Hôpital Erasme

ULB

DIAGNOSIS OF HEREDITARY SPHEROCYTOSIS

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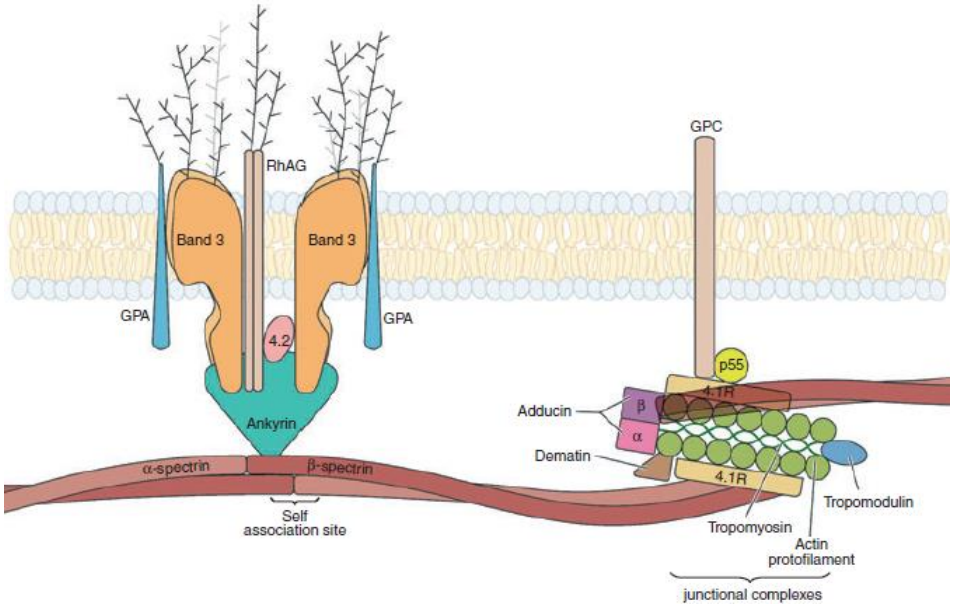
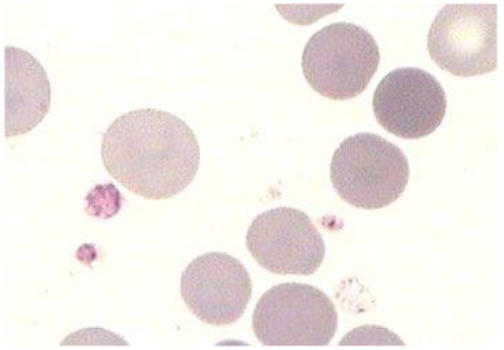
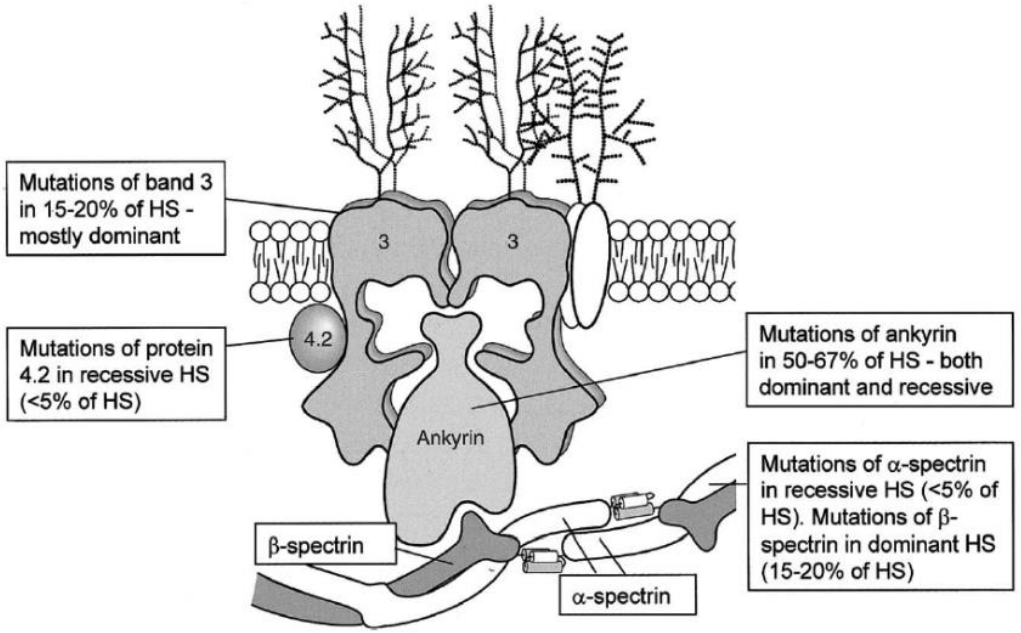
Clinical Biology

24/09/2015

RBC MEMBRANE PROTEINS AND HEREDITARY SPHEROCYTOSIS (HS)







Incidence: 1/2000-1/5000

AD inheritance (2/3); de novo mutations (1/3) or truly AR in 10%

Protein	Gene	Chromosome localisation
Spectrin Ŭ-chain	*SPTA1*	1q22-q23
Spectrin ɓ-chain	*SPTB*	14q23-q24.2
Ankyrin	*ANK1*	8p11.2
Band 3	*EPB3*	17q12-q21
4.1 protein	*EPB41*	1q33-p34.2
4.2 protein	*ELP42*	15q15-q21
7.2b protein	*EPB72*	9q33-q34

An X, Mohandas N, BJH, 2008

Eber S, Lux S, Semin Hematol, 2004

HS: CLINICAL FEATURES AND DIAGNOSIS

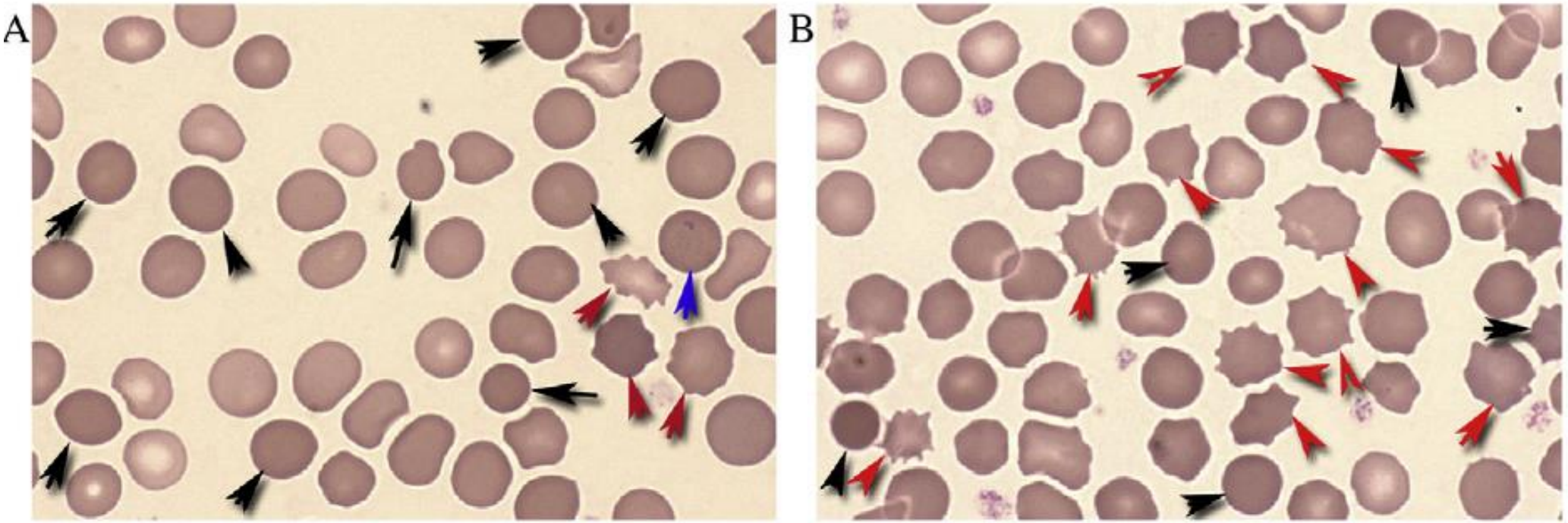
- Heterogeneous group of disorders (clinical severity, protein defect and mode of inheritance)
- Age at diagnosis (neonatal jaundice, after aplastic crisis/ N.B. gallstones and splenomegaly in adult patient)
- Family history (75%)
- Clinical and laboratory features

Parameter	Features
Clinical features	Splenomegaly almost always
Laboratory RBC indices	(↓)Hb, (↑)MCHC, ↑hyperdense cells, ↑RDW, ↑reticulocyte count
Blood film	Abnormal morphology- spherocytes
Direct antiglobulin test	Negative
Evidence of hemolysis	↑bilirubin; reticulocytosis

Guidelines for the diagnosis and management of hereditary spherocytosis-2011 update , BJH, 2011, 156, 37-49

RBC MORPHOLOGY IN HS

L. Da Costa et al. / Blood Reviews 27 (2013) 167–178



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RECHERCHE ET DIAGNOSTIC DE SPHEROCYTOSE HEREDITAIRE

Dépistage : 1 tube **EDTA** 5 ml *(nouveau-né : 1 ml)*
- test de cryohémolyse (code INAMI 553254)
- test à l'éosine-5-maléimide – (facturation au patient : 5 €)

Diagnostic : 1 tube **hépariné** 5 ml *(nouveau-né : 2 ml)* + 1 tube **EDTA**
- electrophorèse des protéines membranaires (facturation au patient : 10 €)
- ektacytométrie : (facturation au patient : 30 €)

- si possible, un **prélèvement des parents** est souhaitable.
- envoyer le(s) prélèvement(s) endéans les 24H (éviter le vendredi) - ne pas centrifuger.

