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Critically Appraised Topic

Optimization of diagnosis Hereditary spherocytosis in general laboratory

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CLINICAL BOTTOM LINE

Hereditary spherocytosis (HS) is the most common congenital hemolytic anemia in Caucasians, affecting approximately 1 in 1000-2000 individuals (Bianchi et al., 2012). The diagnosis of HS is based upon a combination of clinical history, family history, physical examination (splenomegaly, jaundice) and laboratory data.
The traditional laboratory diagnostic test for HS is the osmotic fragility (OF) test. However, OF test is labor-intensive, time-consuming and requires a high volume of blood, at least 2 ml. It also showed low sensitivity and specificity values. For these reasons, attention has turned to other screening tests with greater sensitivity and specificity for HS diagnosis (Bianchi et al., 2012; King et al., 2015; Farias et al., 2016).
According to the published data and our preliminary study, a screening algorithm, based on the automated reticulocyte parameters might be helpful in the screening of HS (first line screening/diagnostic orientation), however the combination with other methods is necessary to ensure effective screening for HS (Lazarova et al., 2014).
Diagnostic guidelines for HS recommend either the cryohemolysis test (CH) or EMA-binding cytometry test as screening methods (second line screening), both tests present equal grades of recommendation and evidence (Bolton-Maggs et al., 2012; Gulbis et al., 2013; Lazarova et al., 2014).
The CH test has superior diagnostic performance compare to OF test, however still requires different steps with manual preparation and necessary strict temperature monitoring during the incubation. The reference range should be carefully evaluated by each laboratory and, the normal reference values for CH test might need to be adjusted.
None of the tests can recognise all cases of HS, its diagnosis is not always strait forward and requires the different investigations (Gulbis et al., 2013; Lazarova et al., 2014).

CLINICAL/DIAGNOSTIC SCENARIO

Hereditary spherocytosis is the most common congenital hemolytic anemia in Caucasians, affecting approximately 1 in 1000-2000 individuals (Bianchi et al., 2012). Seventy-five percent of the cases have a dominant mode of inheritance (King et al., 2013).
The molecular defect is highly heterogeneous involving the genes encoding for spectrin, ankyrin, band 3 and protein 4.2 (Table 1) and the degree of hemolysis varies widely, from fully compensated to transfusion-dependent anemia (Bolton-Maggs et al., 2004; Bianchi et al., 2012).

Table 1: Membrane molecules associated with erythrocyte cytoskeleton (Bolton-Maggs et al., 2004)

Protein	**Band on gel**	**Mr (kD)**	**Gene**	**Chromosomal location**	**Number of exons**
α Spectrin	1	240	*SPTA1*	1q22-q23	52
β Spectrin	2	220	*SPTB*	14q23-q24·1	32
Ankyrin	2·1	210	*ANK1*	8p11·2	42
Band 3 (AE1)	3	90–100	*AE1 (SLC4A1)*	17q21-q22	20
Protein 4·1	4·1	80	*EPB41*	1p36·2-p34	≥22
Protein 4·2	4·2	72	*EPB42*	15q15-q21	13
Glycophorin C	GPC	32	*GYPC*	2q14-q21	4

Bolton-Maggs (2004) recommended that the patients with HS should be graded by their severity of disease (baseline Hb, reticulocyte count, jaundice, level of activity) as ‘mild’, ‘moderate’ or ‘severe’ (for criteria see Table 2). This predicts clinical course and the need for splenectomy (Bolton-Maggs et al., 2004).
Mild HS can be difficult to identify because individuals may have normal haemoglobin and bilirubin concentrations. The presence of spherocytes and a reticulocytosis will support the diagnosis. If there are no spherocytes seen on the film, no abnormalities in the red cell indices, and the reticulocyte count is normal, then a ‘carrier’ state cannot be excluded, but the individual is unlikely to have any clinical sequelae . Asymptomatic or mild HS condition can be exacerbated by an infection (e.g., Parvovirus B19, Herpes 6, CMV, or gastroenteritis) or pregnancy (King et al., 2013).

Table 2: Classification of spherocytosis and indications for splenectomy (modified from Eber et al., 1990; Bolton-Maggs et al., 2004)

Classification	**Trait**	**Mild**	**Moderate**	**Severe**
aemoglobin (g/dl)	Normal	11–15	8–12	6–8
Reticulocyte count %	Normal (<3%)	3–6	>6	>10
Bilirubin (μmol/l)	<17	17–34	>34	>51
Splenectomy	Not required	Usually not necessary during childhood and adolescence	Necessary during school age before puberty	Necessary – delay until 6 years if possible

The diagnosis of HS is based upon a combination of clinical history, family history, physical examination (splenomegaly, jaundice) and laboratory data (full blood count, especially red cell indices and morphology, and reticulocyte count) (Table 3) (Bolton-Maggs et al., 2004).

Table 3: Diagnostic parameters for hereditary spherocytosis (Bolton-Maggs et al., 2004)

Parameter	**Features**
Clinical features	Splenomegaly almost always
Laboratory red cell indices	(↓Hb, ↓MCV, ↑MCHC, ↑% hyperdense cells, ↑RDW, ↑reticulocyte count)
Blood film	Abnormal morphology – spherocytes
Direct antiglobulin test	Negative
Evidence of haemolysis	Raised bilirubin; reticulocytosis

MCV, mean cell volume; MCHC, mean cell Hb concentration; RDW, red cell distribution width.

The osmotic fragility (OF) test

The principle of traditional laboratory diagnostic tests for hereditary red cell membrane defects exploits the reduced surface area-to-volume ratio found in spherocytes. The test measures the rate of red cell lysis in incubation media. The traditional OF test uses concentrations of NaCl, ranging from 0.1 to 0.8 g/dL NaCl (Parpart et al., 1947; King et al., 2015). Spherocytes have less resistance to lysis at each NaCl concentration when compared to normal RBCs (figure 1). Pre-incubation of the whole blood sample for 24 h at 37 °C will enhance the degree of cell lysis and the sensitivity from 68% to 81% on fresh and incubated blood, respectively (King et al., 2013). Unfortunately, sensitivity is lower with compensated HS cases (53% and 64%, respectively, for fresh and incubated blood) (Bianchi et al., 2012; King et al., 2013).

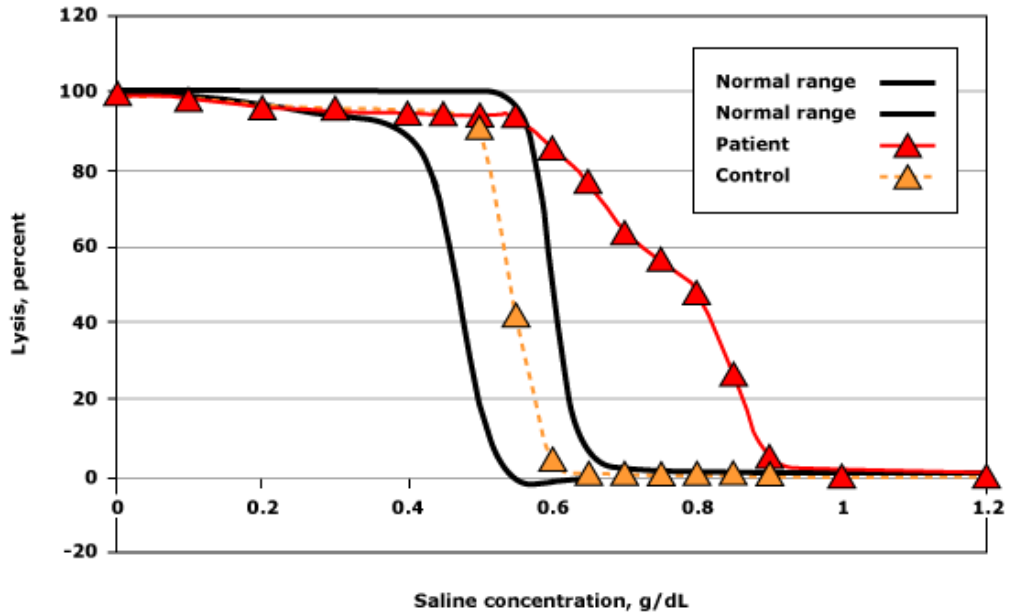

Fig.1: The osmotic fragility test in hereditary spherocytosis
This figure shows results of an incubated osmotic fragility test performed on red cells from an adult patient with hereditary spherocytosis, showing markedly increased osmotic fragility. Note that the patient's red cells were >60 percent hemolyzed at a saline concentration (0.7 g/dL) that did not cause any osmotic lysis in normal, incubated red cells (Mentzer et al., 2017).

