Progress in the Laboratory Diagnosis of Red Cell Membrane Disorders

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Today's Presentation (14 October 2014)

Inherited Haemolytic anaemia

Hereditary Stomatocytosis

Hereditary Spherocytosis

Hereditary Elliptocytosis

Protein defects

Lipid bilayer

Abnormal K^+/Na^+ permeability

Red cell cytoskeleton

Fragile red cells

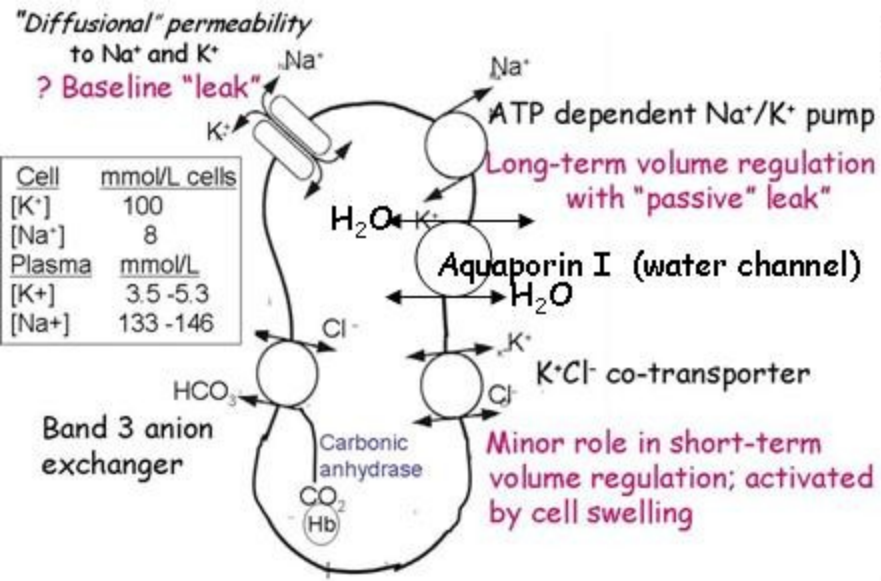
Characteristic features

Laboratory tests for Membranopathy

How to Use the EMA Binding Test

Hereditary Stomatocytoses (HSt)

"cation leak" disorder → volume change



Dominant mode of inheritance
Mild to moderate anaemia
Hb: > 80 g/L; Retics.: ~ 5-10%
Tendency of heavy iron overloading

Blood film: ± stomatocytes or target cells
Wet film: bowl-shaped RBCs (fresh)

	Overhydrated HSt	Dehydrated HSt (DHst)	Cryohydro -cytosis	Familial Pseudohyper K^+
Frequency	1: million births	1:10,000	Rare	Rare
Features	Atypical HS macrocytosis	DHst, DHSt/Pseudo-↑K^+ DHSt/Pseudo-↑K^+ and fetal edema	mild haemolysis stomatocytes ↑↑ RBC swelling + lysis (at 4°C)	Normal haematology Net loss of K^+ at RT
Membrane defects	Rh glycoprotein *Splenectomy not advised	Piezo 1 *Splenectomy not advised	Band 3 Glut-1 ± Stomatin Neurological disorder in subset	ABCB6 (porphyrin transporter)

* Increased risk of venous thromboembolism

Neqas 2014 (mjk)

What is the cause of anaemia?

Neqas 2014 (mjk)

Female patient: 74 y; ? HS

EMA Binding Test (flow cytometry)
Patient's MCF = 57.7 units
Normal (n = 6): 54.8 – 59.1 units

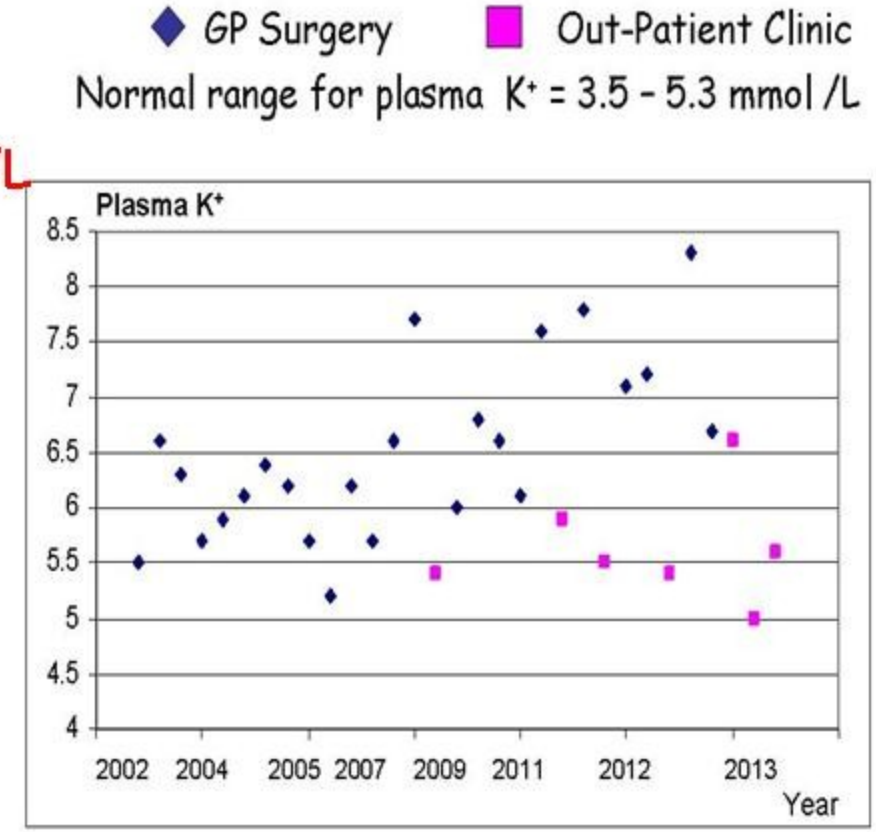
First presented at age 60 with anaemia

Blood film: few spherocytes, polychromasia, target cells

3 years later, plasma K^+ = 5.5 mmol/L

Red cell indices in 2002 - 2013

Hb: 120 – 162 g/L
MCV : 89 – 98 fL
MCH: 32.0 – 32.5 pg
MCHC: 321 – 346 g/L
Retics count: 334, 338
Bilirubin: 15 – 35 μM



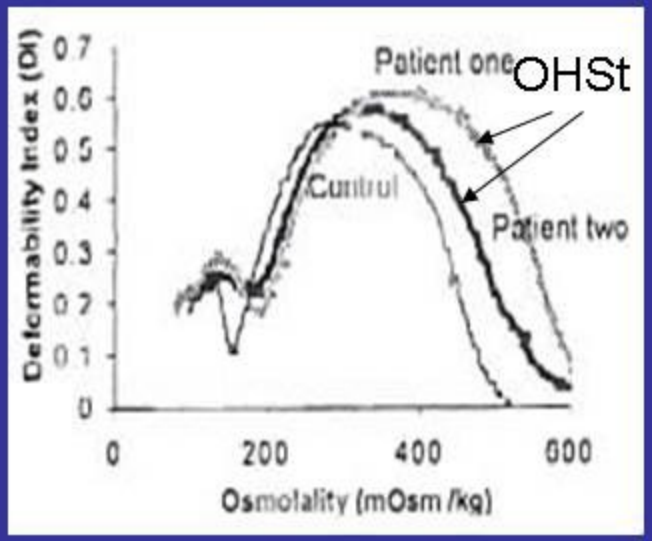
? Familial Pseudo↑K^+ or DHSt

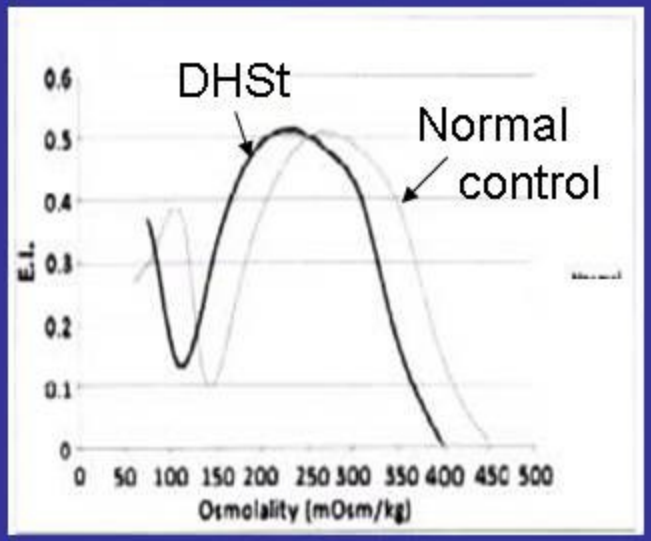
Data collated by Keith Chambers
(Leicester Royal Infirmary, UK)