IDENTIFICATION, DONNEES CLINIQUES ET BIOLOGIQUES
*(ou joindre la biologie) DU PATIENT (**en gras = données indispensables**)*

Nom			
Prénom			
Date de naissance	/ /	**Date de prélèvement**	/ /
Origine géographique	○ Caucasienne ○ Africaine ○ Asiatique ○ Inconnue	**Date de biologie**	/ /
Sexe	○ M ○ F	Bilirubine totale	mg/dL
Histoire familiale	○ oui ○ non	Bilirubine non conj.	mg/dL
Anémie hémolytique	○ oui ○ non	LDH (valeurs de réf.)	U/L
Splénomégalie	○ oui ○ non	Haptoglobine	mg/dL
Lithiase biliaire	○ oui ○ non	Hémoglobine	g/dL
Diabète	○ oui ○ non	GR	$10^6/mm^3$
Ictère néonatal	○ oui ○ non	MCV	fL
Transfusion ? O / N (date de la dernière transfusion :……..)		MCHC	g/dL
Traitement :		MCH	pg
		RDW	%
Commentaire – Lien de parenté		Réticulocytes	/mm³ (val. absolue)
		Morphologie GR	

Version 1- juillet 2013

METHODS FOR LABORATORY DIAGNOSIS OF HS

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- **Screening (+ family history and typical clinical features)**
  - First line
    - RBC morphology on blood smear
    - Hematology parameters
    - Biochemical hemolysis parameters
  - Second line (reduced area-to-volume ratio, increased osmotic fragility)
    - Hypertonic cryohemolysis, acid glycerol lysis test, osmotic fragility test, pink test
    - Eosine-5-maleimide binding
- **Diagnosis**
  - ❑ SDS-PAGE
  - ❑ Ektacytometry with osmotic resistance measurement
  - ❑ Molecular analysis

To increase the negative and positive predictive values of the tests, associate two screening tests, for example the EMA binding test and the acidified glycerol lysis test (Bianchi *et al*, 2012)

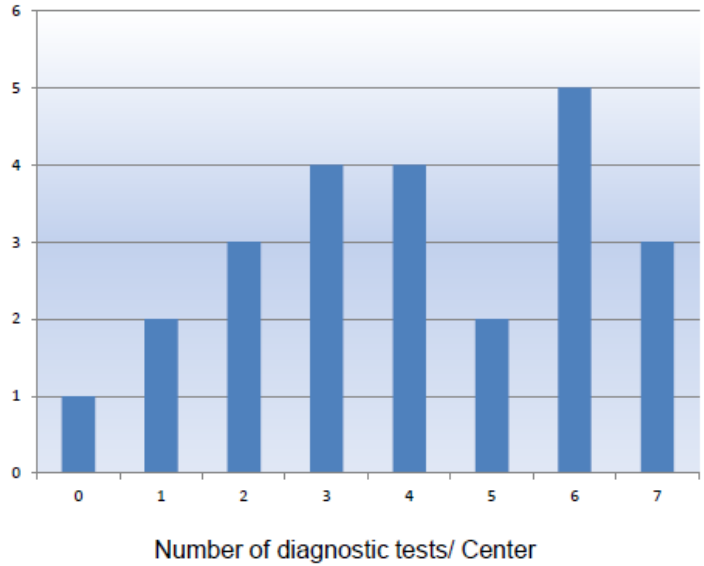
METHODS FOR LABORATORY DIAGNOSIS OF HS

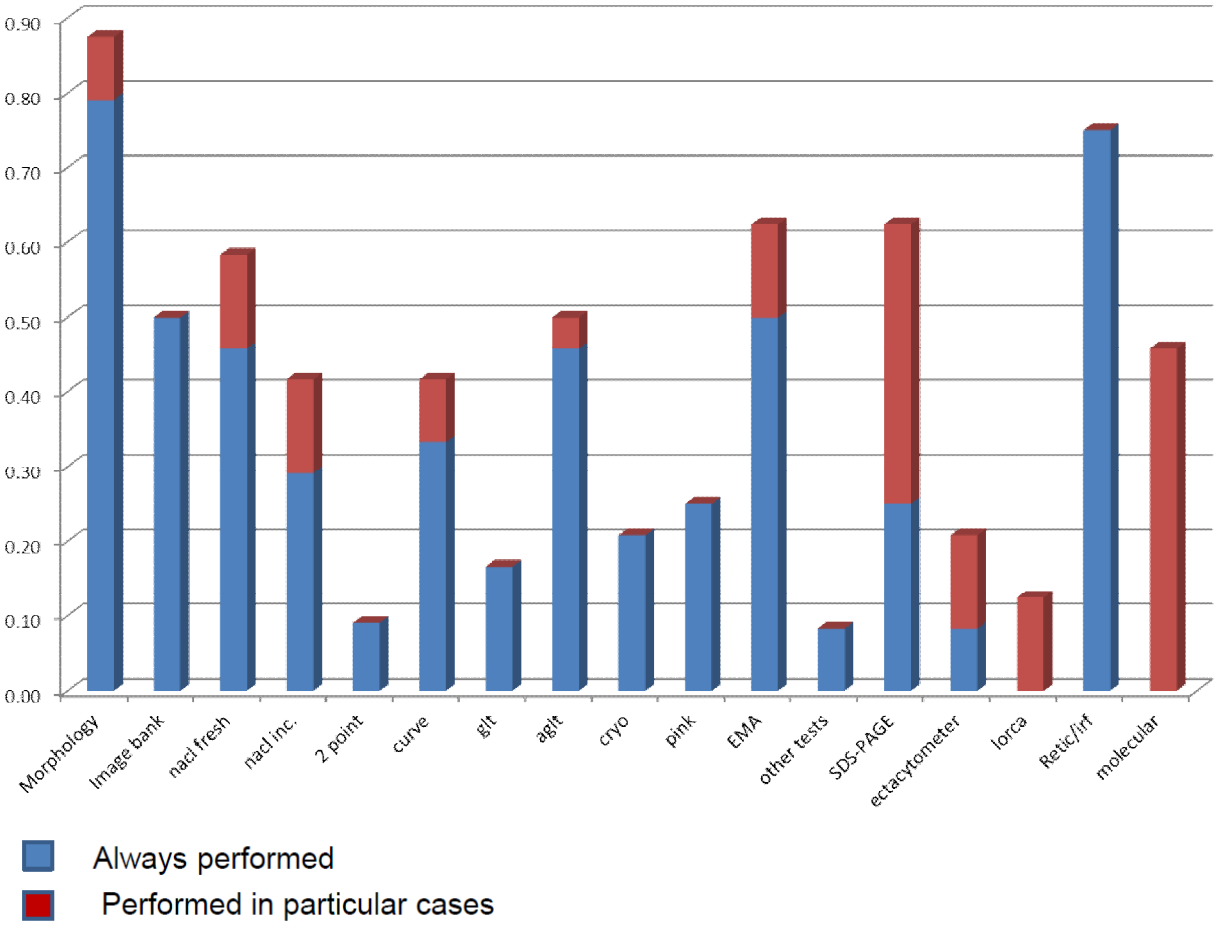
- **European survey on red cell membrane disorders and enzyme defects**

  ENERCA 2013

enerca
European Network for Rare and Congenital Anaemias

Survey on red cell membrane disorders and enzyme defects:
Centres involved: 24





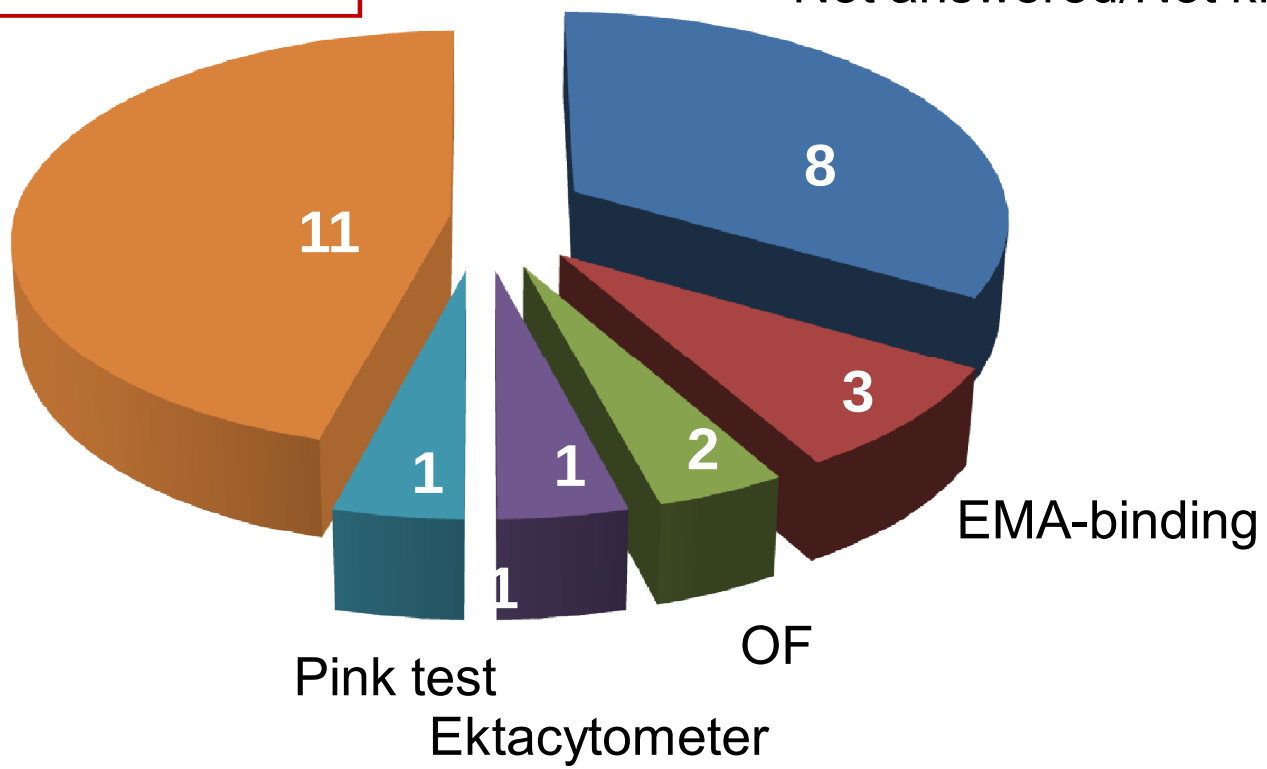
Excluding morphology and hematologic parameters almost always perfomed and molecular characterization always used only in particular cases.