The drawback of the OF test is a lack in specificity, indeed, the OF test cannot differentiate between causes of spherocytosis (immune versus non-immune) (King et al., 2015), as other congenital red cell defects or conditions can also give a positive result (i.e. increased red cell lysis). These include immune hemolytic anemia, recent blood transfusion (i.e. lysis of recently transfused RBCs *ex vivo* due to depletion of ATP in these cells), RBC enzyme deficiencies (e.g. G6PD and pyruvate kinase deficiencies), and unstable hemoglobin variants. The OF test result has to be interpreted together with family history and examination of the peripheral blood smear.

A normal osmotic fragility result does not exclude the diagnosis of HS (King et al., 2015). Cynober et al., (1996) reported that the OF test was normal in 34% of HS samples (Cynober et al., 1996; Mentzer et al., 2017). Incubation of blood specimens for 24 hours in the absence of metabolic substrate accentuates the OF of spherocytes and makes it easier to distinguish HS from other diseases, however, even after incubation, 15 percent of the samples in the above study had normal OF (Cynober et al., 1996; Mentzer et al., 2017). Cell dehydration occurring in the spherocytes of a patient with HS can be one of the causes of normal osmotic fragility results for non-splenectomized HS patients (Cynober et al., 1996; King et al., 2015).

In UZ Leuven the traditional OF test is performed, however, the OF test is labor-intensive, time-consuming, requires a high volume of blood at least 2 ml. It also showed low sensitivity and specificity values (Bianchi et al., 2012; King et al., 2015; Farias et al., 2016). For these reasons, attention has turned to other screening tests with greater sensitivity and specificity for HS diagnosis (Bianchi et al., 2012; King et al., 2015; Farias et al., 2016). In this regard, a review of methods currently used for diagnosis of HS in general laboratory is needed.

QUESTION(S)

1) *Question 1: Can routine hematological parameters (e.g. Reticulocyte Indices) be used in the screening for patients with HS (first line screening)?*
2) *Question 2: Is the cryohemolysis test a suitable alternative for OF test in second line screening of HS?*

SEARCH TERMS

1) *MeSH Database (PubMed): MeSH term: "Hereditary spherocytosis; automated reticulocyte parameters; screening; diagnostic test"*
2) *PubMed Clinical Queries (from 1966; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi): Systematic Reviews; Clinical Queries using Research Methodology Filters (diagnosis + specific, diagnosis + sensitive, prognosis + specific)*
3) *Pubmed (Medline; from 1966), SUMSearch (http://sumsearch.uthscsa.edu/), National Guideline Clearinghouse (http://www.ngc.org/), Institute for Clinical Systems Improvement (http://www.icsi.org), The National Institute for Clinical Excellence (http://www.nice.org.uk/), Cochrane (http://www.update-software.com/cochrane, Health Technology Assessment Database (http://www.york.ac.uk/inst/crd/htahp.htm)*
4) *National Committee for Clinical Laboratory Standards (NCCLS; http://www.nccls.org/), International Federation of Clinical Chemistry (IFCC; http://www.ifcc.org/ifcc.asp), American Diabetes Association (ADA; http://www.diabetes.org/home.jsp), National Diabetes Information Clearinghouse (NDIC; http://diabetes.niddk.nih.gov/), Westgard QC (http://www.westgard.com), Clinical Laboratory Improvement Amendments (CLIA; http://www.cms.hhs.gov/clia/)*
5) *UpToDate Online version 12.2 (2004)*

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Can routine hematological parameters (e.g. Reticulocyte Indices) be used in the screening for patients with HS (first line screening)?

It has been shown that reduced membrane surface area-to-volume ratio and increased haemoglobin concentration are specifically present at the reticulocyte stage in HS but not in normal conditions or in autoimmune haemolytic anemia (Da Costa et al., 2001; Lazarova et al., 2014), thus, the automated reticulocyte parameters could be of great interest for hereditary spherocytosis screening especially if it is able to demonstrate the presence of small dehydrated reticulocytes coexistent with spherocytes (Da Costa et al., 2001; Lazarova et al., 2014).

Screening algorithm, proposed by Lazarova et al., (2014)

Last generation haematological equipment offers new parameters derived from well known RBC parameters and reticulocyte analysis such as mean reticulocyte volume (MRV or MCVr), immature reticulocyte fraction (IRF), reticulocyte haemoglobin equivalent or content (Ret-He or CHr) and reticulocyte distribution width (RDWR), reviewed recently by Piva et al. , 2010 and Lazarova et al., 2014. There are only few published data concerning the utility of those reticulocyte parameters in the screening for HS.
Lazarova et al., (2014) performed an evaluation of automated reticulocyte parameters (i.e MRV, IRF and mean sphered cell volume (MSCV)) available on the Beckman Coulter UniCel DxH800 instrument with regard to their usefulness in HS screening (Lazarova et al., 2014).
Lazarova et al., reported the diagnostic performance of automated reticulocyte haematological parameters (calculation on the bases on 374 cryohaemolysis negative samples and 48 HS positive samples) (Table 4).

Table 4: Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and positive likelihood ratio (+LR) of MSCV, delta (MCV-MSCV), Ret/IRF, MRV and RDWR (Lazarova et al., 2014)

Parameter	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**+LR**
MSCV cut-off ≤76.5	1	0.73	0.32	1	3.6
MSCV cut-off ≤70.2	0.92	0.9	0.54	0.99	9
Delta (MCV-MSCV) ≥10.4	1	0.74	0.34	1	3.8
Delta (MCV-MSCV) ≥18.1	0.92	0.94	0.66	0.99	14.9
Ret/IRF ≥1.53	1	0.54	0.22	1	2.2
Ret/IRF ≥2.58	0.92	0.89	0.50	0.99	8
MRV ≤96.72	1	0.88	0.53	1	8.7
MRV ≤92	0.92	0.94	0.67	0.99	15.5
RDWR ≥26.4	1	0.57	0.23	1	2.3
RDWR ≥28.53	0.92	0.79	0.36	0.99	4.3

From the receiver operating characteristic (ROC) curve analysis, both delta (mean cell volume (MCV)-MSCV) and MRV presented an area under the curve (AUC) of 0.98. At the diagnostic cut-off of 100 % sensitivity, MRV showed the best specificity of 88 % and a positive likelihood ratio of 8,7.
Ret/IRF ratio showed a sensitivity of 92 % and a specificity of 89 % which were comparable to those of delta (MCV-MSCV).
Moreover, Lazarova et al., evaluated the efficiency of the new haematological parameters parameters (RDWR, MSCV, MRV, Ret/IRF) to differentiate HS from other frequent anaemia like autoimmune haemolytic anaemia (AIHA), glucose-6- phosphate dehydrogenase deficiency (G6PD def), betathalassaemia minor, iron deficiency as well as sickle cell anaemia (Hb SS) or haemoglobin S carrier (Hb AS). Patients presenting with these pathologies were tested with standard haematological parameters , cryohemolysis test and new haematological parameters (RDWR, MSCV, MRV, Ret/IRF; Fig. 2. Data of Lazarova study (2014) showed the presence of statistical differences between HS and each of the other pathologies besides AIHA for all parameters except RDWR. The parameters IRF, MRV and MSCV discriminated HS not only from controls and other tested pathologies but also from AIHA contrary to the cryohemolysis test. Indeed, Ret/IRF ratio, MRVand MSCV showed a statistical difference between the HS and AIHA group, ($p<0.0001$ for Ret/IRF ratio and MRV; $p=0.0008$ for MSCV).