Laboratory Testing for Hereditary Stomatocytosis (HSt)

	Overhydrated HSt	Dehydrated HSt
MCV	≥ 100fL (>120 fL o/n)	~ 100 fL
MCHC	decreased	Increased
% hyperdense cells	?	Increased
Plasma [K^+]		elevated
2,3 DPG	Decreased	Decreased
Osmotic Fragility test	Increased (HS-like)	Decreased (Exclusion of Thalassemia)
EMA Binding	Normal or ↑ fluorescence	Normal fluorescence
Ektacytometry	right shifted curve	left-shifted curve

Deformability profiles





Summary (I)

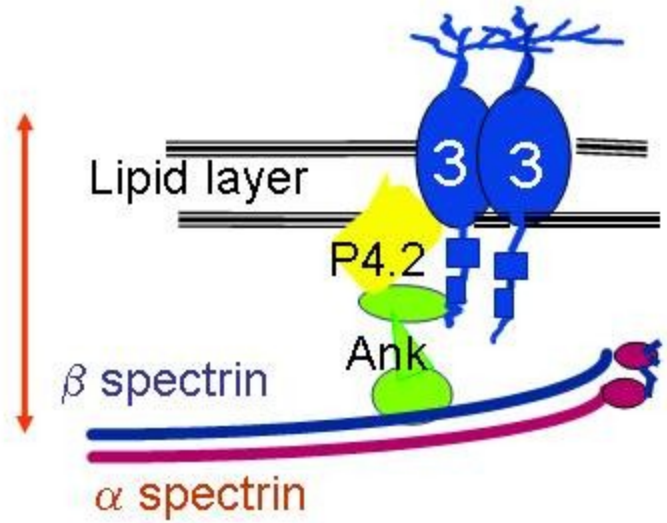
Laboratory diagnosis of Hereditary Stomatocytosis

1. Red Cell morphology: stomatocytes or target cells
2. If (1) is not helpful, review red cell indices reticulocyte count , bilirubin, and plasma [K^+] in U/E
3. Carry out the osmotic fragility test.
   If not available, carry out the following experiment:
   Determine MCV and plasma K^+ level in a normal control and the patient before and after overnight storage at RT and at 4°C.
4. If available, perform molecular analysis to confirm the diagnosis.

* When an "HS" patient is found non-responsive to splenectomy, Re-examine the patient for hereditary stomatocytosis (e.g., OHSt, DHSt).

Hereditary Spherocytosis: heterogeneous in every aspect

Vertical interaction



- **Clinically**

	Hb (g/dL)	Retics (%)	Bb (μM)
Asymptomatic (compensated HA)			
mild	11-15	3-6	17-34
moderate	8-12	>6	>34
severe transfusion dependent anaemia	6-8	10	>51

Hydrops fetalis reported but rare occurrence (one parent must have HS)

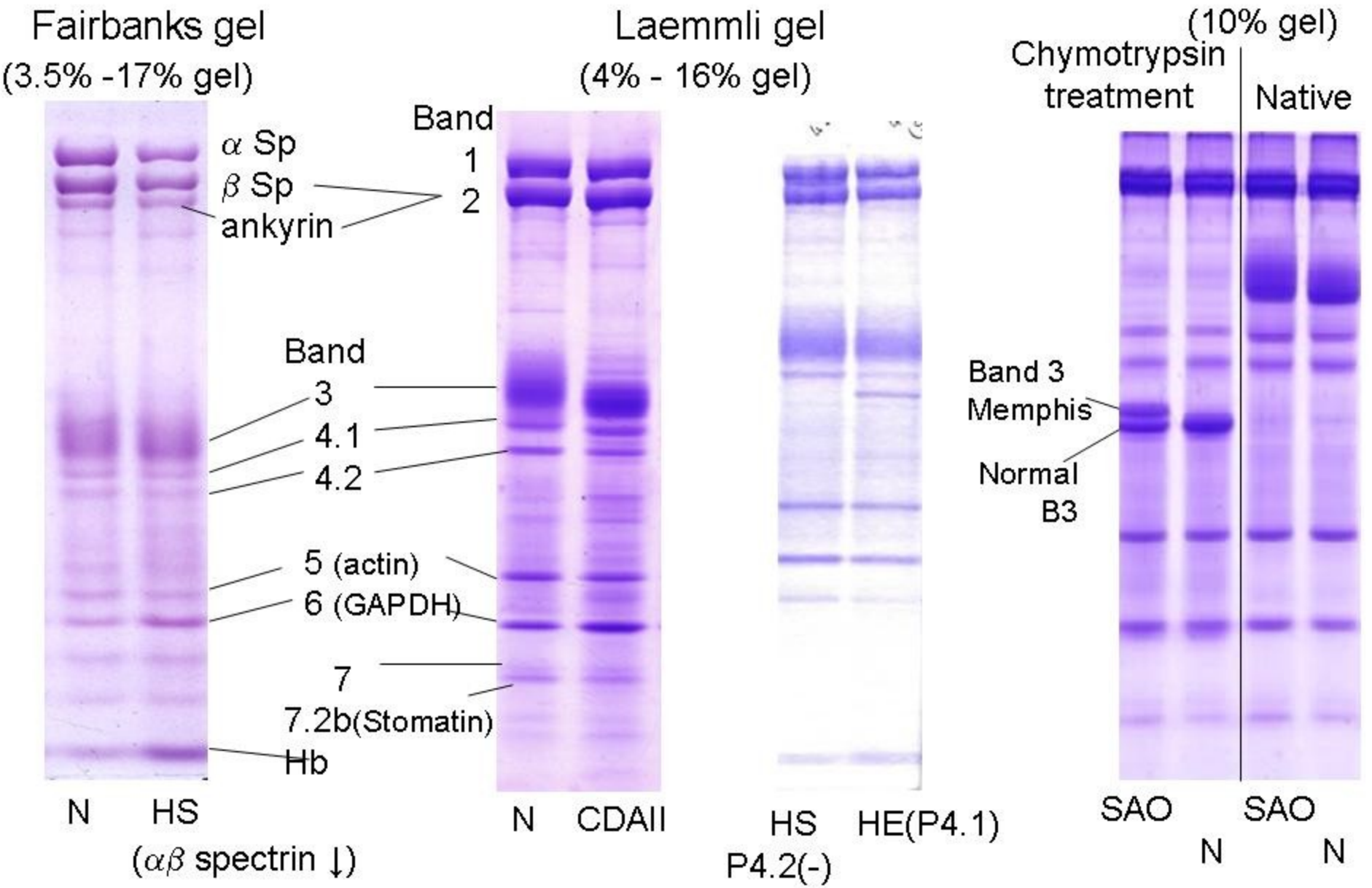
- **Biochemically**: more than one protein affected
- **Genetically:** more than one gene affected

Mode of Inheritance:

Dominant: Proband – mild to moderate HA
Parent(s) – family history

Recessive or Non-dominant HS: Proband - Severe HA (infancy /childhood) regular transfusion, early splenectomy
Parents: clinically normal, subtler RBC abnormality

SDS-polyacrylamide gel electrophoresis of erythrocyte membrane proteins



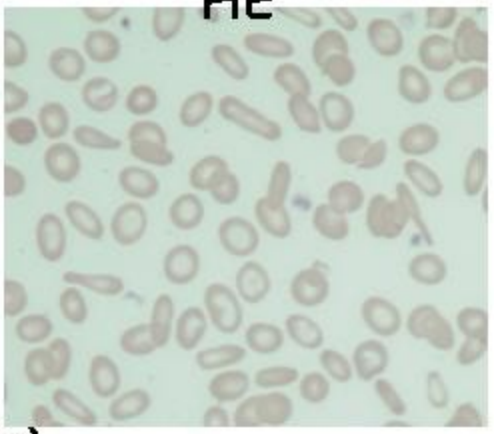
Neqas 2014 (mjk)