- **RBC morphology: 1st step**
- **44% osmotic fragility**
- **50% AGLT**
- **60% (15/25) EMA-binding in all cases with suspected hemolytic anemia**
- **5 centers use ektacytometry**
- **50% SDS-PAGE and/or molecular biology for atypical cases as a 2nd step**

Method with best specificity and sensitivity

Combination of tests

RBC Morphology +EMA
EMA+AGLT
AGLT+ Cryo
RIA+EMA+AGLT
OF +EMA+Cryo
Cryo+EMA+SDS
EMA+pink+OF
RBC Morphology + Pink
RIA+AGLT+OF
EMA+AGLT+SDS



- ✓ 50% of centers use 4 to 6 different tests
- ✓ Only 3 centers use 1 or 2 tests for diagnostic work-up
- ✓ **No consensus on the best test (or combination of tests) to use** in the **screening/diagnosis** of red cell membrane disorders

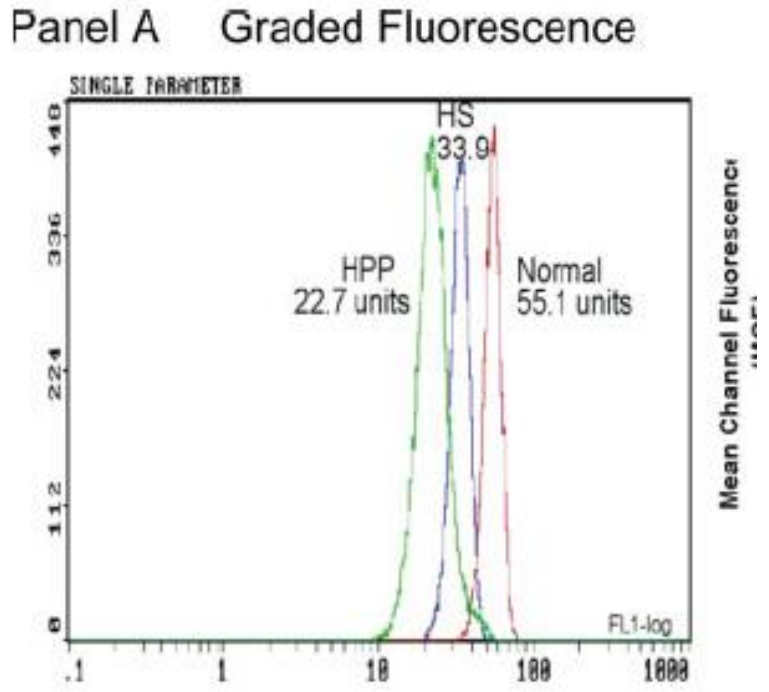
PERFORMANCE OF SCREENING TESTS

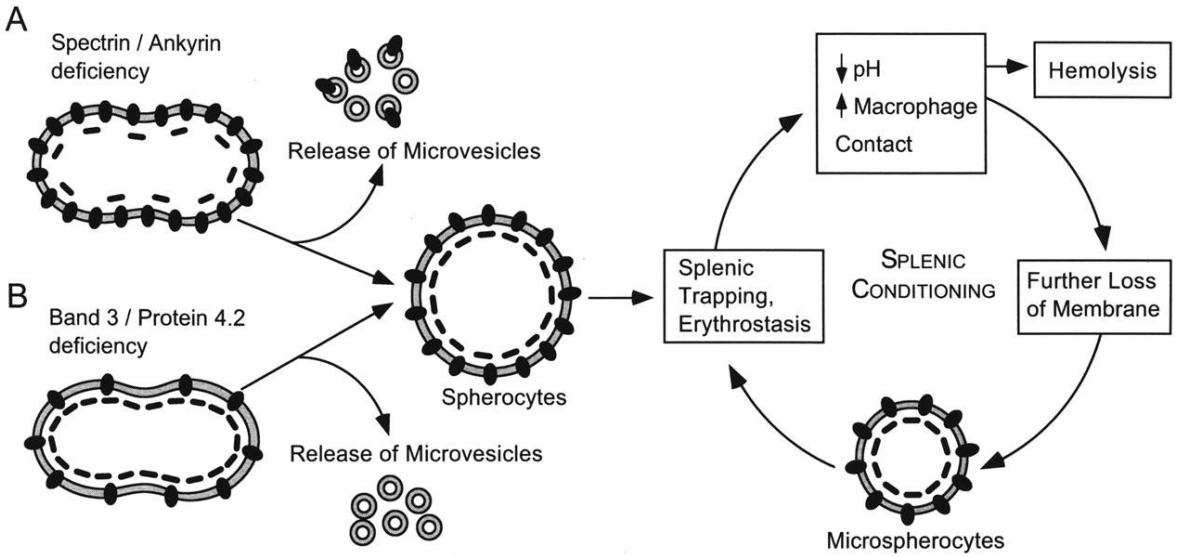
Test	Sensitivity	Specificity	Reference(s)
Osmotic Fragility (OF)	Fresh = 68 % 24 h Incubn = 81 %	Not given Not given	Bianchi *et al*. 2012
Compensated HS:	Fresh = 53 % 24 h Incubn = 64 %	Not given	
Acid Glycerol Lysis Time (AGLT)	95%	Not given	Bianchi *et al*. 2012
Cryohemolysis	100 %	86 %	Streichman *et al* (1990)
EMA Binding	89 % - 99.1%	92.7 % - 99.1%	King *et al*. (2000) Stoya *et al* (2006) Girodon *et al*. (2007) Bianchi *et al* (2012)

References: Bianchi *et al*. (2012) Haematologica **97**, 516
Streichman et al. (1990) Am J Hematol. **35**, 104. Stoya *et al*. (2006) Acta Haematol. **116**, 185.
King et al. (2000) Br J Haematol. **111**, 924. Girodon *et al*. (2007) Br J Haematol. **140**, 464.

EMA

Eosin-5-maleimide





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	EMA Binding test (Data from Bicêtre, Bristol, Mayo, and Milan laboratories)					
Result for normal controls	Lab (SD)	Instrument	n	[1] MCF(SD)	[2] Ratio (SD)	[3] %Reduction
	Bristol [4]	FC500	120	53.9 (1.9)	1.00 (0.04)	*0 %
		Canto II	120	12412 (638)	1.01 (0.05)	*0 %
	Bicêtre[5]	Navios	391	70.4 (5.2)	1.00 (0.04)	0 % (4%)
	Milan[5]	Canto II	1445	10830 (2290)	1.00 (0.05)	0% (5%)
	Mayo	FACSCaliburs	213	629.2 (34.4)	1.00 (0.05)	* 0%
HS	Reduced fluorescence					
	Lab	Instrument	n	MCF(SD)	Ratio (SD)	%Reduction (SD)
	Bristol[4]	FC500	43	42.9 (4.7)	0.78 (0.09)	* -22%
		Canto II	43	9660 (1098)	0.77 (0.08)	* -23%
	Bicêtre[5]	Navios	228	53.5 (8.4)	0.75 (0.11)	-25% (-11%)
	Milan[5]	Canto II	381	7800 (1900)	0.75 (0.1)	-27% (-10%)
	Mayo	FACSCaliburs	213	444.3 (34.4)	0.71 (0.05)	* -29%

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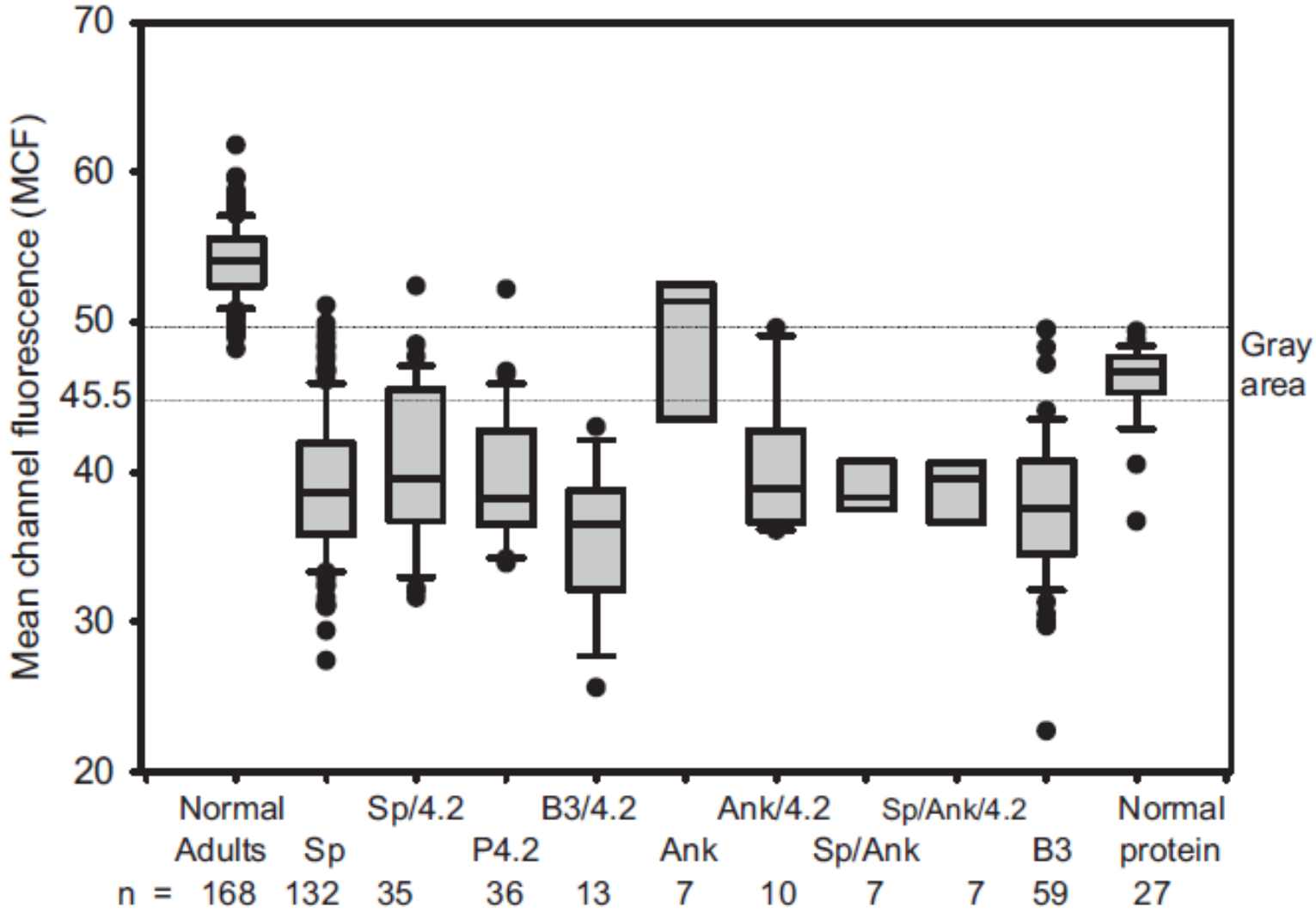