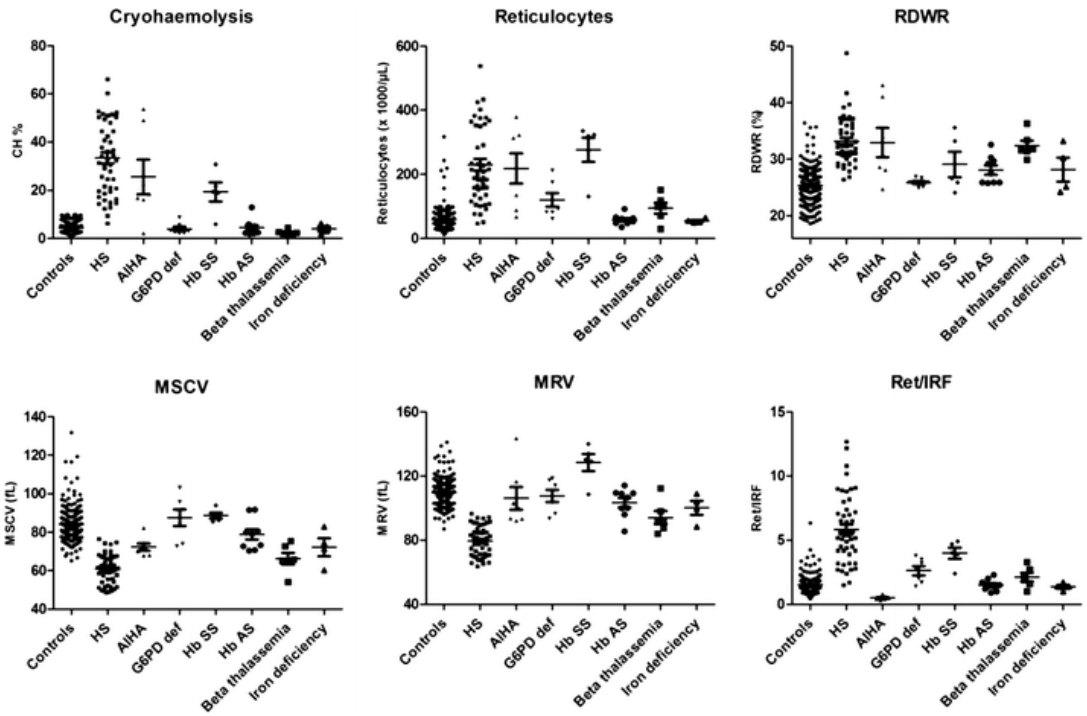


Fig. 2 Comparison of screening test results in hereditary spherocytosis patients (HS, n=48), controls (n=213) and in patients with anaemia of different origins Results are presented as mean ± SEM (Lazarova et al., 2014)
Hereditary spherocytosis patients (*HS*, $n = 48$), controls ($n = 213$), patients with anaemia of different origins: autoimmune haemolytic anaemia (*AIHA*, $n = 7$), G6PD-deficient patients ($n = 7$), homozygotes for haemoglobin S (*Hb SS*, $n = 5$), heterozygotes for haemoglobin S (*Hb AS*, $n = 9$), patients with beta-thalassaemia minor ($n = 6$) and patients with iron deficiency ($n = 4$).

The screening algorithm, proposed by Lazarova et al., is as follows: if MSCV <70.2 fL or delta (MCV-MSCV) >10.4 fL and/or MRV <96.7 fL, the cryohemolysis test is performed, and if the latter is >10 %, the confirmatory SDS-PAGE is realised.

Screening algorithm, proposed by Mullier et al., (2011)

Mullier et al., (2011) proposed a diagnostic tool (Table 5) based on the physiopathology of HS. Indeed, in HS, the loss of surface area is already present at the circulating reticulocyte stage. Thus, the automated reticulocyte parameters could be of great interest for hereditary spherocytosis screening (Mullier et al., 2011). Red cell and reticulocyte counts and indices (percentage of microcytes (MicroR), percentage of hypochromic cells (%Hypo-He), reticulocyte counts, and percentage of immature reticulocytes) and haemoglobin values were determined using XE-2100 (n=15) and XE-5000 (n=30; Sysmex, Kobe, Japan). %Hypo-He is a parameter analysed in the reticulocyte channel of the XE-5000 (Fig 3) .

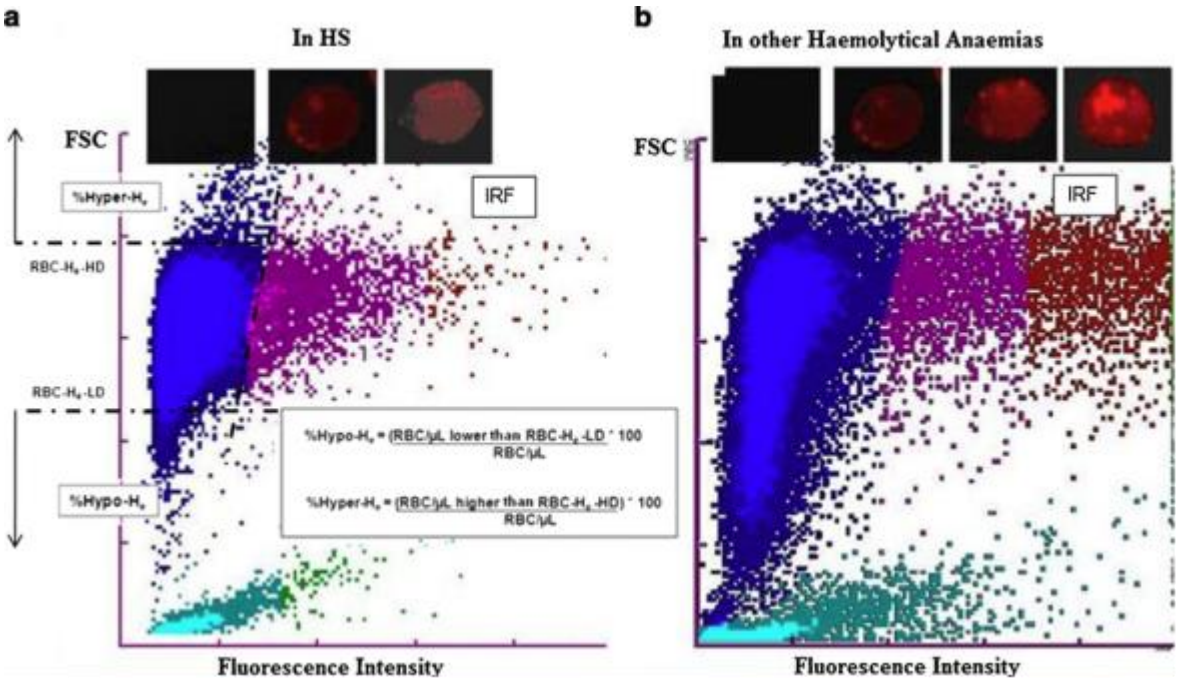


Fig.3 Reticulocytes channel on Sysmex XE-5000CM. a Hereditary spherocytosis: reticulocytosis with decreased immature reticulocytes fraction (IRF). Hypochromic erythrocytes (%) and hyperchromic erythrocytes are also shown. The basis for analysis of these parameters is the mean haemoglobin content of all the measured red blood cells (RBC-He) analysed in the reticulocyte channel. Red blood cells with a mean haemoglobin content lower than 17 pg, corresponding to the low discriminator for RBC-He (RBC-He-LD), are classified as hypohaemoglobinized cells, whereas the hyper-haemoglobinized red blood cell population contains cells with a haemoglobin content higher than to 49 pg, the high discriminator for RBC-He (RBC-He-HD).

b Other haemolytical anaemias: reticulocytosis with normal IRF. Hypo-He hypochromic erythrocytes, Hyper-He hyperchromic erythrocytes (Mullier et al., 2011).