Hereditary Elliptocytosis (HE)

Mostly asymptomatic or mild:
i.e., no anaemia nor splenomegaly.
Reticulocyte count: normal to slightly raised

	Frequency	Protein defect
Caucasians	1:5000 births	↓ Protein 4.1
Black populations	1:100 births	Spectrin mutation(s)

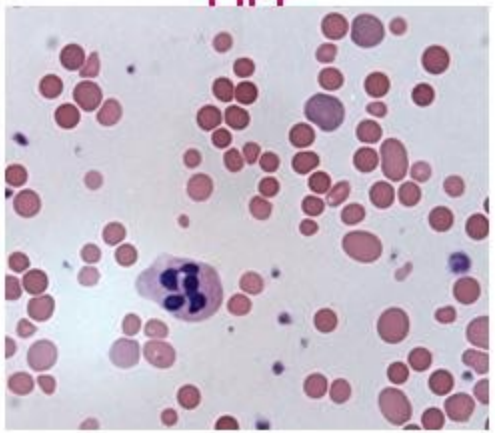
HE



severe haemolysis → Haemolytic HE (severe): P4.1 null

severe haemolysis → Hereditary Pyropoikilocytosis

HPP



Hydrops fetalis (rare)

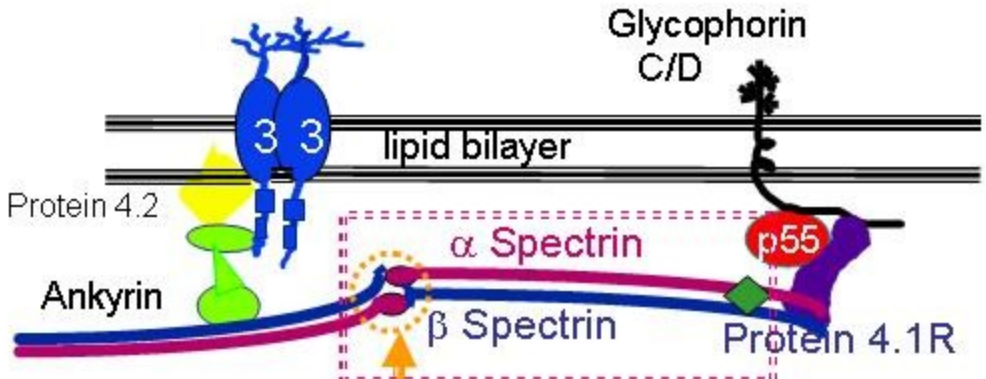
HPP (MCV 50 – 60 fL), transfusion dependent.
It is often diagnosed in 1-2 years of life

Mild HE with transient poikilocytosis in Infancy

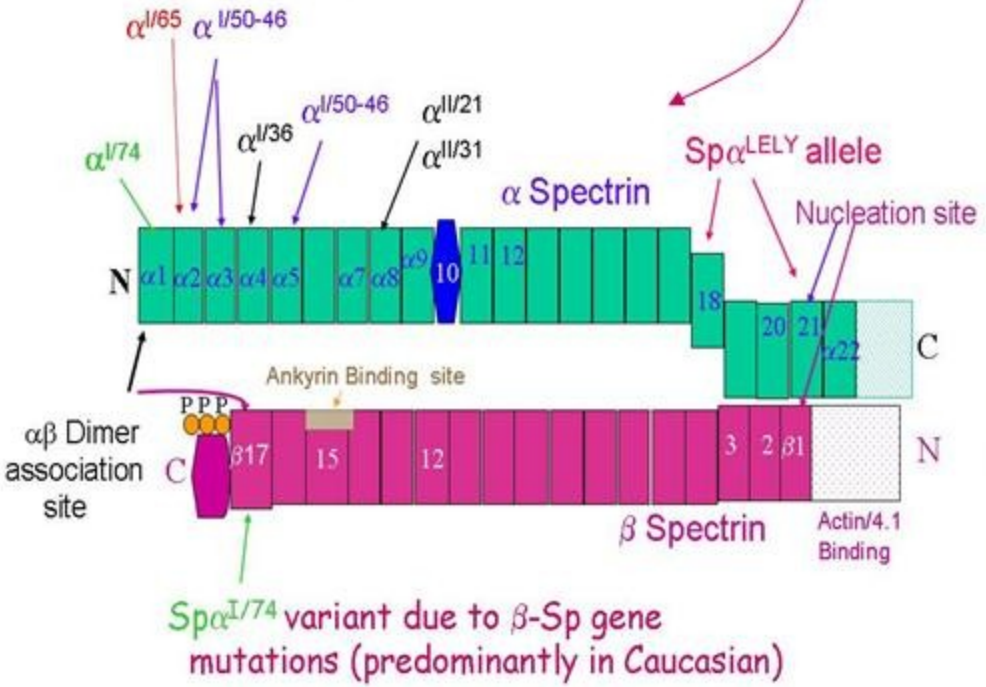
First 12 months: Severe haemolytic anaemia, elliptocytes, poikilocytosis may require regular blood transfusion

By age 1 yr: Haemolysis and poikilocytosis decline
Clinical picture of compensated mild HE similar to that of the affected parent

Confirmatory tests for HE/HPP

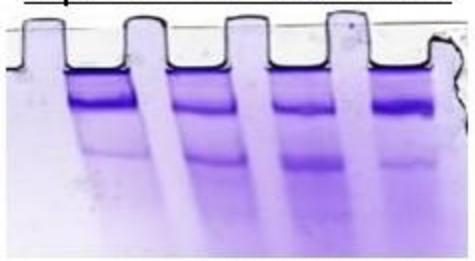


Most Sp mutations in Tetramerisation site



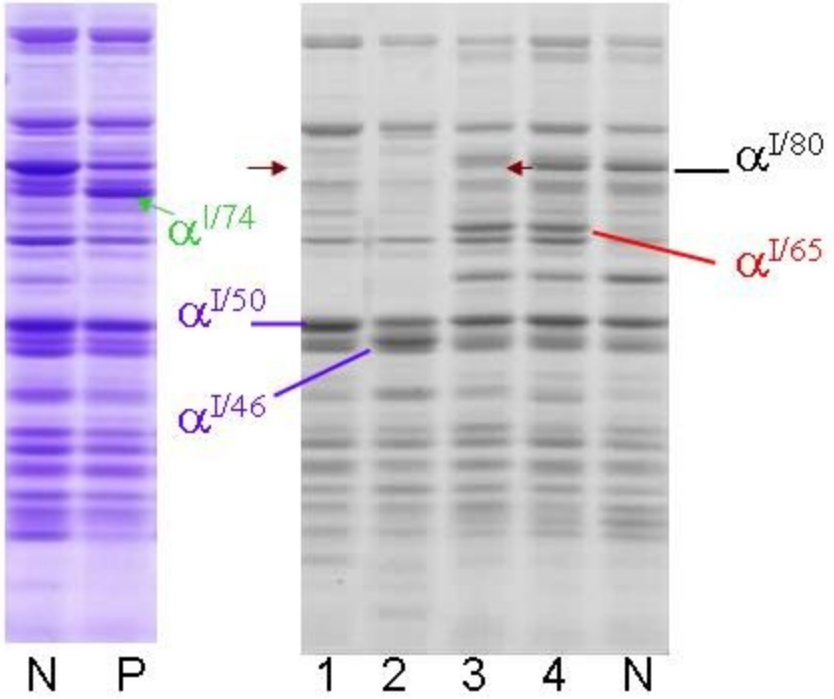
	HE	HPP
Sp dimer	↑	↑↑
Sp variants	yes	yes

Sp Dimer content



N HE HPP N

Limited trypsin digestion of spectrin



Summary II

"Rough Guide" for the diagnosis of Hereditary Spherocytosis and Hereditary Elliptocytosis/H. Pyropoikilocytosis