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    - Eosine-5-maleimide binding

Parameter	Instrument	Clinical use	Ref
RBC extended parameters **%Hypo, %Hyper** **%Micro, %Macro; MicroR, MacroR** **LHD%, MAF**	ADVIA 2120 XE 2100 Cell-Dyn Sapphire LH 750, DxH 800	• Restricted erythropoiesis (iron deficiency, beta thalassemia, ACD) • Latent iron deficiency • **Hereditary spherocytosis**	Bovy 2005 Urrechaga 2009, 2011 Maconi 2009 Piva 2010 Ermens 2012, Osta 2012 Rooney 2014, Ng 2014
Immature reticulocyte fraction **IRF**	ADVIA 2120 XE 2100 Cell-Dyn Sapphire Pentra 120 DX LH 750, DxH 800	• Classification of anemias • Early identification of BM regeneration/engraftment • Early monitoring of response to treatment in anemia • **Hereditary spherocytosis**	Buttarello 2002 Torres Gomez 2001, 2003 Noronha 2003
Mean reticulocyte volume **MRV, MCVr**	ADVIA 2120 Pentra 120 DX LH 750 DxH 800	• Diagnosis of iron-deficient erythropoiesis • Early monitoring of response to treatment in anemia • Early signs of erythropoietic recovery • Epo abuse in sports • **Hereditary spherocytosis**	D'Onorfio 1995 Brugnara 1998 Cappelletti 2006 Mullier 2011 Morkis 2014 Lazarova 2014
Reticulocyte Hb content (equivalent) **CHr, Ret He**	ADVIA 2120 XE 2100	• Restricted erythropoiesis (iron deficiency, chronic inflammation) • Latent iron deficiency • Monitoring response to Fe or Epo treatment in CKD	Thomas 2002, 2005 Buttarello 2004 Fishbane 2001 Ullrich 2005 Brugnara 2006 van Santen 2011 Joosten 2013
Reticulocyte distribution width **RDWR-CV (SD), RDWr**	ADVIA 2120 LH 750, DxH 800	• Restricted erythropoiesis (iron deficiency, beta thalassemia, ACD) • **Hereditary spherocytosis**	Oustamanolakis 2011 Lazarova 2014
Other **RSf (red cell size factor)** **MSCV (mean sphered red cell volume)**	LH 750 DxH 800	• Iron deficiency with or without chronic inflammation • **Hereditary spherocytosis**	Urrechaga 2010, Ng 2014 Broseus 2010 Lazarova 2014 Liao 2014

Corata 2014

Not standardized; reference intervals: method-dependent

AUTOMATED RETICULOCYTE PARAMETERS AND HS

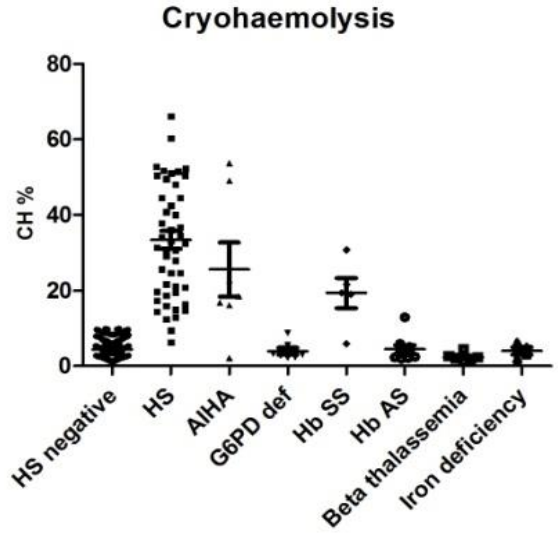
- **Ret/IRF**
  - ↑reticulocytes without an equally ↑IRF: useful in HS diagnosis (Mullier *et al*, 2011)
- **MSCV** mean sphered corpuscular volume (Beckman Coulter):
  - whole RBC population volume; hypo-osmotic conditions of the ghosting solution for Hb leaking out before reticulum definition; RBC swelling
  - **MSCV<MCV**; HS could be suggested with a sensitivity of 100% (Chiron *et al*, 1999, GEN.S. Coulter)
  - **delta (MCV-MSCV)** >9.6 fl, HS suspected and DD with AIHA by anti-globulin test is proposed (Broseus *et al*, 2010, LH 750)

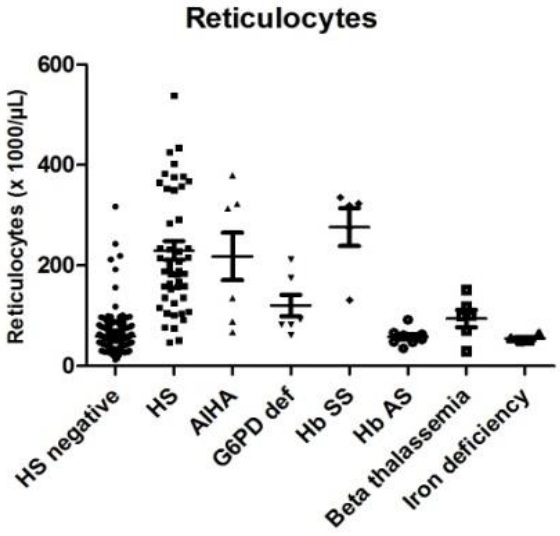
Automated reticulocyte parameters HS: our experience

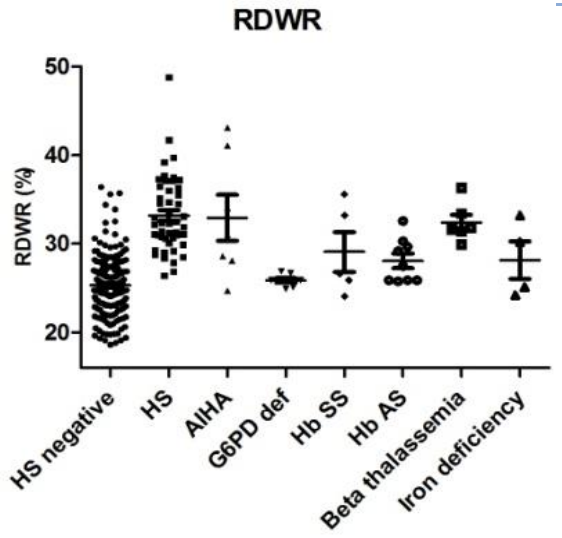
- MSCV, MRV, RDWR and IRF (DxH 800, Beckman Coulter)
  - Reference values for our population
  - Diagnostic performances for HS compared to the cryohaemolysis test
  - Efficiency to differentiate HS from other conditions that affect erythropoiesis

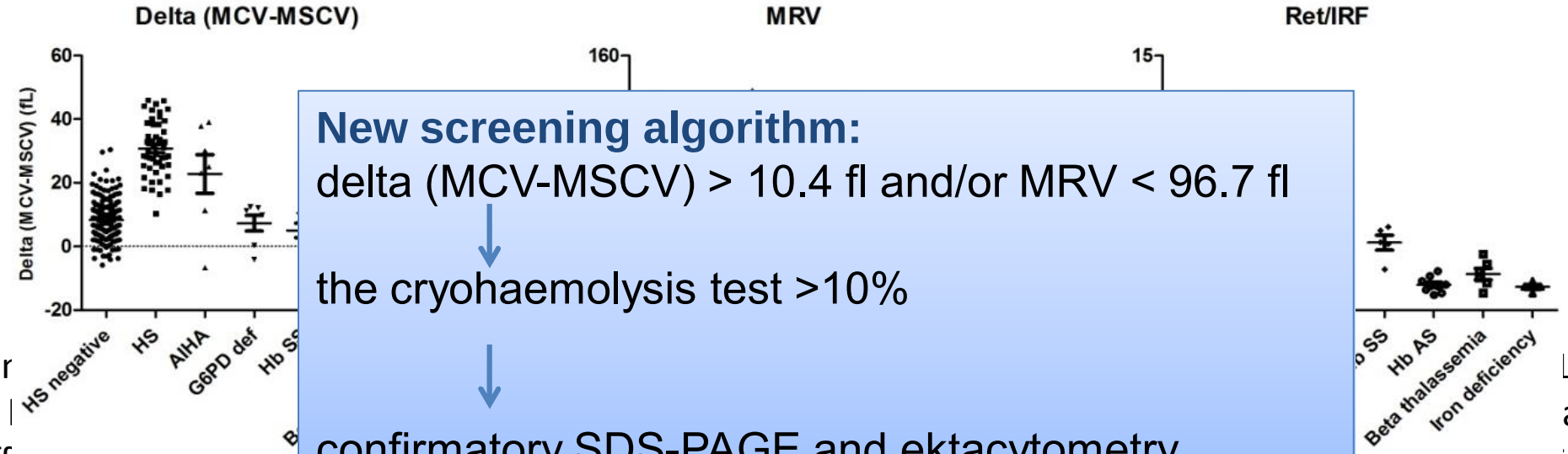
New screening algorithm for HS

HS AND OTHER CONDITIONS AFFECTING THE LEVEL OF ERYTHROPOIESIS









New screening algorithm:

