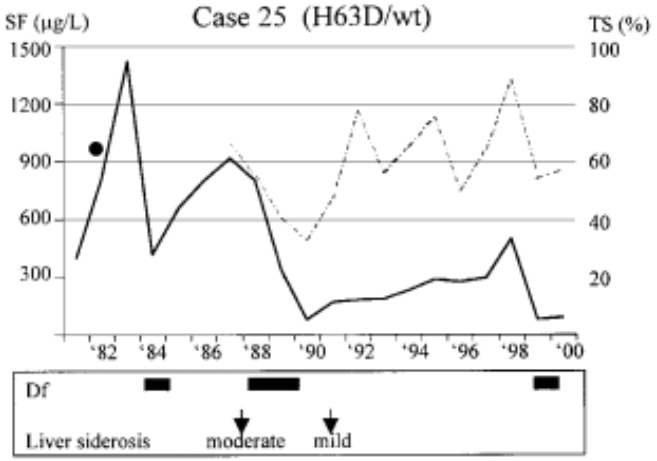
Learning objectives of the webinar

- ✓ Pathophysiology and phenotypic variability of PK deficiency
- ✓ Diagnostic approaches to PK deficiency
- ✓ The genotype-phenotype correlation in PK deficiency

PKD Clinical Heterogenity

Case 1

Man	75 yrs
Diagnosis	31 yrs (1975)
Hb	12.5 g/dL
Retic	123 10^9/L
Serum ferritin	810 ul/L
Splenectomy	No
Transfusions	No
HFE genotype	H63D/wt



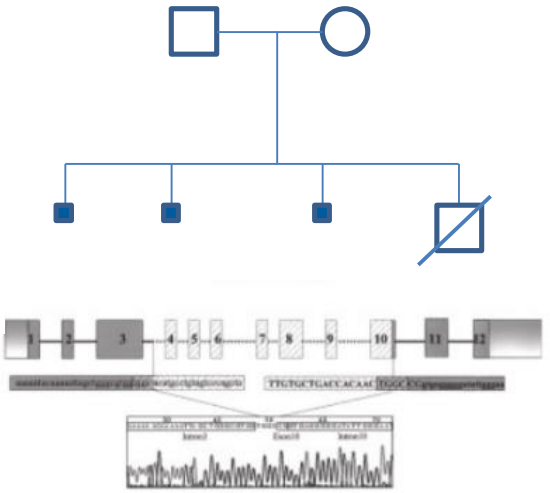
***PKLR* genotype** c.1675T/c.1456T

Zanella et al, 2001

PKD Clinical Heterogenity

Case 2

Male	1 day
Diagnosis at birth	
Death	at birth
Hb	8,9 g/dL
Retic	na
Serum ferritin	>4000 ul/L
ExTx	Yes



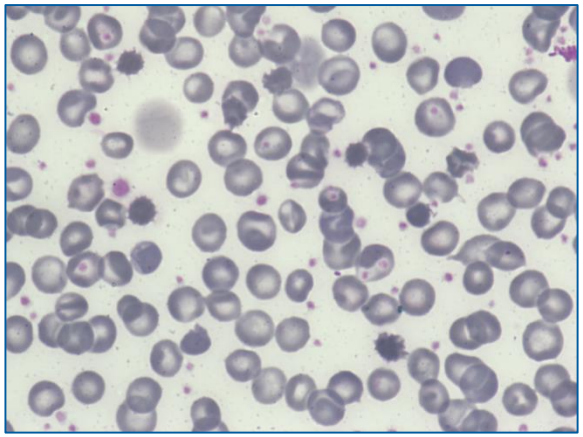
Fermo et al, 2005

***PKLR* genotype**
c.409G>A/c.283+1914_1434del

PKD Clinical Heterogenity

Case 3

Male,	17 yrs
Diagnosis	13 yrs
Previous diagnosis	CDAII (SEC23B normal)
Hb (birth)	8 g/dL
Hb (1 month)	3g/dL
TX	2U/mo
Splenectomy	8 yrs
Hb (post splen)	8.1g/L
TX (12 yrs)	2U/mo

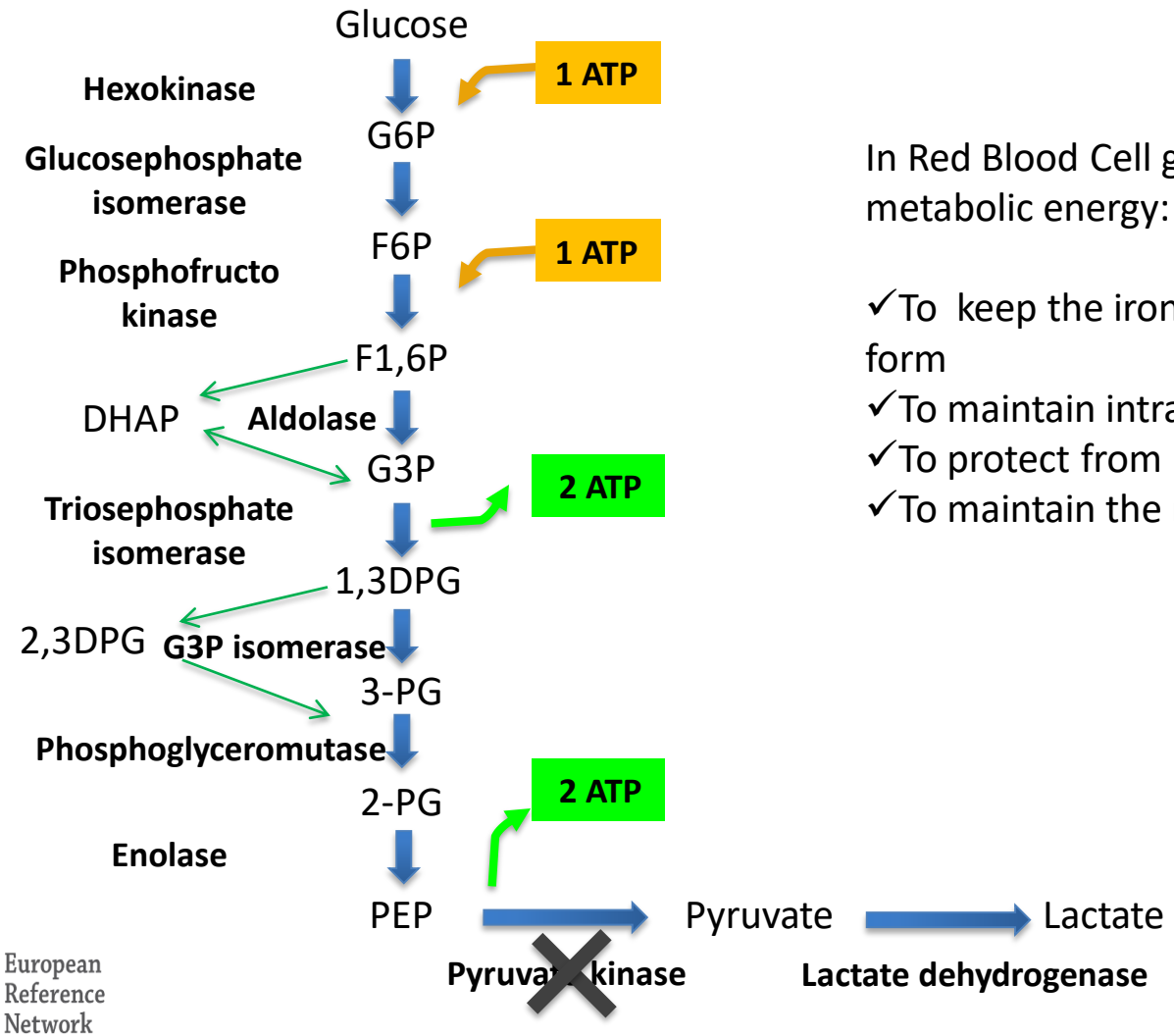


PK activity	6.0 IU/gHb (11.9-16.7)
***PKLR* genotype**	c.1528C>T/ c.1528C>T

Fermo et al, 2005

Al-Samkari H, et al, The variable manifestations of disease in pyruvate kinase deficiency and their management. Haematologica. 2020

