Additional erythrocytic and reticulocytic parameters helpful for diagnosis of hereditary spherocytosis: results of a multicentre study

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Running short title: NEW DIAGNOSTIC PARAMETERS FOR HEREDITARY SPHEROCYTOSIS

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Abstract

Background: Hereditary Spherocytosis (HS) is characterized by weakened vertical linkages between the membrane skeleton and the red blood cell's lipid bilayer, leading to the release of microparticles. All the reference tests suffer from specific limitations. The aim of this study was to develop easy to use diagnostic tool for screening of hereditary spherocytosis based on routinely acquired haematological parameters like percentage of microcytes, percentage of hypochromic cells, reticulocyte counts, and percentage of immature reticulocytes.

Design and methods: The levels of Hb, MCV, MCHC, Reticulocytes (Ret), Immature Reticulocytes Fraction (IRF), Hypochromic Erythrocytes (Hypo-He) and Microcytic Erythrocytes (MicroR) were determined on EDTA samples on Sysmex instruments from a cohort of 45 confirmed SH. The HS group was then compared with haemolytical disorders, microcytic anaemia, healthy individuals and routine samples (n: 1488).

Results: HS is characterized by a high Ret count without an equally elevated IRF. All 45 HS have Ret > 80000/µl and Ret(/µl)/IRF (%) greater than 7.7 (rule 1). Trait and mild HS had a Ret/ IRF ratio greater than 19. Moderate and severe HS had increased MicroR and MicroR/Hypo-He (rule 2). Combination of both rules gave Predictive Positive Value and Negative Predictive Value of respectively 75% and 100% (n: 1488), which is much greater than single parameters or existing rules.

Conclusions: This simple and fast diagnostic method could be used as an excellent screening tool for HS. It is also valid for mild HS, neonates, ABO incompatibilities and overcomes the lack of sensitivity of electrophoresis in ankyrin deficiencies.

Keywords: hereditary spherocytosis, reticulocyte, microparticles, ankyrin

Introduction

Hereditary Spherocytosis (HS) [1-3] is the most common cause of inherited chronic haemolysis in northern Europe and North America where it affects one person in 2000. HS refers to a group of heterogeneous regenerative haemolytic anaemias characterised by a loss of membrane surface area, leading to reduced deformability due to defects in proteins in the erythrocytic membrane (ankyrin, band 3 protein, β-spectrin, α-spectrin, or protein 4.2). Osmotic resistance, hypertonic cryohaemolysis test, eosin-5-maleimide (EMA) binding in flow cytometry, Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) and ektacytometry are the reference tests for the diagnosis of HS. However, all tests suffer from specific limitations. Osmotic fragility test lacks of sensitivity and specificity [3]. Hypertonic cryohaemolysis test and EMA test are not specific and can also detect red cells with rare membrane disorders, such as aberrant band 3 proteins, a change in intracellular viscosity and temperature-sensitive monovalent cation transport [2]. Moreover, in mild or atypical cases, difficult interpretation is likely to occur. SDS-PAGE lacks sensitivity to very mild or asymptomatic HS carriers [2], and a high reticulocyte count might mask a reduction in ankyrin-1 in SDS-PAGE [4] whereas an ankyrin-1 defect represents 40-65% of HS in the USA and Europe [3]. A key test for HS, osmotic gradient ektacytometry, is only available in specialised laboratories. 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 [2]. 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. Other causes of

haemolytic anaemia should be excluded, particularly autoimmune haemolytic anaemia caused by warm (IgG) or cold (IgM) autoantibodies and also alloimmune haemolytic anaemia in the event of ABO incompatibility in neonates. Autoimmune haemolysis (AIHA) can usually be excluded by a negative direct anti-globulin test (DAT). Sometimes, there are difficulties in interpreting DAT-negative haemolytic anaemias in individuals with no family history, particularly in adults. In some cases, the density of attached autoantibody may be too low for detection by DAT. Polyspecific DAT reagents may also fail to detect some autoantibodies, particularly IgA autoantibodies. Moreover, diagnosis is often more difficult during the neonatal period than later in life, for many reasons [2,3].