delta (MCV-MSCV) > 10.4 fl and/or MRV < 96.7 fl

↓

the cryohaemolysis test >10%

↓

confirmatory SDS-PAGE and ektacytometry

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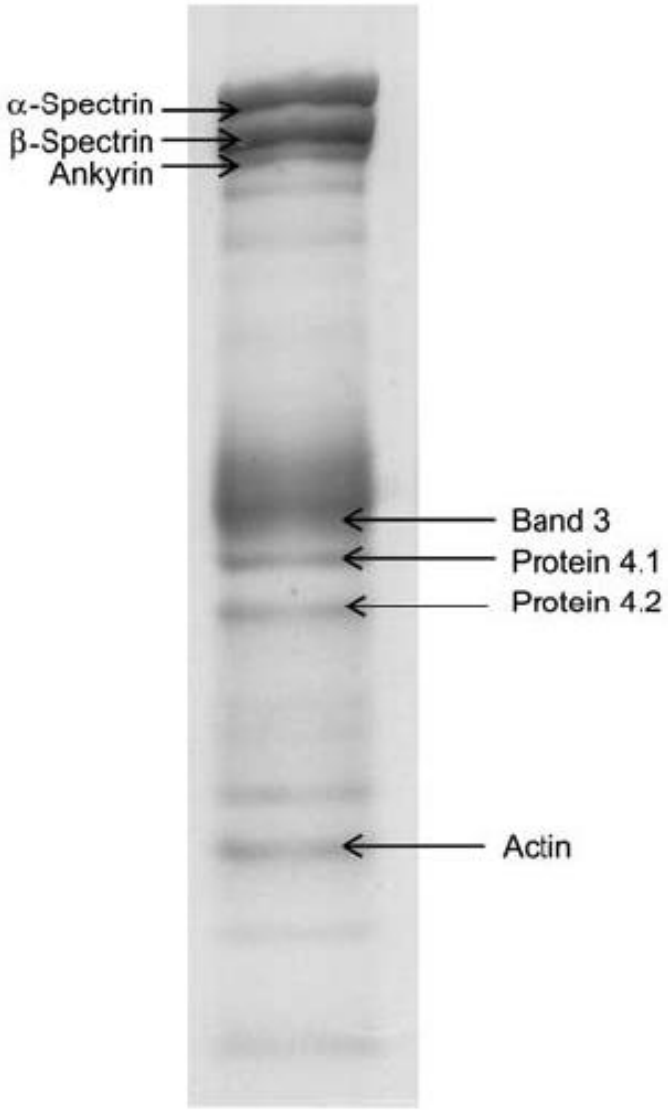
- **Diagnosis**
  - ❑ SDS-PAGE
  - ❑ Ektacytometry with osmotic resistance measurement
  - ❑ Molecular analysis

DIAGNOSTIC TESTS: SDS-PAGE (FAIRBANKS)

- Determines the extent of membrane deficiency
- Lack of sensitivity to very mild 'carrier' HS
- Recommended if:
  - Clinical phenotype more severe than predicted from RBC morphology
  - RBC morphology is more severe than predicted from parental blood film
  - Equivocal or borderline results of the screening test
  - Dg is not clear prior to splenectomy



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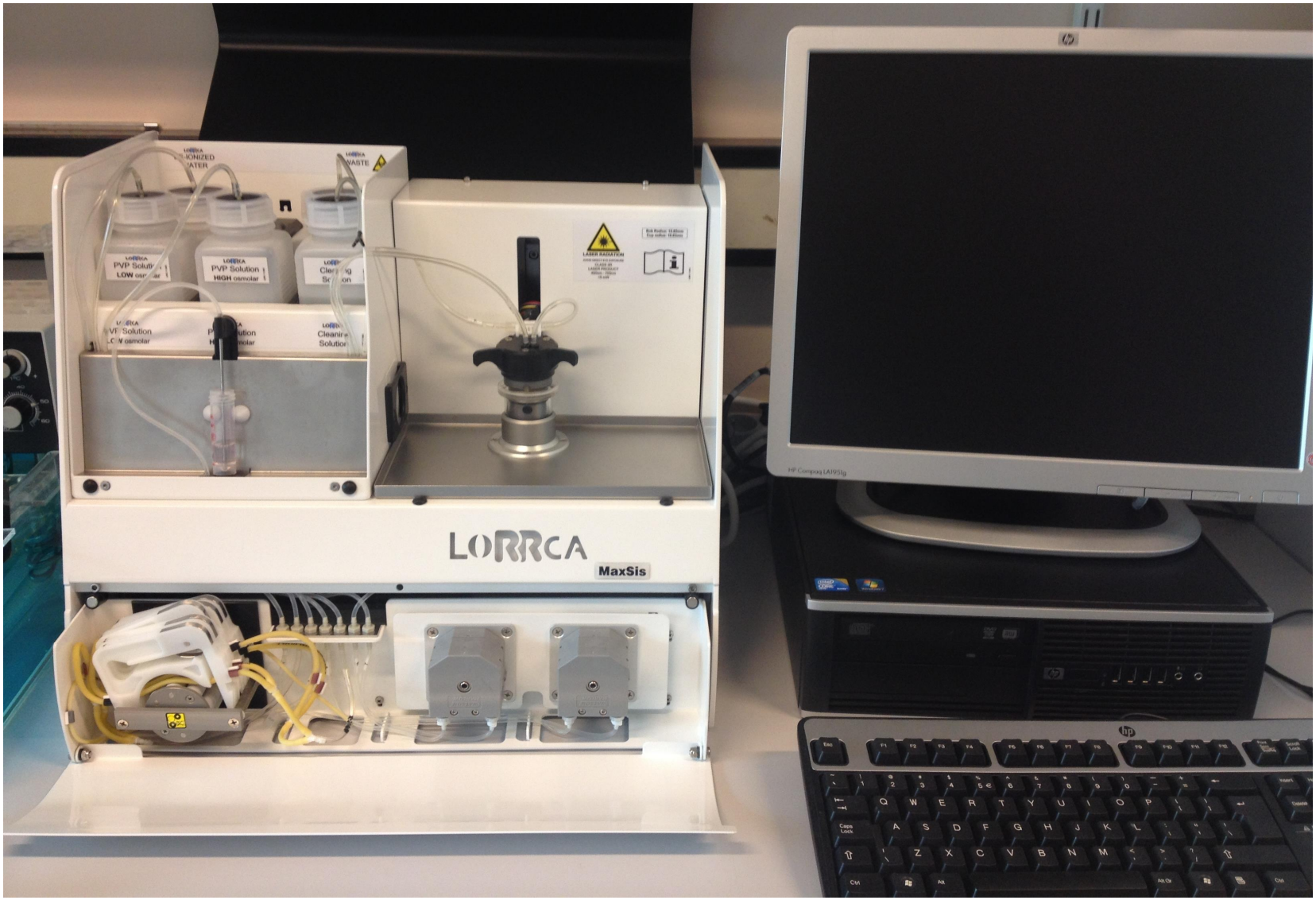
(b)

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

DIAGNOSTIC TESTS: EKTACYTOMETER



DIAGNOSTIC TESTS: EKTACYTOMETER

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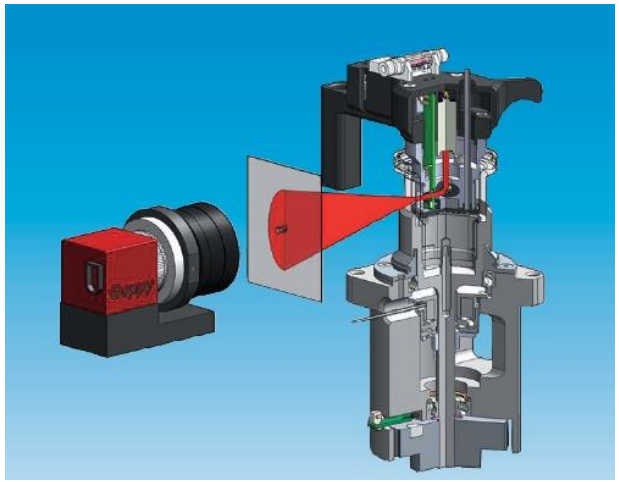
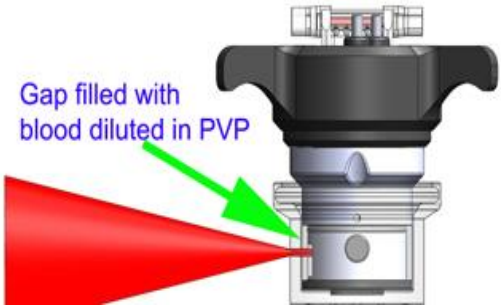
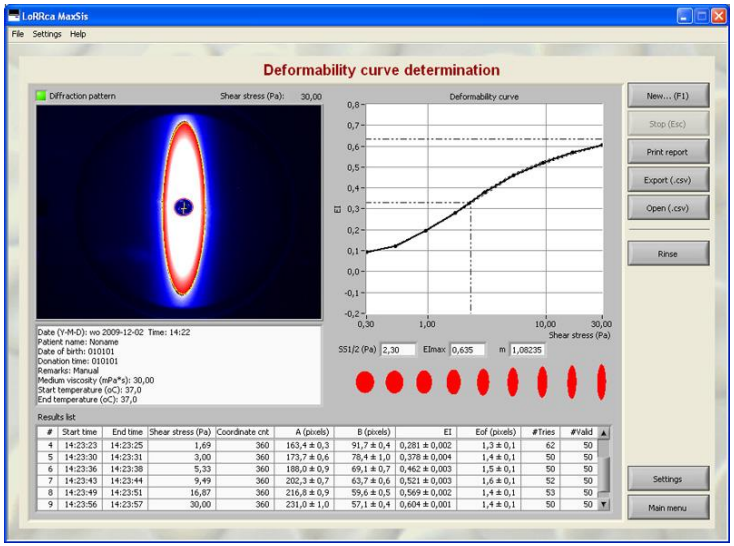
Shear Stress between concentric cylinders, laser beam diffraction measurement