The Embden-Meyerhof Pathway



In Red Blood Cell glycolysis is the main source of metabolic energy:

- ✓To keep the iron of hemoglobin in the functional form
- ✓To maintain intracellular ions concentration
- ✓To protect from oxydative stress
- ✓To maintain the red cell shape

PK deficiency pathophysiology

Mutation in *PKLR* gene

↓ PK activity
↑ PK instability

⇩

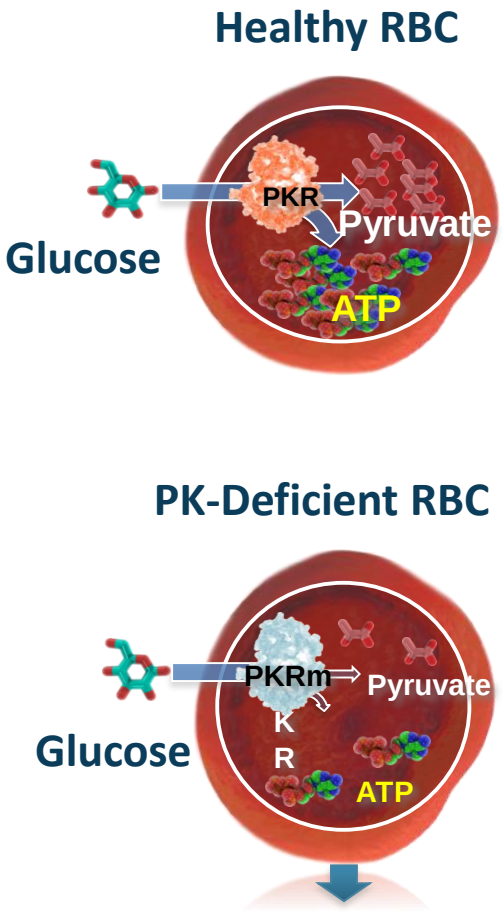
Inefficient glycolysis
↓ ATP generation

⇩

RBC membrane integrity /deformability
Premature removal from the circulation

⇩

Extravascular hemolysis
Chronic hemolytic anemia



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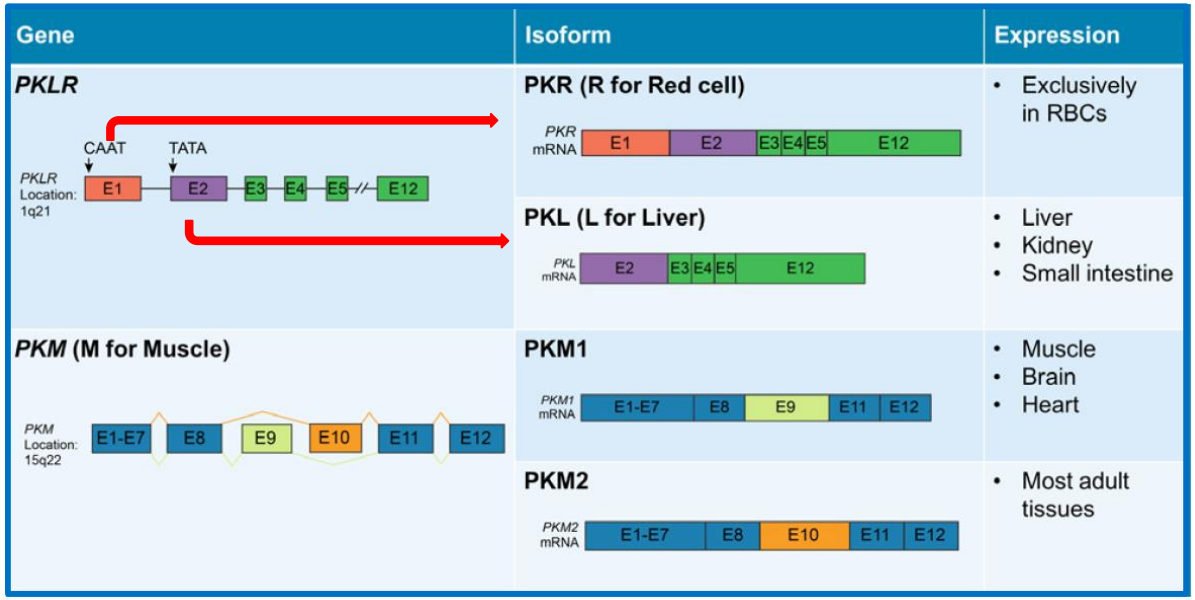
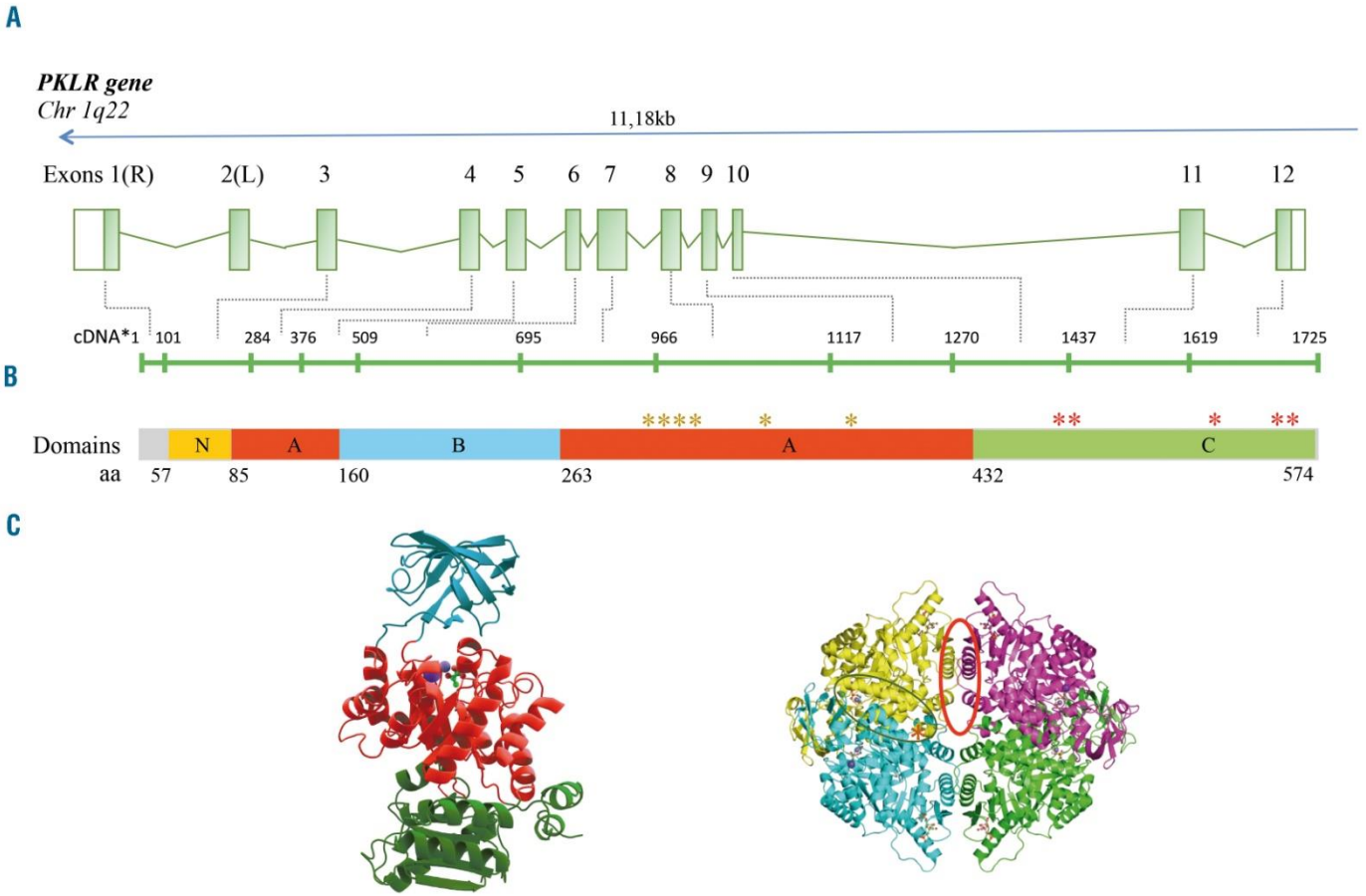
PK Deficiency: Genetics



Derived from Israelsen and Semin (2015).