In conclusion, the development of simple, fast, accurate, sensitive, and specific diagnostic laboratory tests for hereditary spherocytosis is a real challenge [2,3]. Some authors recommend automated red cell indices to predict or identify HS. Increased parameters such as reticulocytosis, % microcytes [5,2,6], combination of increased Mean Corpuscular Haemoglobin Concentration (MCHC) with increased red distribution width (RDW) [2,7] , combination of increased RDW with hyperdense cells (Hyperchromic cells) [2,8], combination of increased MCHC with increased hyperdense cells [2], decreased red cell and reticulocyte surface area [9], discordance between the Cell Haemoglobin Concentration Mean (CHCM) and MCHC [8] or Mean Spherized Corpuscular Volume (MSCV) lower than Mean Cell Volume (MCV) [10] are used for HS screening. However, most of these parameters are mean parameters and none of the tests is efficient for HS screening, especially in mild forms (without anaemia, reticulocytes <6% and bilirubin 17.4-34.2µM) or carriers of an asymptomatic trait (without anaemia, slight reticulocytosis (1.5-3.0%) and slightly reduced haptoglobin) [3]. Although the main molecular defects in hereditary spherocytosis are

heterogeneous, one common feature of the erythrocytes in this disorder is weakened vertical linkages between the membrane skeleton and the lipid bilayer and its integral proteins [3]. When these interactions are compromised, loss of cohesion between bilayer and membrane skeleton occurs, leading to destabilisation of the lipid bilayer and the release of skeleton-free lipid vesicles (or microvesicles or microparticles). Furthermore, one previous study [9] showed that different mechanisms lead to reduced membrane surface area in hereditary spherocytosis and some forms of AIHA. Indeed, in HS, but not in AIHA, the surface area loss is already present at the circulating reticulocyte stage. As a consequence of the release of such microparticles, the percentage of microcytes should be higher in HS. Since HS affects reticulocytes, the total reticulocyte count and Immature Reticulocytes Fraction could be of great interest. Therefore, we decided to study reticulocyte parameters to develop a diagnostic tool on 2 haematological analysers: XE-2100 and XE-5000 (Sysmex, Kobe, Japan).
The study objectives were i) to characterize a cohort of confirmed HS patients in terms of reticulocytosis and IRF ratio, and MicroR/Hypo-He ratio; ii) to study the efficiency of the combination of those 2 rules to screen confirmed HS cases and to differentiate HS from other haemolytic disorders, microcytic red cell disorders, healthy subjects and a routine haematological database; iii) to compare this tool with current existing rules, and iv) to assess the new method in neonates and in trait and mild forms.

Design and methods

Subjects

Between January 2008 and May 2009, the diagnosis of 45 HS patients (22 women, 23 men, mean age 13.1, from 1 day to 76 years old) was confirmed by clinical data and laboratory diagnosis; flow cytometry (eosin-5'-maleimide assay) and/or ektacytometry and/or SDS-

PAGE electrophoresis as shown in Table 1. They were classified according to the haemoglobin level as severe (n= 6, Hb <8g/dl), moderate (n= 27, Hb 8-12 g/dl) and mild (n= 12, Hb > 12g/dl). Three of the 45 cases of HS were splenectomised before analysis (patients 1, 11 and 12 in Table 1).

The HS group was compared with a group of 108 patients suffering from various haemolytical disorders, such as ABO incompatibility (n=4), Thrombotic Thrombocytopenic Purpura (TTP, n=4), HUS (Haemolytic Uremic Syndrome, n=3), Drepanocytosis (n=5), PNH (Paroxysmal Nocturnal Haemoglobinuria, n=4), Glucose-6-PhosphateDeHydrogenase (G6PDH) deficiency (n=1), Pyruvate kinase (PK) deficiency (n=1), HbE + β-thalassemia (n=3), β-thalassemia (n=3) , haemolytic anaemia due to leukemias (n=7), lymphomas (n=12), myelomas (n=4), cold agglutinins (n=4) and others haemolytical disorders (n=53).

Furthermore, we compared the HS group with a group of 93 microcytic anaemia patients (64 with iron deficiency and 29 with functional iron deficiency), with a control group of 61 healthy individuals and 1230 samples from the routine haematological database.

Finally, one membranopathie other than hereditary spherocytosis (ie a xerocytosis) was included.

Methods

HS diagnostic tests

In addition to the clinical data, serum bilirubin, hypertonic cryohaemolysis test [2], flow cytometry (Eosin-5' maleimide assay) [11,1,12], SDS-PAGE electrophoresis [1] and ektacytometry [6] were performed on HS patients. Osmotic fragility tests were not performed

routinely since gradient ektacytometry is available in our laboratory which is considered superior to tests of osmotic fragility.

