FONDAZIONE IRCCS
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Sistema Sanitario Regione Lombardia

Investigating the Haemolytic Patient

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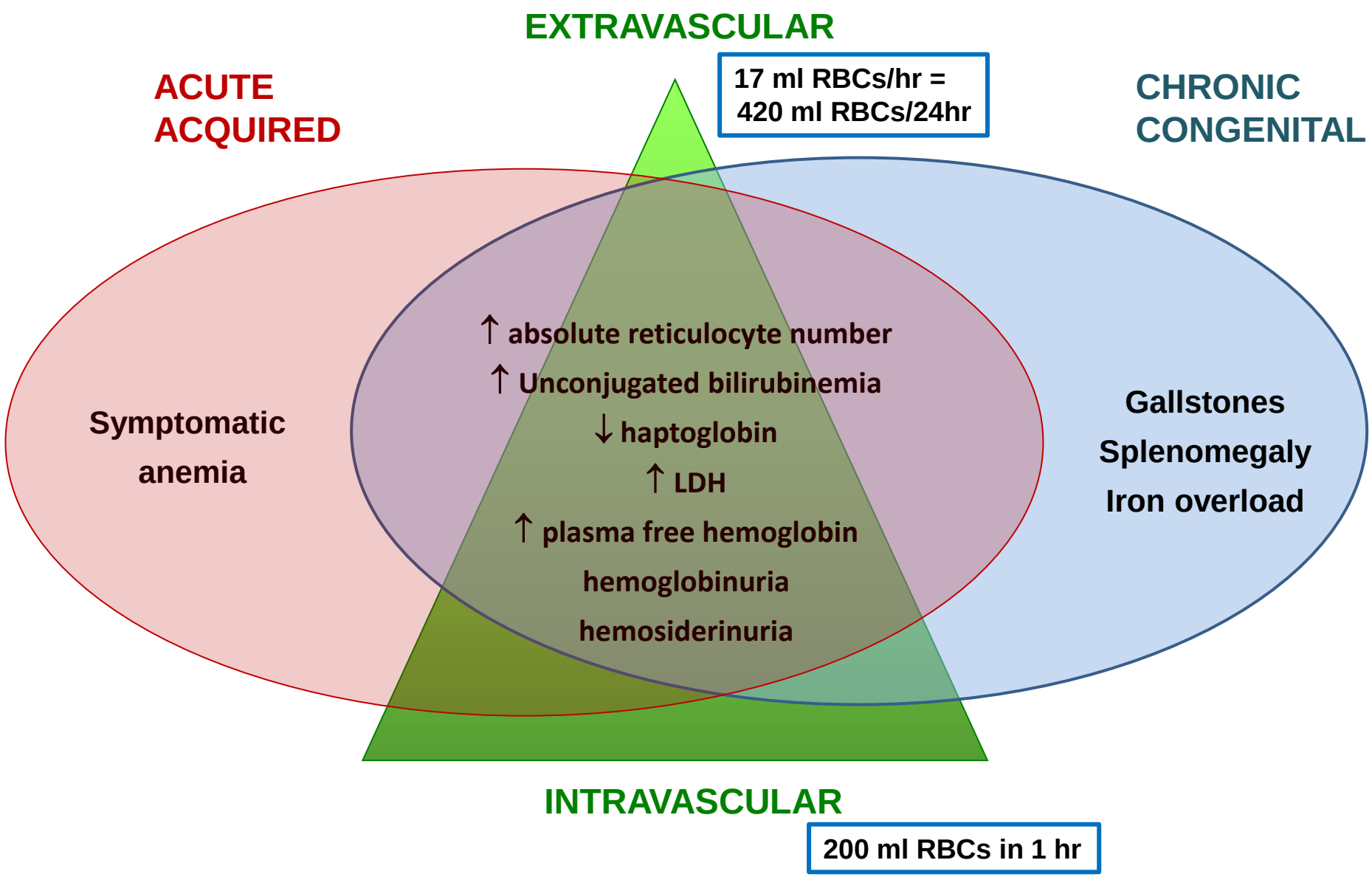
Fondazione IRCCS Ca' Granda Ospedale Maggiore Milano

UK NEQAS Haematology 22nd Annual Participants' Meeting
October 9th, 2019

Diagnostic aspects of:

- ✓ Red cell membrane defects
  - hereditary spherocytosis
  - defects of permeability and cell volume regulation
- ✓ Defects of red cell metabolism
  - pyruvate kinase deficiency
- ✓ Targeted Next Generation Sequencing panels

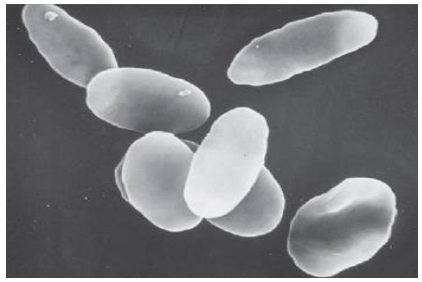
Haemolysis: RBC destruction



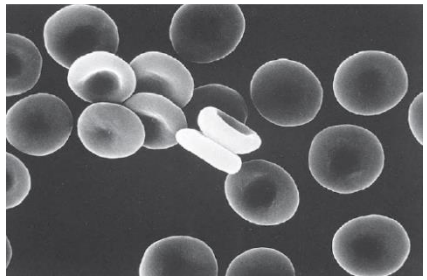
Major causes of congenital red cell disorders

- Disorders of hemoglobin / unstable hemoglobins *e.g.* HbS

- Defective structure and/or function/permeability of the red cell membrane, *e.g.* hereditary elliptocytosis



- Disorders of red blood cell metabolism, *e.g.* pyruvate kinase deficiency

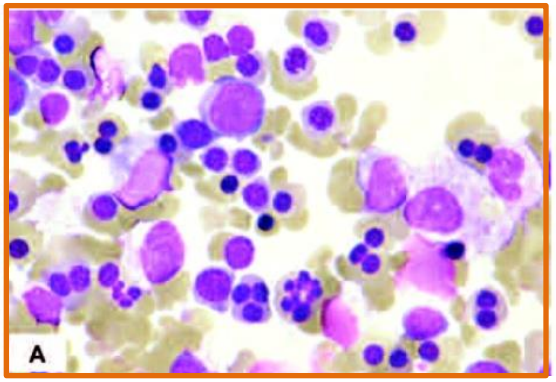


Rare /very rare diseases

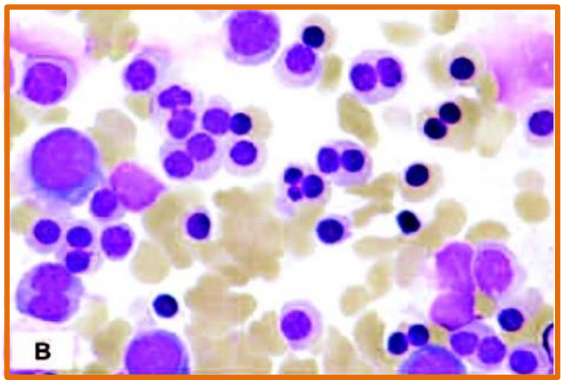
Congenital dyserythropoietic anemia (CDA)

Heterogeneous group of hemolytic anemias characterized by ineffective erythropoiesis and by distinct morphological abnormalities of erythroblasts in the bone marrow.