- The *PKLR* gene is located on chromosome 1(1q21)
- Transcription of *PK* tissue-specific promoters yields PKR and PKL
- The cDNA of PKR is 2060 bp long and codes for 574 amino acids
- PKR is expressed exclusively in red blood cells

PKLR gene



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Bianchi and Fermo, Haematologica 2020

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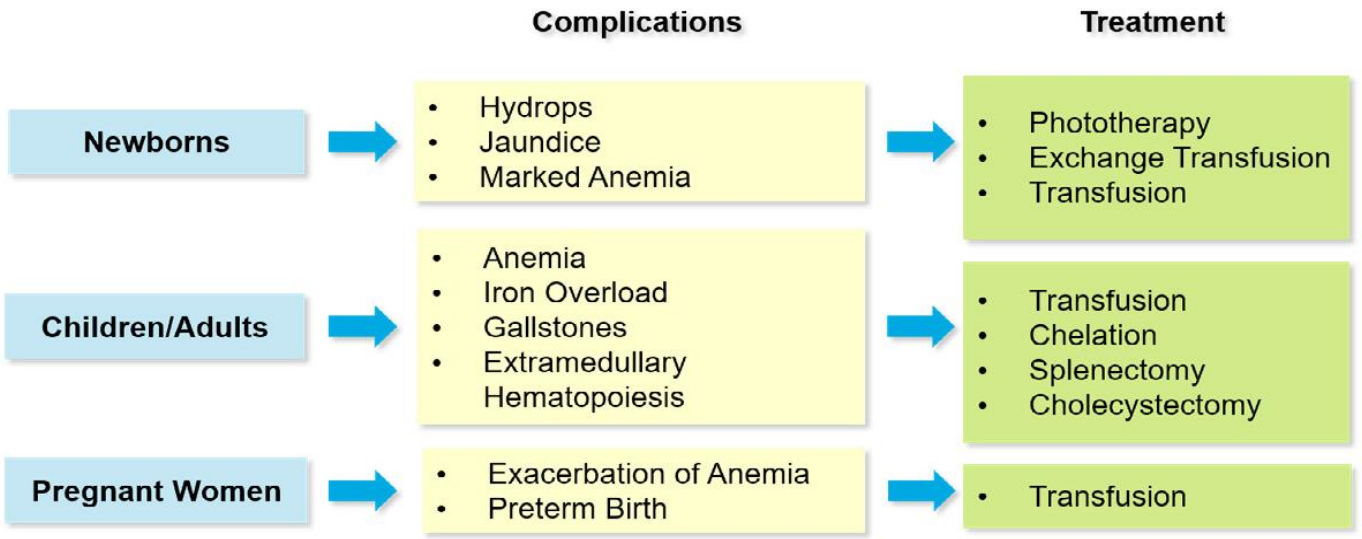
PK deficiency: a rare form of congenital hemolytic anemia

- ✓ The most common glycolytic defect causing non-spherocytic hemolytic anemia
- ✓ Autosomal recessive inheritance pattern
- ✓ World-wide distribution
- ✓ Heterogeneous clinical presentation

Clinical presentation and complications



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Zanella, et al. 2007; Grace, et al 2018, Iolascon A et al 2017, Al-Samkari et al 2020

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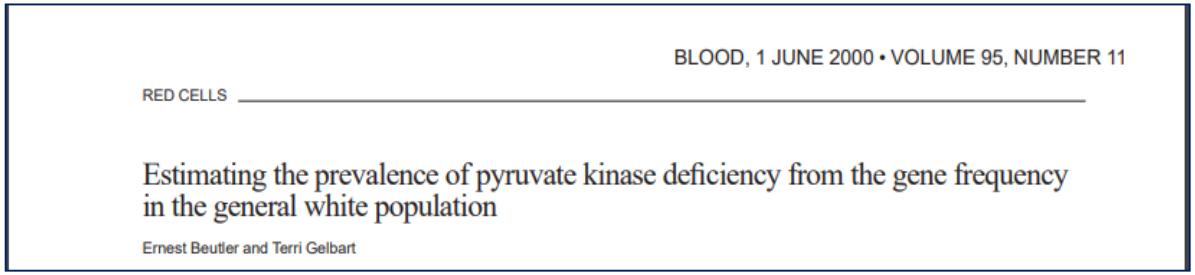
How rare is PK deficiency?



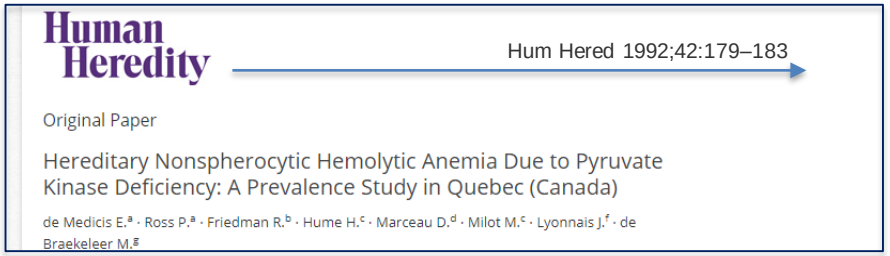
BLOOD, 1 JUNE 2000 • VOLUME 95, NUMBER 11

RED CELLS

Estimating the prevalence of pyruvate kinase deficiency from the gene frequency in the general white population

Ernest Beutler and Terri Gelbart

PKD genetic prevalence:
1:20.000



Human Heredity

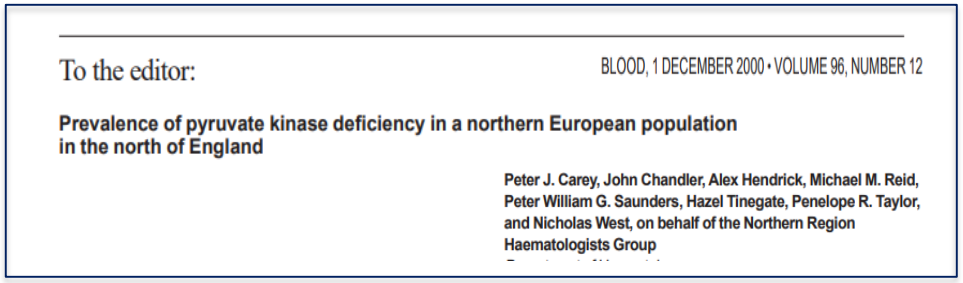
Hum Hered 1992;42:179–183

Original Paper

Hereditary Nonspherocytic Hemolytic Anemia Due to Pyruvate Kinase Deficiency: A Prevalence Study in Quebec (Canada)

de Medicis E.[a] · Ross P.[a] · Friedman R.[b] · Hume H.[c] · Marceau D.[d] · Milot M.[c] · Lyonnais J.[f] · de Braekeleer M.[g]

PKD prevalence in different provinces of Quebec:
1:51,000-1:454,000



To the editor:

BLOOD, 1 DECEMBER 2000 • VOLUME 96, NUMBER 12

Prevalence of pyruvate kinase deficiency in a northern European population in the north of England

Peter J. Carey, John Chandler, Alex Hendrick, Michael M. Reid, Peter William G. Saunders, Hazel Tinegate, Penelope R. Taylor, and Nicholas West, on behalf of the Northern Region Haematologists Group

PKD prevalence in Northern EU populations:
1:300,000

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Beutler & Gelbart, 2000; De Medicis et al, 1999; Carery et al 2000

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Prevalence of pyruvate kinase deficiency: A systematic literature review

Matthew H. Secrest[1] | Mike Storm[2] | Courtney Carrington[1] | Deborah Casso[3] | Keely Gilroy[2] | Leanne Pladson[2] | Audra N. Boscoe[2]

The prevalence of clinically diagnosed PK deficiency is likely between 3.2 and 8.5 per million in Western populations, while the prevalence of diagnosed and undiagnosed PK deficiency could possibly be as high as 51 per million.