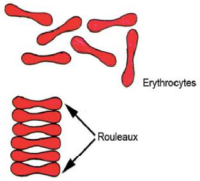
- **RBC Deformability (elongation),** stress dependent
- **Stability**, time dependent

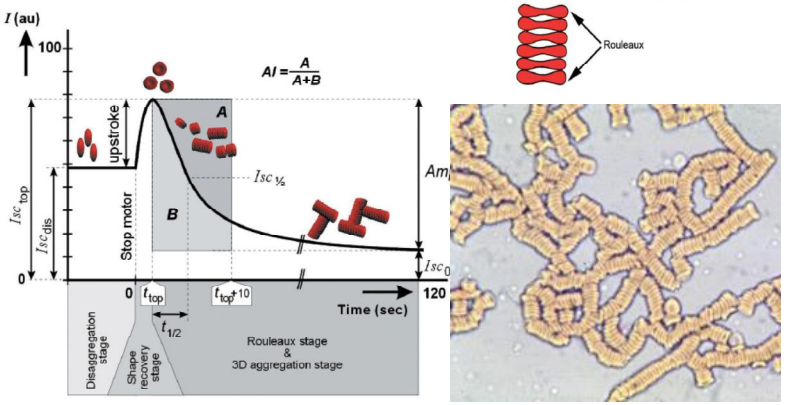


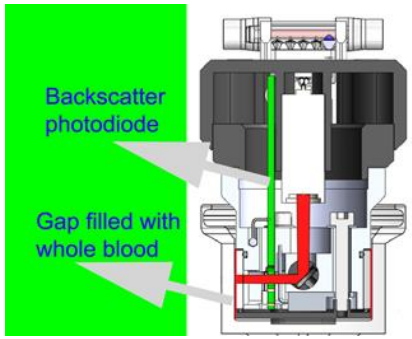


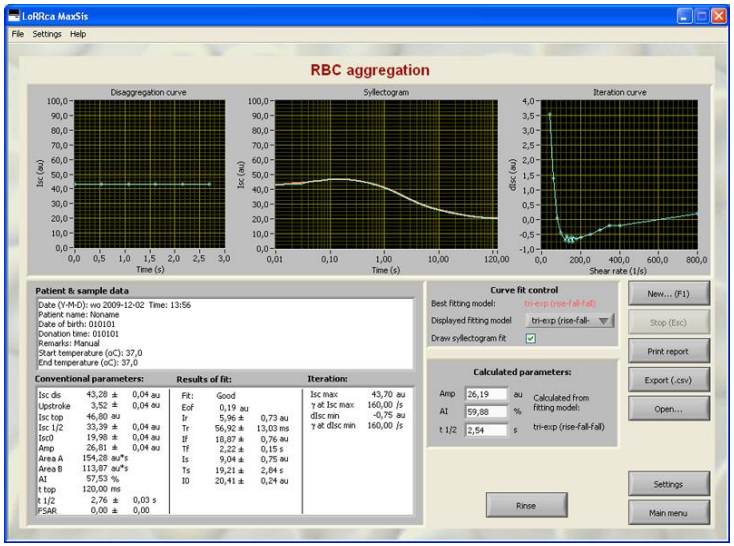


- **RBC aggregation**



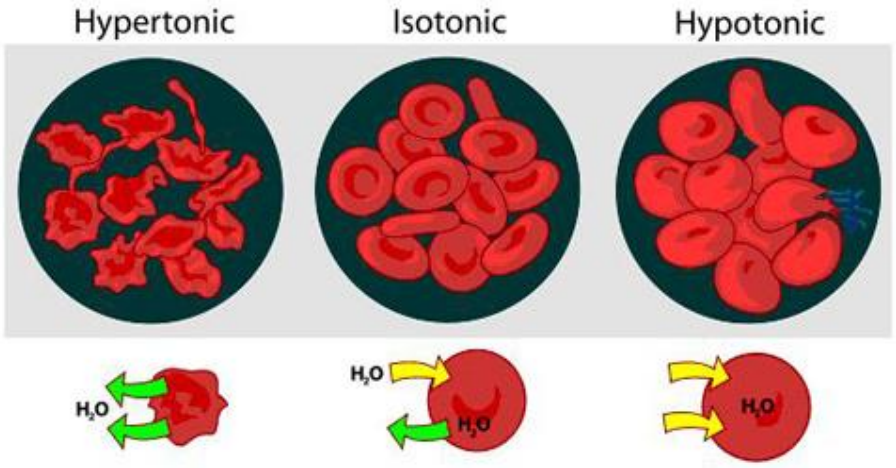


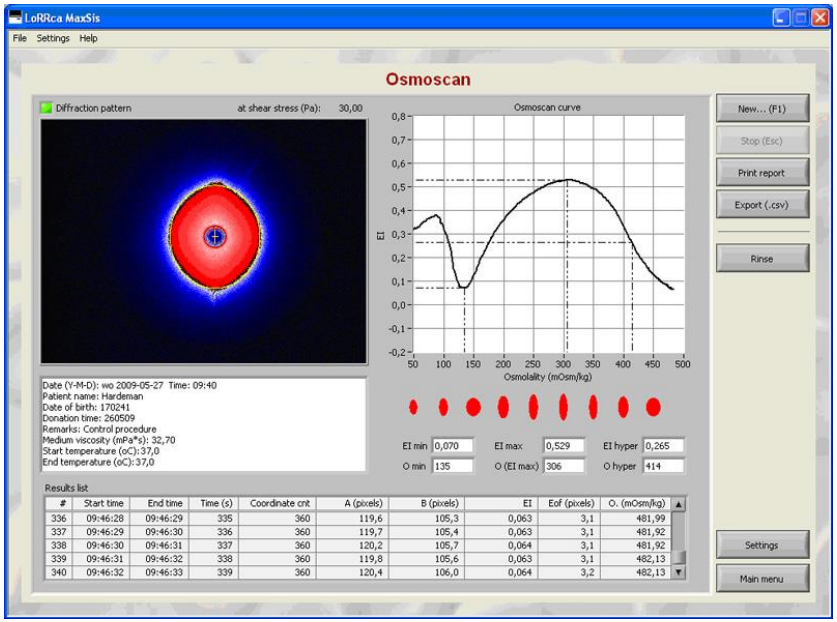


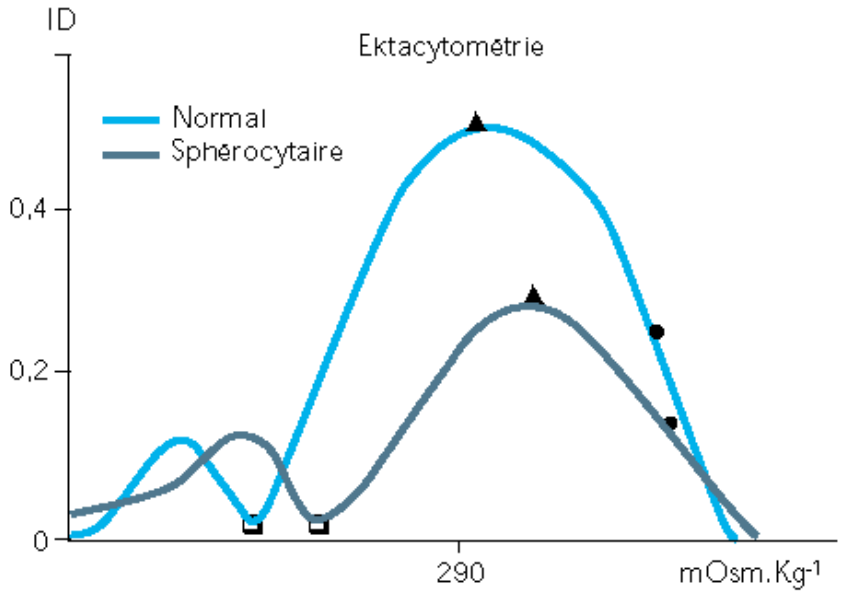


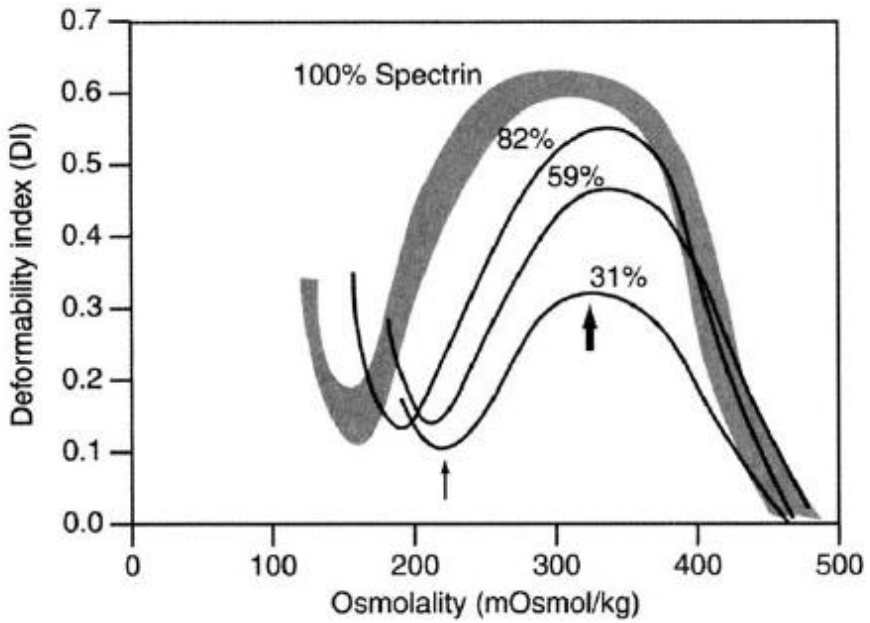
DIAGNOSTIC TESTS: EKTACYTOMETER

- **Osmoscan and RBC deformability**