	HS	*HE/HPP*
Family history	Affected parent (dominant)	One parent must have HE
	Normal parents (recessive/nd)	Rare to have normal parents
Best indicator of disease severity	Microcytes (hyperdense)	Microcytes (dehydrated, mature RBCs) HPP: MCV 50-60fL Important to exclude thalassemia or Co-inheritance of HE/thalassemia
Blood film	Spherocytes (dominant)	Elliptocytes
	various sizes (recessive/nd)	Poikilocytes

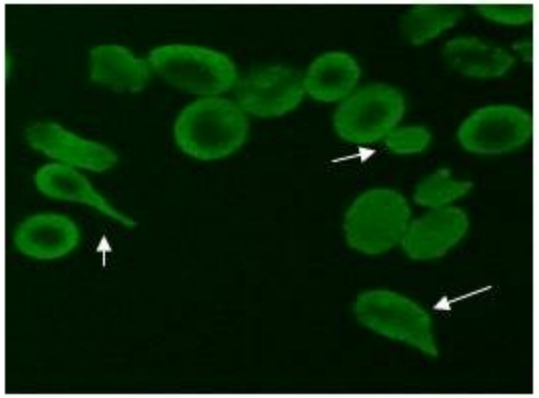
Neas 2014 (mjk)	Screening tests		Confirmatory test
Spectrophotometry (i) OF test (ii) Acid Glycerol Lysis Time Test (iii) Cryohemolysis Test	**Ektacytometry** of RBC Or , LORCA (laser-assisted optical rotational cell analyser)	**Flow cytometry** The Eosin-5-Maleimide (EMA) Binding test	**SDS-Polyacrylamide gel electrophoresis** of erythrocyte membrane proteins
(i) 1 and 24 hours (ii) 1-2 hours (iii) 1-2 hours	About 2- 3 hours	About 2 hours	3- 5 days for HS or HE(P4.1) HPP: 10 -12 days
Same day blood collection and testing	Same day testing (preferred), or blood stored at 4°C o/n	Maximum 3 days when stored in refrigerator	Prepare membranes within 24h collection Testing within 1 week-membranes @-80°C
False positive results for certain red cell conditions unrelated to membranopathy	Ektacytometer setup takes time. Analysis time: 10 minutes	92.7% sensitivity 99.1% specificity for HS. Also detects SAO, some CDAII cryohydrocytosis	Normal proteins in 10% HS ↓Ankyrin not detected in patient with reticulocytosis

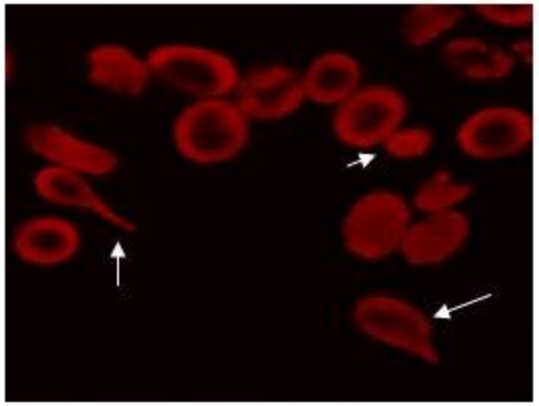
How to use the EMA Binding Test

5 μl washed packed RBCs
25 μl EMA (0.5 mg/ml PBS)
Mix, Incubate at room temperature
for 1 hour in the dark

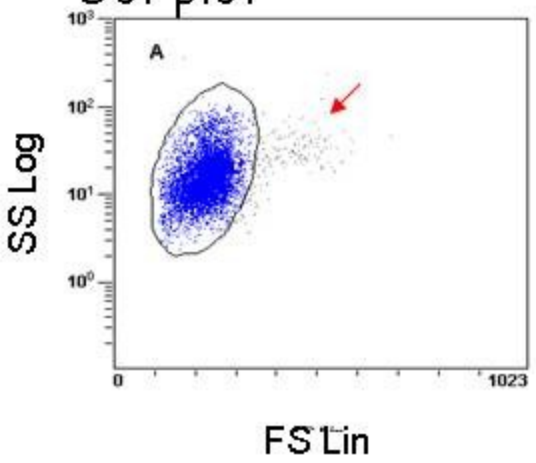
Pulse spin, remove supernatant
Wash cell pellet with PBS/BSA, 2x
Re-suspend pellet in PBS/BSA
FACS analysis: Ex. λ = 488 nm
(15,000 events) Detector: FL-1

Patient Sample (confocal)

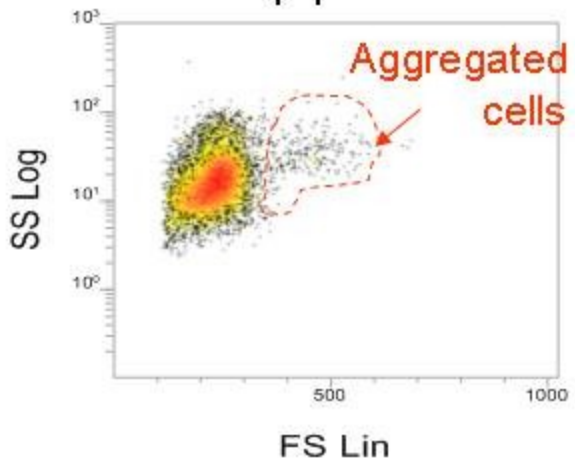




Dot plot



Density plot



Requirement for blood specimen

Whole blood anti-coagulated in EDTA.K_2

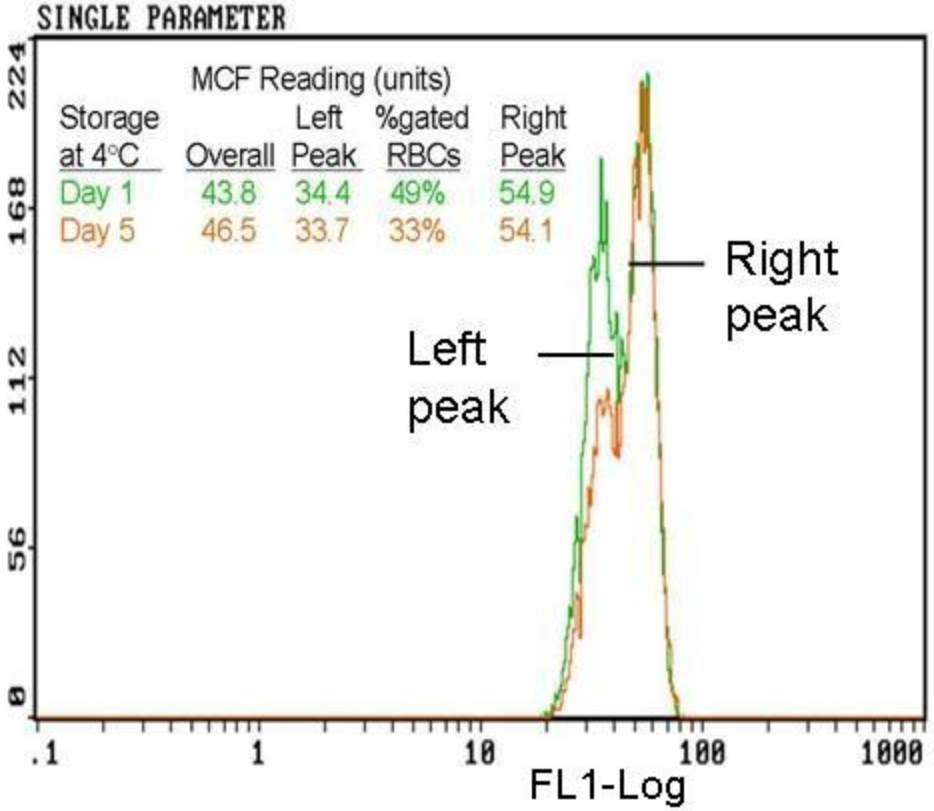
Keep specimen at 4°C to minimize red cell lysis: endogenous proteases in RBCs can degrade membrane proteins.

Testing must be carried out within 3 days of blood collection.