~5-15-fold difference between genetic estimates and diagnosed cases

Secrest et al, Eur J Hematol 2020;105:173–184.
EurobloodNet surveys – White Book, ENERCA reccomendation

Factors influencing diagnosis

- ✓ **Recessive transmission**
- ✓ **Lack of knowledge of the disease**:

"The diagnosis of PK deficiency is made only when the physician considers the possibility and has red cell enzyme assays performed." (E. Beutler)

✓**Heterogeneous clinical phenotype (mild forms - intrauterin death?)**

- ✓ **Technical problems:**
  - Recent transfusions
  - WBCs/platelet contamination
  - Increased reticulocyte number
  - Variants displaying *in vitro* normal enzyme activity

Is PK deficiency misdiagnosed / underdiagnosed?

Am J Hematol. 2018 May;93(5):672-682. doi: 10.1002/ajh.25058. Epub 2018 Feb 24.

Multi-gene panel testing improves diagnosis and management of patients with hereditary anemias.

Russo R[1,2], Andolfo I[1,2], Manna F[1,2], Gambale A[1,2], Marra R[1,2], Rosato BE[1,2], Caforio P[1,2], Pinto V[3], Pignataro P[2], Radhakrishnan K[4,5], Unal S[6], Tomaiuolo G[7], Forni GL[3], Iolascon A[1,2].

"45.5% of the probands originally classified as CDA exhibited a conclusive diagnosis of chronic anemia due to enzymatic defects, mainly due to mutations in *PKLR* gene."

Eur J Haematol. 2018 Sep;101(3):297-304. doi: 10.1111/ejh.13097. Epub 2018 Jun 25.

Targeted next generation sequencing for the diagnosis of patients with rare congenital anemias.

Shefer Averbuch N[1,2], Steinberg-Shemer O[1,2], Dgany O[3], Krasnov T[3], Noy-Lotan S[3], Yacobovich J[1,2], Kuperman AA[4,5], Kattamis A[6], Ben Barak A[7], Roth-Jelinek B[8], Chubar E[9], Shabad E[10], Dufort G[11], Ellis M[2,12], Wolach O[2,13], Pazgal I[2,14], Abu Quider A[15], Miskin H[16,17], Tamary H[1,2].

"Genetic diagnosis was achieved in 13 out of 21 patients (62%). Six patients were diagnosed with pyruvate kinase deficiency (28,5%). The mean lag time from presentation to diagnosis was over 13 years."

Received: 19 July 2018 | Revised: 19 October 2018 | Accepted: 20 October 2018
DOI: 10.1002/ajh.25325

WILEY AJH

TEST OF THE MONTH

Addressing the diagnostic gaps in pyruvate kinase deficiency: Consensus recommendations on the diagnosis of pyruvate kinase deficiency

Paola Bianchi[1] | Elisa Fermo[1] | Bertil Glader[2] | Hitoshi Kanno[3] | Archana Agarwal[4] | Wilma Barcellini[1] | Stefan Eber[5] | James D. Hoyer[6] | David J. Kuter[7] | Tabita Magalhães Maia[8] | Maria del Mar Mañu-Pereira[9] | Theodosia A. Kalfa[10] | Serge Pissard[11] | José-Carlos Segovia[12,13] | Eduard van Beers[14] | Patrick G. Gallagher[15] | David C. Rees[16] | Richard van Wijk[17] | with the endorsement of EuroBloodNet, the European Reference Network in Rare Hematological Diseases

Global PK deficiency International expert group (2016)
(24 experts from 20 different Expert Centres)

↓

Survey on diagnostic methodologies

↓

Forum discussion
7 Centres from EU, 5 from USA, and 1 from Asia

↓

Consensus diagnostic recommendations
Algorithm for the diagnosis of PK deficiency

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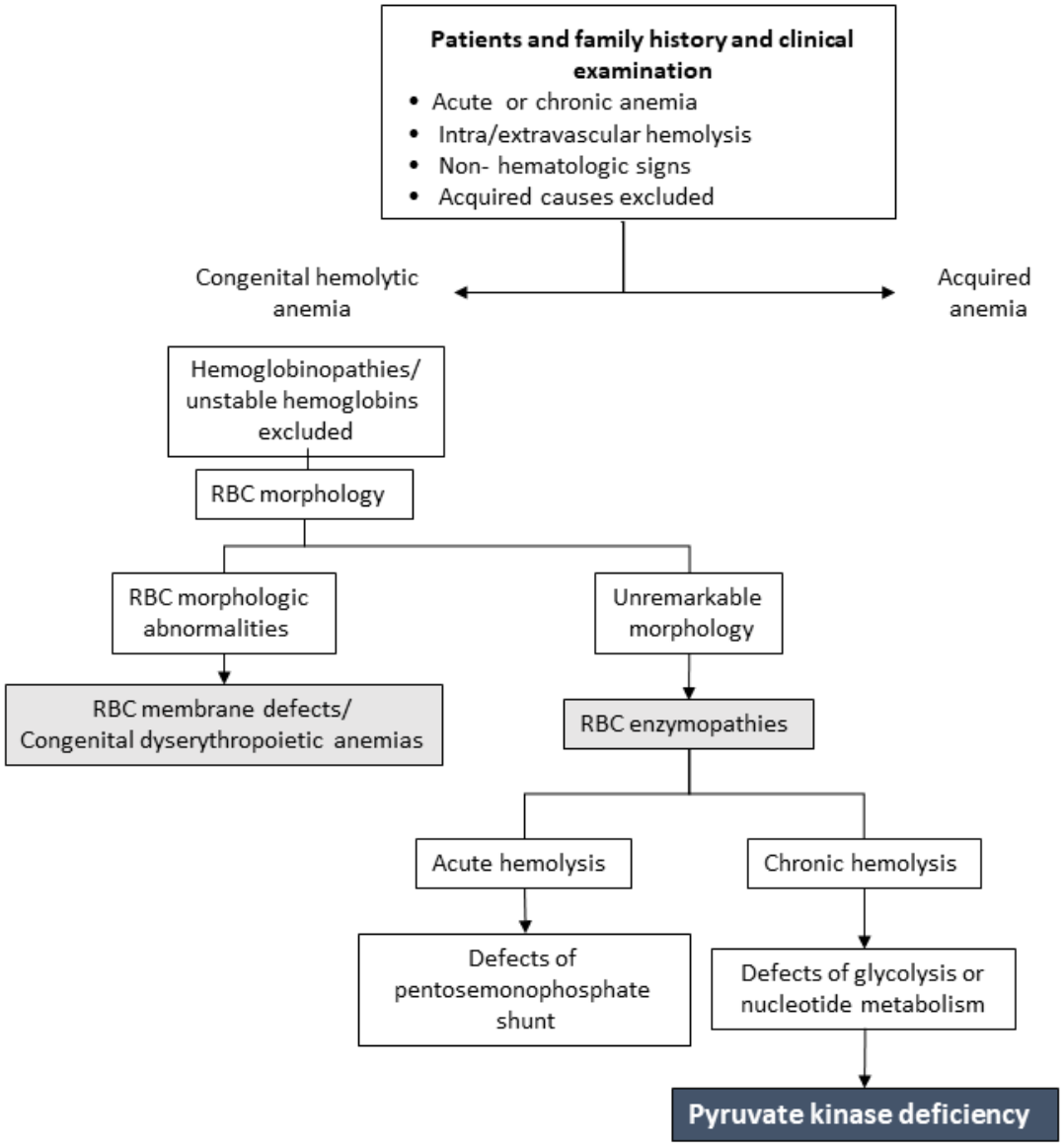
	Recommendation	Evidence
Clinical presentation	PK deficiency may be suspected in: - patients with variable chronic anaemia and/or splenomegaly and/or jaundice, with normal or near-normal red cell morphology. - transfusion dependent cases of unknown aetiology - haemolytic patients with unexplained severe neonatal indirect hyperbilirubinemia - presence of high reticulocyte number in splenectomised patients with no diagnosis	Mean: 95% Median: 100% (75-100)
Clinical data	-Information on clinical history (both recent as well as from infancy, ie neonatal jaundice), family history should always be requested together with samples, as well as the time of last blood transfusion	Mean: 98.6% Median:100% (90-100)
Laboratory data (mandatory in bold)	-**Complete blood count** -**RBC morphology** -Markers of haemolysis (**reticulocyte count, LDH, unconjugated bilirubin,** haptoglobin[1,2])	Mean: 97% Median:100% (90-100)
Differential diagnosis	Acquired haemolytic anaemia, membranopathies, CDAs, unstable haemoglobins, red cell enzymopathies other than PK deficiency should be excluded (See Figure 5)	Mean: 92.1% Median: 100% (50-100)