As shown in Table 5, the diagnostic algorithm includes a precondition to screen all cases of HS (Rule1), and a second rule (Rule 2) taking into account the severity reflected by the degree of anaemia.

Table 5: Diagnostic algorithm (Mullier et al., 2011)

Rule		**Parameters**		
Rule 1	Precondition	Ret ≥80,000/µl and Ret/IRF >7.7		
Rule 2	Severity	Trait or mild HS Hb>12 g/dl	Moderate HS 8 g/dl ≥ Hb ≤ 12 g/dl	Severe HS Hb <8 g/dl
		Ret/IRF ≥19	MicroR ≥3.5% and MicroR/Hypo-He ≥2.5	MicroR ≥3.5% and MicroR/Hypo-He ≥2

Ret reticulocytes (/µl), *IRF* immature reticulocytes fraction (%), *HS* hereditary spherocytosis, *Hb* haemoglobin, *MicroR* microcytic erythrocytes (%), *Hypo-He* hypochromic erythrocytes (%)

Rule 1: Ret and Ret/IRF ratio
All 45 confirmed cases of HS in the Mullier study (2011) had reticulocytes >80 $X10^9$/L and a reticulocytes (10^9/L)/immature reticulocytes fraction (%) (Ret/IRF) ratio higher than 7.7. This limit is used as a precondition for the screening of all the cases of HS.

Rule 2: Ret/IRF ratio or MicroR/Hypo-He ratio
Mullier et al., (2011) reported that the severity of the disease shown by Hb level is due to the intensity of release of microparticles, which is reflected by MicroR, the best indicator of HS severity (Bolton-Maggs et al., 2004). Therefore, MicroR was included in rule 2, only for moderate and severe cases of HS (Mullier et al., 2011).

Trait and mild HS
All trait and mild cases (Hb >12 g/dl, $n = 12$) of HS had a Ret/IRF ratio higher than 19. The screening of trait and mild HS based on this method is certainly a major advance. Indeed, interpretation of hypertonic cryohemolysis test and EMA binding in flow cytometry is often difficult in mild cases (Mullier et al., 2011).

Moderate and severe cases of HS
For moderate and severe cases of HS, MicroR and MicroR/Hypo-He were combined. The optimal cut-offs in cases of Hb between 8 and 12g/dl were 3.5% and 2.5, respectively. When Hb was lower than 8 g/dl, the optimal cut-offs were 3.5% and 2.0, respectively (Mullier et al., 2011).

The performances of the HS diagnostic tool proposed by Mullier et al., (2011) were compared with single parameters and existing rules, as shown in Table 6.
The area under the curve (AUC), sensitivity, specificity, predictive positive value and negative predictive value were respectively 0.997 (95% confidence interval 0.992–0.999), 100%, 99.3%, 75% and 100%. This diagnostic tool is therefore much more efficient than single parameters (Ret/ IRF index).

Table 6: Efficiency of the HS diagnostic tool and comparison with single parameters and existing rules

Parameter	AUC (95% CI)	Cut-off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
MCHC (g/dl)	0.735 (0.711–0.758)	34.7	73.3	72.6	5.1	99.3
MicroR (%)	0.744 (0.721–0.766)	7.8	56.7	84.8	7.0	99.0
RDW-CV (%)	0.684 (0.659–0.708)	18.1	55.2	80.6	5.6	98.9
MCHC and RDW-CV	0.678 (0.653–0.702)	Positive	37.9	97.6	24.4	98.7
Hyper-He (%)	0.750 (0.726–0.772)	0.5	55.2	82.1	6.0	98.9
MCHC and Hyper-He	0.714 (0.690–0.738)	Positive	44.8	98.1	32.5	98.8
RDW-CV and Hyper-He	0.642 (0.617–0.667)	Positive	34.5	94.0	10.6	98.6
MicroR/Hypo-He ratio	0.743 (0.720–0.764)	4.0	76.7	65.6	4.3	99.3
Ret (10^9/L)	0.938 (0.925–0.950)	103.5	93.3	83.6	10.3	99.8
Ret/IRF ratio	0.976 (0.967–0.983)	9.7	96.7	89.6	15.9	99.9
HS diagnostic tool	0.997 (0.992–0.999)	Positive	100.0	99.3	75.0	100.0

AUC area under the curve, *95% CI* 95% confidence interval, *PPV* predictive positive value, *NPV* negative predictive value, *MCHC* mean corpuscular haemoglobin concentration (g/dl), *MicroR* microcytic erythrocytes (%), *RDW-CV (%)* red blood cells distribution width–coefficient of variation, *Hyper-He* hyperchromic erythrocytes (%), *Hypo-He* hypochromic erythrocytes (%), *MicroR/Hypo-He* microcytic erythrocytes/hypochromic erythrocytes, *Ret/IRF* reticulocytes/immature reticulocytes fraction [10^9/(L×%)], *HS* hereditary spherocytosis (Mullier et al., 2011)

Mullier et al., (2011) evaluated the efficiency of diagnostic tool to screen confirmed HS cases to differentiate HS from other haemolytic disorders, iron deficiencies, healthy individuals and controls (Figure 4-6). They reported that Ret/IRF ratio is highly efficient to discriminate confirmed mild and even moderate HS cases from patients suffering from various haemolytical disorders (n=108), patients with microcytic anaemia (n=93 whose 64 with iron deficiency and 29 with functional iron deficiency), healthy individuals (n=61) and samples from the routine haemotological database (n=1230). However, Mullier et al., reported that addition of MicroR/Hypo-He (Fig.5) and MicroR (Fig.6) is required to discriminate severe HS from some haemolytical disorders and some healthy individuals, respectively.

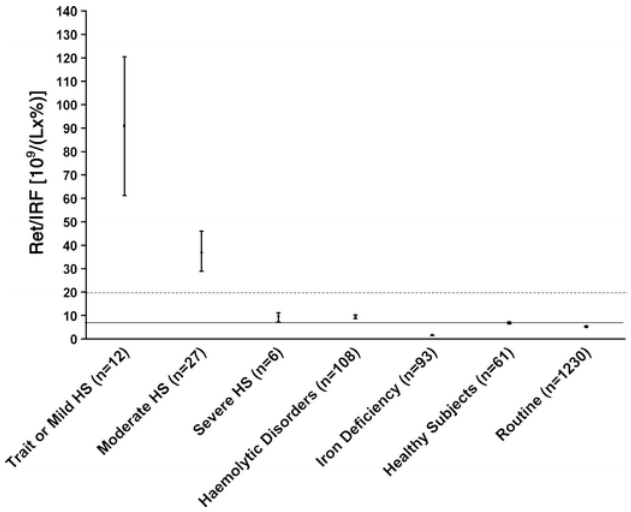


Fig. 4 Distribution of Ret-IRF ratios (mean±SEM) among trait or mild HS (n=12), moderate HS (n=27), severe HS (n=6), haemolytic disorders (n=108), iron deficiency (n=93), healthy subjects (n=61) and routine haematological database (n= 1230). The cut-offs defined in the diagnostic tool are also shown: 7.7 for rule 1 (continuous line) and 19.9 for rule 2 (dotted line)(Mullier et al., 2011)