Delay in testing RBC

The most fragile RBCs will disintegrate first on prolonged storage.

The EMA result will not give a true picture of the patient's RBC composition.



Blood Transfusion and the EMA Binding test

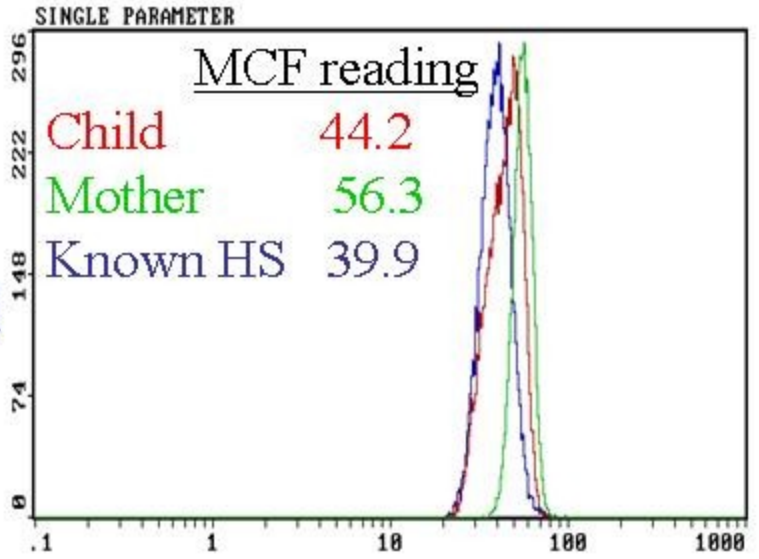
1. Patient on regular transfusions:
   (i.e. @ 5 to 6 week-intervals)
   Ask the clinician to withhold blood transfusion as long as possible before taking a specimen for testing

   OR, try to "wean" the patient off blood transfusion before testing

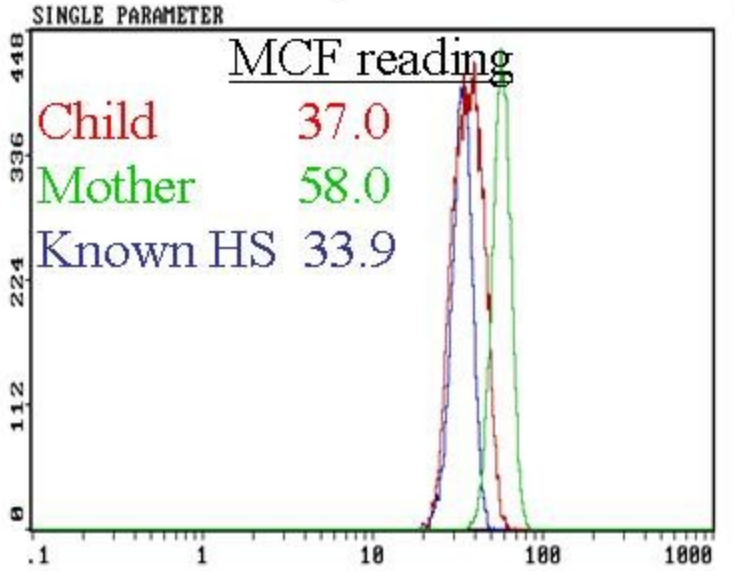
2. Recently transfused patient:
   Wait for 2 months for complete clearance of residual transfused RBCs

10 yr old child (F) : 4 units of blood

1 month **post-transfusion**



4 months **post-transfusion**



Summary statistics and data for EMA binding to red cells

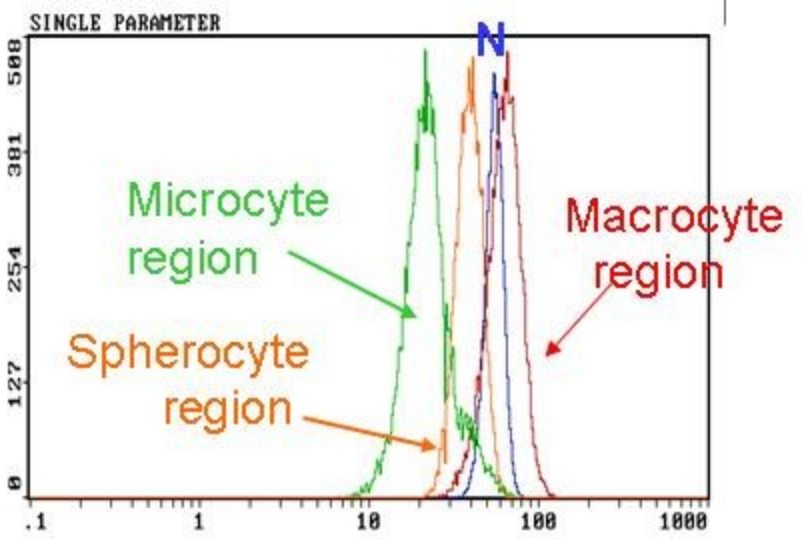
The cut-off MCF for distinguishing between normal and HS = 45.5 units

Group	N	MCF reading Mean	SD	Median	IQuart Range
Normal	180	53.91	3.24		
HS (Sp ↓)	41	36.88	4.25		
HS (B3 ↓)	19	36.02	4.98		
HS (P4.2 ↓)	36			39.20	5.00
HS (Ank ↓)	3	51.97	1.33		
HE (P4.1↓+Sp)	25	50.99	4.03		
HPP	5	26.90	2.50		
Immune HA	10	53.72	4.14		
Fe deficiency	18	52.41	3.45		
Chr renal failure	14			51.80	2.80
β Thalassemia	57			57.35	3.20

Ref.: King *et al.* (2000) *Br. J. Haematol.* **126**,455.

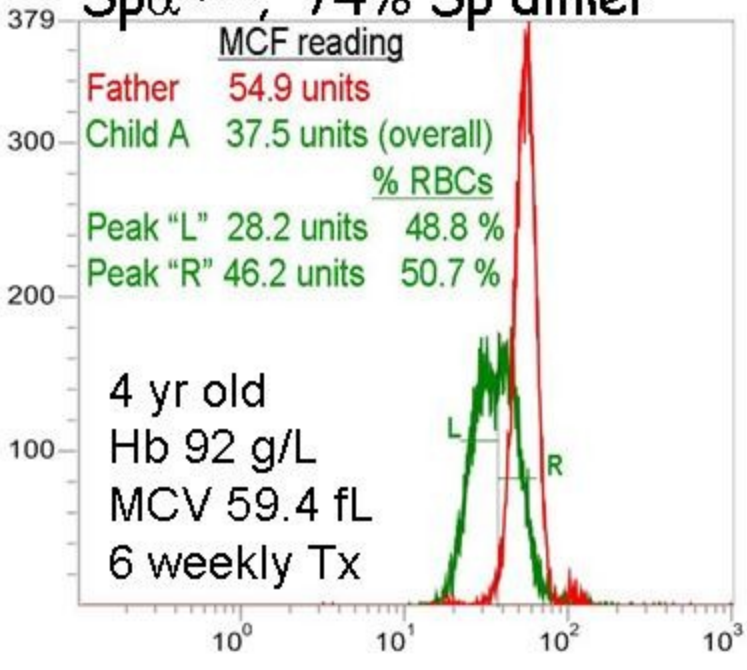
Caveat (EMA): Exclusion of

	n	Fluorescence
SAO	4	36.6±2.7 units
CDAII	26	49.4±4.7 units
CHC	3	36.0±3.5 units
HS	16	40.4±3.3 units
Normal	13	55.4±3.0 units