Bianchi et al, AJH 2018



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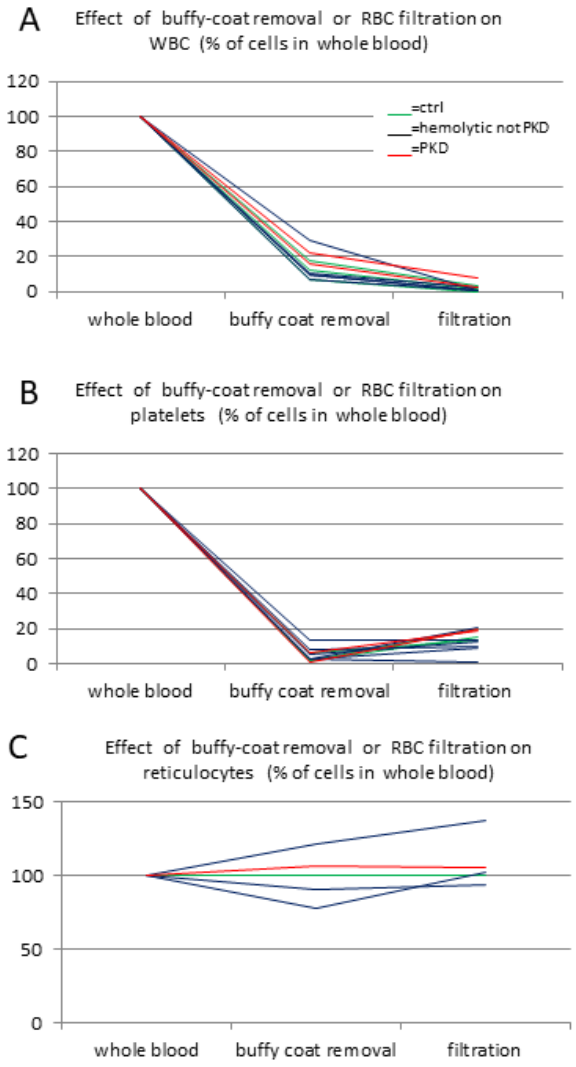
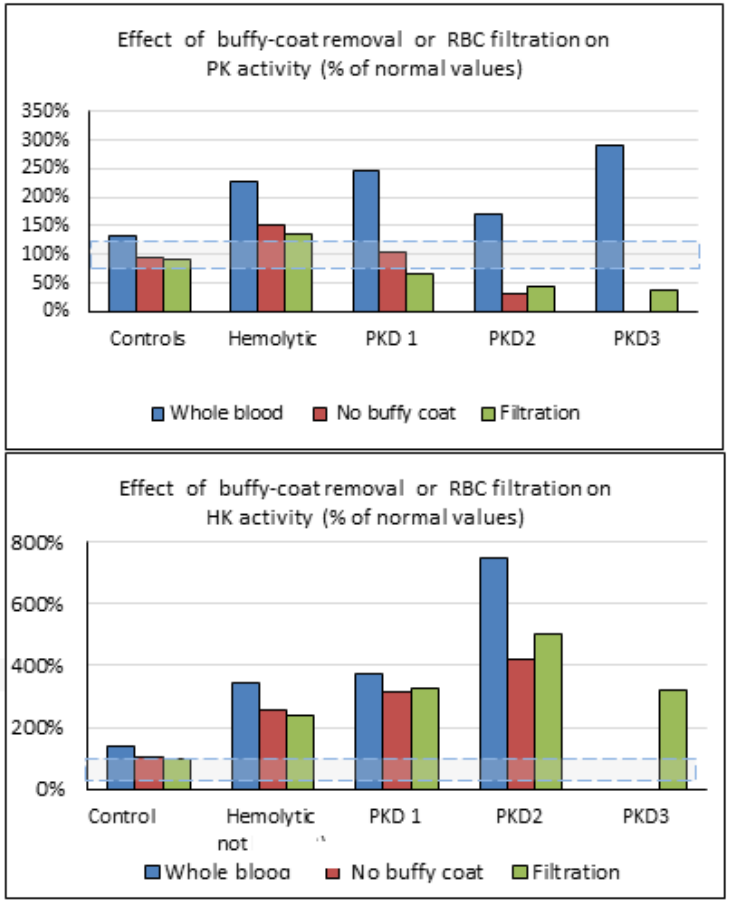
Laboratory diagnosis of PK deficiency

Biochemical testing		
Reference test for biochemical assay	RBC PK activity assay by spectrophotometry (Beutler, 84)	Mean: 98.7% Median: 100% (80-100)
Storage time of sample	PK enzyme assay may be considered stable at 4° C until up to 21 days after collection[3]. A maximum of 14 days storage is recommended if PK activity is related to HK activity due to different stability of HK activity	Mean: 95% Median: 100% (80-100)
Sample anticoagulant	ACD; EDTA, CPD, Heparin could be considered for the enzyme assay (Beutler, 84): EDTA is the main anticoagulant used in daily practice.	Mean: 100% Median: 100%
Sample preparation	Purification on α-cellulose/microcrystalline cellulose column is recommended. Buffy coat removal may be considered as an alternative. PK enzyme activity cannot be performed on whole blood	Mean: 96.7% Median: 100% (80-100)
Reticulocytes interference	Reticulocyte number must be taken into account when interpreting results of PK enzyme assay, particularly when of low-normal PK activity levels. Results could be compared with enzyme activities obtained from a control sample with the same degree of reticulocytosis, or by calculating the ratio of PK activity to another cell age dependent enzyme (e.g. hexokinase).	Mean: 96.1% Median: 100% (70-100)
Interference of donor red blood cells	The enzyme assay should be performed as far as possible after a red cell transfusion. The laboratory should record the time since transfusion. A minimum of 50 days from last transfusion is considered a "safe" period for testing of PK activity, leading to an estimated donor RBC contamination of about 7-14%. Results of enzyme activity need to be interpreted with caution in transfused patients[4].	Mean: 96.9% Median: 100% (60-100)
Confirmatory tests	In case of decreased PK activity, sequencing of *PKLR* gene is highly recommended to confirm the diagnosis	Mean: 88.3% Median: 100% (10-100)