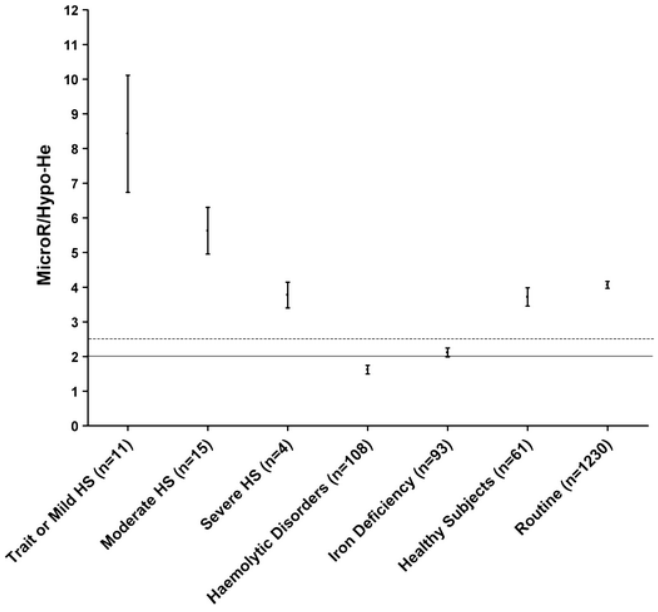


Fig.5 Distribution of MicroR/Hypo-He ratios (mean ± SEM) among trait or mild HS ($n = 11$), moderate HS ($n = 15$), severe HS ($n = 4$), haemolytic disorders ($n = 108$), iron deficiency ($n = 93$), healthy subjects ($n = 61$) and routine haematological database ($n = 1,230$). The cut-offs defined in the diagnostic tool are also shown: 2.5 for moderate HS (*continuous line*) and 2.0 for severe HS (*dotted line*) (Mullier et al., 2011)

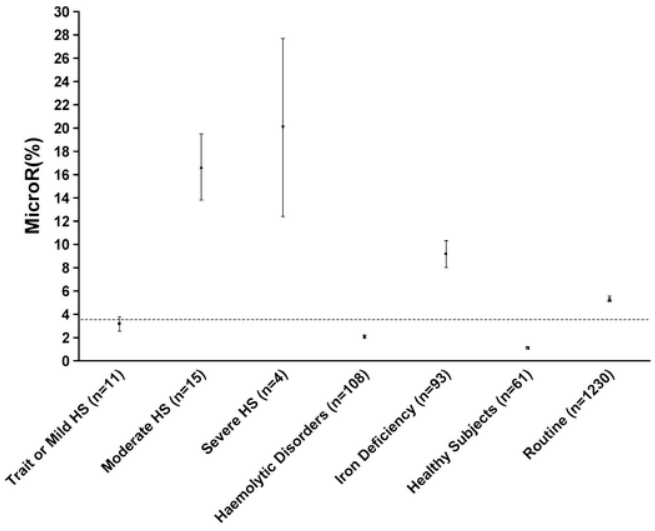


Fig. 6 Distribution of MicroR (mean ± SEM) among trait or mild HS ($n = 11$), moderate HS ($n = 15$), severe HS ($n = 4$), haemolytic disorders ($n = 108$), iron deficiency ($n = 93$), healthy subjects ($n = 61$) and routine haematological database ($n = 1,230$). The cut-off defined in the diagnostic tool is also shown: 3.5 for moderate and severe HS (*dotted line*) (Mullier et al., 2011)

Validation of Mullier's Screening algorithm by Persijn et al., 2012.

Persijn et al., (2012) retrospectively evaluated the value of the Mullier's diagnostic tool and reported much lower sensitivity of 76% and specificity of 98% , PPV of 26.8% and NPV of 99.8%. They missed 6/25 patients with known HS: 3 due to lower reticulocyte counts (range = 50.6–69.2 × 10^9/L), possibly because they were all splenectomised, and 3 due to lower percentage of microcytic erythrocytes (MicroR, range = 2.6–3.4%). Persijn et al., (2012) reported that all non-splenectomised patients had reticulocyte counts of >140 × 10^9/L . Since the primary goal of the Persijn's study was to identify undiagnosed patients and as splenectomy evokes a decrease in reticulocyte count, Persijn et al., adapted the HS diagnostic tool: reticulocytes ≥100 × 10^9/L instead of ≥80 × 10^9/L and MicroR ≥2.6% instead of ≥3.5%. The new approach leads to a sensitivity of 100% for non-splenectomised HS patients and 84% for all HS patients with respectively a PPV of 42.6% and 43.8% . When the screening rule is positive, the presence of spherocytes was evaluated on a blood smear and if clinically suspected for HS or without clear diagnosis, the flow cytometric eosin-5-maleimide (EMA) test was performed. Persijn et al., reported that one month after implementation, 9/731 individuals were flagged positive. Four individuals were suspected of HS of which three had a positive EMA test.

The stability of the reticulocyte indices

The stability of the automated parameters was analysed by Lazarova et al., (2014) for different conservation temperatures, 4 and 20°C. The stability of all automated parameters for 24 h at 4 °C was warranted by statistically non-significant Student t test; the same was observed at 20 °C for Ret/IRF and MRV. On the opposite, MCV, MSCV and delta (MCV-MSCV) presented statistically significant Student t test in the 24-h stability study at 20 °C with respective mean differences of 3.13, 7.14 and 10.3

Retrospective study based on UZ Leuven LAG Data

Our laboratory uses Sysmex - XE-5000 haematological analysers.
Our purpose was to investigate the reliability of automated reticulocyte parameters which were proposed by Mullier et al., (2011) as a screening tool for HS.
We conducted a single-institution retrospective study of samples tested for HS using the OF test between 2012 and 2016. We identified the patients by searching the UZ-Leuven electronic database. We found 24 samples from patients with HS (confirmed by clinical data and laboratory tests) .

Fig.7 and Fig.8 illustrate the distribution of Reticulocytes and Ret/IRF respectively in the group of samples from patients with HS and in all samples.

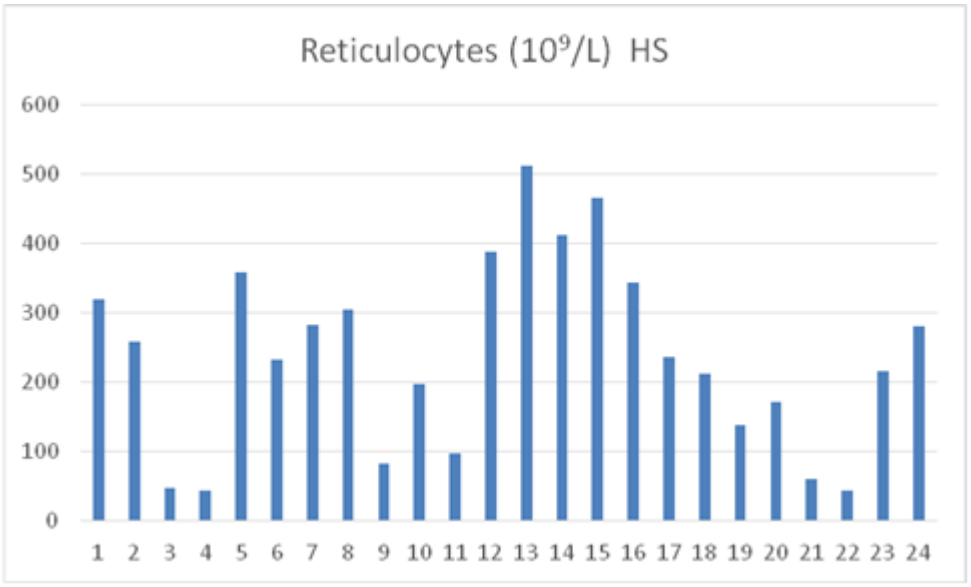


Figure 7A. Distribution of Reticulocytes in the group of samples from patients with HS