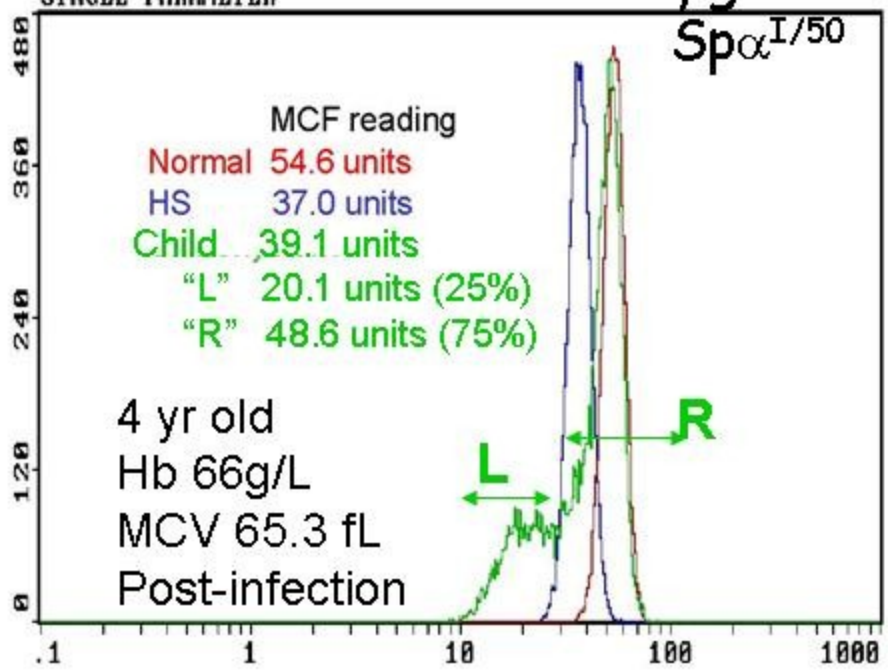
HPP Homozygote

Spα$^{I/74}$; 74% Sp dimer

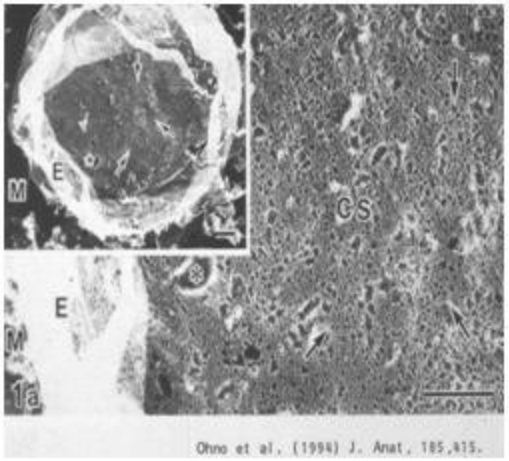


HE Heterozygote

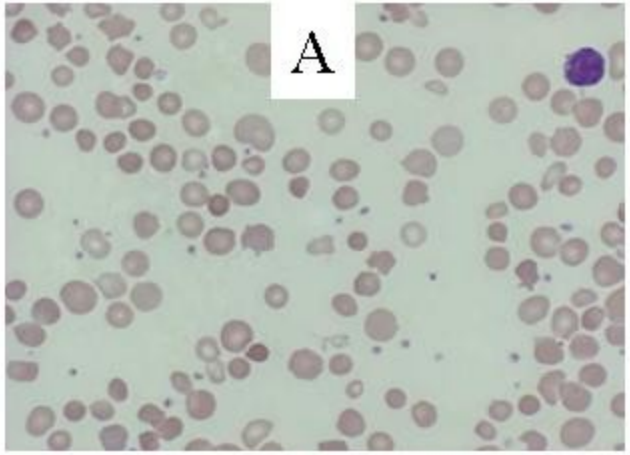
Spα$^{I/50}$

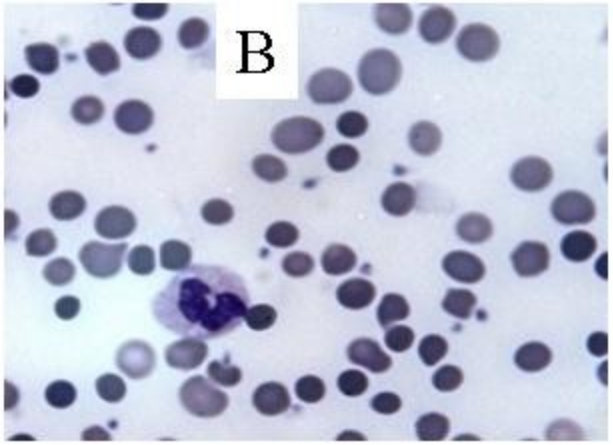


EM of RBC skeleton



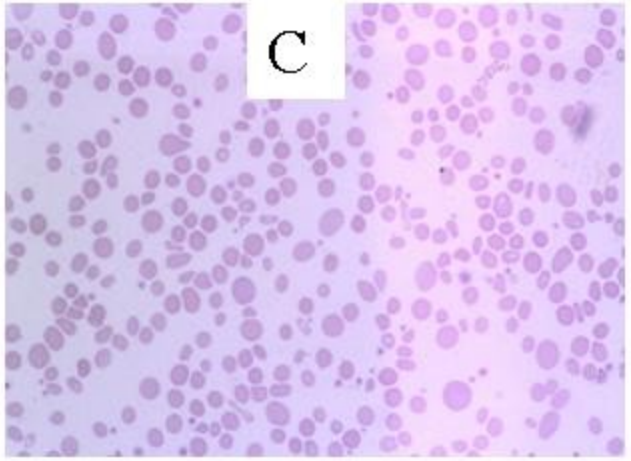
<u>Function of the cytoskeleton</u>
Strength (≈ osmotic fragility)
Deformability (elasticity)
Cell shape