Haematological measurements (XE-2100 and XE-5000)

Blood was drawn in Venosafe® terephthalate polyethylene tubes (Terumo Europe, Leuven, Belgium) containing dipotassium ethylenediaminetetraacetic acid (EDTA K2). Samples were taken during normal diagnostic follow-up. No additional sampling was performed. All the measurements were performed within 24 hours of blood sampling. Red cell morphology on the blood smears was evaluated by optical microscopy. Red cell and reticulocyte counts and indices and haemoglobin values were determined using XE-2100 (n= 15) and XE-5000 (n= 30) (Sysmex, Kobe, Japan). These two automated blood cell analysers combine a red semiconductor laser technique with different polymethine dyes to produce a 5-part differential WBC count and reticulocyte measurement. The minimal volumes required for Cell Blood Count (CBC) and reticulocyte measurements are 40 µl (capillary mode), 130µl (manual mode) or 200 µl (automatic mode). Parameters of interest provided by both haematological analysers are: Haemoglobin (Hb, (g/dl)), Mean Corpuscular Volume (MCV, fl), Mean Corpuscular Haemoglobin Concentration (MCHC, (g/dl)), Reticulocytes (RET, (giga/l)) and Immature Reticulocytes Fraction (IRF, (%)) and the specific XE-5000 parameters Hypo-H_e, Hyper-H_e, MicroR and MacroR [13]. In addition, we calculated systematically the 2 following parameters: the MicroR/Hypo-He ratio and the Ret/IRF ratio ((10^9)/ (l* %)).

%HYPO-H_e and %HYPER-H_e are unique parameters analysed in the reticulocyte channel of the XE-5000. The basis for analysis of these parameters is the mean haemoglobin content of

all the measured red blood cells analysed in the reticulocyte channel. Red blood cells with decreased haemoglobin content (< 17pg) are classified as hypo-haemoglobinized cells, whereas the hyper-haemoglobinized red blood cell population contains cells with an increased haemoglobin content (>49pg) (Figure 1a)). The RBC cell size distribution curve shows an almost Gaussian distribution in healthy human blood. By applying two discriminators, one in the lower (<60fl) and one in the upper area (>120fl), a microcytic and a macrocytic population are analysed.

These parameters are sensitive for a small percentage of abnormal cells and therefore more sensitive than mean values.

Statistical methods

Sensitivity, specificity, ROC curves, and predictive values were calculated using Medcalc version 11.1.1.0 (Gent, Belgium). Comparison of Ret-IRF ratios (Figure 2), MicroR-HypoHe ratio (Figure 3) and MicroR (Figure 4) 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) was performed using GraphPad Prism® software Version 4. Results were presented as mean +/- standard error of the mean (SEM).

Results

All HS showed a high reticulocyte count without an equally elevated Immature Reticulocytes Fraction (Figure 1). The 45 confirmed cases of HS have reticulocytes > 80 10^9/l and a Reticulocytes (10^9/l) / Immature Reticulocytes Fraction (%) (Ret/IRF) ratio higher than 7.7 (Figure 2). As shown in Table 2, this limit is used as a precondition for the screening of all the

cases of HS. The combination of reticulocytosis with index reticulocytosis/IRF is more discriminating than the combination of reticulocytosis with low IRF (data not shown). Moreover, all trait and mild cases (Hb >12g/dl, n= 12) of HS have a Ret/ IRF ratio higher than 19 (Figure 2). Consequently, as shown in Table 2, this cut-off is used as rule 2 for the screening of trait and mild HS.

For moderate and severe cases of HS, MicroR was included since this parameter reflects the severity of the disease [2]. Furthermore, for those patients, MicroR and HypoHe were combined. Indeed, a preliminary study showed that combining MicroR and the MicroR/Hypo-He ratio led to the elimination of more non-HS cases than MicroR alone. ROC Curve analysis showed that the optimal cut-offs for MicroR and MicroR/Hypo-He in cases of Hb between 8 and 12 g/dl, were 3.5% and 2.5, respectively. When Hb was lower than 8 g/dl, the optimal cut-offs for MicroR and MicroR/Hypo-He were 3.5% and 2.0, respectively.

We then evaluated the efficiency of our diagnostic tool to screen confirmed HS cases to differentiate HS from other haemolytic disorders, iron deficiencies, healthy individuals and controls. Figure 2 showed 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). The HS prevalence was 1.98% in our population. However, as illustrated in Figures 2-4, addition of MicroR/Hypo-He (Figure 3) and MicroR (Figure 4) is required to discriminate severe HS from some haemolytical disorders and some healthy individuals, respectively.