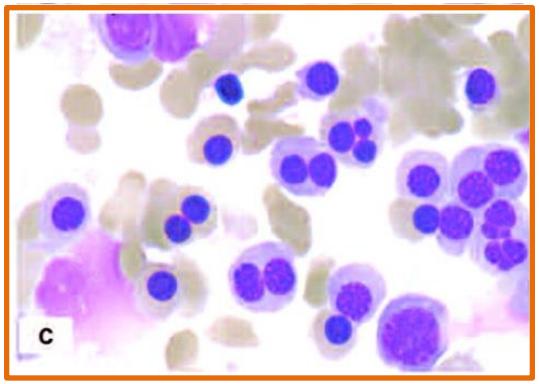
CDA type I



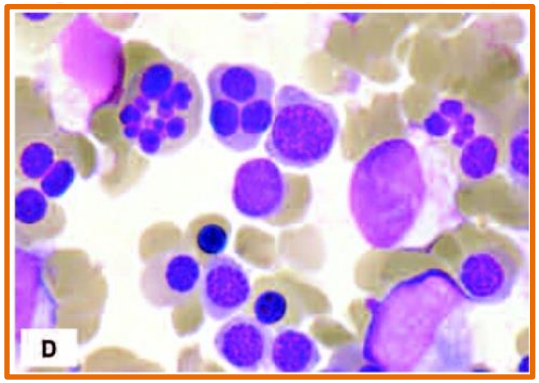
CDA Type II



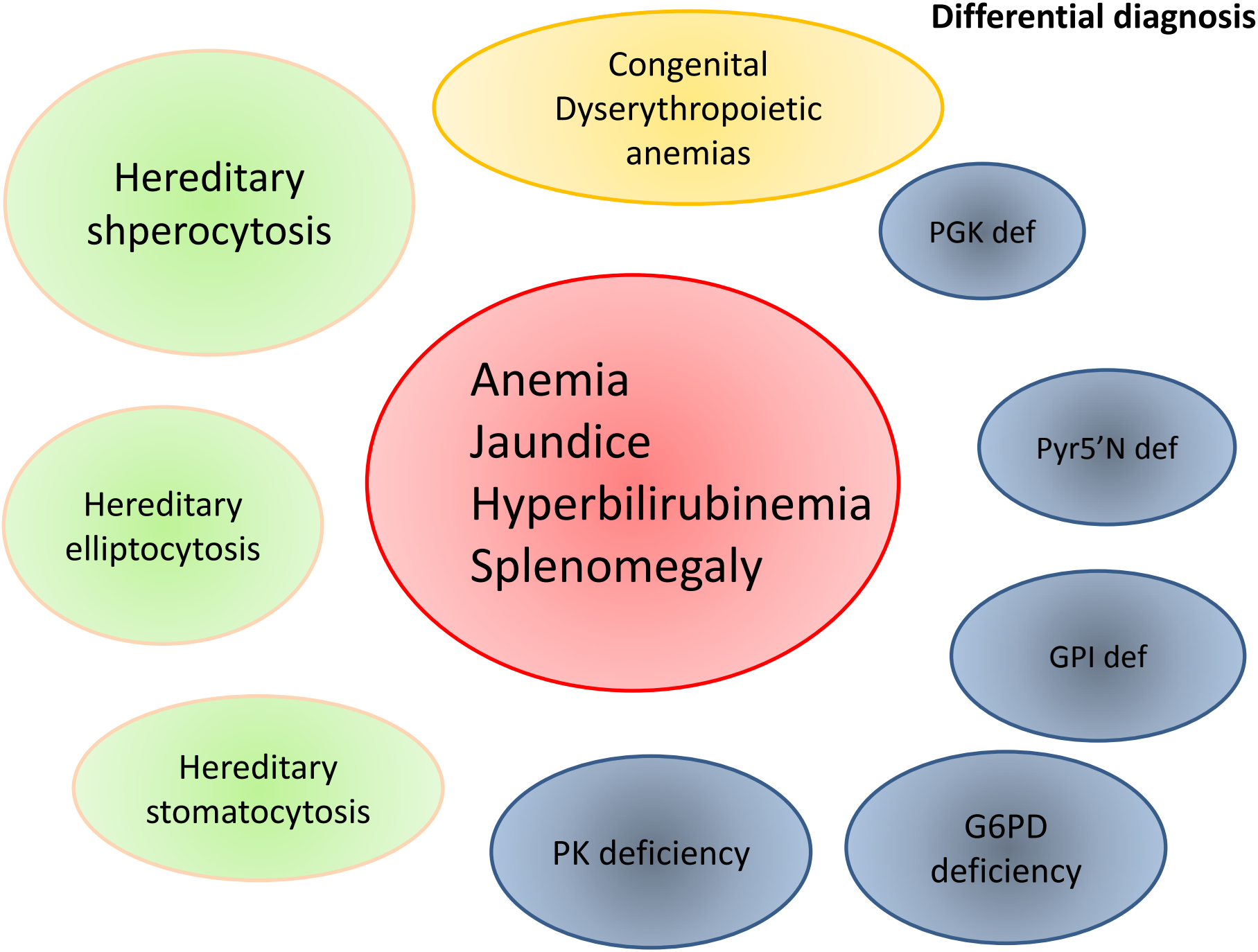
CDA type III

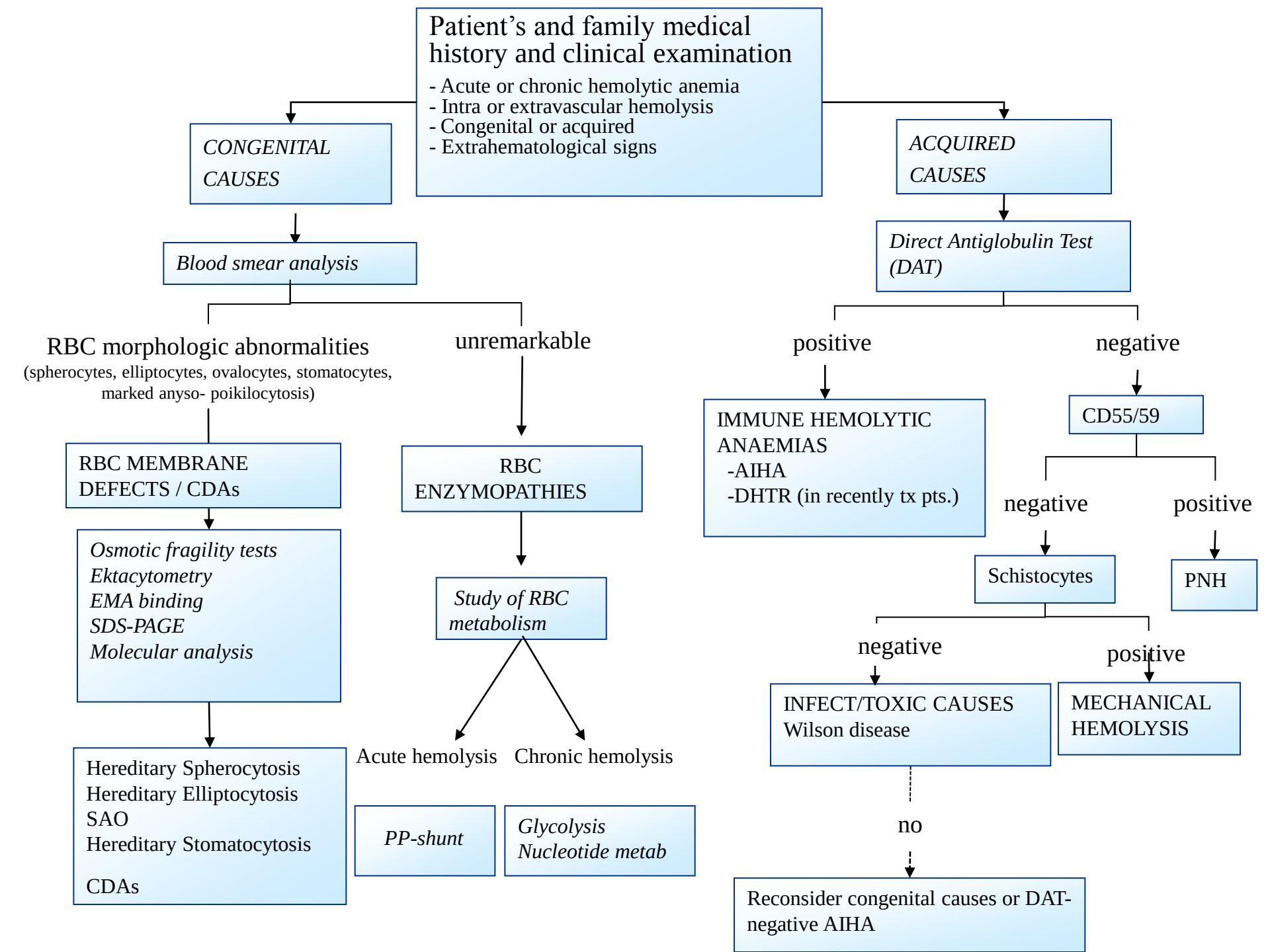


CDA Type IV



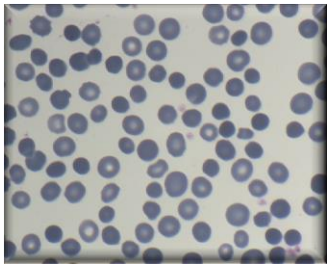
Differential diagnosis



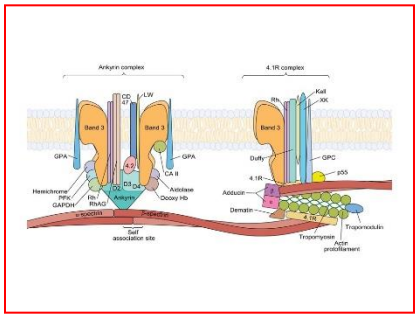

Patient's and family medical history and clinical examination
- Acute or chronic hemolytic anemia
- Intra or extravascular hemolysis
- Congenital or acquired
- Extrahematological signs
CONGENITAL CAUSES
ACQUIRED CAUSES
Blood smear analysis
Direct Antiglobulin Test (DAT)
RBC morphologic abnormalities
(spherocytes, elliptocytes, ovalocytes, stomatocytes, marked anyso- poikilocytosis)
unremarkable
positive
negative
RBC MEMBRANE DEFECTS / CDAs
RBC ENZYMOPATHIES
IMMUNE HEMOLYTIC ANAEMIAS
-AIHA
-DHTR (in recently tx pts.)
CD55/59
negative
positive
Osmotic fragility tests
Ektacytometry
EMA binding
SDS-PAGE
Molecular analysis
Study of RBC metabolism
Schistocytes
PNH
negative
positive
Hereditary Spherocytosis
Hereditary Elliptocytosis
SAO
Hereditary Stomatocytosis
CDAs
Acute hemolysis
Chronic hemolysis
INFECT/TOXIC CAUSES
Wilson disease
MECHANICAL HEMOLYSIS
PP-shunt
Glycolysis
Nucleotide metab
no
Reconsider congenital causes or DAT-negative AIHA

CBC
Markers of hemolysis
Coomb's test
HPLC Hb

Clinical and patient's family history

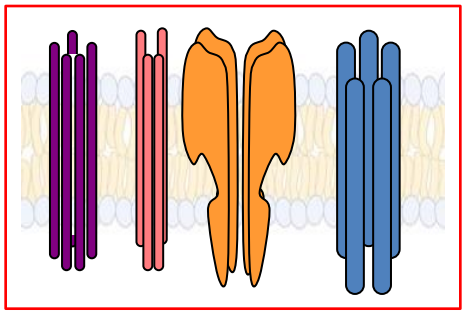


RBC cytoskeleton



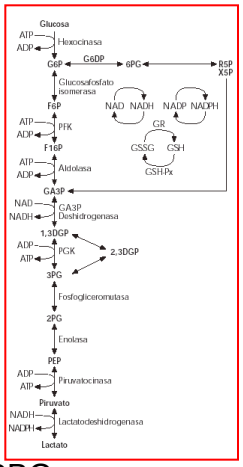
Osmotic fragility tests
EMA-binding test
SDS-PAGE
Molecular testing

RBC hydrations defects



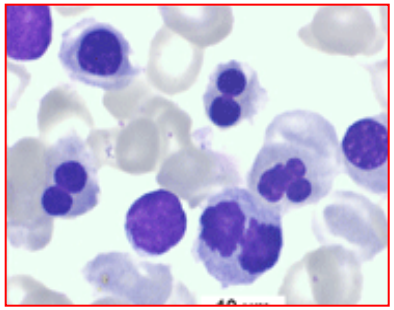
Molecular testing

RBC metabolism



RBC enzyme assays
Molecular testing

BM failure - CDAs



BM evaluation
EMA-binding test
SDS-PAGE Hypogl. Band3
Molecular testing

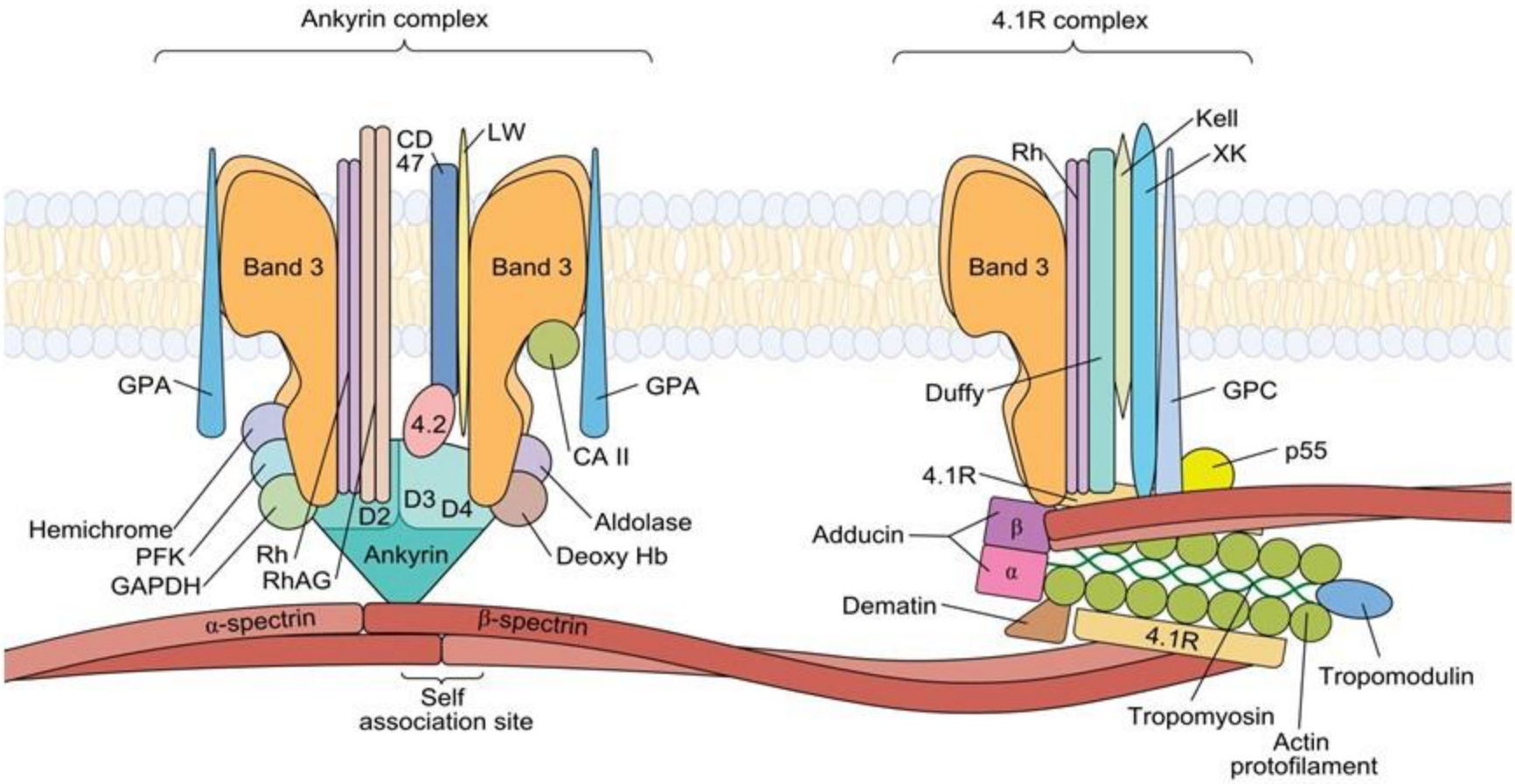
NGS

Diagnostic aspects of:

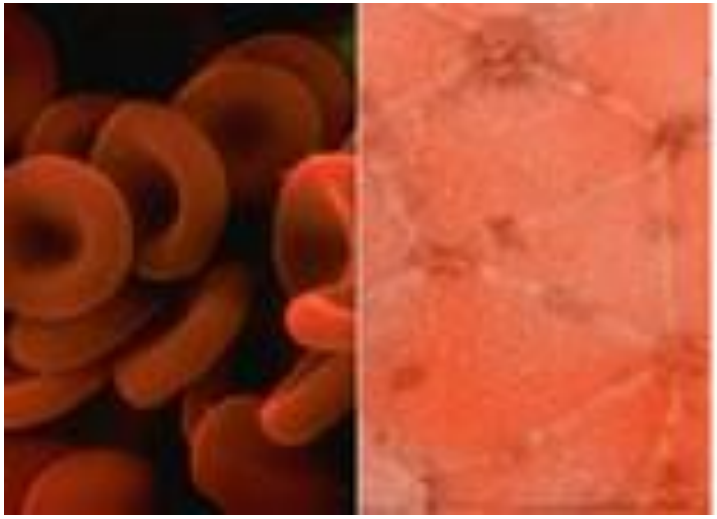
- ✓ Red cell membrane defects
  - hereditary spherocytosis
  - defects of permeability and cell volume regulation

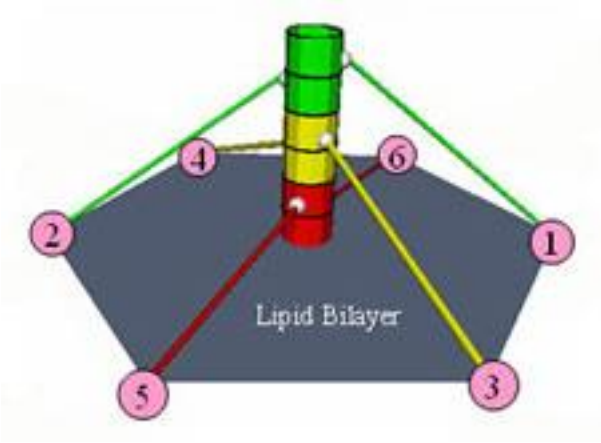
- ✓ Defects of red cell metabolism
  - pyruvate kinase deficiency
  - Targeted Next Generation Sequencing panels

Red cell membrane disorders



From: Bianchi P, M Narla, Post Graduate Hematology 7 eds. , 2015

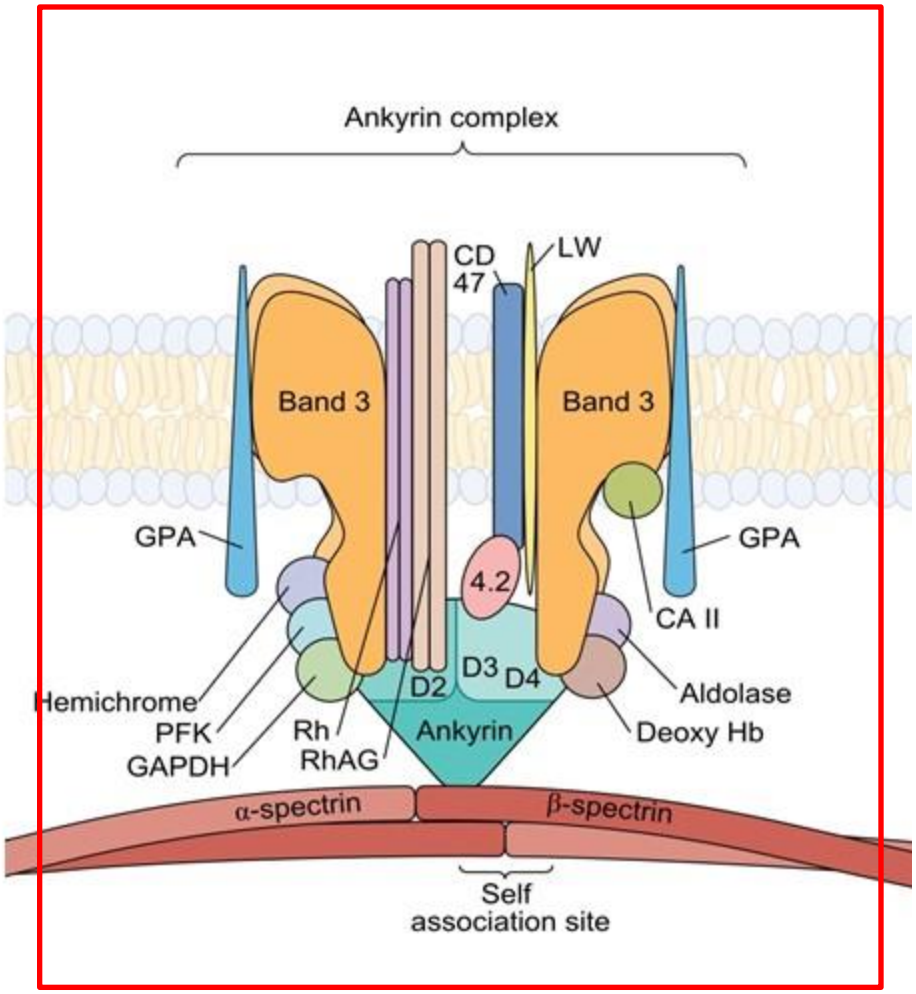




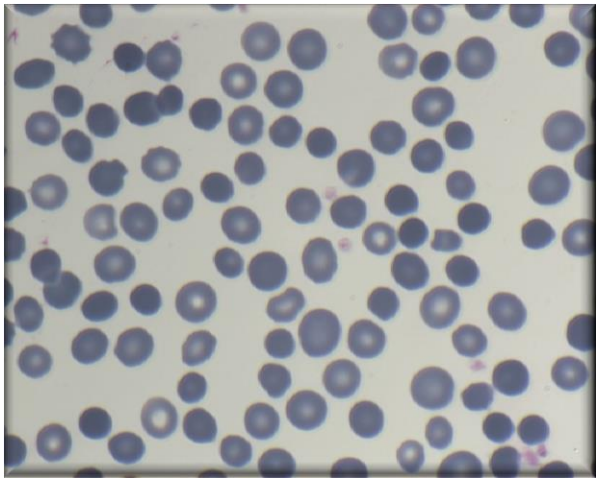
The six spectrin fibers attach at precise positions on the proto-filament. The more a red blood cell is mechanically deformed, the more likely individual proto-filaments will rotate like baseball bats swung over home plate, which in this case is the lipid layer of a cell membrane.