Laboratory diagnosis of PK deficiency – WBC influence





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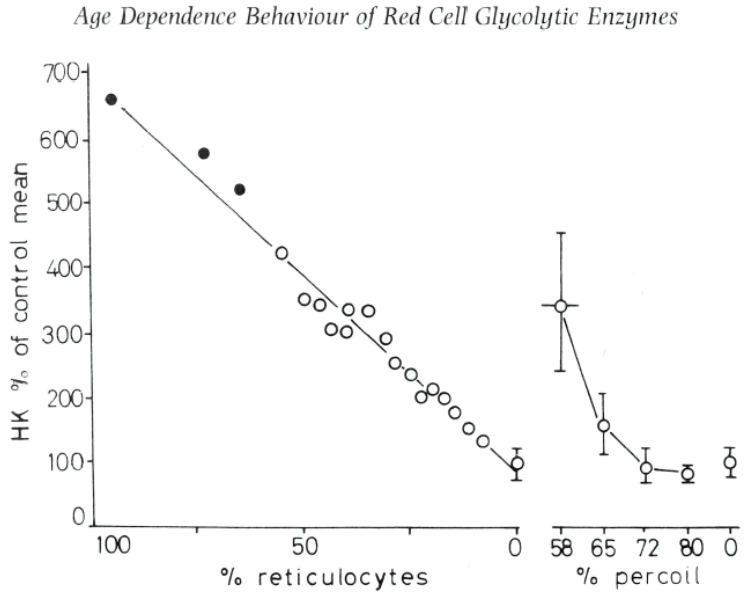
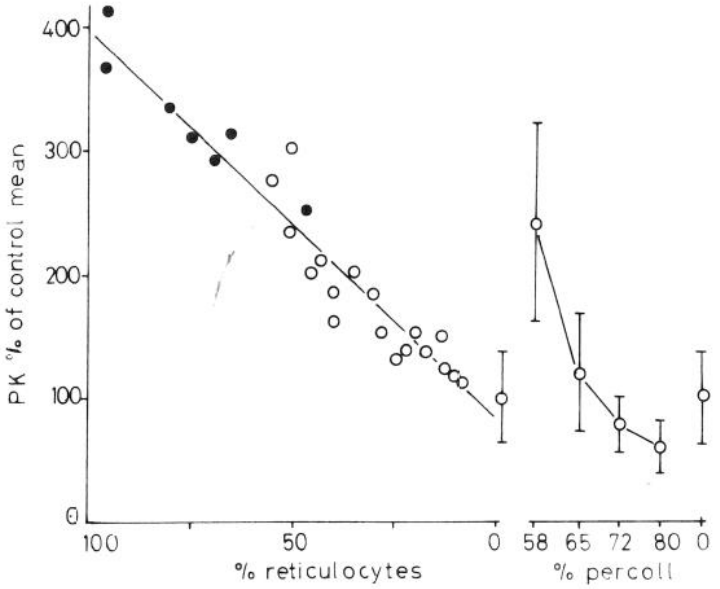
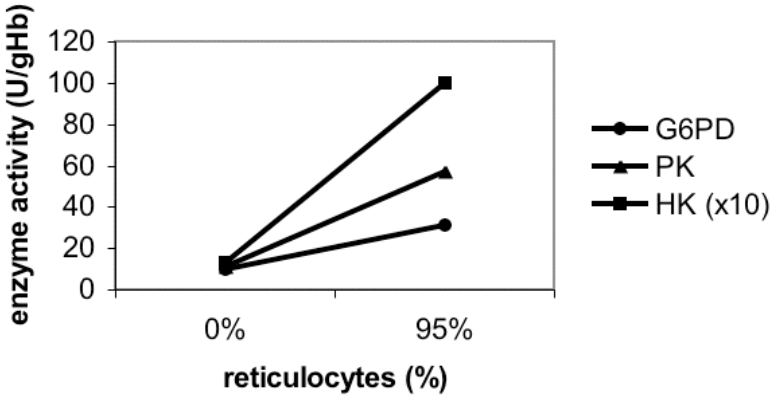
Laboratory diagnosis of PK deficiency – Influence of reticulocytosis





Jansen et al. Br J Haematol 1985; 61: 51-59



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PKDiagnosis European Working Group
PKD DIAGNOSIS EUROPEAN WORKING GROUP
"Towards harmonization of Diagnosis of PK deficiency in EU"
Paris, April 18th, 2019

Slides by R. Van Wijk , UMCU, NL

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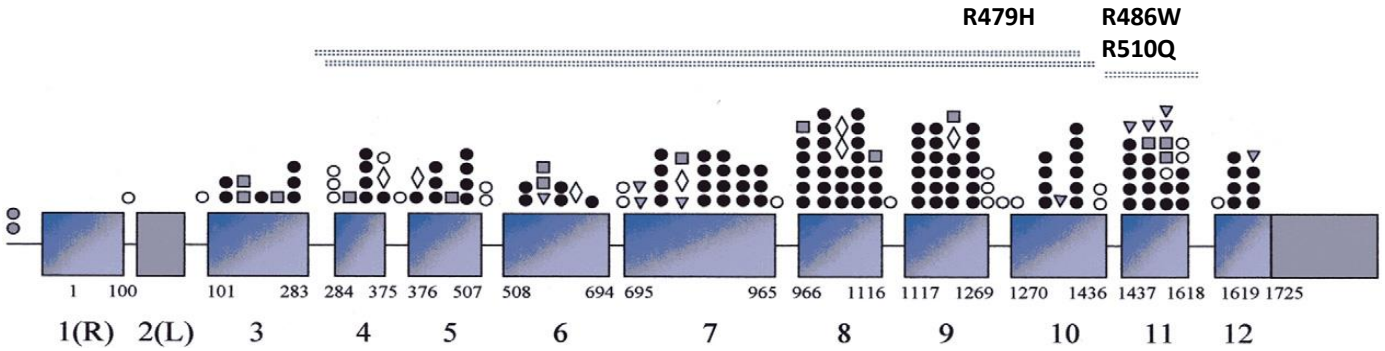
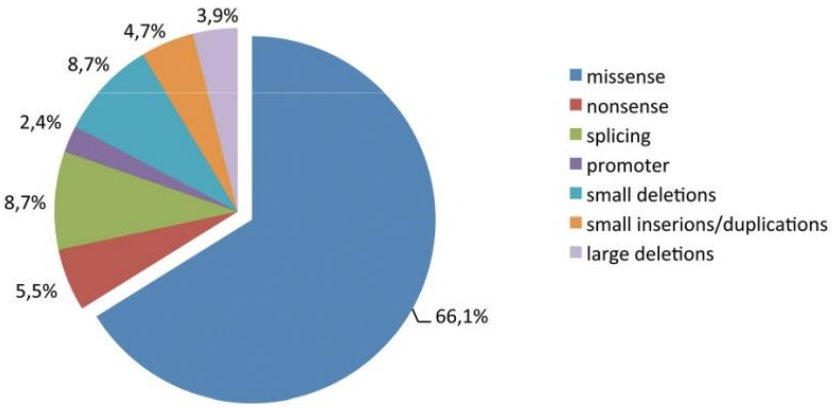
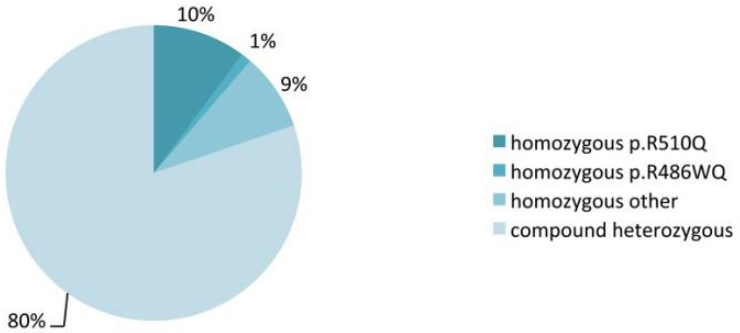
Laboratory diagnosis of PK deficiency – Molecular testing

Molecular testing		
Indication	-Molecular testing is highly recommended to confirm a suspected case of PK deficiency based on decreased enzyme activity. -Molecular testing of *PKLR* gene by Sanger is suitable for patients with (relatively) decreased PK activity - Use of NGS panels is a reliable alternative method for diagnosis of PK deficiency. It is particularly relevant for: - neonates (if family study is not available) - transfusion dependent patients/recently transfused patients - samples with prolonged shipping times	Mean: 91.2% Median: 100% (10-100)
PKLR genotype discrepancies	In case of genotype discrepancies (patients with suspected PKD and one or none mutations detected) further investigation are required: -Assays for detection of large deletions -Re-evaluation of other causes of haemolysis by specific tests or NGS platform In absence of any mutation and decreased PK activity: - NGS tools or, *KLF1* gene mutations should be considered	Mean: 92.5% Median: 100% (40-100)

Laboratory diagnosis of PK deficiency – Molecular testing







Unknown intronic variants

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. ASH Meeting 2018

Zanella, et al. 2007; Kanno, H., et al. 1991. Bianchi et al, 2020

Laboratory diagnosis of PK deficiency – NGS

Reference	Method	N. of genes analyzed	N. of cases studied with CHA	PKD diagnosis	New diagnosis and number and type of mismatched diagnoses
Svidnicki et al, 2018	t-NGS	35	36	2	2 new PKD
Jamwal et al , 2020	t-NGS	55	43	8	8 new PKD
Qin et al, 2020	WES	n.a.	4	4	4 new PKD
	t-NGS	76	21[a]	6	3 new PKD 2 CDA→ PKD 1 DBA→PKD
Kedar et al, 2019	t-NGS	76	21[b]	6	4 new PKD 2 CDA→PKD
	t-NGS	34 and 71	74[c]	7	7 CDA→PKD
Shefer Averbuch, 2018	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. [a]All transfusion-dependent patients. [b]No diagnosis despite extensive laboratory investigations. [c]Suspected 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.