Eber S, Lux S, Semin Hematol, 2004

OSMOSCAN CURVE

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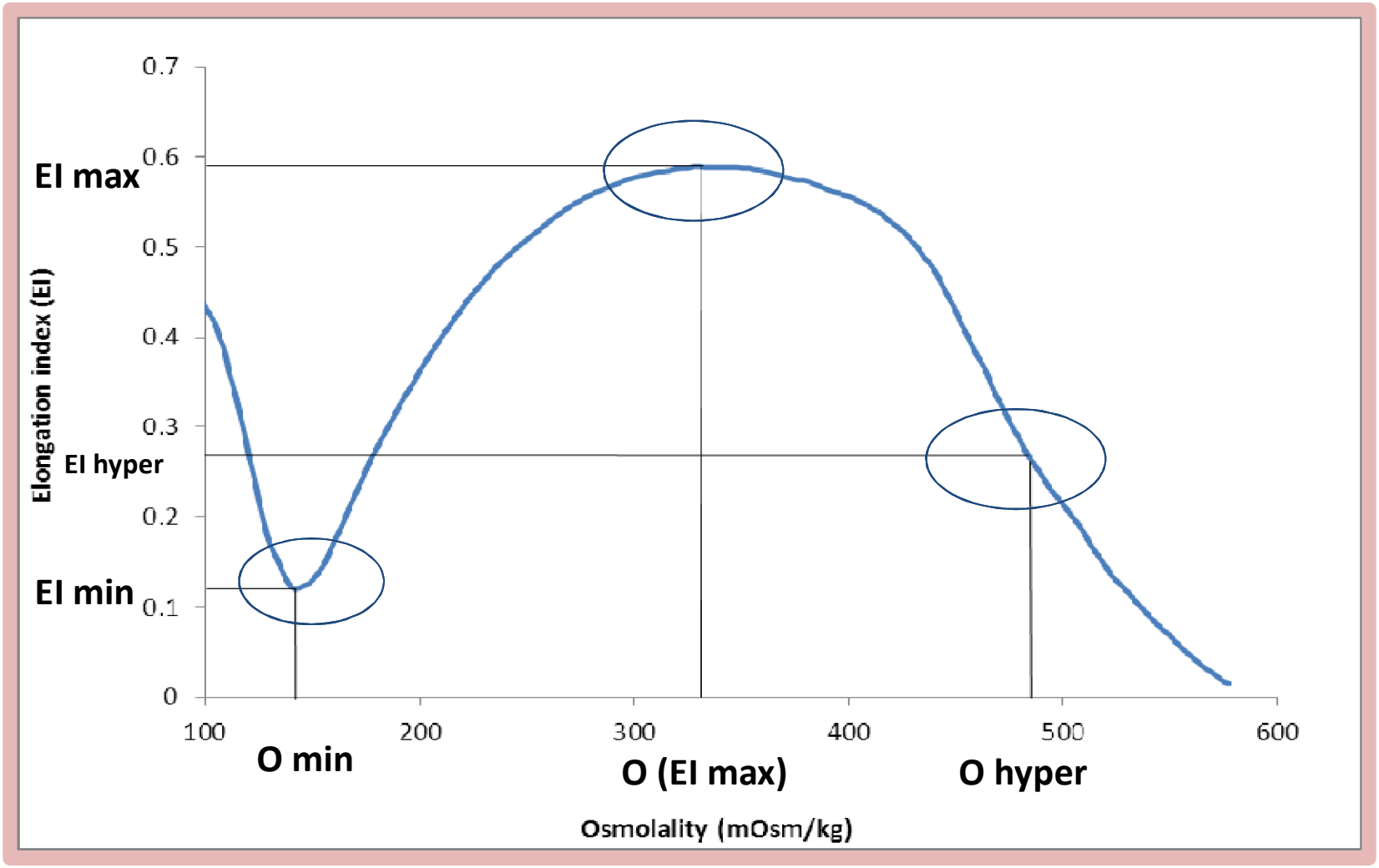
Analysis of the RBC deformability in changing osmotic environment with applied constant sheer stress.



DIAGNOSTIC TESTS: EKTACYTOMETER

Hôpital Erasme ULB

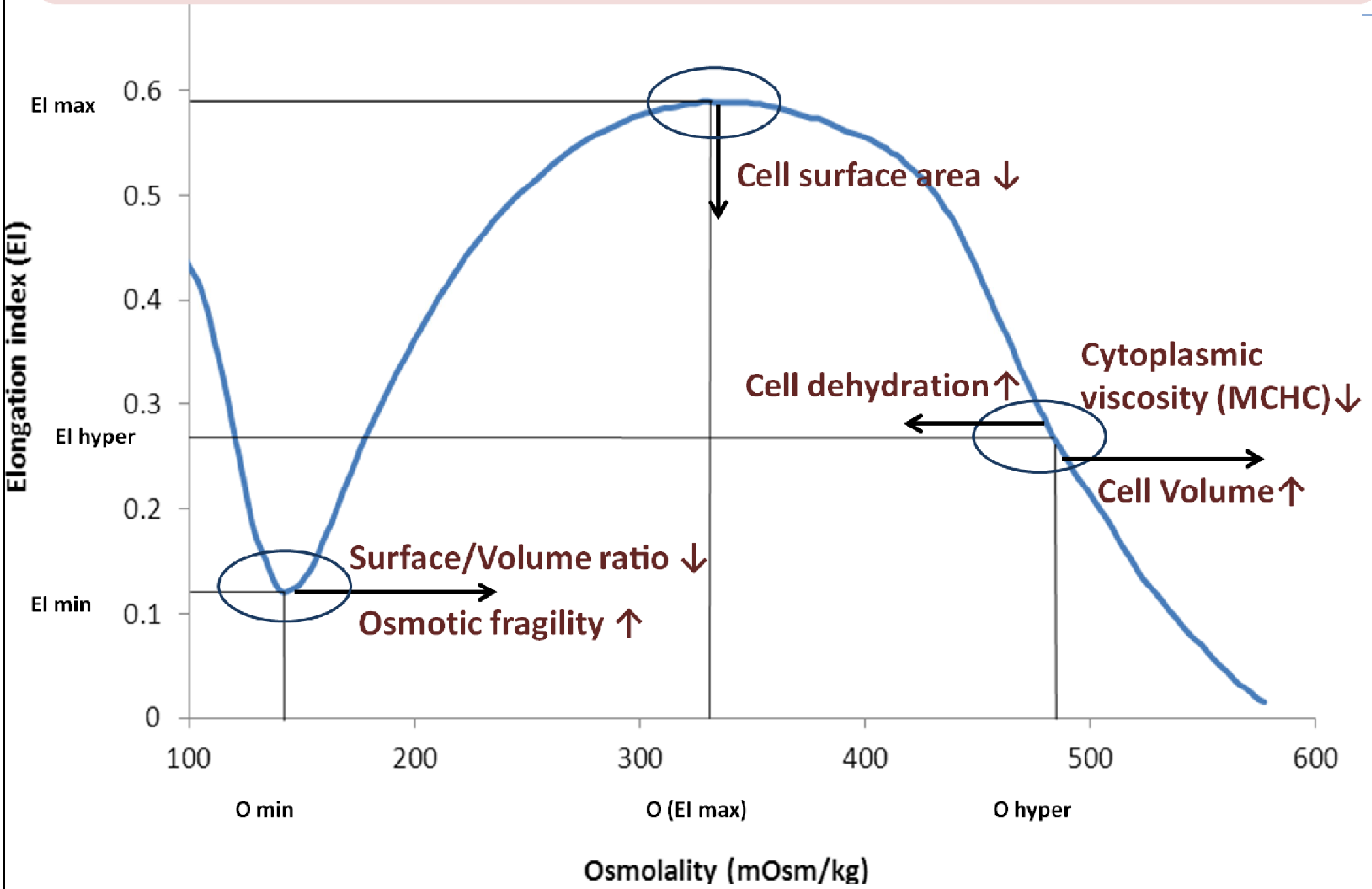
Intra assay variability

%	EI min	Omin	EI max	0 (EI max)	EI hyper	O hyper	Area
CV (5x1)	3	1.2	0.2	1	0.2	1.4	0.3
Mean CV (4x2)	1.2	2.3	0.4	2.2	0.3	0.7	0.3