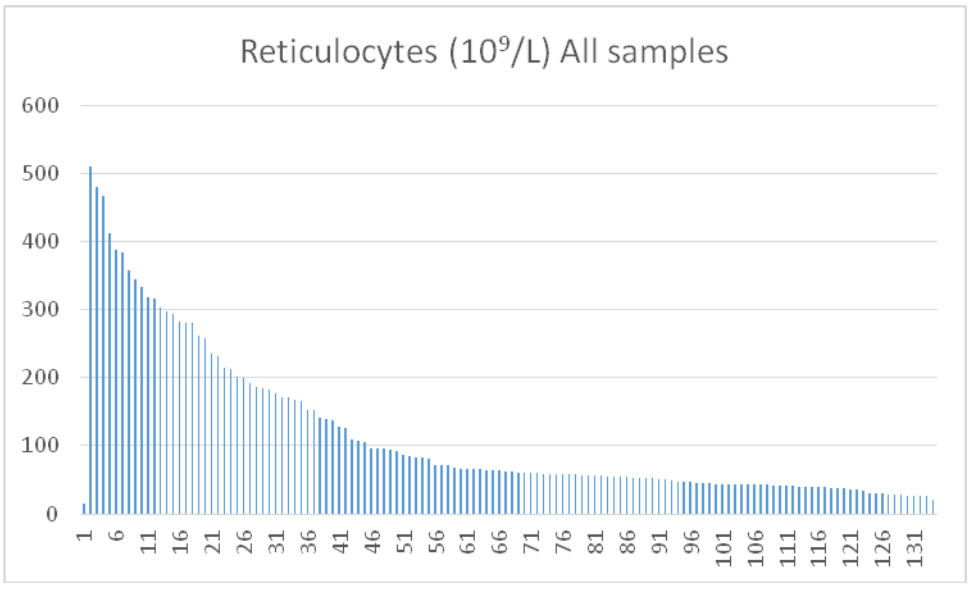


Figure 7B. Distribution of Reticulocytes in all samples

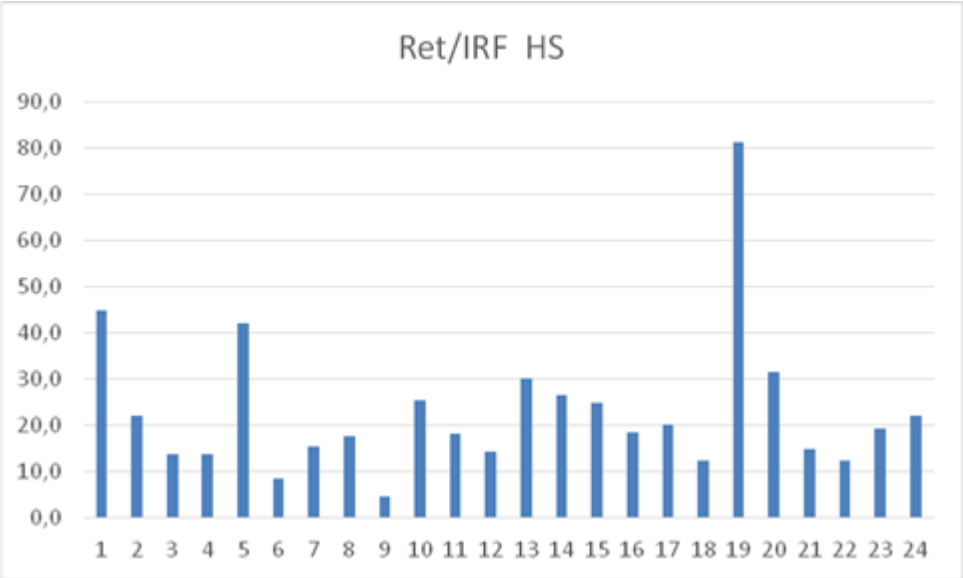


Fig. 8A Distribution of Ret/IRF in the group of samples from patients with HS

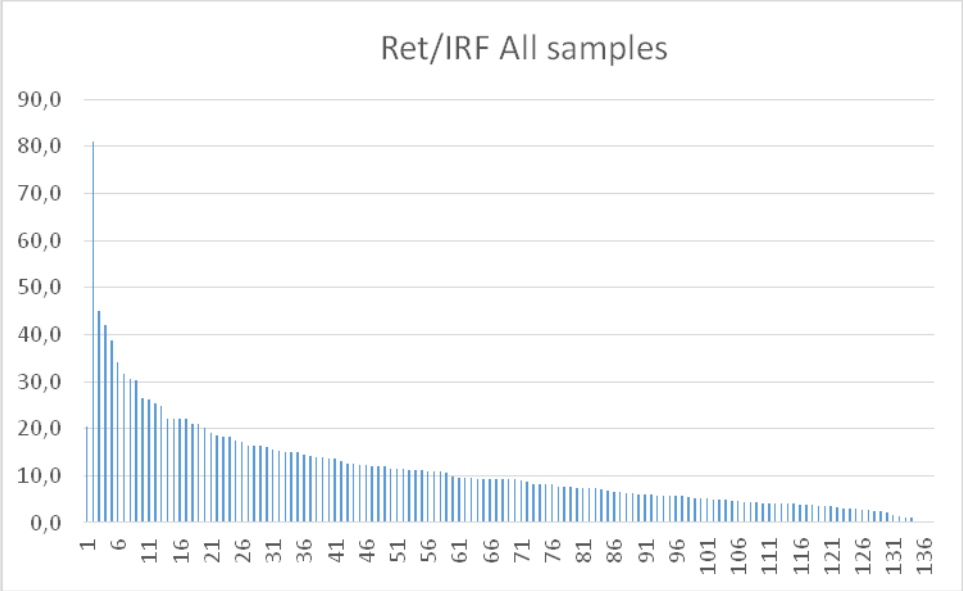


Fig. 8B Distribution of Ret/IRF in all samples

Rule1:
Applying Rule 1 of the screening algorithm, proposed by Mullier et al., (2011), we could identify HS in 19 from 24 samples, collected from patients with confirmed HS. However, Rule 1 of the screening algorithm could not identify HS in 5 samples from patients with confirmed HS (samples N3; N4; N9; N21 and N22 figure 7 and 8). Based on the clinical data, the mentioned 5 samples were collected from the patients with mild HS. Ret/IRF indexes were less than 19 in all 5 samples. We have therefore observed some discrepancy between our and Mullier's data, i.e. even mild cases of HS in Mullier's study had a Ret/IRF ratio higher than 19.

Rule2:
The data regarding the MicroR and Hypo-He research reticulocytes parameters for samples tested for HS by using OF test between 2012 and 2016 was not available. These research parameters were not reported and not recorded in LIS data base).
To be able to apply the rule 2, we adapted our LIS together with the LIS-team. The data regarding the MicroR and Hypo-He research reticulocytes parameters are now prospectively collected.
The analysis will be performed in the near future.

Limitations

Unfortunately, IRF is measured by different methods in the various haematology analysers automates, so data are difficult to compare (Lazarova et al., 2014).

Conclusion

In conclusion, the automated reticulocyte parameters might be helpful in the screening for HS (diagnostic orientation), however, the combination with another methods remains necessary to ensure the reliable screening for HS (Lazarova et al., 2014).

Diagnostic guidelines for HS diagnosis, those from the British Committee for Standards in Haematology, recommend either the cryohemolysis test or EMA-binding cytometry test as screening methods, both tests presenting equal grades of recommendation and evidence (Bolton-Maggs et al., 2012; Lazarova et al., 2014).

Cryohemolysis test

The cryohemolysis test (CH) is based on an increased susceptibility of HS red cells to rapid cooling from 37 to 0 °C in hypertonic conditions (King et al., 2013).
As the test does not depend on the surface area-to-volume ratio but on the integrity of proteins of the membrane, it gives normal results in spherocytosis secondary to autoimmune hemolytic anemia (Rivera et al., 2006; Crisp et al., 2011). However, opinions concerning routine utilization of CH test for diagnosis are controversial (because of different results reported by several authors (Crisp et al., 2011). Streichman and Gescheidt (1998) reported 90% specificity and 100% sensitivity, but these values were not obtained by ROC curves. On the contrary, Mariani et al., (2008) found a remarkably lower sensitivity (53%), but in their study, CH test was performed only in 33 out of 300 HS patients. Crisp et al., (2011) reported 96% specificity and 79% sensitivity.