The performances of the HS diagnostic tool were compared with single parameters and existing rules, as shown in Table 3. 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) or the existing rules (reticulocytosis, % microcytes [5,2,6], combination of increased MCHC with RDW [2,7] , combination of increased RDW with hyperdense cells (Hyperchromic cells) [2,8], combination of increased MCHC with increased hyperdense cells [2]). The negative predictive value is excellent (>98.5 %) for all parameters or rules. However, the second best rule in term of positive predictive value is the combination of MCHC and Hyper-He with a PPV of only 32.5% (Table 3).

Finally, regarding ABO incompatibilities (n=4), all had Ret/IRF ratio higher than 7,7 but none had Ret/IRF ratio higher than 19,9, MicroR higher than 3,5 or MicroR/Hypo-He higher than 2.

Discussion

The diagnostic tool proposed in the present study is based on the physiopathology of HS. Indeed, one common feature of the red blood cells in this disorder is weakened vertical linkages between the membrane skeleton and the lipid bilayer leading to the release of skeleton-free lipid vesicles. In HS, the loss of surface area is already present at the circulating reticulocyte stage. We thus decided to study reticulocyte parameters to develop a diagnostic tool on 2 haematological analysers: XE-2100 and XE-5000 (Sysmex, Kobe, Japan).

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

Rule 1: Ret and Ret/IRF ratio. All 45 confirmed cases of HS have reticulocytes > 80 10^9/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. 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 [2]. Therefore, MicroR was included in rule 2, only for moderate and severe cases of HS.

Trait and mild HS

All trait and mild cases (Hb >12g/dl, n= 12) of HS have 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 cryohaemolysis test and EMA binding in flow cytometry is often difficult in mild cases. Moreover, SDS-PAGE suffers from many limitations: it lacks of sensitivity to very mild or asymptomatic HS carriers [2], and a high reticulocyte count might mask a reduction in ankyrin-1 in SDS-PAGE [4,3]. Patients 3, 4 and 6 (Table 1), for whom HS was confirmed by clinical data and flow cytometry, illustrate the lack of sensitivity of SDS-PAGE.

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 12 g/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.

Neonates and ABO incompatibilities

Interestingly, the method was also valid for the 4 neonates (2 severe, 1 moderate and 1 mild) and the 4 cases of ABO incompatibility patients. The screening of HS in such individuals is

another major advance of this method. Indeed, the diagnosis is often difficult during the neonatal period for several reasons, including infrequent splenomegaly, variable and usually not severe reticulocytosis, few spherocytes in their peripheral blood smears (whereas spherocytes (≤ 1%) are commonly seen in neonatal blood films in the absence of disease), and the fact that neonatal red blood cells are more osmotically resistant than adult cells. Furthermore, at this age, the difference between an ABO incompatibility patient and an HS patient is very difficult to detect [14,15].

We then evaluated the efficiency of our diagnostic tool to screen confirmed HS cases, healthy individuals and to differentiate HS from other haemolytic disorders and iron deficiencies. The Area Under the Curve (AUC), sensitivity, specificity, predictive positive value and negative predictive value were respectively 0.997, 100%, 99.3%, 75% and 100%. This diagnostic tool is therefore much more efficient than single parameters (Ret/IRF index) or the existing rules (reticulocytosis, % microcytes [5,2,6], combination of increased MCHC with RDW [2,7] , combination of increased RDW with hyperdense cells (Hyperchromic cells) [2,8], combination of increased MCHC with increased hyperdense cells [2]).
As HS is a low prevalence disease, predictive values are more relevant parameters than sensitivity and specificity. The negative predictive value is excellent (>98,5%) for all parameters or rules. However, the second best rule in term of positive predictive value of MCHC and Hyper-He with a PPV of only 32.5% (Table 3). Besides its very good performances, this diagnostic method is also inexpensive, corresponding to a Cell Blood Count (CBC) and parameters linked to reticulocytes and red blood cell parameters (Hb, MCV, MCHC, RET, IRF, Hypo-He, MicroR).