“Scientists Discover Secret Behind Human Red Blood Cell's Amazing Flexibility” . By Rex Graham, Jacobs School of Engineering , October 2005

Hereditary spherocytosis



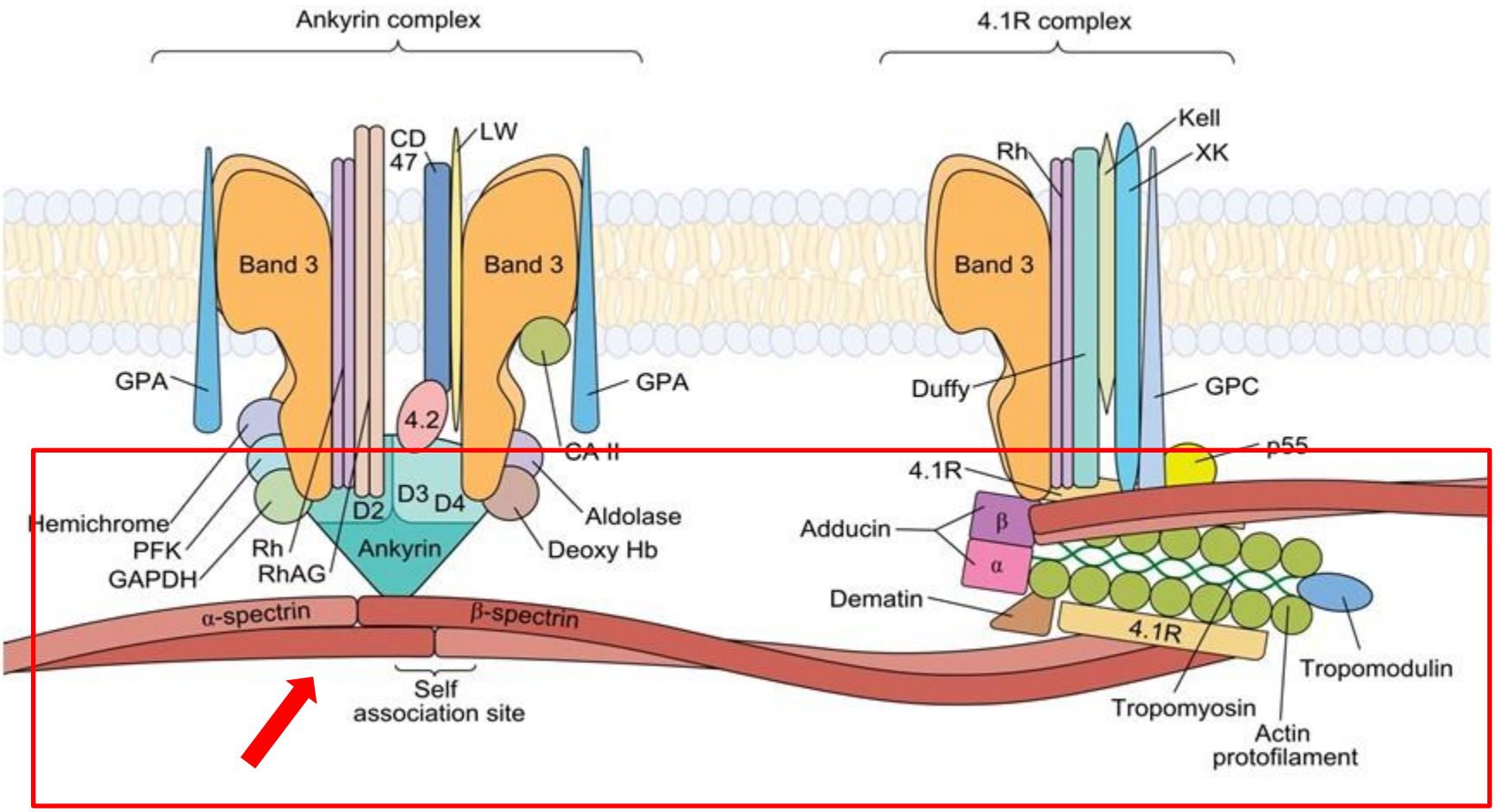
Hereditary spherocytosis



Prevalence 1:2000

Definition: **Hereditary spherocytosis (HS)** is a genetically determined chronic haemolytic anaemia characterized by the spherical shape of the affected red cells.

Hereditary elliptocytosis



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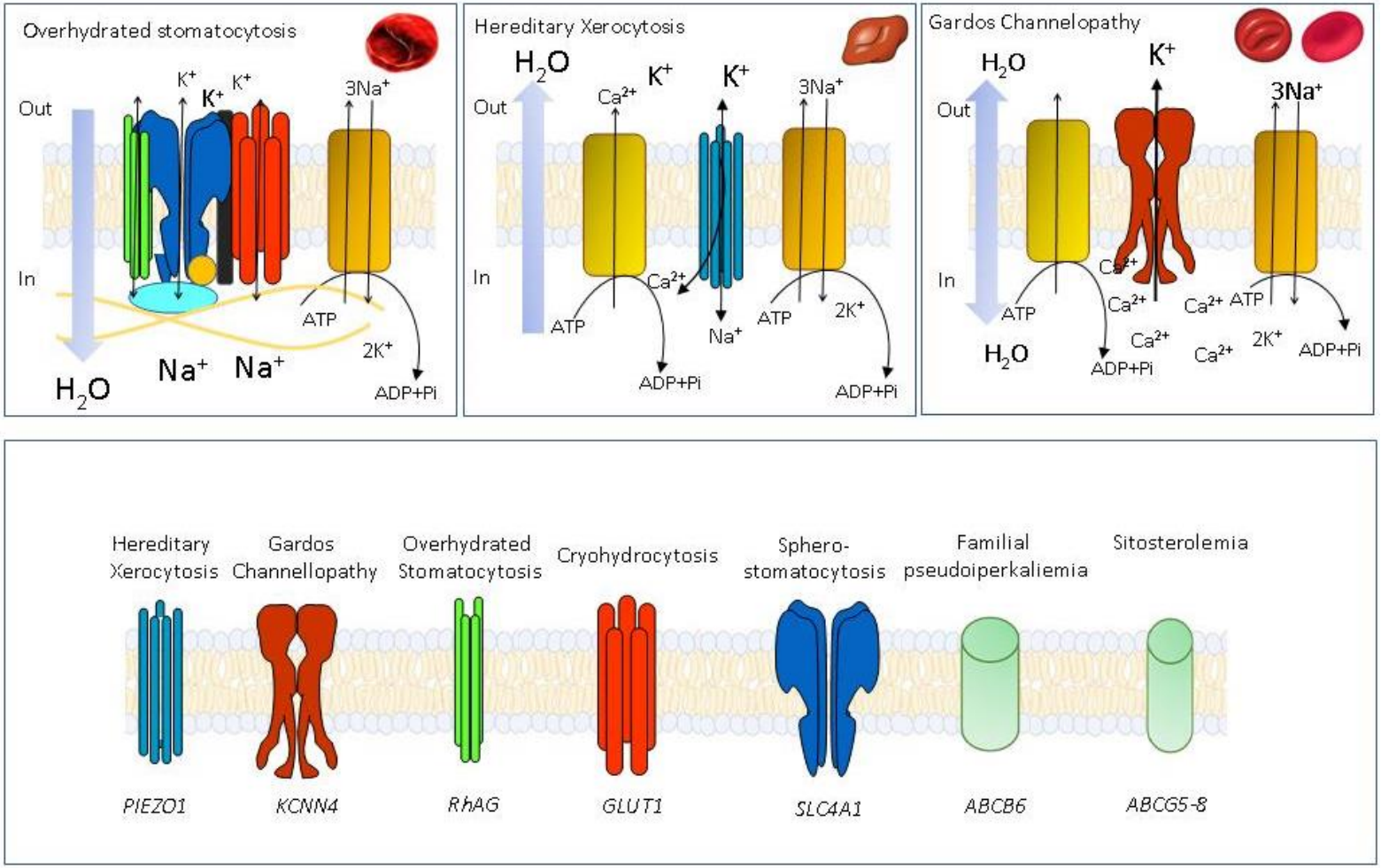
From: Bianchi P, M Narla, Post Graduate Hematology 7 eds. , 2015

RBC hydration defects



Modified from Badens &Guizouran, 2016
Fermo et al, 2018

Protein	Gene	Position	Function	Phenotype
α-spectrin	*SPTA1*	1q23.1	Membrane skeletal network	HS HE HPP
β-spectrin	*SPTB*	14q23,3	Membrane skeletal network	HS HE
Ankyrin	*ANK1*	8p11.21	Vertical interactions	HS
Protein Band 3	*SLC4A1*	17q21.31	•Anion exchange channel •Link to glycoltytic enzymes •Veritcal interactions	HS SAO HSt
Protein 4.2	*EPB42*	15q15.2	Stabilize band3/ankyrin complex	HS
Protein 4.1	*EPB41*	1p35.3	Stabilize spectrin-ankyrin contact	HE
Glycophorin C	*GYPC*	2q14.3	Gerbich - blood group	HE
FAM38A	*PIEZO1*	16q24.3	Mechanosensitive ion channel	HX Polycythemia
Gardos channel KCa3.1	*KCNN4*	19q13.31	Potassium Calcium-Activated Channel	HSt
Rh associated Glycoprotein	*RHAG*	6p12.3	Rh -blood group	OHSt
GLUT1	*SLC2A1*	1p34.2	Glucose transporter	CHC
ABC transporter Superfamily	*ABCB6*	2q35	Porphyrin transporter	Fam. PHYK

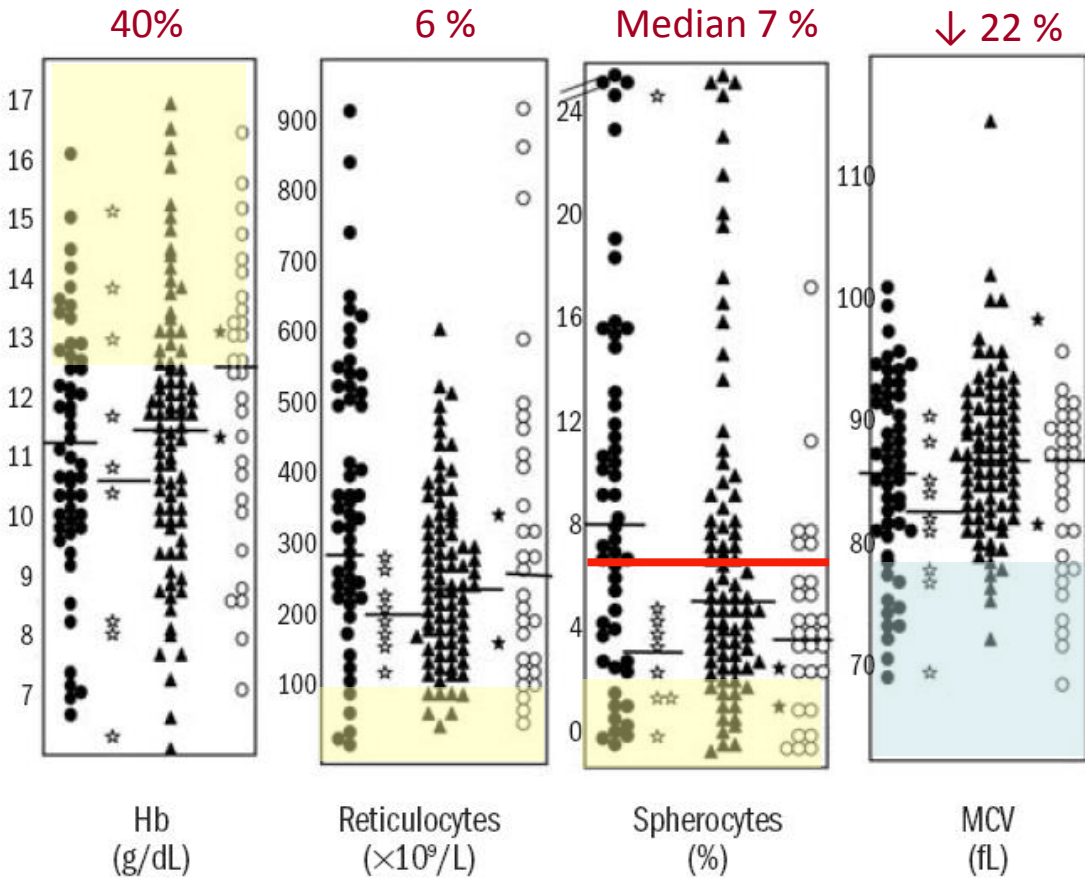
HS: I level laboratory investigations

ICSH guidelines for the laboratory diagnosis of nonimmune hereditary red cell membrane disorders. King et al Int J Lab Hematol. 2015

The laboratory diagnosis of HS is based upon a combination of clinical history, family history, physical examination and laboratory data

Clinical features:	Splenomegaly, jaundice
Laboratory cell indices:	↓ Hb, ↓MCV, ↑MCHC, ↑RDW
Direct antiglobulin test:	Negative
Evidence of hemolysis:	↑ Unconjugated bilirubin, ↑ Absolute reticulocytes number Consumed atoglobulin
Blood film:	Anisopoichylocytosis, spherocytes

HS: Haematological parameters



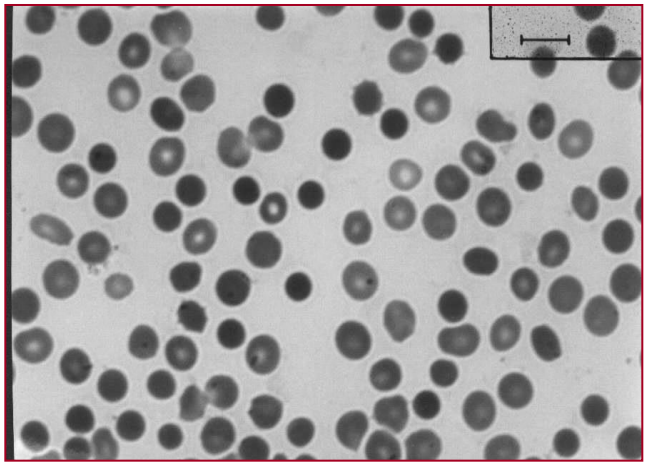
Not always standard hematologic parameters give specific diagnostic indications!