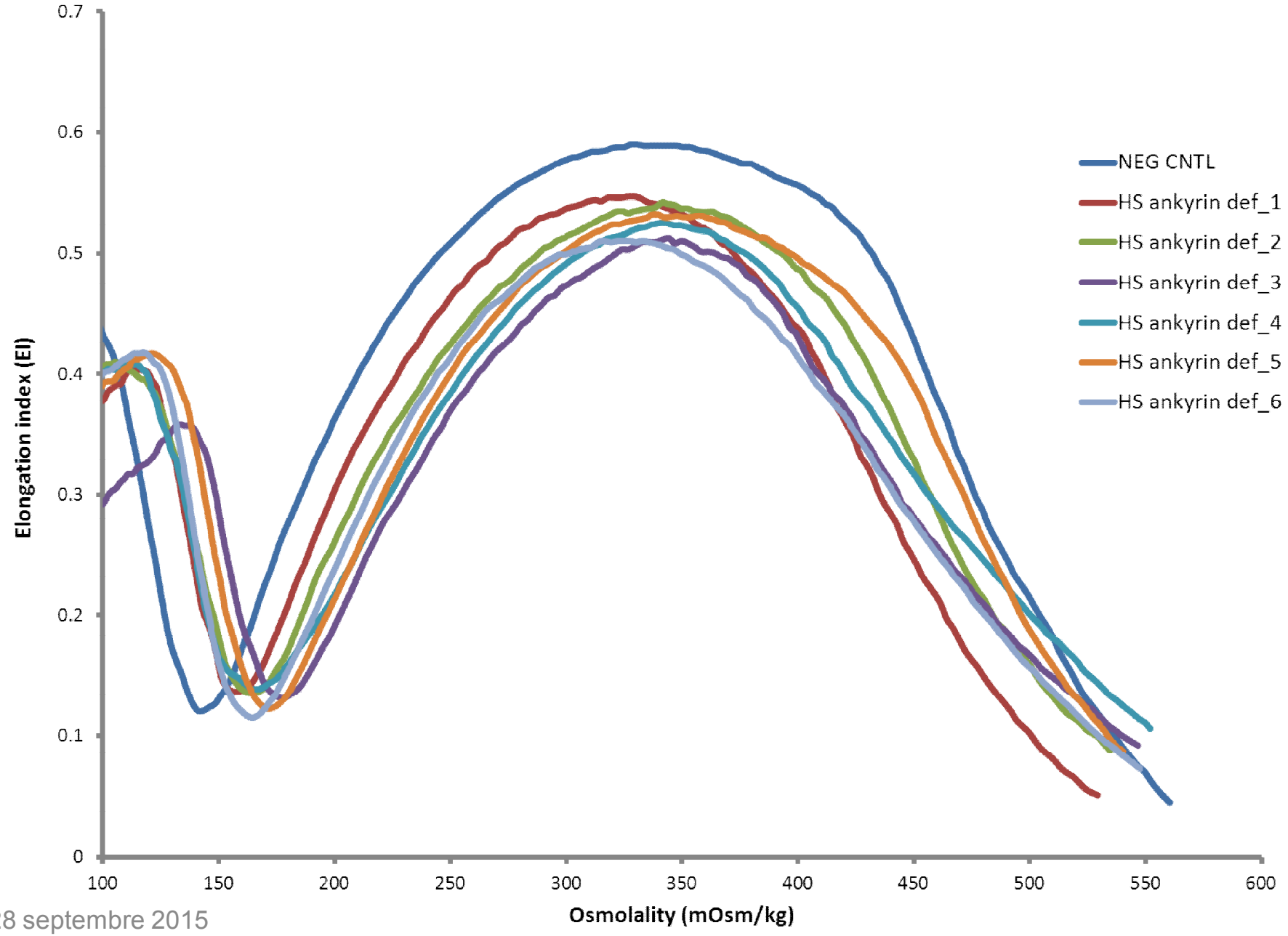
Inter assay variability depending on time of storage, temperature and anticoagulant

Parameter	Stability in days after blood sampling			
	4°C EDTA	4°C ACD	20°C EDTA	20°C ACD
O min	1-5	1-7	1-2	1-7
EI max	1-3	1-7	1-2	1-3
O (EI max)	1-5	1-7	1	1-4
O hyper	1-4	1-7	1	1-2
AUC	1-5	1-7	1-3	1-3

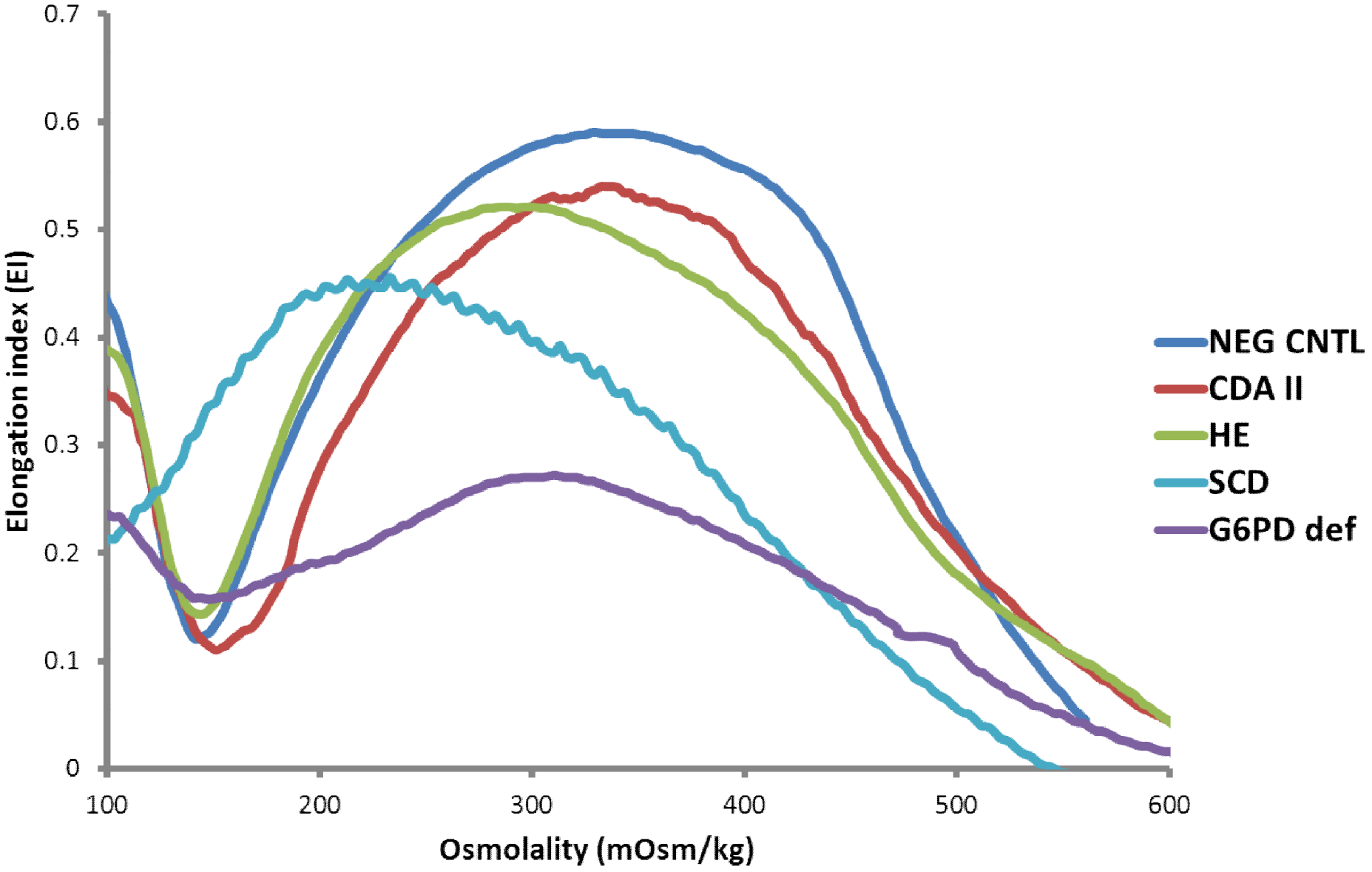
Interpretation of osmoscan shifts in terms of cellular alterations



OSMOSCAN PROFILES OF HS



OSMOSCAN PROFILES OF OTHER RBC PATHOLOGIES



Sample conditions

- EDTA samples are stable during 72h 4°C
- ACD samples are stable during 72h 20°C, and up to one week 4°C

Interpretation

- Typical patterns of osmoscan curves for different RBC pathologies, responding to the patho-physiological mechanisms

Conclusions

- The LoRRca *MaxSis* instrument is of added value for rapid automated HS diagnosis in specialized laboratories

INVESTIGATIONS STEPS FOR DIAGNOSIS OF HEREDITARY SPHEROCYTOSIS

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Clinical features Mode of inheritance Inherited, chronic disease	Family history Individual history neonatal jaundice episodes of anemia and/or acholuric jaundice gallstones splenomegaly
First line laboratory tests Confirm hemolysis Confirm erythropoietic answer Exclude autoimmune anemia Exclude enzymopathy Search for HS characteristics	**Unconjugated bilirubin, haptoglobin, LDH** **Reticulocyte count** **Coombs test** **G6PD, PK and GPI activities*** **Blood smear: spherocytes** **RBC indices: MCHC, MSCV, MRV, IRF**

Gulbis, B., Lazarova, E., Cotton, F., & Ferster, A. (2013) Journal du Pédiatre Belge, 15(4), 258-261

INVESTIGATIONS STEPS FOR DIAGNOSIS OF HEREDITARY SPHEROCYTOSIS

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Second line laboratory tests	
Search for osmotic fragility	Osmotic fragility test not recommended or in combination with other screening tests
Search for RBC membrane protein deficiency	Cryohaemolysis, EMA binding test
Diagnostic test if: • Normal cryohaemolysis or EMA binding test but HS suspected • Absence of family history • Doubtful diagnosis before splenectomy • Heterogeneous clinical expression in relatives • Severe forms of HS	SDS-PAGE and Ektacytometry
Complex and severe clinical situations requesting genetic counselling	DNA analysis

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CONCLUSIONS

- Clinical features, family history
- Laboratory investigation
  - Screening tests (morphology, reticulocyte parameters; EMA, CH)
  - Confirmatory tests (ektacytometry and/or SDS-PAGE)
- Clinician-biologist communication