Practical implementation

In order to gain insight with applicability of the CH test, a visit to the reference laboratory was organized.
The cryohemolysis test is performed as reported by Lazarova et al., 2014. Briefly, 50 μL of washed red cells was dispensed into 2 mL of hypertonic preheated (37 °C) sucrose solution. Following 10 min of incubation at 37 °C, each tube was transferred to 0–4 °C incubation for another 10 min. The tubes were then centrifuged, and the absorbance of the supernatant was measured at 540 nm [DO TEST]. In parallel, 25 μL of washed red cells was completely lysed in 2 mL of distilled water and served as 100 % lysis value [DO 100 % lysate]. The percentage value of cryohemolysis was obtained by the formula: % cryohemolysis=[DO TEST]/ [DO 100 % lysate]×50.
All measurements were done in duplicate, and a control sample was run in each experiment. We use the ranges of the cryohemolysis test, reported as follows: a result <10 % was considered as negative, between 10 and 15 % as suspicious and >15 % as positive (Iglauer et al., 1999; Lazarova et al., 2014).

Stability

The stability was tested (during the test phase of the study) at different storage temperatures: 4-8 °C and room temperature (RT) by using blood sampled from 5 healthy volunteers. The CH tests were repeatedly performed at day 0-1-2-3, either at room temperature or at 4-8°C. We accepted an error of ± 15%. The results are summarized in Table 7A. Based on these results the storage of samples at 4-8°C is required to ensure stability of the sample.

The stability experiment was repeated after the optimization of the protocol (strict control of the temperature during the incubation) by using blood sampled from 5 healthy volunteers and 1 patient's sample. The CH tests were repeatedly performed at day 0-1-4-5 (storage temperatures: 4-8 °C). The results are summarized in Table 7B. The observed CVs were between 9 and 25%. We concluded that the maximum storage time at 4-8 °C is 72 hr.

Table 7A.: Stability 72hr (test phase)

	4°C						
	0hr	**24hr**	**48hr**	**72hr**	**%CV**	**SD**	**agreement 72 hr vs 0 hr**
	24/01/2017	**25/01/2017**	**26/01/2017**	**27/01/2017**			
healthy volunteer 1	1,44	1,68	1,28	1,42	11%	0,17	99%
healthy volunteer 2	1,50	1,76	1,18	1,80	18%	0,29	120%
healthy volunteer 3	1,33	1,69	1,31	1,73	15%	0,23	130%
healthy volunteer 4	1,73	1,72	1,50	1,66	7%	0,11	96%
healthy volunteer 5	1,44	1,74	1,47	1,30	12%	0,19	90%

	RT						
	d0	**24hr**	**48hr**	**72hr**	**%CV**	**SD**	**agreement 72 hr vs 0 hr**
	24/01/2017	**25/01/2017**	**26/01/2017**	**27/01/2017**			
healthy volunteer 1	1,44	1,76	2,97	2,65	33%	0,72	184%
healthy volunteer 2	1,50	2,26	2,65	2,40	22%	0,50	160%
healthy volunteer 3	1,33	2,27	2,47	3,11	32%	0,73	233%
healthy volunteer 4	1,73	2,15	2,50	2,19	15%	0,32	127%
healthy volunteer 5	1,44	1,98	2,11	2,76	26%	0,54	191%

Table 7B: Stability 120h (after optimization the protocol)

	4°C						
	d0	**24hr**	**96hr**	**120hr**	**%CV**	**SD**	**agreement 120 hr vs 0 hr**
	02/03/2017	**03/03/2017**	**06/03/2017**	**07/03/2017**			
healthy volunteer 11	4,2	5,07	4,47	4,32	9%	0.39	103%
healthy volunteer 12	3,95	4,77	3,8	3,12	17%	0.68	79%
healthy volunteer 13	17,59	19,04	18,86	13,87	14%	2.4	79%
healthy volunteer 14	15,98	17,83	15,8	13,55	11%	1.75	85%
healthy volunteer 15	6,3	7,74	6,13	4,05	25%	1.52	64%
VKA648	4,73	6,74		5,75	18%	1.01	122%

Intra-run variation

Intra-run validation was tested by using EDTA plasma collected from a healthy volunteer. Intra –run variation was 14% (Attachment 1).

Comparison of CH test versus OF test

Comparison of CH test versus OF test was performed by using EDTA samples from 15 healthy volunteers and 13 patients (Table 8). Measurements performed during the test phase are marked with the asterisk *in Table 8.
During the test phase of the study a false negative result of the CH test (7,18%) was observed in the sample collected from the patient with known HS. It could be explained by insufficient temperature monitoring during the incubation steps. Therefore we optimized our protocol in such a way that the strict temperature monitoring is performed during the incubation steps at 37C° and at 0-4°C.
Surprisingly, healthy volunteers 13 and 14 had high cryohemolyse percentage (17.59% and 15,98% respectively), however, the OF test was normal in both cases. According to the reference range for CH test (Lazarova et al., 2014), healthy volunteers 13 and 14 had positive CH test for HS. Since all other and supplementary investigations are negative, we are currently investigating the reason for these probably false positive results. These observations suggest that the reference range should be carefully evaluated by each laboratory and, based on verification by UZ Leuven lab, normal reference values for CH test might need to be adjusted.
The major limitation of our study is that we did not have sufficient number of samples from patients with HS. We are currently in the process of a prospective data collection with an aim to compare the CH test and the results of OF test, obtained from patients with suspected HS.

Table 8: Comparison of CH test versus Osmotic fragility test (preliminary data)

	CH (%)	OF	Comments
*healthy volunteer 1	1,44	normale osmotische resistentie	
*healthy volunteer 2	1,5	normale osmotische resistentie	
*healthy volunteer 3	1,33	normale osmotische resistentie	
*healthy volunteer 4	1,73	normale osmotische resistentie	
* healthy volunteer 5	1,44	normale osmotische resistentie	
*healthy volunteer 6	1,63	normale osmotische resistentie	
*healthy volunteer 7	2,01	normale osmotische resistentie	
*healthy volunteer 8	1,99	normale osmotische resistentie	
*healthy volunteer 9	1,851	normale osmotische resistentie	
* healthy volunteer 10	2,104	normale osmotische resistentie	
healthy volunteer 11	4,2	normale osmotische resistentie	
healthy volunteer12	3,95	normale osmotische resistentie	
healthy volunteer 13	17,59	normale osmotische resistentie	
healthy volunteer 14	15,98	normale osmotische resistentie	
healthy volunteer 15	6,3	normale osmotische resistentie	
***67598789**	7,18	verlaagde osmotische resistentie incubatie niet uitgevoerd	HS
*64717408	1,77	normale osmotische resistentie	
*60227532	7,07	normale osmotische fragiliteit	
*71878987	2,72	normale osmotische fragiliteit	
62316347	4,24	verhoogde osmotische fragiliteit	Thalassemie
61044264	1.8	normale osmotische resistentie	
60215522	6.48	normale osmotische resistentie	
85585974	4,4	normale osmotische resistentie	
60744587	35	verlaagde osmotische resistentie incubatie niet uitgevoerd	AIHA
83574426	3,6	normale osmotische resistentie	
84836584	2,3	normale osmotische resistentie	
83570283	7,2	normale osmotische resistentie	
63721500	2,4	verhoogde osmotische resistentie	Thalassemie

*The measurements performed during the test phase of the study

Comparison of CH test results, performed at Reference Center Erasmus Hospital and UZ Leuven

Since a false negative result of CH test was observed in one sample, collected from a patient with HS, a comparison study between our laboratory and the Reference Center Erasmus Hospital was set up. We simultaneously tested 5 samples from healthy volunteers as well as 3 patients samples (Table 9). We did not notice discordant results between both laboratories (positive vs negative). However, the" inter-laboratory" CV was between 9.3 % and 80.7% (mainly due to 1 sample (see bold on Table 9)). The unacceptable high CV could be partially explained by the methodology of the CH method which require different steps with manual preparation and necessary strict temperature monitoring during the incubation, transport and condition of the storage.
At present, we will further collect the data with an aim to compare the CH test results vs OF test results obtained from the patients with suspected HS.