Mariani et al 2008

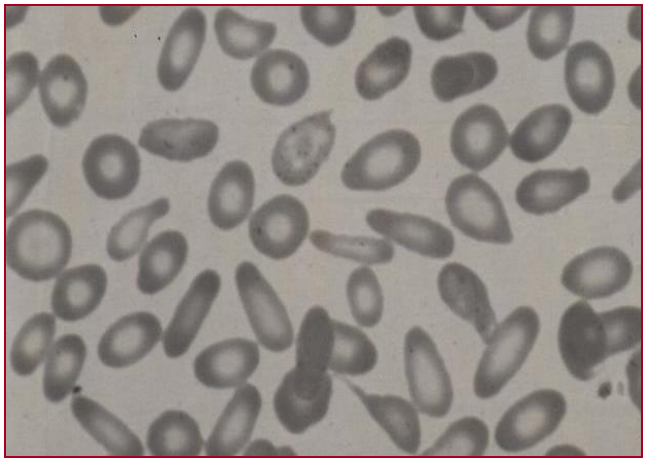
Automated red cell parameters in the prediction of HS

Danise et al 2001	- RDW/HDW ratio significantly greater in CDA II than HS - CHDW/CHDWr ratio significantly lower in CDA II than HS *RDW= anisocytosis; HDW= anisochromia; CHDWr= cell Hb content of reticulocytes*	p<0.0002 p<0.0002
M. Chiron, et al 1999	HS samples MSCV < MCV *Mean Spherized Corpuscular Volume, assessed during the retics count procedure under hypoosmotic conditions)*	Sensitivity 100% Specificity 93.3%
Brosèus, et al 2010	Delta MCV-MSCV >9.6fL Beckman coulter cell analyzer	
Da Costa et al 2001	Reticulocyte volume <100fL HS (except for neonates) Advia H*3 Bayer	
Mullier F. et al 2011	Hs screening index [%MicroR%/HypoHe]: < 4 *%MicroR: (% of particularly small erythrocytes <60 fL) %Hypo-He: (% of erythrocytes with particularly low Hb)* Spherocytosis quotient [Reticulocytes/IRF (Immature Reticulocyte Fraction)] Sysmex analyzer	Sensitivity 94.4% Specificity 94.3%
Persijn L et al 2012	Modification of Mullier algorithm Sysmex analyzer	Sensitivity 100%
Lazarova , et al 2014	mean reticulocyte volume (MRV) immature reticulocyte fraction (IRF) Delta MCV-MSCV Beckman Coulter cell analyser	Sensitivity 100% Specificity 88%
Bobée V et al, 2018	Hb, retics, IRF, MicroR, and %HypoHe (47 HS, 17 PKD) Sysmex XE-500 analyzer	Sensitivity 100% Specificity 92.1%

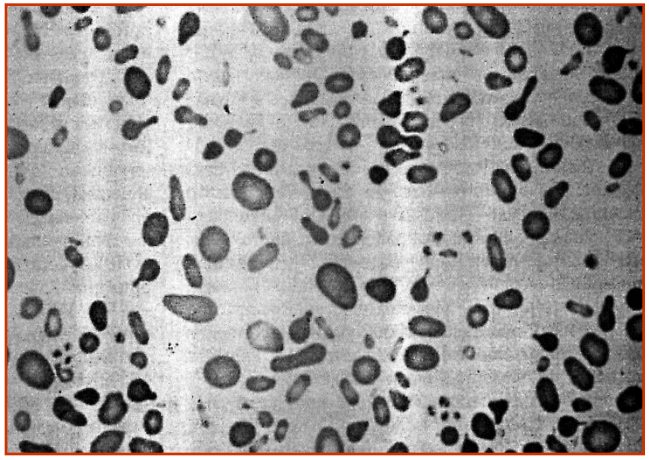
Red cell morphology



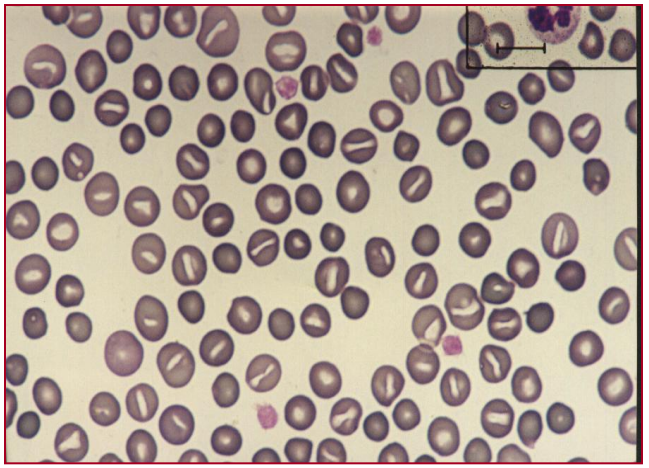
Hereditary spherocytosis (HS)
1:2000 Dom.Tr (75% of cases)



Hereditary elliptocytosis (HE)
1:4000 Dom. Tr



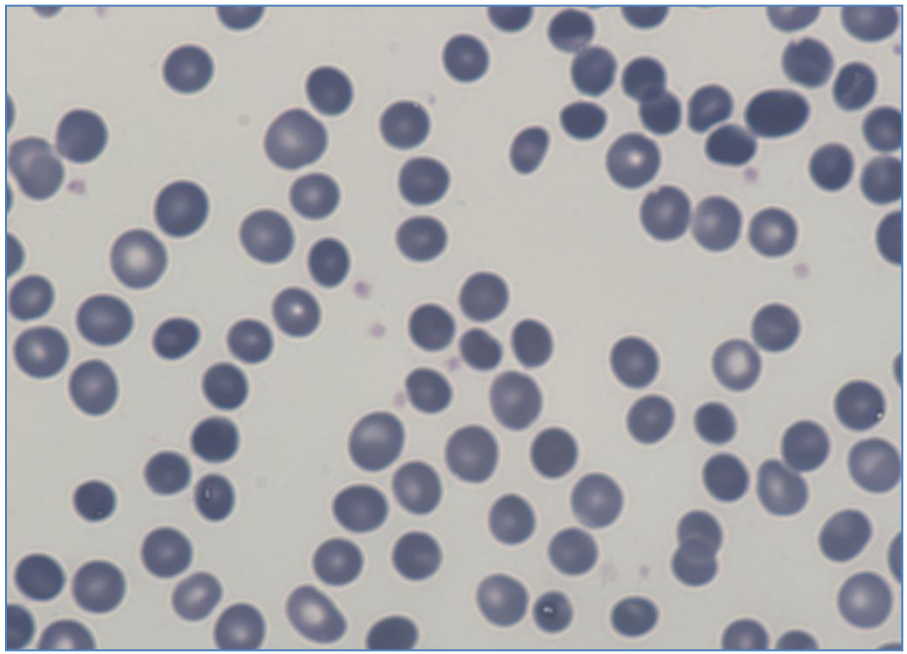
Hered. Pyropoikilocytosis (HPP)
Non-Dom. Tr



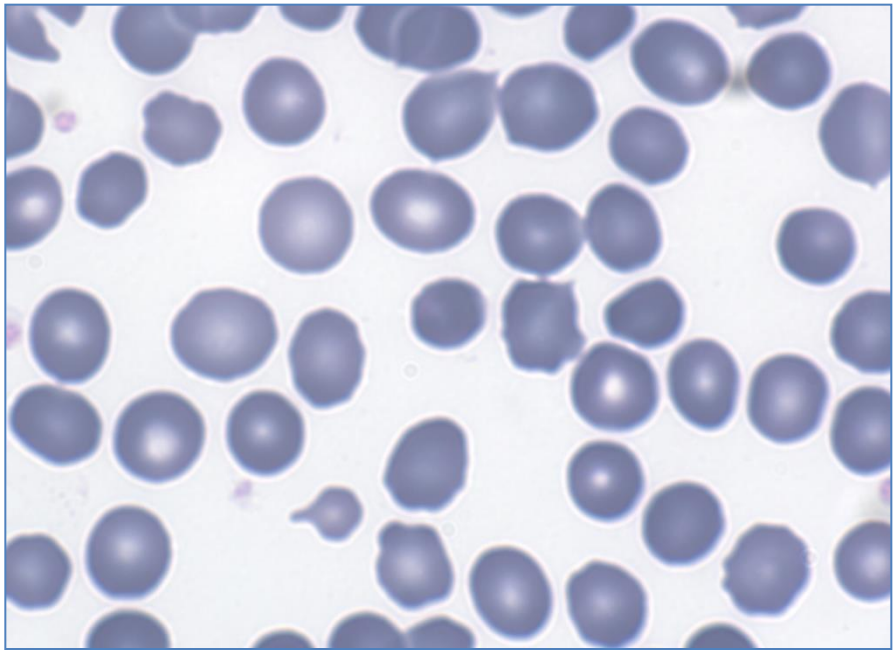
Hereditary stomatocytosis (HSt)
1:50000 – 1:100000 Dom. Tr

RED CELL MORPHOLOGY
Differential diagnosis

Autoimmune hemolytic anemia



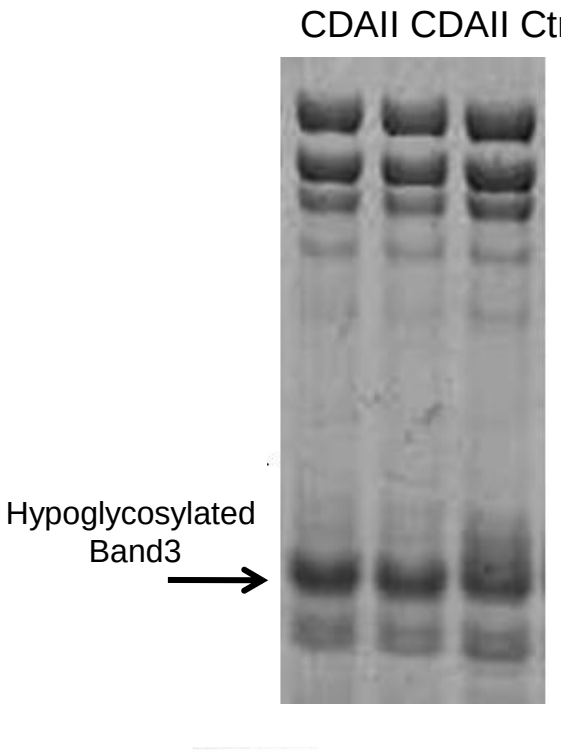
Congenital dyserythropoietic anemia type II



HS: Specific tests

Osmotic fragility (OF) test (Parpart et al, 1947)
Acidified glycerol lysis test (AGLT) (Zanella et al, 1980) **The Pink test** (Vettore & Zanella, 1984)
Hypertonic cryohaemolysis test (Streichman & Gescheidt, **1998**)
Eosin-5-maleimide (EMA) binding (King et al, 2000)

SDS-PAGE of red cell membrne proteins



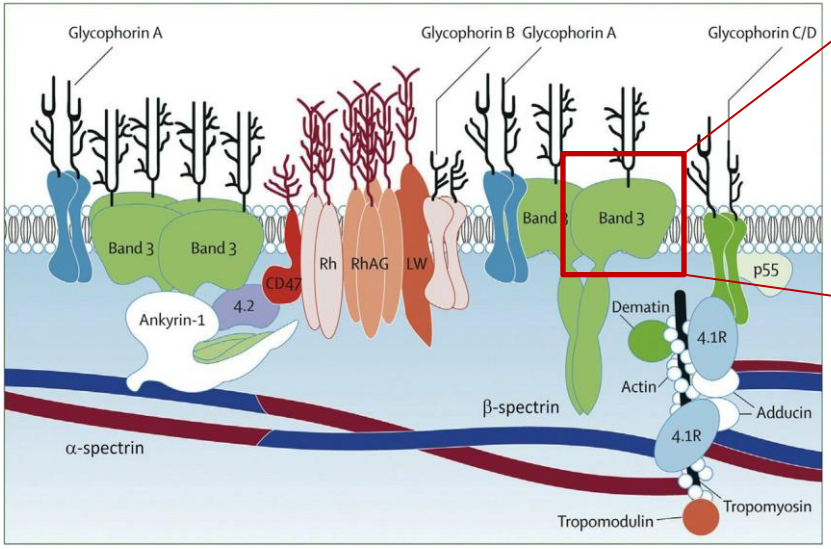
EMA BINDING TEST

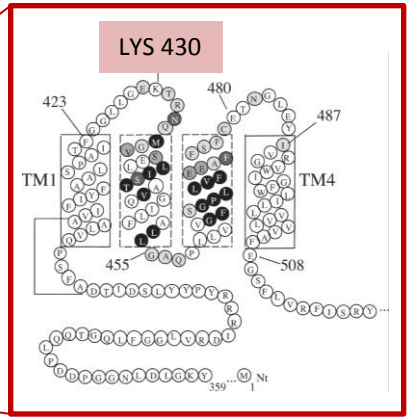
British Journal of Haematology, 2000, **111**, 924–933

Rapid flow cytometric test for the diagnosis of membrane cytoskeleton-associated haemolytic anaemia

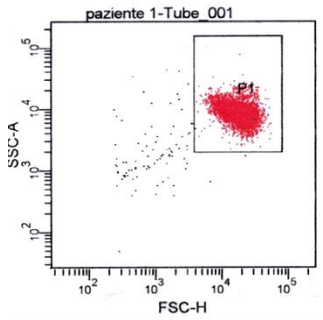
MAY-JEAN KING,[1] JUDITH BEHRENS,[2] CHRIS ROGERS,[3] CLARE FLYNN,[4] DAVID GREENWOOD[5] AND KEITH CHAMBERS[6]
[1]*International Blood Group Reference Laboratory, Bristol,* [2]*Department of Haematology, St. Helier Hospital, Carshalton,*
[3]*Research and Development Support Unit, Southmead Hospital, Bristol,* [4]*Department of Haematology,*
St. Mary's Hospital, London, [5]*Department of Haematology, Southmead Hospital, Bristol, and*
[6]*Department of Haematology, Leicester Royal Infirmary, Leicester, UK*

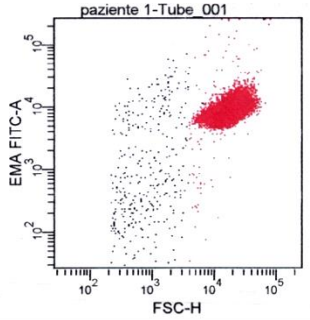
Received 12 June 2000; accepted for publication 13 July 2000