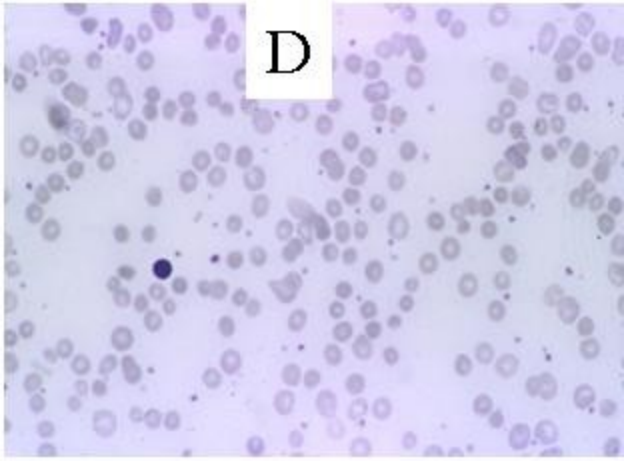


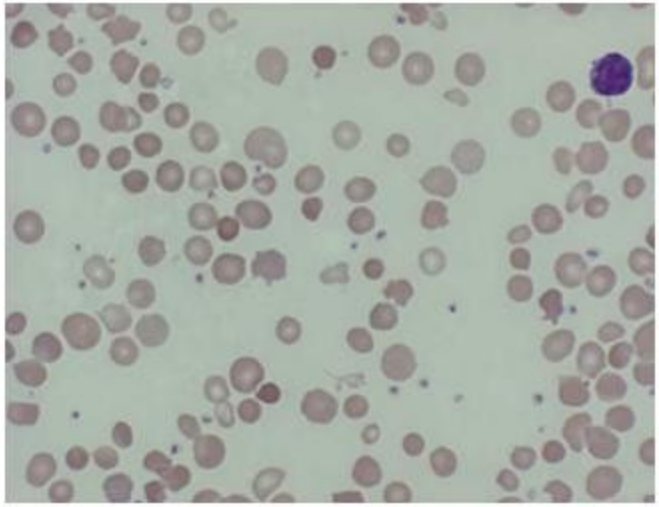


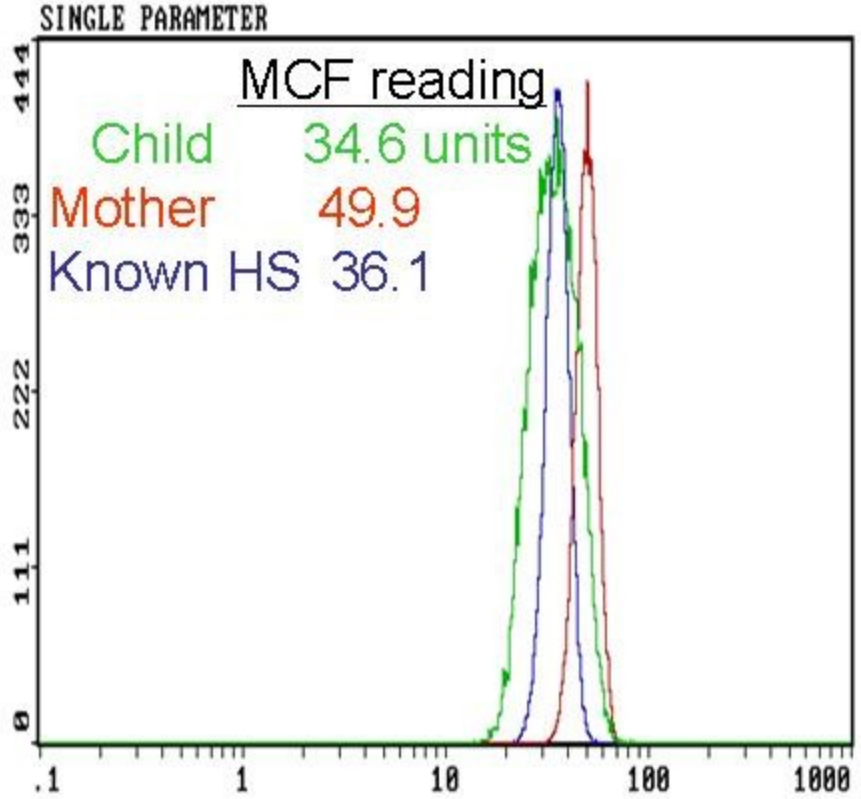
What are these blood films?
A = ?
B = ?
C = ?
D = ?

HS (Recessive)
HS (Typical)
HE / HPP
DAT(+) RBCs









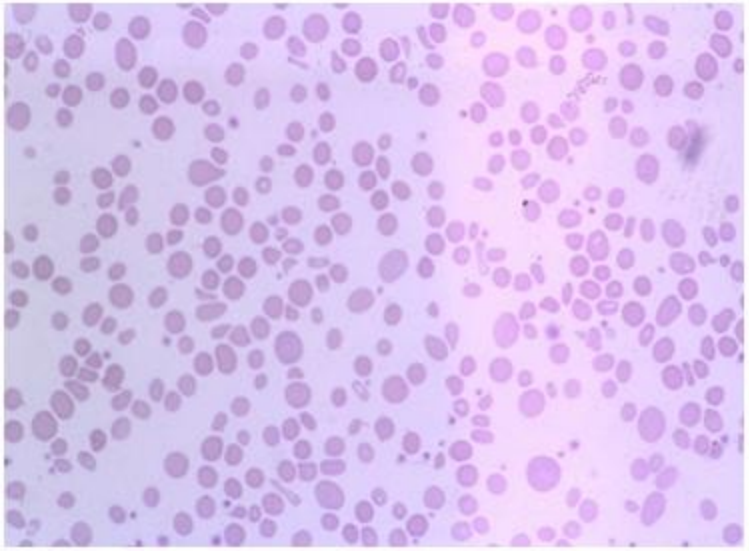
Child : age 6 months (Caucasian)
Blood film: poikilocytosis and red cell fragmentation, spherocytes
No requirement for transfusion
No family history of haemolytic anaemia

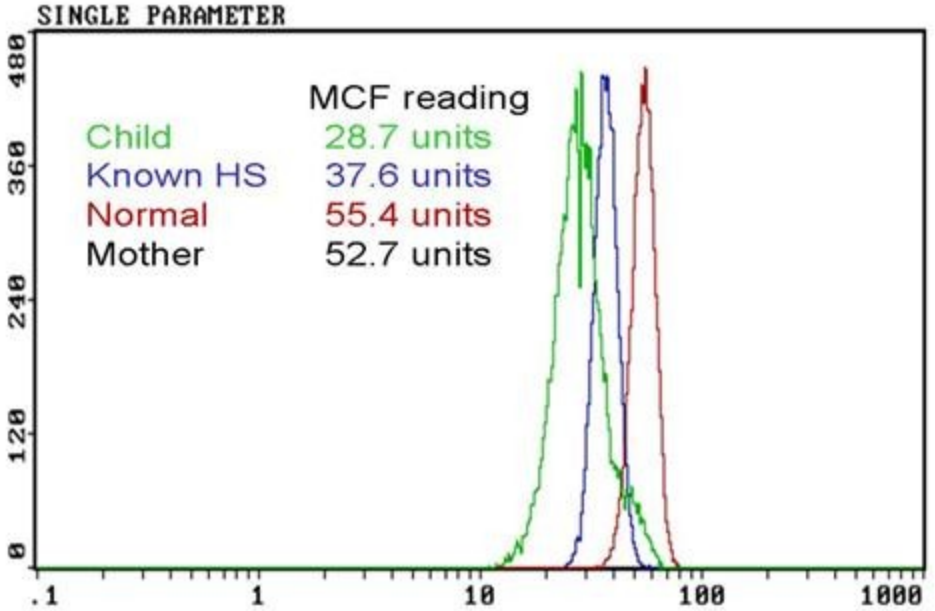
	Child	Mother	Father
Hb:	84.0 g/L	143.0	149.0
MCV:	*71.3 fL*	*89.4*	*82.1*
MCHC:	343.0 g/L	328.0	337.0
MCH:	24.4 pg	29.3	27.7
RDW:	29.7 %	12.6	13.5
Retics:	12.6 %	1.8	1.2

- SDS-PAGE:
  Child: α and β spectrin reduction
  Parents: normal membrane proteins

- PCR for Spα^{LEPRA} polymorphism
  Spα^{LEPRA} allele found in child

Neqas 2014 (mjk)





Child: age 7 months (Afro-Caribbean)

	Child	Mother
Hb:	107.0	112.0 g/L
MCV:	*47.7*	83.4 fL
MCHC:	324.0	340.0 g/L
MCH:	15.4	28.3 pg
RDW:	32.4	17.3 %
Retics:	5.7	1.6 %
Sp dimer	47.8%	36.0 %
Sp$\alpha^{I/46\text{-}50}$	Homozygote	Heterozygote

Neqas 2014 (mjk)

Family L

Father and daughter (D) have occasional spherocytes
Son (1, index) has raised bilirubin and jaundice. **?HS**

	Father	Mother	Son (1)	Son (2)	D	Normal Range
Os Fragility	6.0		5.3	5.0	5.3	4.65 - 5.90
EMA	45.1	51.7	47.7	47.0	48.1	51.5 - 56.5
Hb (g/L)	161		140	150	142	
MCV (fL)	84.0		78.0	80.0	82.0	
MCHC (g/L)	342		355	348	346	
Retic. count	71.0		31.0	49.0	42.0	
RDW (%)	11.8		12.1	11.5	11.6	

Grandfather: diagnosed to have HS in his late 50's

Paternal uncle: splenectomised and his children have HS.

Comment: useful to exclude Gilbert's syndrome in son (1)

Father and children are likely to have a mild HS

Question: is the finding of mild HS clinically significant?

Red blood cells giving high MCF readings

	EMA (MCF units)	Hb (g/L)	MCV (fL)	MCH (pg)
RBCs with high MCV (mainly alcoholics, n = 16)	61.2±3.9		108±5.2	
PK deficiency	70.0	83	112.0	36.1
	70.7	107	118.0	37.0
CDA type I	70.6	106	112.7	36.1
	64.5	114	130.0	42.2
Immune HA	[a] 73.0	47	130.0	37.3
	[b] 60.2	56	108.6	33.9
Dehydrated HSt	63.8	148	99.0	-
Overhydrated HSt	70.4	111	120.0	36.7
	68.2	109	116.5	39.5