Russo R, et la 2018

Roy et al, 2016

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Bianchi et al, Haematol, 2020

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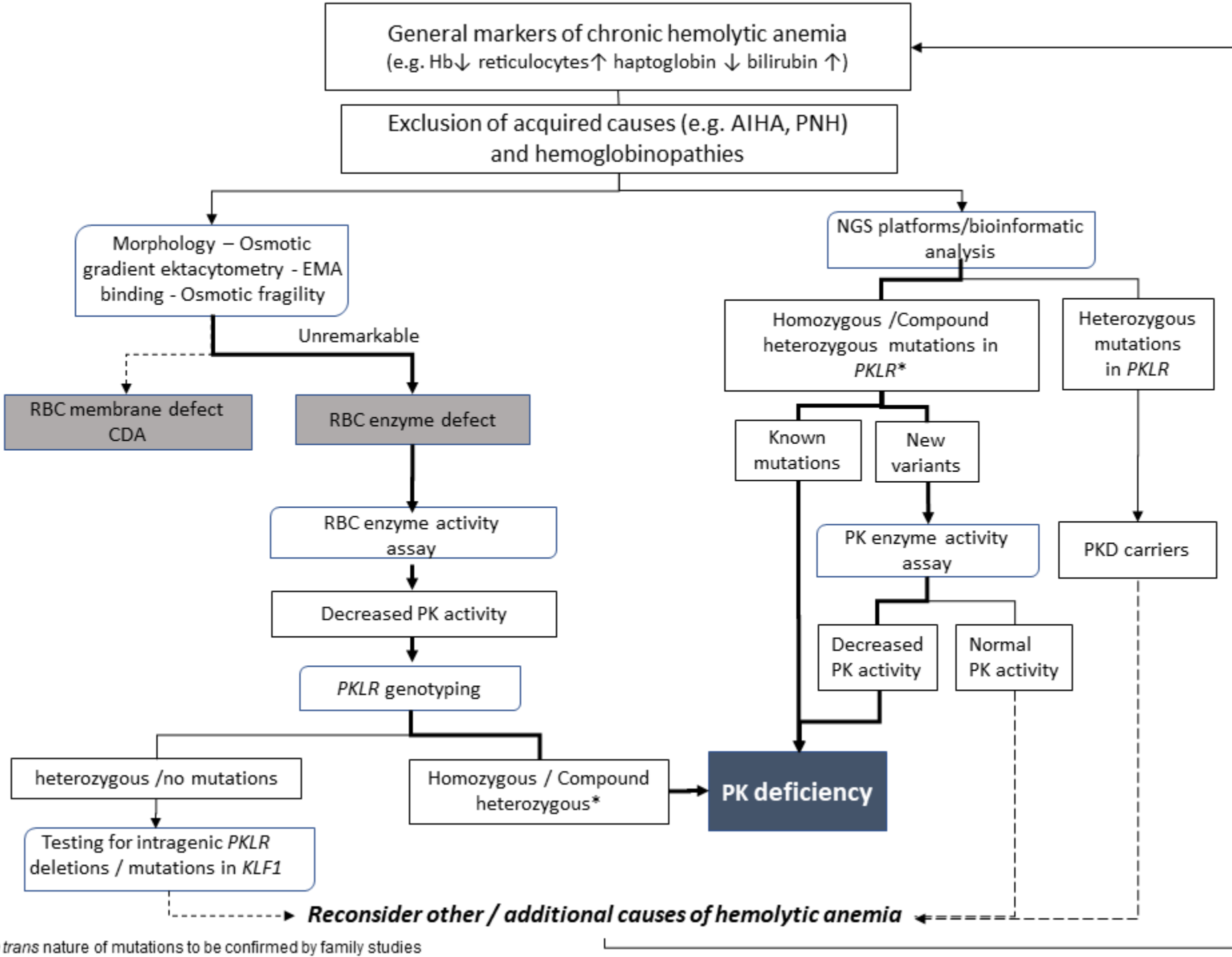
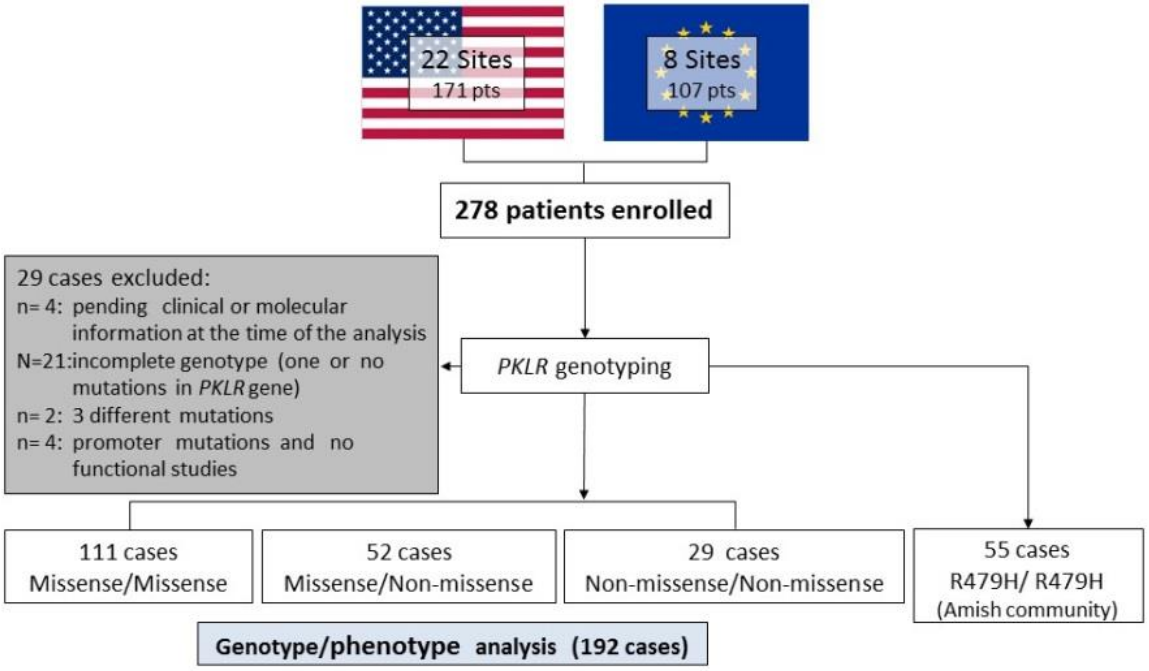

General markers of chronic hemolytic anemia
(e.g. Hb↓ reticulocytes↑ haptoglobin ↓ bilirubin ↑)
Exclusion of acquired causes (e.g. AIHA, PNH) and hemoglobinopathies
Morphology – Osmotic gradient ektacytometry - EMA binding - Osmotic fragility
Unremarkable
RBC membrane defect CDA
RBC enzyme defect
RBC enzyme activity assay
Decreased PK activity
PKLR genotyping
heterozygous /no mutations
Testing for intragenic PKLR deletions / mutations in KLF1
Homozygous / Compound heterozygous*
PK deficiency
NGS platforms/bioinformatic analysis
Homozygous /Compound heterozygous mutations in PKLR*
Heterozygous mutations in PKLR
Known mutations
New variants
PK enzyme activity assay
PKD carriers
Decreased PK activity
Normal PK activity
Reconsider other / additional causes of hemolytic anemia
* In trans nature of mutations to be confirmed by family studies