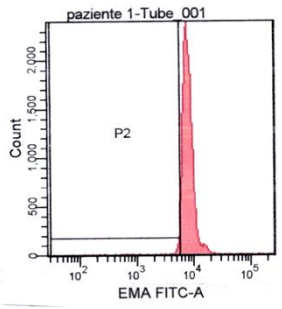




Sensitivity = 92,7%
Specificity = 99,1%







Sensitivity of diagnostic tests according to clinical phenotype

Articles and Brief Reports

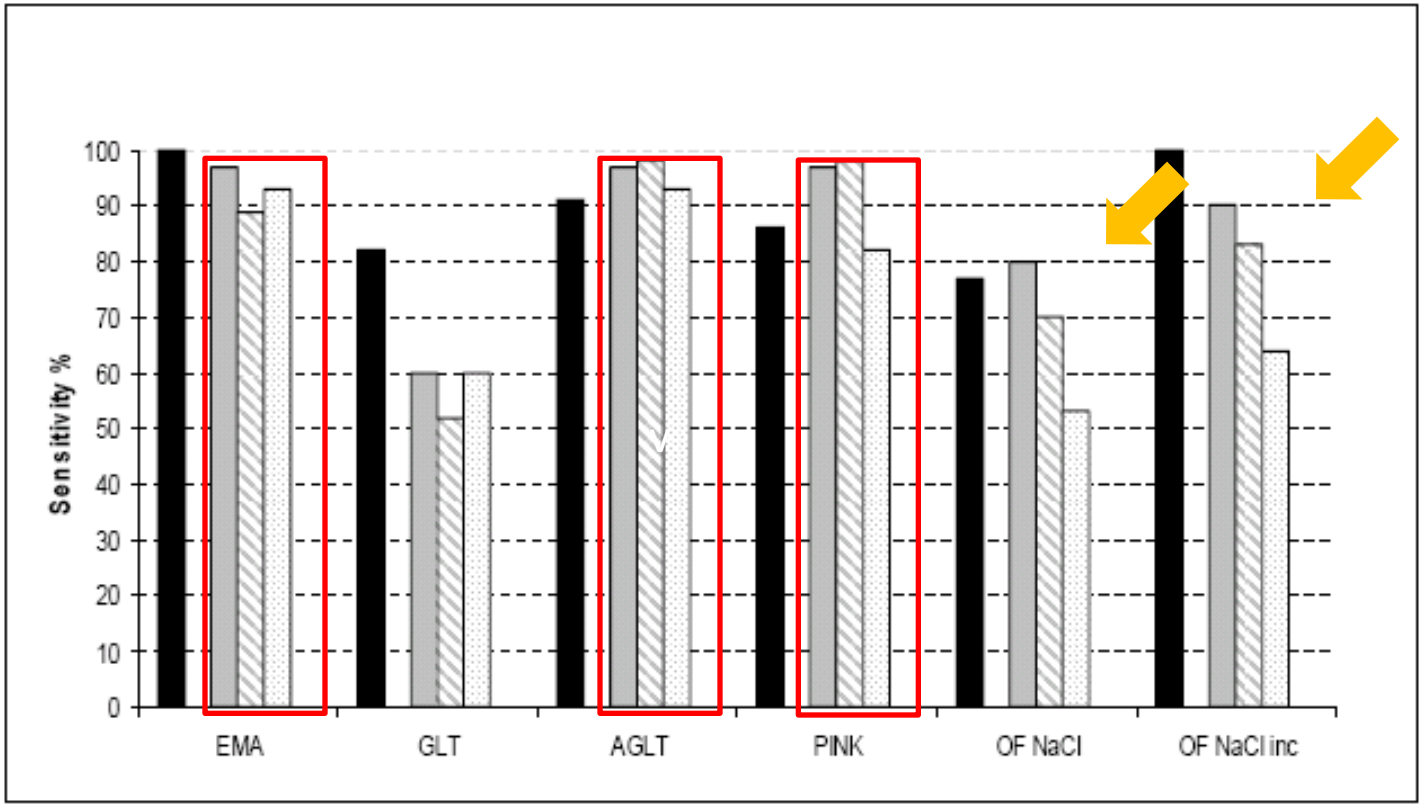
Red Cell Disorders

Diagnostic power of laboratory tests for hereditary spherocytosis: a comparison study in 150 patients grouped according to molecular and clinical characteristics

Paola Bianchi,[1] Elisa Fermo,[1] Cristina Vercellati,[1] Anna P. Marcello,[1] Laura Porretti,[2] Agostino Cortelezzi,[1,3] Wilma Barcellini,[1] and Alberto Zanella[1]

[1]U.O. Ematologia 2, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy; [2]Centro di Medicina Trasfusionale, Terapia Cellulare e Criobiologia, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milano, Italy, and [3]U.O. Ematologia 1 e Centro Trapianti di Midollo, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico e Università degli Studi di Milano, Milan, Italy

Sensitivity = 93 %
Specificity = 98 %



Diagnostic power of laboratory tests for hereditary spherocytosis: a comparison study in 150 patients grouped according to molecular and clinical characteristics

Paola Bianchi,[1] Elisa Fermo,[1] Cristina Vercellati,[1] Anna P. Marcello,[1] Laura Porretti,[2] Agostino Cortelezzi,[1,3] Wilma Barcellini,[1] and Alberto Zanella[1]

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150 HS

87 altre anemie

Sensibilità per HS

EMA	93%
GLT	61%
AGLT	95%
Pink	91%
OF (NaCl fresh)	68%
OF (NaCl inc.)	81%

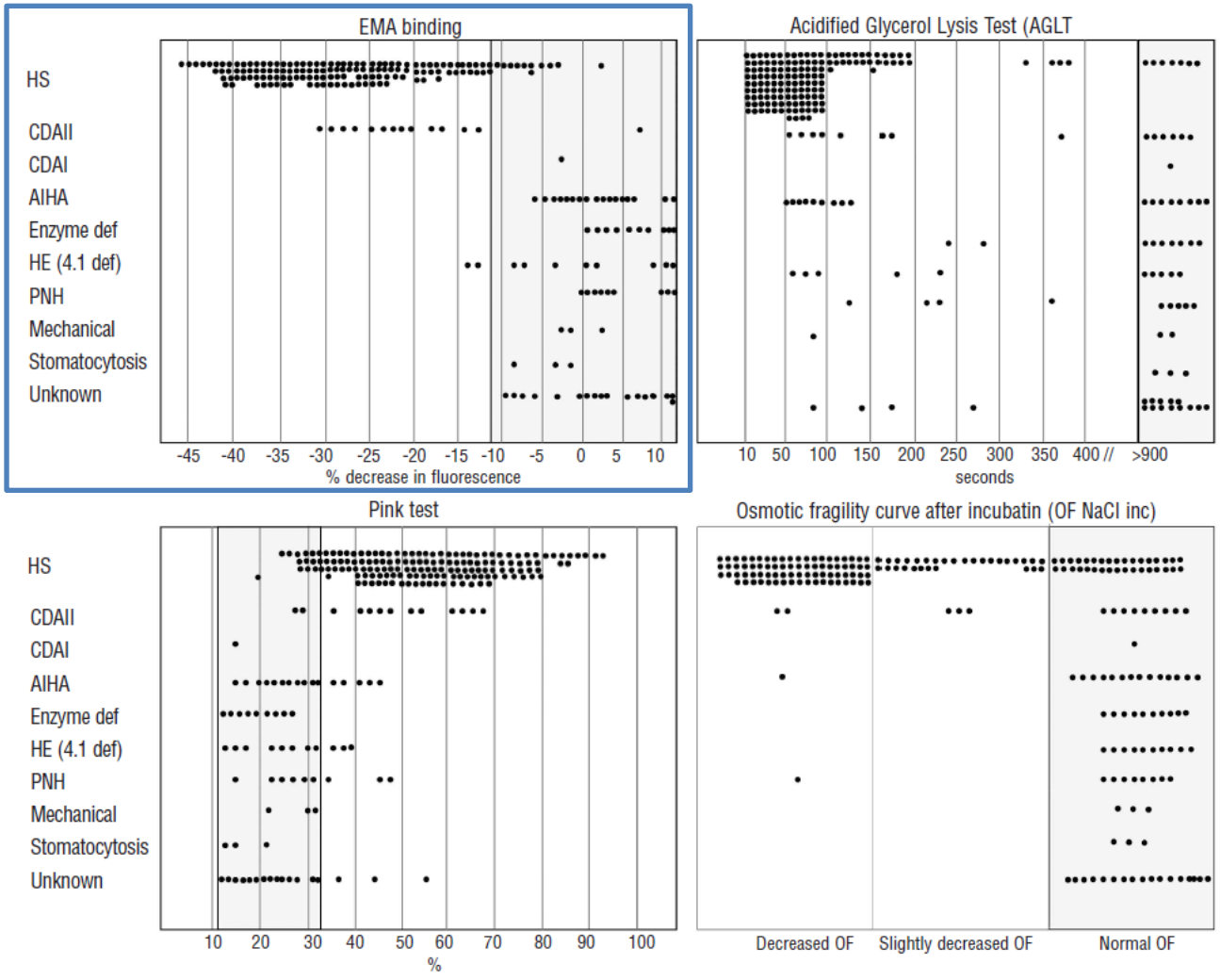
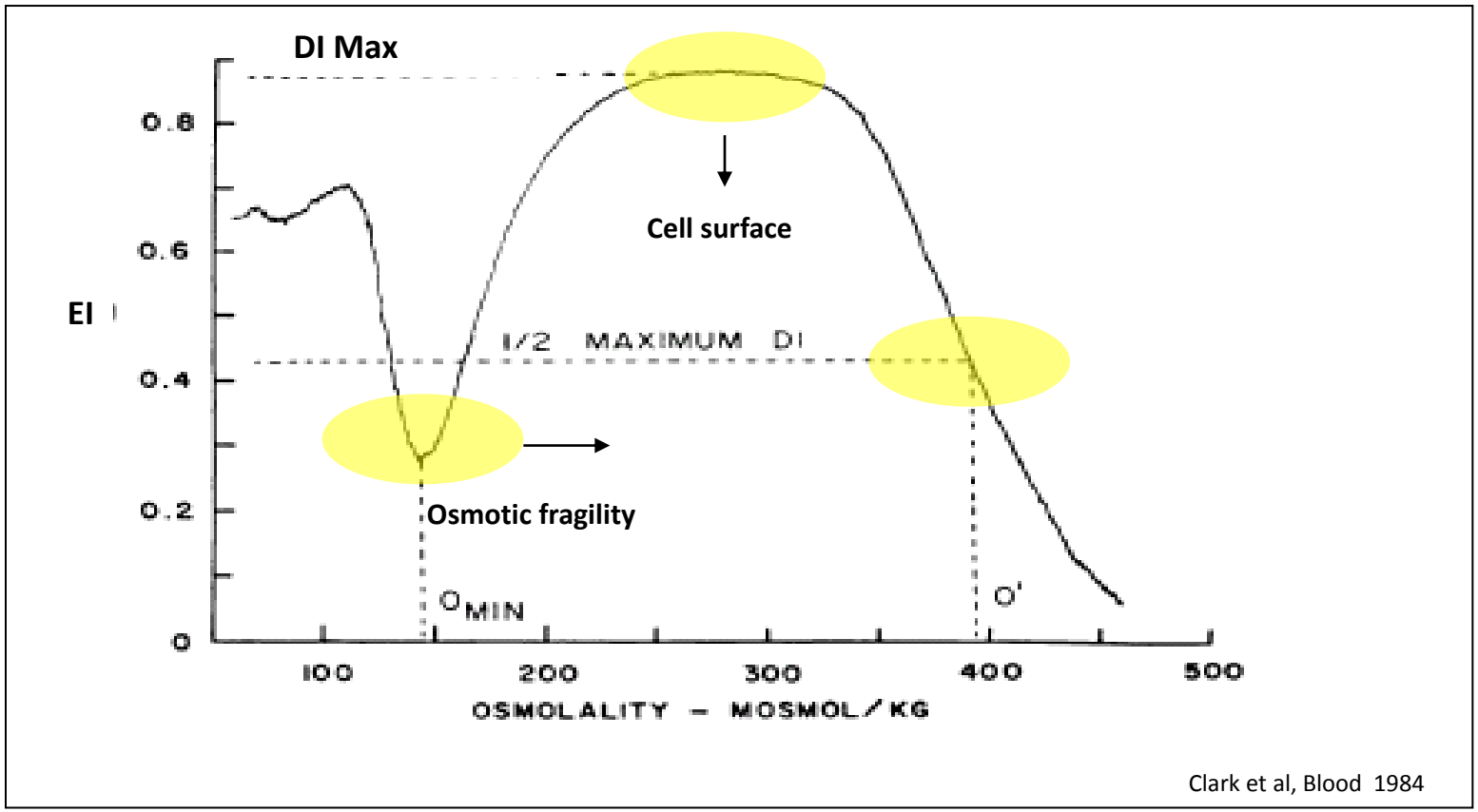


Figure 2. Results of individual diagnostic tests in patients with hemolytic anemias other than hereditary spherocytosis (HS), compared with those with HS. The shaded area represents normal reference intervals. CDA: congenital dyserythropoietic anemia; AIHA: autoimmune hemolytic anemia; HE: hereditary elliptocytosis; PNH: paroxysmal noctural hemoglobinuria

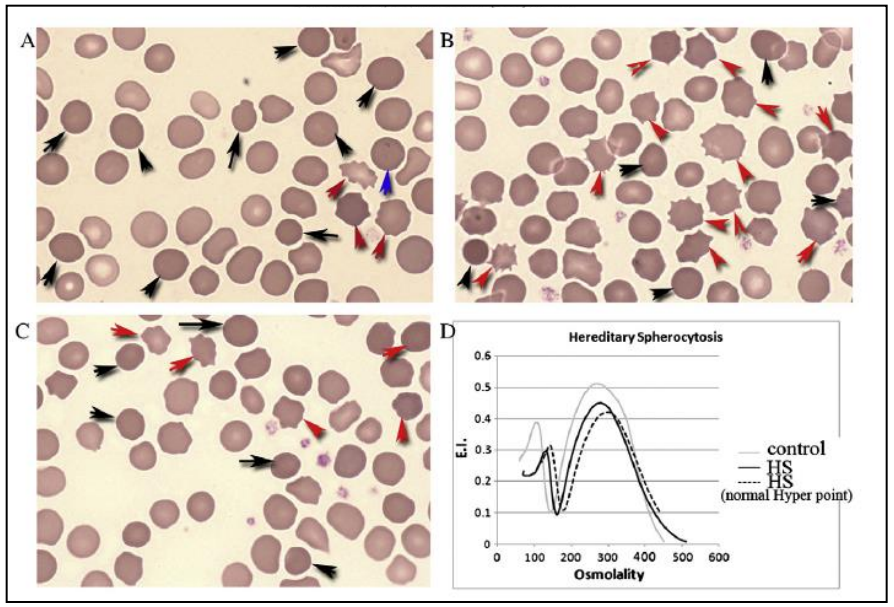
Ektacytometry - Laser-assisted Optical Rotational Cell Analyzer LoRRca MaxSis