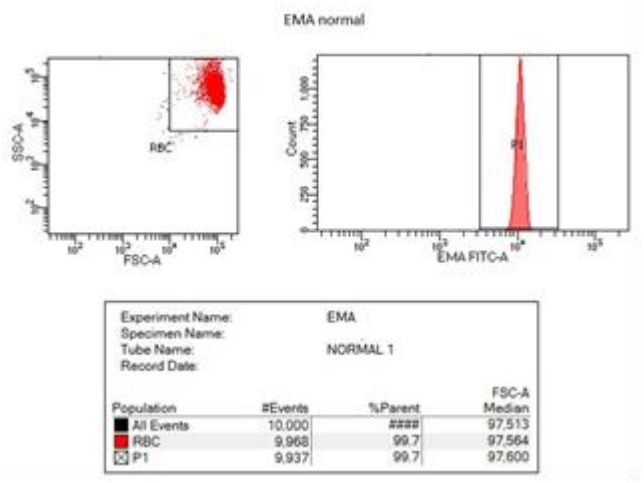
Table 9: Comparison of CH test results, performed simultaneously in UZ Leuven and Erasmus Hospital

	CH% Leuven	**CH% Brussel**	**Concordance of the CH Results: Yes/No**
healthy volunteer 11	4,2	3,6	Yes
healthy volunteer12	3,95	2,4	Yes
healthy volunteer 13	17,59	14,2	Suspicious Leuven vs Positive Brussel
healthy volunteer 14	15,98	14	Positive Leuven vs Suspicious Brussel
healthy volunteer 15	6,3	4,4	Yes
VKA648	4,73	6,1	Yes
VKA069 HS	**16,4**	**60**	**Yes**
60744587 AIHA	35,2	29,6	Yes

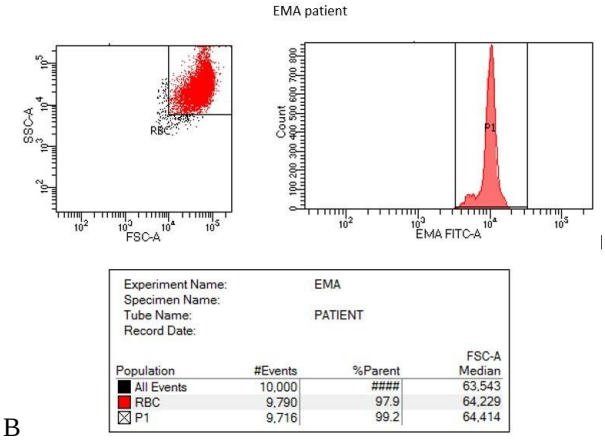
<10% negative, between 10 and 15 % as suspicious and >15 % as positive

Eosin-5'-maleimide (EMA) binding test

The EMA binding test uses flow cytometry to determine the amount of fluorescence (reflecting EMA bound to specific transmembrane proteins) derived from individual red cells (Fig 9 A, B) (King et al., 2004; Bolton-Maggs et al., 2012). A decrease of these molecules on the surface of red blood cells has been shown in hereditary spherocytosis. EMA binding to these proteins (band 3, CD47, RhAG and Rh polypeptide), which are closely associated with the red cell cytoskeleton, underpins the high specificity of this test (http://www.inesss.qc.ca/accueil.html). Results are expressed as decreased fluorescent intensity of red cells labeled with EMA (Fig.9B) (Gulbis et al., 2013).



A



B

Fig. 9 A-B. An EMA (eosin-5′-maleimide) binding test is performed and this shows reduced binding consistent with hereditary spherocytosis https://teamhaem.com/blog/page/4/

The test can be done using a small volume of sample (min 100µl), which makes it suitable for children and neonates (King et al., 2000).
Moreover, results are not affected by recent transfusions since the method discriminates different red cell populations (King et al., 2008; Bianchi et al., 2012).
A further advantage of the EMAbinding test is that results are not influenced by shipping or storage for up to 6 days at 4°C (Guitton et al., 2008; Bianchi et al., 2012).
The clinical performance of the EMA test for detecting hereditary spherocytosis is good in most of the studies, with sensitivities higher than 95% and specificities higher than 85% (reviewed by Gulbis et al., 2013; http://www.inesss.qc.ca/accueil.html). According to the results of six studies, the area under curve ranges from 0.873 to 0.99. (reviewed by http://www.inesss.qc.ca/accueil.html).

Limitations

The method of reporting the results of the EMA binding test has not been standardized; some laboratories report the results as absolute mean fluorescence intensities (MFIs) of EMA in patient RBCs, whereas others use percentages of normal controls (reviewed by Park, et al., 2014).
Indeed, the cut-off values vary and are expressed in different ways; i.e., as a percent decrease of mean fluorescent intensity (11%, 17%, >17%, 18%, 19.5%, 21% and 80%), mean fluorescent intensity (0.80, 400 and 10,126 MFI) or MFI units (45.5, 48.2 and 91.5 MFI) http://www.inesss.qc.ca/accueil.html. Some authors report a grey area between 16% and 21% (Mackiewicz et al., 2012; ttp://www.inesss.qc.ca/accueil.html) .
If the majority of the patient's red cells are phenotypically normal without significant loss of the EMA binding membrane proteins (as in the case of mildly affected HS), the fluorescence results obtained may be indeterminate (Bolton-Maggs et al., 2012).

Conclusion

In Conclusion, none of the tests can recognise all cases of HS, its diagnosis is not always strait forward and requires the different investigations (Gulbis et al., 2013; Lazarova et al., 2014).
Consequently, first, when selecting an appropriate test for HS, the sensitivity and specificity of the test for HS, the complexity of the protocol, and the total cost of instrument(s) and its maintenance should be taken into consideration (Bolton-Maggs et al., 2004; Mullier et al., 2011). Secondly, diagnosis of HS is currently based on a combination of clinical and family histories, physical examination (for splenomegaly and jaundice) and laboratory data including blood count, especially red cell indices, reticulocyte count and red cell morphology (Mullier et al., 2011).
Taking into account published data and our observations, the diagnostic tool based on the automated reticulocyte parameters can be useful in the first line screening for the HS.
The CH test has superior diagnostic performance compare to OF test, however still requires different steps with manual preparation and necessary strict temperature monitoring during the incubation. The reference range should be carefully evaluated by each laboratory and, the normal reference values for CH test might need to be adjusted.

The following Investigations steps have been proposed for laboratory diagnosis of HS by Gulbis et al., 2013; Mullier et al., 2011; Persijn et al., 2012; Lazarova et al., 2014

I Screening (General laboratory)

Screening (+ family history and typical clinical features)
First line

- RBC morphology on blood smear
- Hematology parameters /Screening algorithm, based on the automated reticulocyte parameters
- Biochemical hemolysis parameters

Second line (reduced area-to-volume ratio, increased osmotic fragility)

- Hypertonic cryohemolysis, osmotic fragility test
- Eosine-5-maleimide binding

II Diagnosis (Reference centers)

- SDS-PAGE
- Ektacytometry with osmotic resistance measurement
- Molecular analysis

COMMENTS

TO DO/ACTIONS

1) To collect MicroR and Hypo-He data regarding the research reticulocytes parameters and apply the Rule 2 of the screening algorithm

2) At present, we are collecting the data with aim to compare the CH test results vs OF test results obtained from the patients with suspected HS

3) Verification reference-value for CH test

ATTACHMENTS

Attachment 1

Intra-assay variation

	Within-run opwerking berekend	**Within-run toestel berekend**	**Within-run opwerking sucrose Abs**	**Within-run opwerking totale hemolyse Abs**	**Within-run toestel sucrose Abs**	**Within-run toestel totale hemolyse Abs**
#1	2%	2%	0,063	1,499	0,056	1,436
#2	3%	2%	0,074	1,414	0,056	1,436
#3	2%	2%	0,058	1,669	0,056	1,436
#4	2%	2%	0,059	1,804	0,056	1,436
#5	2%	2%	0,064	1,429	0,056	1,436
#6	2%	2%	0,069	1,545	0,056	1,436
#7	2%	2%	0,062	1,489	0,056	1,436
#8	2%	2%	0,060	1,578	0,056	1,436
#9	2%	2%	0,064	1,531	0,056	1,436
#10	2%	2%	0,056	1,435	0,056	1,436
%CV	14%	0%	9%	8%	0%	0%