Genotype-Phenotype Correlation and Molecular Heterogeneity



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Bianchi et al, AJH 2020

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Genotype-Phenotype Correlation

	NM/NM, N=29 Median (Range)	M/NM, N=52 Median (Range)	M/M, N=111 Median (Range)	p-value+
Age at diagnosis (years)	0.4 (0-10.9) n=29	0.7 (0-42.3) n=50	1.3 (0-60.3) n=105	0.049
Hemoglobin (g/dl)**	7.9 (6.5-8.9) n=14	8.4 (6.4-12.8) n=21	9.2 (4.3-12.3) n=40	0.003*
Total number of lifetime transfusions	65 (3-991) n=27	25 (1-721) n=38	16 (1-1915) n=81	0.0013*
Maximum ferritin (ng/ml)	1787 (423-13,409) n=22	604 (22-8,220) n=37	573 (31-9,679) n=75	<0.0001*
PK enzyme activity normalized to patient-specific normal range (%)	-41.6 (-152.4-15.2) n=18	-51.9 (-211.1-64.4) n=24	-69.6 (-485.7-117.6) n=60	0.16

Response to splenectomy

	No response (Hb <8 g/dl) n=31	Partial response (Hb 8-<11 g/dl) n=110	Complete response (Hb ≥11 g/dl) n=7	p
Genotype				
M/M	29%	59%	100%	0.0017
M/NM	32%	26%	0%	0.0005
NM/NM	39%	16%	0%	0.5

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Grace, 2018; Bianchi et al, AJH 2020

Genotype-Phenotype Correlation

Perinatal Course and Pregnancy Outcomes of Patients by *PKLR* Mutation Type

	Missense/Missense N=113 patients	Missense/ Non-Missense N=52 patients	Non-Missense/ Non-Missense N=30 patient	P value**
Characteristics	**n**	**n**	**n**	
Perinatal	28/100 (28%)	15 /48 (31%)	11/28 (39%)	0.26
Complications/Treatment				
In utero transfusions	12/26 (46%)	8/15 (53%)	4/10 (40%)	1
Hydrops	3/26 (12%)	5/14 (36%)	1/11 (9%)	1
Exchange transfusion	34/80 (42%)	17/40 (43%)	9/21 (43%)	1
Pregnancy Outcomes/Management (data presented per pregnancy)*	n=29 pregnancies* 16 female patients	n=13 pregnancies* 6 female patients	n=7 pregnancies* 3 female patients	
Normal birth - Full-term	20/29 (69%)	8/13 (62%)	5/7 (71%)	1
Pre-maturity	3/29 (10%)	0/13 (0%)	2/7 (29%)	0.24
Transfusions during pregnancy	9/21 (43%)	3/6 (50%)	5/5 (100%)	0.043

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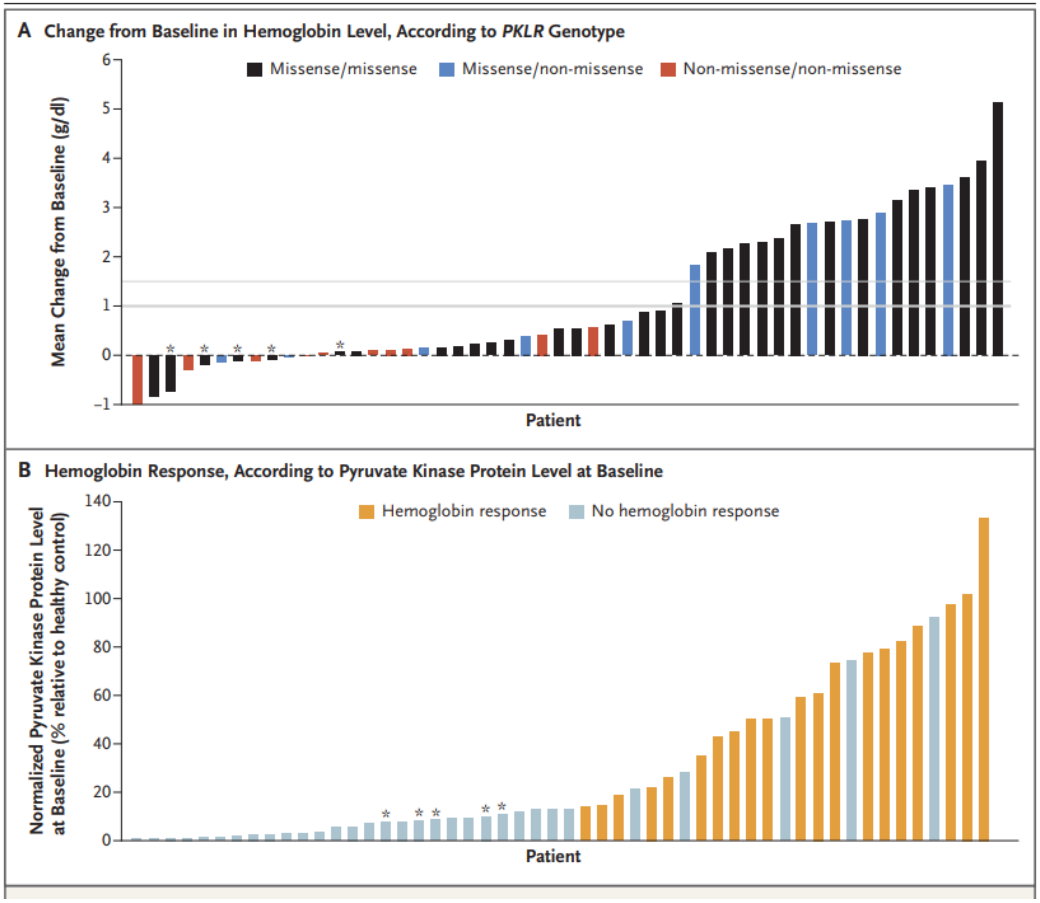
Grace et al, 2018; Bianchi et al, AJH 2020

Thursdays Webinars

Genotype-phenotype correlation and new drugs

Safety and Efficacy of Mitapivat in Pyruvate Kinase Deficiency



Grace et al, NEJM 2019

Genotype-phenotype correlation in patients underwent BMT

	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. [1]Directly involved in the fructose 1,6 bisphosphate activator. [2]Directly involved in the substrate and cation binding sites. [3]Residues directly involved in the allosteric site and catalytic center. [4]Highly unstable. [5]Proximity of the substrate-binding site.

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Van Straaten, et al, Haematol, 2018 Bianchi et al, Haematol 2020

Thursdays Webinars

External Quality Assessment scheme for PK activity assay

New PK scheme proposal: UK NEQAS

- European collaboration: essential because of small numbers of laboratories in each country
- Performance assessment for quantitative assay
- Could develop to include molecular methods
- Development phases:
  - Survey material development
    - Storage, stability, volumes etc.
  - Recruitment of interested participants
  - Small scale survey with selected labs
  - Pilot exercise(s) to refine scheme design
  - Development of performance assessment methods

UK NEQAS
International Quality Expertise

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PKDiagnosis European Working Group

PKD DIAGNOSIS EUROPEAN WORKING GROUP

"Towards harmonization of Diagnosis of PK deficiency in EU"

Paris, April 18th, 2019

Thursdays Webinars

Take home messages

1. PKD diagnosis: to monitor complicances; to have access to new therapies
2. 2. Biochemical testing and molecular characterizaton are complementary approaches, not alternative
3. Always consider differential diagnosis
4. Careful costs/benefits evaluation before chosing the diagnostic approach

Biochemical assay:

😐 € / 2 hours

☹ Other laboratory tests to exclude other conditions

NGS:

☹ €€-€€€ / 15-30 days

😐 Diagnosis of wide spectrum of defects