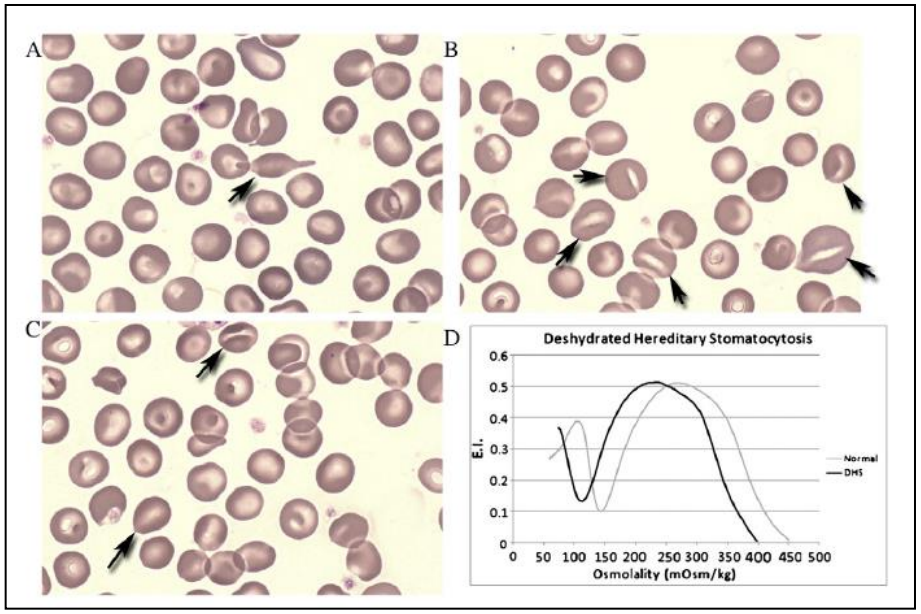
Clark et al, Blood 1984

High repeatability, riproducibility

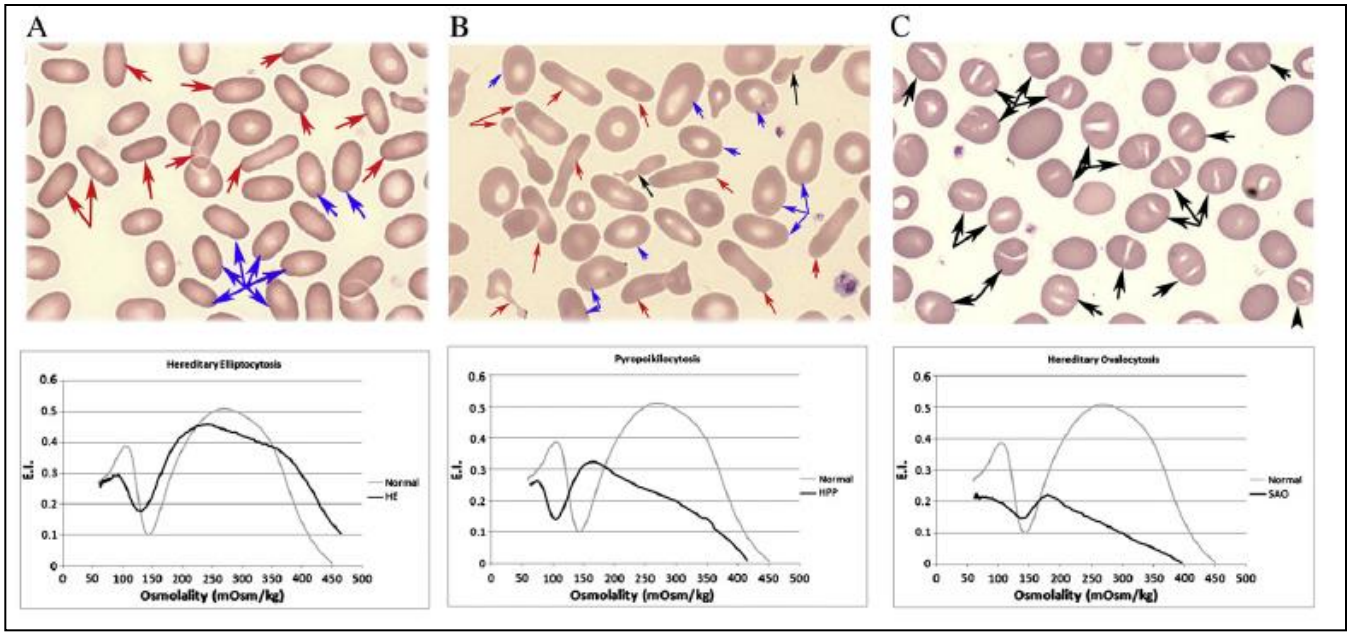
Hereditary Spherocytosis



Dehydrated Stomatocytosis



Hereditary Elliptocytosis

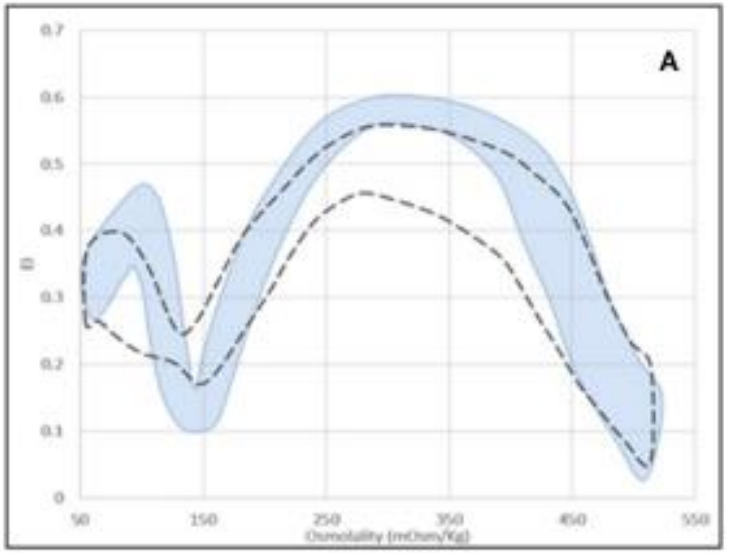


L. Da Costa, 2013

Use of Laser Assisted Optical Rotational Cell Analyzer (LoRRca MaxSis) in the Diagnosis of RBC Membrane Disorders, Enzyme Defects, and Congenital Dyserythropoietic Anemias:
A Monocentric Study on 202 Patients.

Zaninoni A, Fermo E, Vercellati C, Consonni D, Marcello AP, Zanella A, Cortelezzi A, Barcellini W, Bianchi P

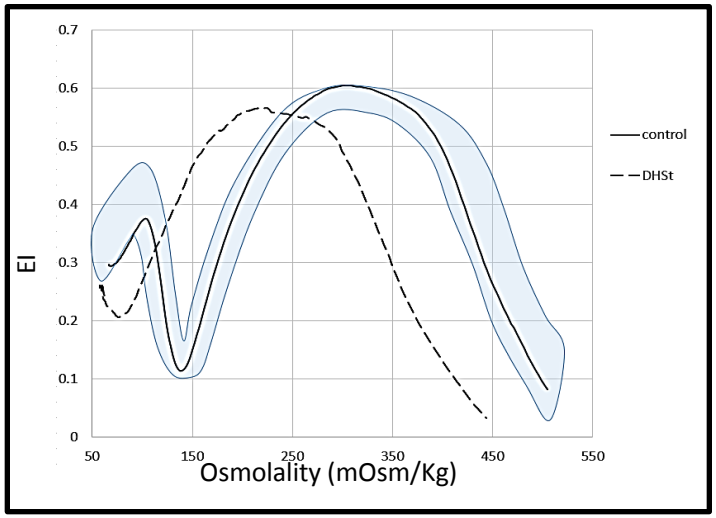
140 patients affected by RBC membrane disorders, 37 by enzymopathies, and 16 by CDAII
- All the HS regardless the biochemical defect, showed altered Osmoscan curves,
- Hereditary elliptocytosis (HE) displayed a trapezoidal curve and decreased EImax.
- Dehydrated hereditary stomatocytosis (DHSt) caused by *PIEZO1* mutations was characterized by left-shifted curve



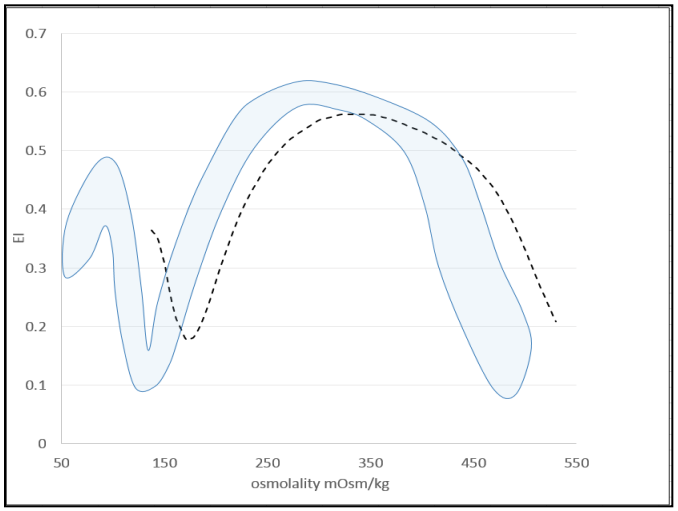
Effect of splenectomy of RBC of patients with hemolytic anemias

Diagnostic power of ektacytometry in the diagnosis of RBC hydrations defects

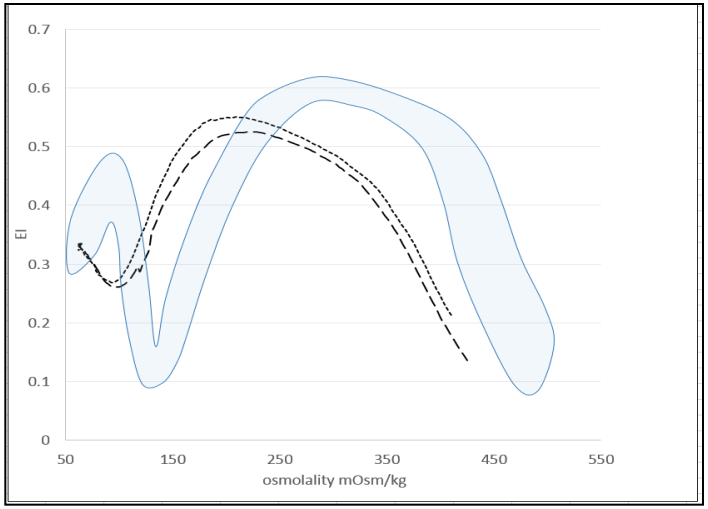
Hereditary xerocytosis



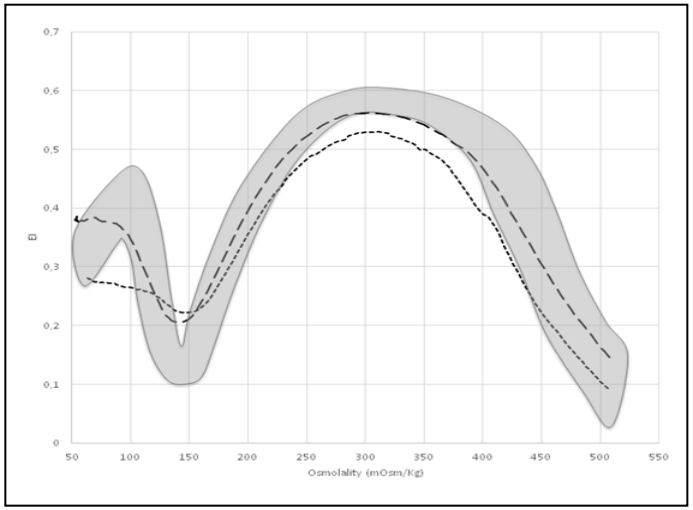
Overhydrated stomatocytosis



β-thal trait



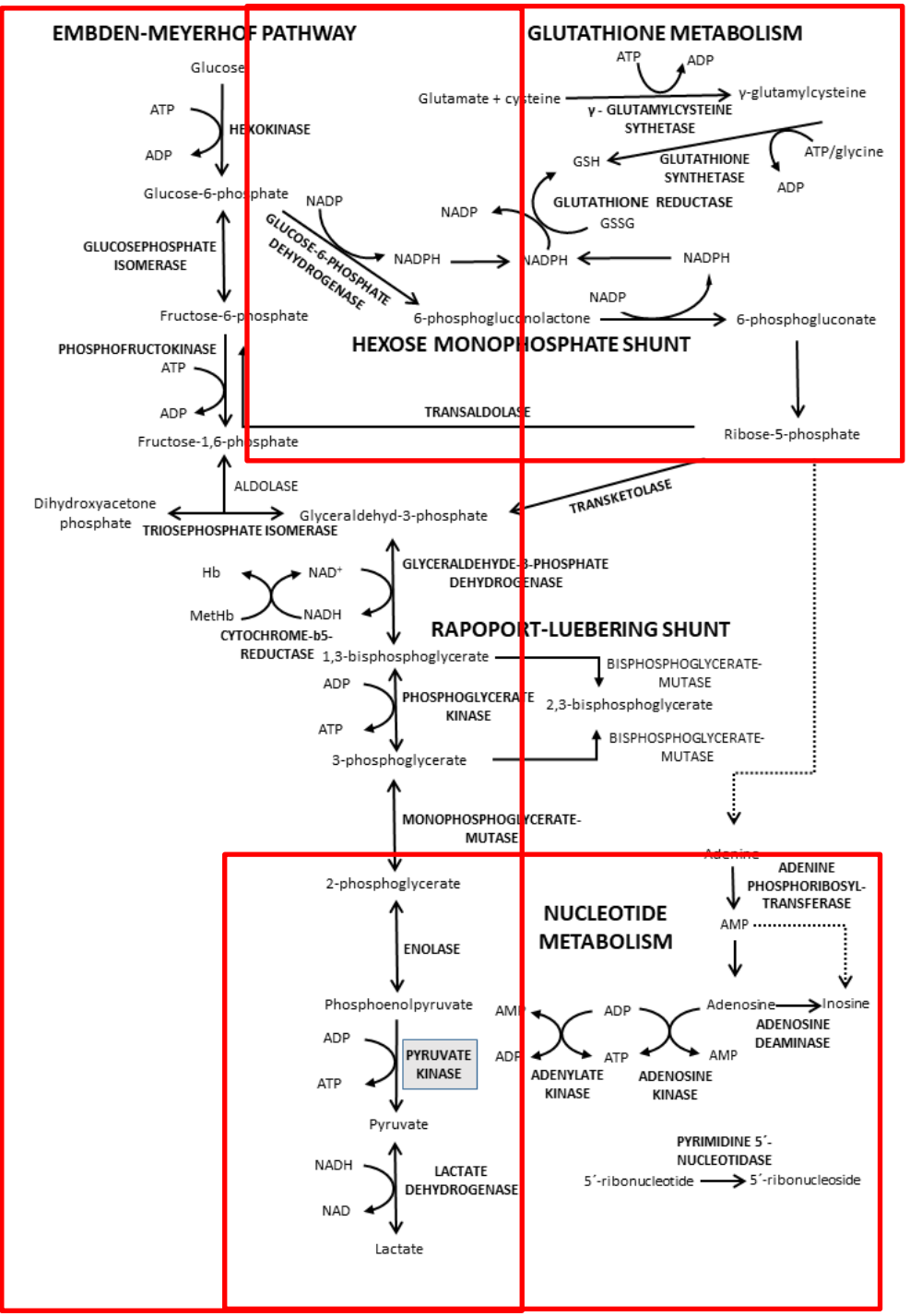
Gardos Channel variants



Diagnostic aspects of:

- ✓ Red cell membrane defects
  - hereditary spherocytosis
  - defects of permeability and cell volume regulation
- ✓ Defects of red cell metabolism
  - pyruvate kinase deficiency
- ✓ Targeted Next Generation Sequencing panels

Congenital hemolytic anemias due to RBC enzyme defects



Methemoglobinemia

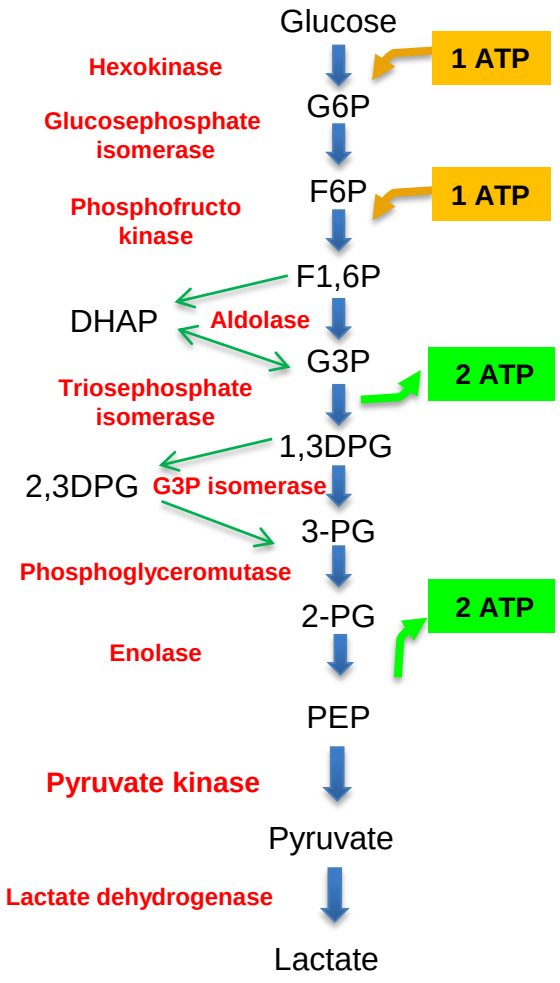
Erythrocytosis

Hemolytic anemia
(acute o chronic)