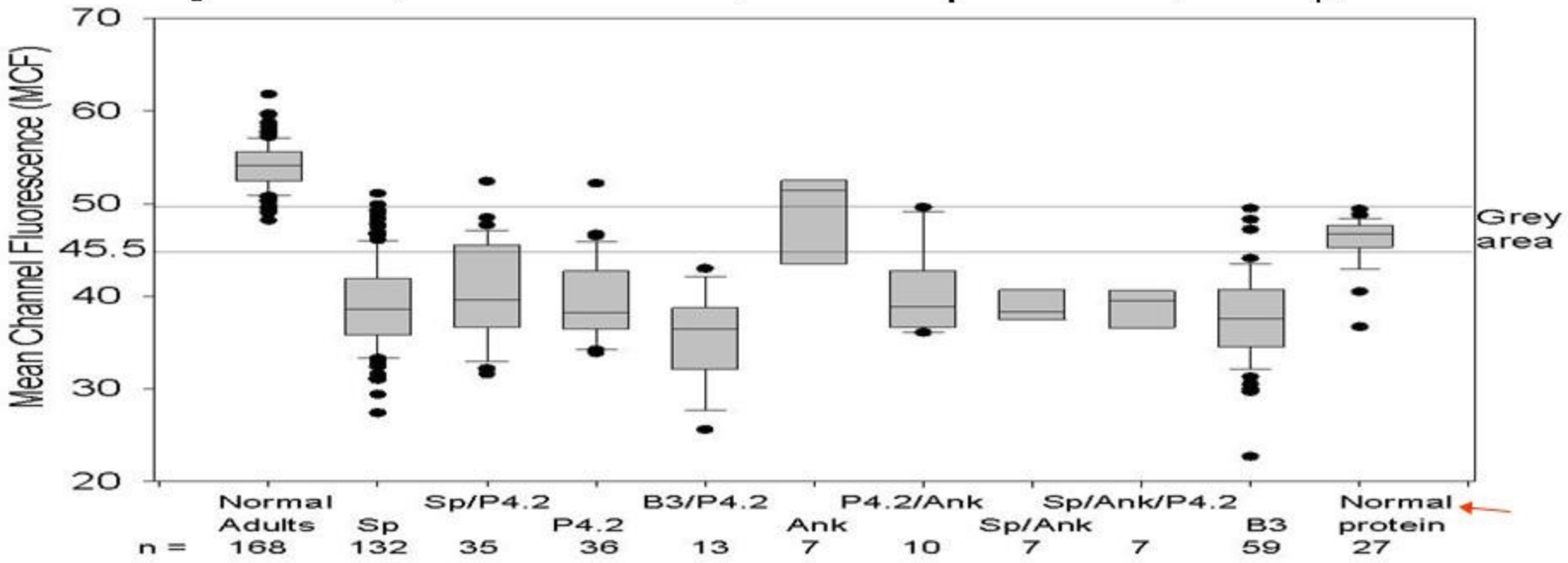
[a]:HDN (anti-D); [b]: cold agglutinin (CMV)

Macrocytosis → ↑ fluorescence

Membrane protein deficiencies (SDS-PAGE)

	Milan			Naples	
	Bristol	+ spleen	Splenectomy	Families	Patients
n	333	259	41	220	580
Spectrin	39%	31%	41%	15%	16%
Ankyrin	6%	3%	10%	60%	56%
Band 3	22%	54%	46%	17%	19%
P4.2	11%	1%	0%	0.4%	0.2%
Normal prot.	8%	11%	3%	8%	9%
Sp/P4.2	11%	n/a	n/a	n/a	n/a

EMA Binding results for HS subsets (based on protein deficiency)

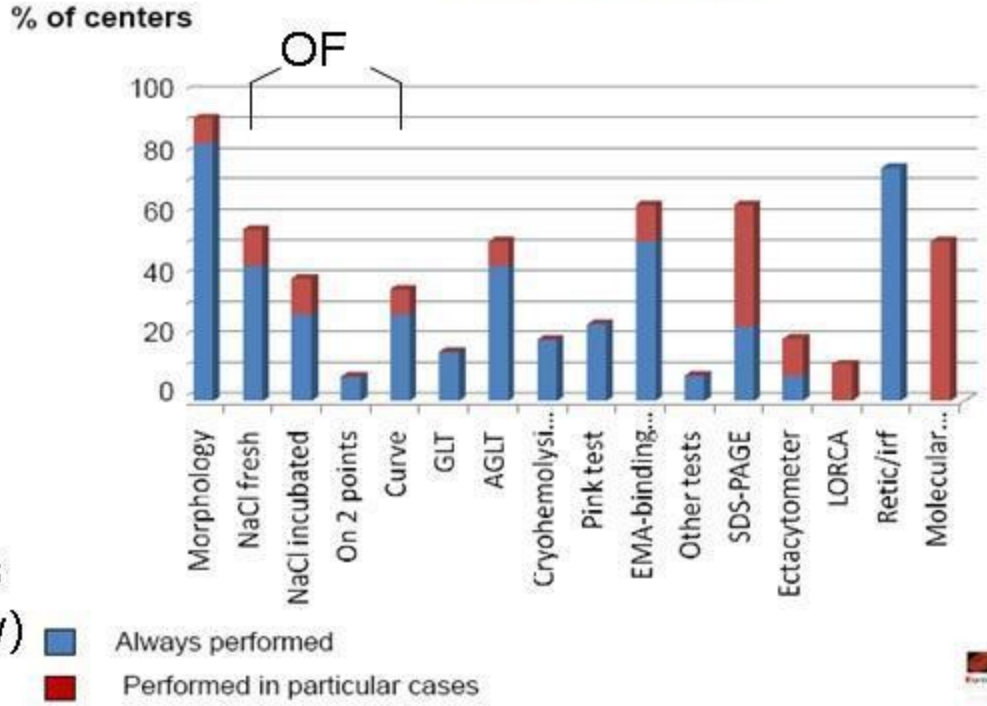


enerca

European Network for Rare and Congenital Anaemias

Survey on red cell membrane disorders and enzyme defects
Centres involved: 25

Use of diagnostic tests performed for diagnosis of red cell membrane defects



enerca

ENERCA III project
Task group 6: Membranopathies
Prof. Alberto Zanella (Milan, Italy)
Paola Bianchi (Milan, Italy)

Survey results e-mailed to all participants in August 2012

Aim: an overview of diagnostic tests used for the diagnosis of RBC membrane disorders

Final aim to establish ENERCA diagnostic recommendations

Comments:

- RBC morphology: 1st step for most centers
- 44% use Osmotic Fragility (OF)
- 50% of centers perform AGLT
- 60% of centers (15/25) perform the EMA-Binding test in all cases with **suspected hemolytic anemia**
- 5 centers use ektacytometry or LORCA (laser-assisted optical rotational cell analyser)
- 50% centers use SDS-PAGE and/or molecular biology for atypical or severe cases as a 2nd step

Interpretation of results

	Cytometer 1	Cytometer 2	Cytometer 3
Normal adults	11861.5 ± 883.51 (n = 20)	53.9 ± 3.2 (n = 180)	478.3 ± 44.4 (n = 110)
HS range	7949.3 ±1304.1 (n =20)	36.8 ±4.2 (n= 41)	340.4 ±33.9 (n= 58)
Cut-off HS and Normal	10126.0	45.5	400.0

Disadvantage: difficult to compare test results from different models of flow cytometer

Harmonisation of Result Presentation (EMA Binding Test)

Hunt *et al.* (2014) Cytometry B DOI:10.1002/cyto.b.21187

	FC500	Canto II
Mean ratio (HS, n= 43)	0.782 (SD = 0.086)	0.774 (SD = 0.085)
Optimal Cut-off ratio	0.918	0.925
Specificity	98.7%	97.1%
Sensitivity	95.6%	100%
Gray area ratio	0.868 – 0.918.	0.859 – 0.925

Summary III

Laboratory Diagnosis of Membrane Disorders

Initial Assessment (Clinical/Laboratory Interface)

- **Clinical history**: chronic: ? since birth
  acute: ? Recent, post-infection
- **Family history and ethnicity**
- **Evidence of haemolytic anaemia**
  - **Blood transfusion history**
  - **splenomegaly, gallstones**
- **RBC morphology**
- **Other laboratory tests results**:
  exclusion of other haemolytic anaemias;
  $[K^+]$ in U/E

Screening tests

optional

- Osmotic fragility (OF) Test/ EMA
- Acid Glycerol Lysis Time (AGLT) Test/EMA
- Ektacytometry/ EMA
- EMA Binding Test
- Cryohemolysis test

SDS-PAGE

optional

- protein deficiency
- abnormal band 3 (CDA II)

Molecular testing (cDNA or gDNA sequencing of protein genes)