The type and degree of haemolysis in CNSHA depends on:

- the metabolic cycle involved
- the relative importance of the affected enzyme
- the functional properties of the mutant enzyme with regard to kinetic abnormalities and/or instability
- the ability to compensate for the enzyme deficiency by over-expressing isoenzymes or using alternative pathways

The Embden-Meyerof 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

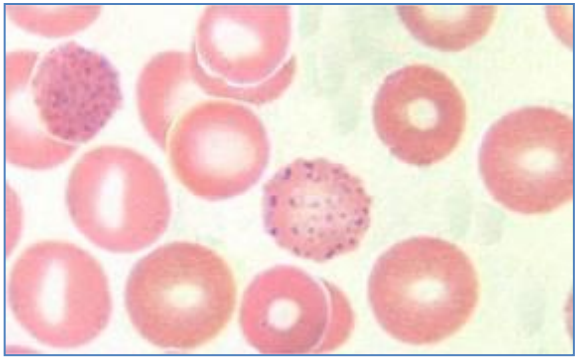
Enzyme	Gene	Position	N. of cases	Phenotype
Embden-Meyerof pathway				
Hexokinase	*HK1*	10q22.1	20 cases	CNSHA
Glucosephosphate isomerase	*GPI*	19q13.11	>50 fam	CNSHA Mental retardation?
Phosphofructokinase	*PFK-M* *PFK-L*	12q13.11 21q22.3	~75 cases	Erythrocytosis, minimal hemolysis, Tarui disase, muscle disease
Aldolase	*ALDOA*	16p11.2	6 cases	CNSHA, mental retardation Dysmorphism
Triosephosphate isomerase	*TPI1*	12p13	~75 cases	CNSHA, neuromuscular disease, Infections
Phosphoglycerate kinase	*PGK1*	X13.3	40 cases	CNSHA, neuromuscular disease
Pyruvate kinase	*PKLR*	1q22	>500 fam	CNSHA

Diagnosis of RBC enzyme defects

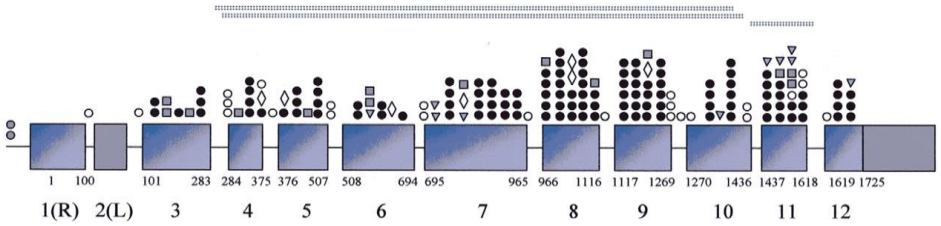
- Morphological analysis usually unremarkable, except in P5N deficiency
- Demonstration of the specific enzyme defect by measuring red blood cell enzyme activities (Beutler, 84)
- Other clinical symptoms may be helpful (*e.g.* neuromuscular symptoms, myopathy)
- DNA analysis is required to confirm the diagnosis

Diagnostic pitfalls

- Contamination with donor RBCs in transfused patients
- Incomplete leukocyte removal
- Reticulocyte number
- Storage and shipment of samples *e.g.* instability of PFK, TPI
- Mutant with normal catalytic activity "*in vitro*"



Pyrimidine 5'nuleotidase def (P5'-N)



Molecular heterogenity of *PKLR* gene in PK deficiency (>300 variants)

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

Received: 19 July 2018 | Revised: 19 October 2018 | Accepted: 20 October 2018
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WILEY AJH

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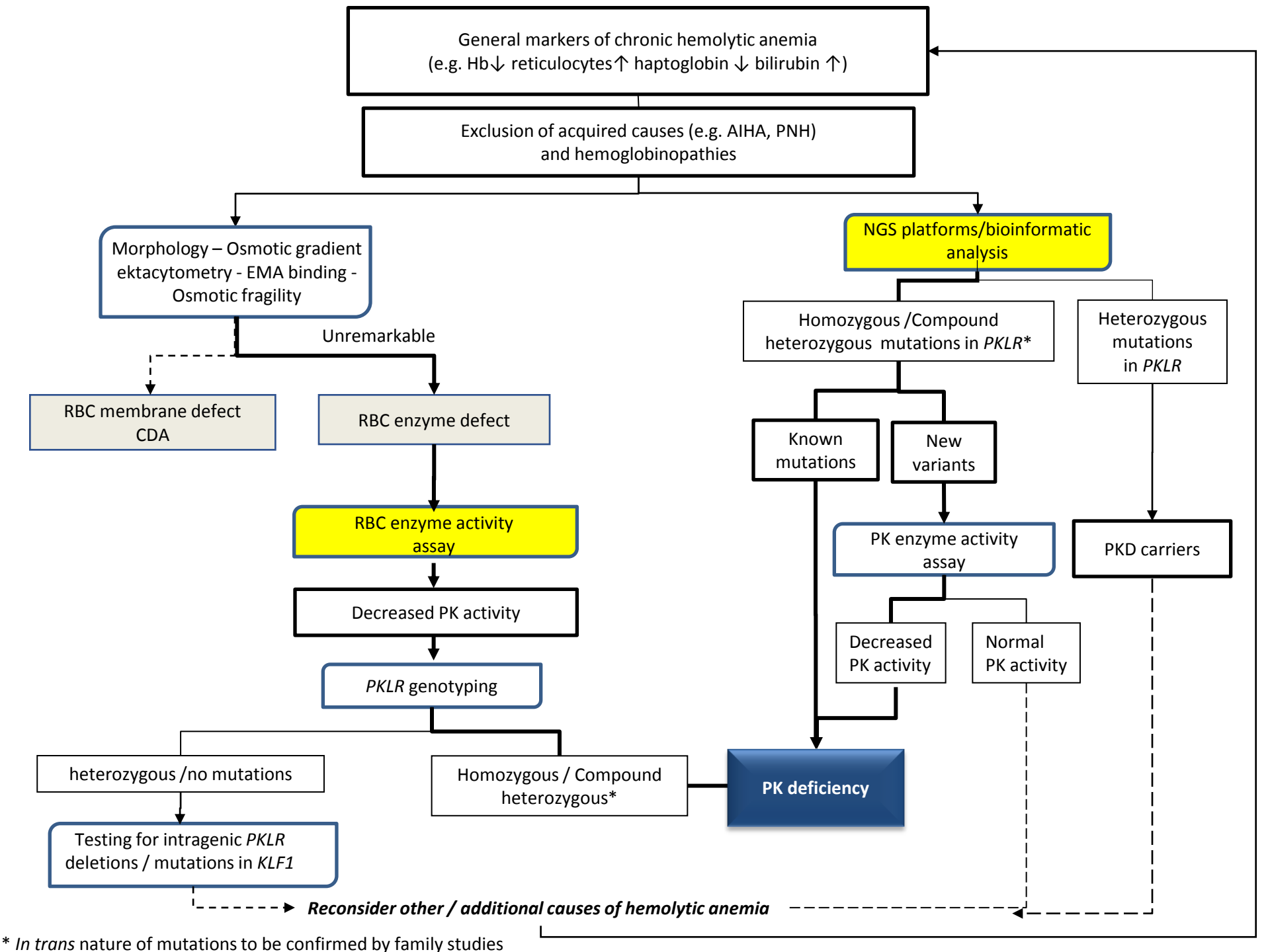
	Recommendation	Evidence
Clinical presentation	PK deficiency may be suspected in: - patients with variable chronic anaemia and/or splenomegaly and/or jaundice, with normal or near-normal red cell morphology. - transfusion dependent cases of unknown aetiology - haemolytic patients with unexplained severe 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)

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)

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)


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

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

EuroBloodNet European Reference Networks

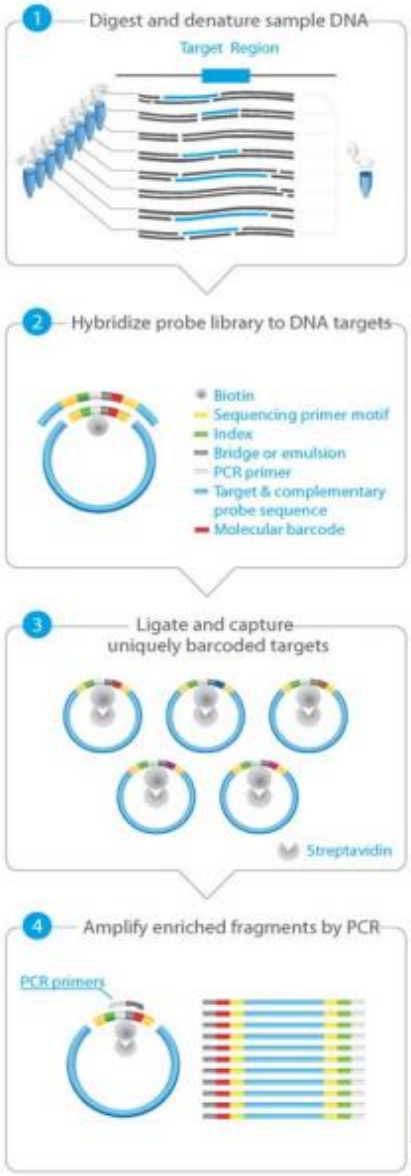
Slides by Barbara de la Salle UK-NEQAS

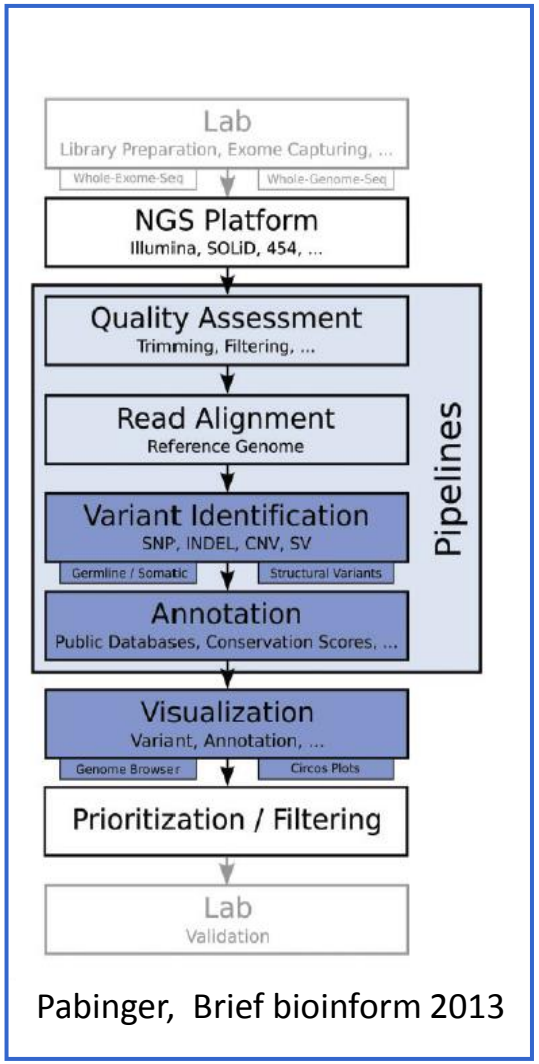
Diagnostic aspects of:

- ✓ Red cell membrane defects
  - hereditary spherocytosis
  - defects of permeability and cell volume regulation
- ✓ Defects of red cell metabolism
  - pyruvate kinase deficiency
- ✓ Targeted Next Generation Sequencing panels

Targeted Next Generation Sequencing panels

HaloPlex HSTarget Enrichment System Agilent





Pabinger, Brief bioinform 2013

Sequencing :MiSeq Illumina

Targeted Next Generation Sequencing panel

	Genes	Cases	Results
Li Y, et al. Zhonghua Xue Ye Xue Za Zhi. 2018.	217	46	60.9% (41%)
Russo et al. Am J Hematol , 2018	34-71	74	64.9% (45.8%)
Agrawal AM et al, Br J Haem 2016	28	17	70%
Roy et al . Br J Haematol. 2016	33	57	38.6% (22%)

40 genes -Targeted Next Generation Sequencing panel

Libraries were obtained by: HaloPlexHS Target Enrichment System Kit (Agilent)
MiSeq platform (Illumina).
Amplicons 15766
Coverage: 99.31%

Gene	Ref. Sequence	Gene	Ref. Sequence
ABCB6	NM_005689	GSS	NM_000178
ABCG5	NM_022436	HK1	NM_033497
ABCG8	NM_022437	KCNN4	NM_002250
ALAS2	NM_001037967	KIF23	NM_138555.2
AK1	NM_000476	KLF1	NM_006563.3
ALDOA	NM_000034	NT5C3A	NM_016489.12
BPGM	NM_001293085	PFKL	NM_001002021
C15ORF41	NM_001130010	PFKM	NM_000289.5
CDAN1	NM_138477	PGK1	NM_000291.3
CYB5R3	NM_000398	PGM1	NM_001172819
ENO1	NM_001201483	PKLR	NM_000298.5
EPB41	NM_004437.3	PIEZO1	NM_001142864.2
EPB42	NM_000119.2	RHAG	NM_000324.2
G6PD	NM_000402	SEC23B	NM_006363.4
GATA1	NM_002049	SLC2A1	NM_006516
GCLC	NM_001498.3	SLC4A1	NM_000342.3
GCLM	NM_001308253	SLC25A38	NM_017875.2
GPI	NM_000175.3	SPTA1	NM_003126.2
GPX1	NM_000581.2	SPTB	NM_000347.5
GSR	NM_000637	TPI1	NM_000365.5

FONDAZIONE IRCCS CA' GRANDA
OSPEDALE MAGGIORE POLICLINICO
Sistema Sanitario Regione Lombardia

Fermo et al, EHA 2018

Targeted Next Generation Sequencing panel

131 cases congenital hemolytic anemia
109 families

46 families
no diagnosis after extensive investigations

1 Sideroblastic anemia (*ALAS2*)
1 Sitosterolemia (*ABCG5*)
5 Hst
2 *PIEZO1*
3 *KCCN4*
6 Enzyme defects
2 *PKLR*
1 *HK1*
3 *G6PD*

28%

15 families
Recents transfusions/ shipping

1 CDAII
4 Enzyme defects
2 *PKLR*
1 *TPI*
1 *CYB5R3*

33%

48 families
confirm haematological/biochemical diagnosis

32 confirmed dignosis

3 changed diagnosis
HS>>HSt (*PIEZO1*)
HPP>> severe HS
OHst >> sitosterolemia (*ABCG8*)

13 not confirmed at molecular level
--4 Hst
--4 HE
--2 HS
--2 atypical CDA
-- PK def

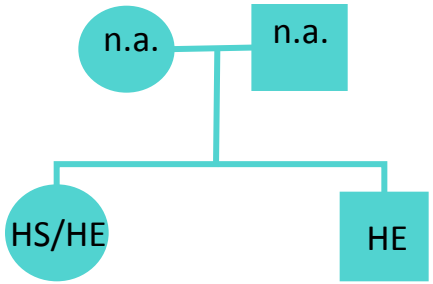
73%

Diagnosis in 53/109 unrelated patients, 48% of cases

FONDAZIONE IRCCS CA' GRANDA
OSPEDALE MAGGIORE POLICLINICO
Sistema Sanitario Regione Lombardia

Fermo et al, unpublished

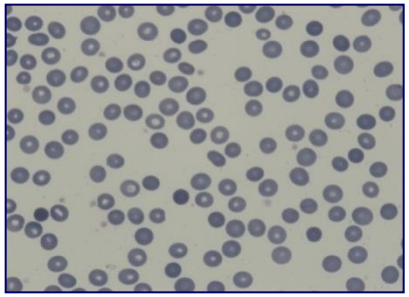
Molecular investigations: contribute to diagnosis

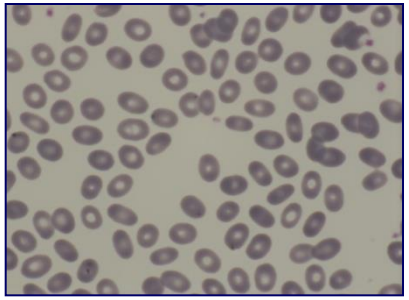


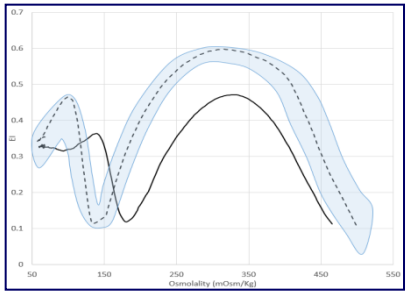
F11-817
SPTA1: L154F/wt
SLC4A1 : S510R/wt
EMA-binding: ↓↓
SDS-Page: spectrin ↓

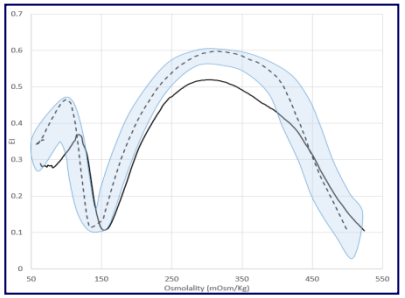
F11- 816
SPTA1: L154F/wt

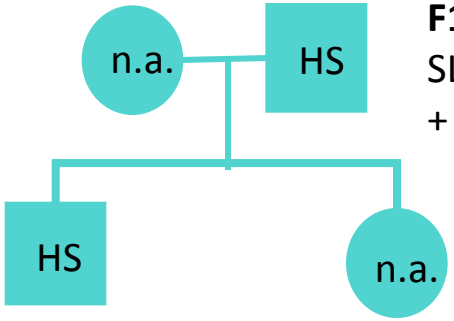
EMA-binding: Norm
SDS-Page: Norm











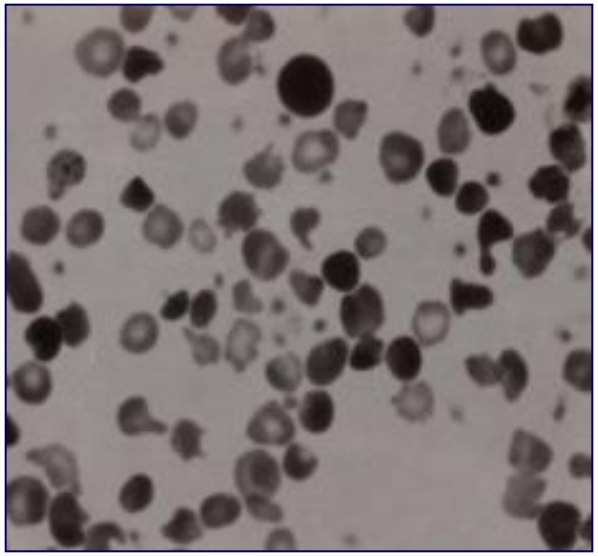
F13-776
SLC4A1
+ R490H

F13-723
SLC4A1
Abnormal splicing
+ R490H
+ QPLLPQ186Q

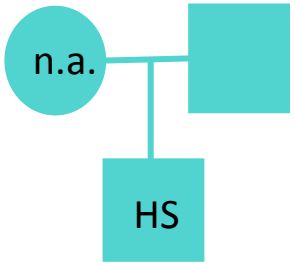
* Post splenectomy

	F13 -723*	F13-776
Hb (g/dL)	13.5	12.3
Retic (abs n.)	176	183
MCV (fL)	108	97.3
Ferritin	817	181
Osm fragility	↓	↓
Ema-binding	↓↓	n.a.
SDS-PAGE	Bd3 38% ↓	Bd3 17 %↓
Transfusions	Tx until spl	No
Splenectomy	Yes 6yr	No
Other sympt.	Priapism	No

Molecular investigations: contribute to diagnosis



F12-73
Healthy
SPTA1:
R1047X



F12-74
Healthy
SPTA1: abn splicing
+Lely

F12-73
SPTA1:
R1047X
+abn splicing
+Lely

	F12 -73
Hb (g/dL)	7.8*/11.2
Retic (abs n.)	415
MCV (fL)	73
Ferritin	69
Osm fragility	↓
Ema-binding	↓↓
SDS-PAGE	αSp 68%↓; Ank 56% ↓
Transfusions	Tx until spl
Splenectomy	Yes 7yr

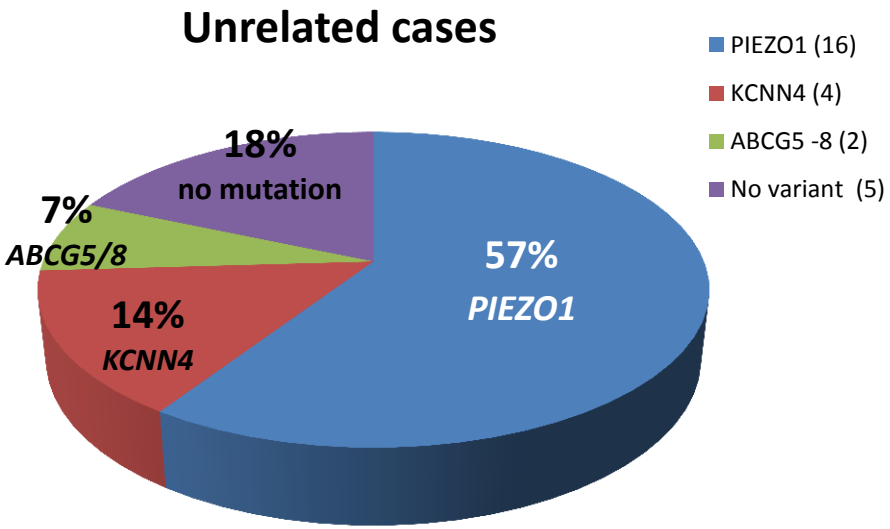
* Pre splenectomy

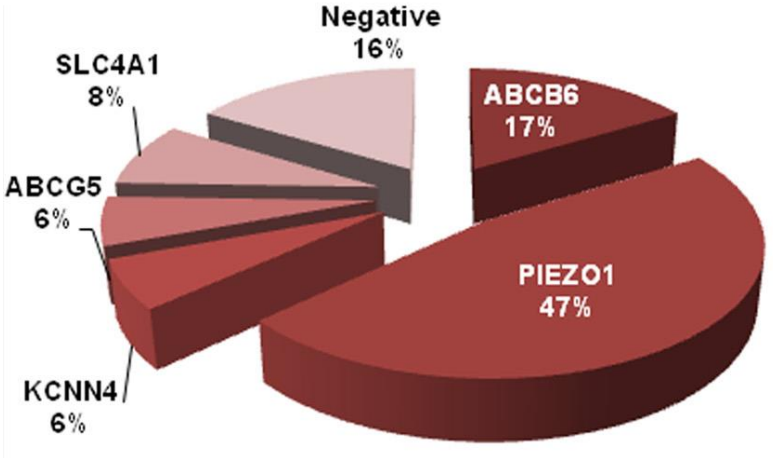
Diagnosis of RBC hydrations defects: 41 cases (27 fam)

Molecular abnormality	N. Cases	Families
PIEZO1	23	16
KCNN4	11	4
ABCG5	1	1
ABCG8	1	1
No mutation	5	5

PIEZO1	
E2496ELE (4)	M605I
R2456H (10)	
V598M	
A2003T (2)	
E2489D (2)	
L2192I (2)	

VUS!





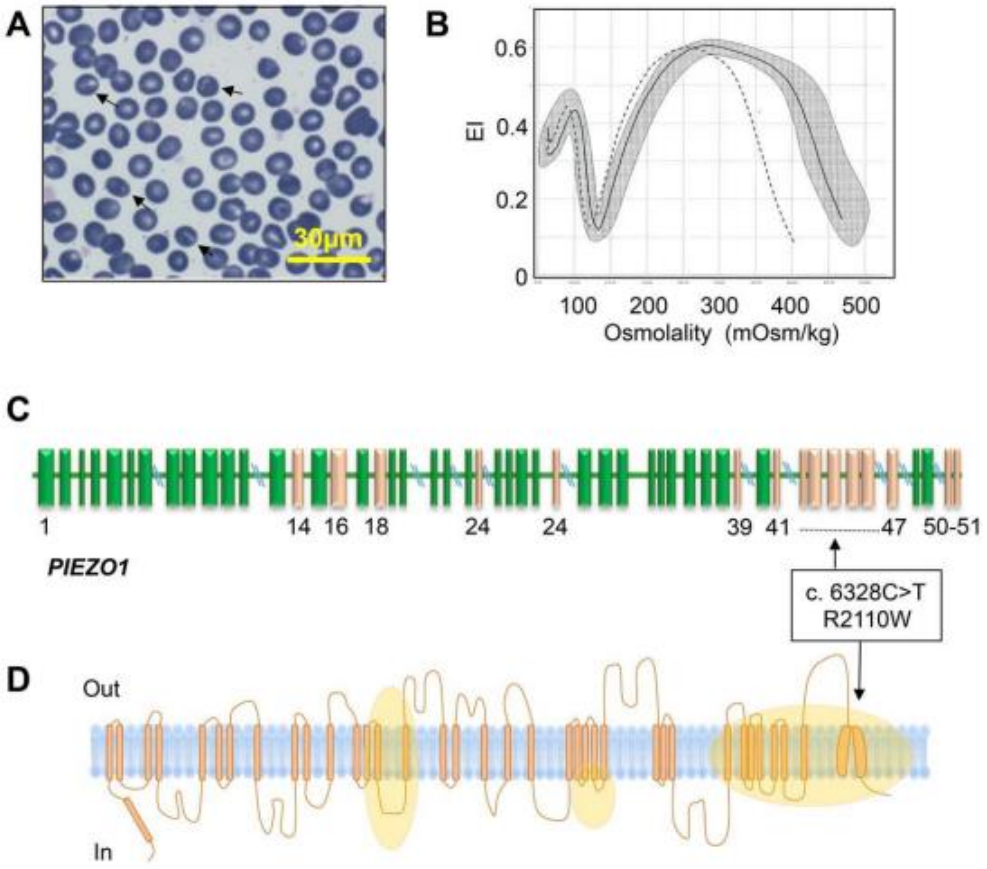
Andolfo et al, 2018

New variants – Functional studies

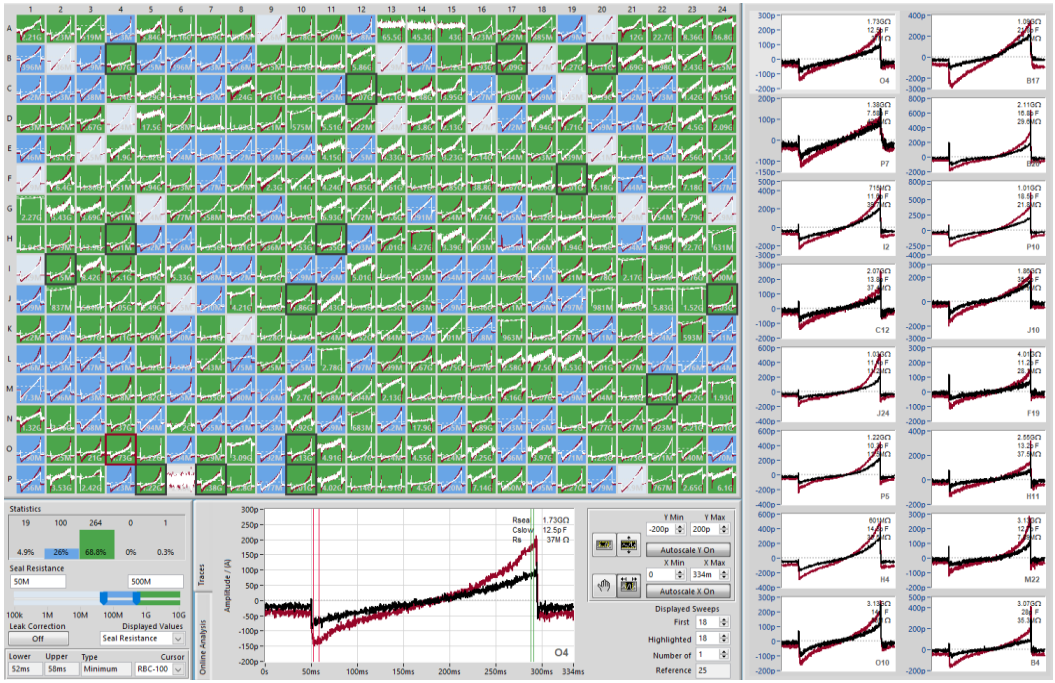
A novel gain-of-function mutation of Piezo1 is functionally affirmed in red blood cells by high-throughput patch clamp

GM. Rotordam, et al Haematologica 2018

	Patient R2110W	Reference values
Age (years)	43	
Transfusions	no	
Splenomegaly	no	
Hb (g/dL)	16.9	13.4-17.5
MCV (fL)	80.9	80-94
MCHC (g/dL)	39.1	31-37
Reticulocytes (x10^9/L)	193	20-100
RBCs morphology	7% stomatocytes	
Unconj. bilirubin (mg/dL)	0.66	<1
Serum ferritin (ng/mL)	546	30-400
AGLT	>900	>900
Pink test	7	11-33
NaCl osmotic fragility	decreased	
EMA binding test	normal	



Functional studies – Single cell patch clamp screening assay



- ✓ A patch clamp based high throughput screening assay (SyncroPatch 384/768PE (Nanion Technologies, Munich, Germany) for Piezo1 activity.
- ✓ It is the first electrophysiologic single-cell based screening performed on RBCs demonstrating the Piezo1 gain-of-function mutation directly on RBCs and providing a putative routine approach for detecting functional (Piezo1) channel mutations as the molecular cause of rare anaemia that can become a standard method in specialised haematological centres.

Take home message

- ✓ The laboratory diagnosis of congenital haemolytic anaemias is based upon a combination of clinical history, family history, physical examination and laboratory data
- ✓ Identification if a mimimun panel of tests for the diagnosis of these diseases to be standardized
- ✓ EMA binding test for the diagnosis of HS
- ✓ NGS may represent a comprehensive diagnostic method, however not all cases at the moment can be disgnosed but this approach alone.

Thanks!

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