From a technological point of view, the method contains a combination of reticulocytosis with a decrease in IRF, due to lower fluorescence intensity (Figure 1). To perform the IRF measurement, reticulocytes are labeled with a fluorescent dye (polymethine and oxazine) in a Ret-Search (II) reagent that results in the cell entry and staining of mRNA reticulocytes. Several hypotheses could explain a high reticulocyte count without an equally elevated Immature Reticulocytes Fraction observed in HS. The first hypothesis is an insufficient entry of the fluorescence dye into the defective cells, leading to an abnormal ratio between the two parameters. Indeed, the loss of red cell membrane proteins (band-3 protein, ankyrin, spectrin) could disturb the response of spherocytic cells to permeabilisation. The staining reaction is thus abnormal at the time of measurement. This results in decreased staining of RNA and normal IRF as seen in the RET scattergram shown in Figure 1. A lower RNA concentration in the RBC implies increased maturity, so that immature reticulocytes will be falsely classified as a more mature fraction (MFR or LFR). Consequently, the highly fluorescent immature reticulocytes containing the most RNA are decreased. The second hypothesis is the early loss of cell surface area during HS. The maturation of reticulocytes into erythrocytes is associated with a loss of intracellular organelles, including mitochondria, endoplasmic reticulum, golgi apparatus and endocytic vesicles. There is also extensive remodeling of the plasma membrane, with progressive loss of various membrane proteins through the release of exosomes. These lost proteins include the transferrin receptor, flotillin, Glut-4, CD47, actin, Hsc70, aquaporin-1 (AQP-1) and adhesion receptors such as β1-integrin [16]. In HS, the loss of cell surface area at the reticulocyte stage [9] could explain that one or multiple of these compounds normally stained by the fluorescent dye in the reticulocyte channel are less marked in cases of HS.

Limitations of this study are that first, these results have been only validated on XE-2100 and XE-5000 instruments. In a near future, a similar method will be developed for other haematological analysers. Detection of abnormalities in the reticulocyte maturation could improve the current performances on Advia 2120 (Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA) and LH750/DXH800 (Beckman Coulter Inc, Miami, FL, USA) based respectively on the discordance between MCHC and CHCM [17,8] and the difference between MCV and MSCV [18,10,19,20]. The second limitation is that, at present it is unknown whether the same phenomena concerning Ret-IRF-ratio and Micro-Hypo-ratio can be observed in other types of membranopathy like xerocytosis and related cryohydrocytosis. This differential diagnosis is particularly important since splenectomy is virtually contraindicated in xerocytosis [21]. However, the only included xerocytosis was positively screened by the precondition (rule 1) since the Ret-IRF ratio was higher than 7,7 (12,6). But, a Hb of 14,4g/dl allowed to exclude the diagnosis of HS with the rule 2 since the Ret-IRF ratio was lower than 19.

Conclusions

We have developed and validated of an original diagnostic tool of hereditary spherocytosis based on reticulocyte-derived microparticles release. This diagnostic tool could be used routinely as an excellent screening method for the diagnosis of HS. This is in line with recommendations proposed by some authors in recent reviews [3]. This rapid method also works on mild SH and in neonates, and overcomes the lack of sensitivity of SDS-PAGE in ankyrin deficiencies. This proposed tool in combination with clinical data could make the diagnosis of HS easier by reducing the number of expensive and time-consuming confirmation tests.

Authorship and Disclosures.

F.M. and B.C conceived the idea and designed the study protocol; F.M and B.C analyzed the Sysmex data and together with N.B and Y.C analyzed cryohemolysis and flow cytometric data. E.L, O.F and L.D analyzed ektacytometry data and together with F.S contributed with provision of study material or patients. F.M and B.C collected, assembled data and interpreted the data; F.M performed statistical analysis; F.M, B.C, C.C and J-M.D wrote the manuscript; and all authors reviewed and approved the manuscript. The authors declare no competing financial interests.

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Table 1: Red Cell Indices, serum bilirubin, cryohaemolysis, flow cytometry, SDS-PAGE and ektacytometry for HS patients

Patient (sex)	Age	Hb (g/dl)[A]	MCV (fL)[B]	MCHC (g/dl)[C]	RET (10^9/l)[D]	IRF (%)[E]	Ret/ IRF	MicroR (%)[F]	Hypo-He (%)[G]	MicroR/Hypo-He	Indirect Bilirubin (mg/dl)	Cryohaemolysis (%)	Flow cytometry (%)	SDS-PAGE[H]	Ektacytometry
1(F)[I]	53y[K]	14,4	93,3	36,3	124,7	3,2	39	1,1	0,7	1,6	0,99	14,5	19,6	ND[L]	ND
2(F)	5mon[M]	8,4	71,1	35,1	252	8,6	29,3	23,9	4,8	5	1,16	13,8	33,3	Spectrin and ankyrin deficiency	ND
3(F)	2mon	8,7	79,6	33,9	228,7	21,2	10,8	9,2	2	4,6	ND	16,3	21,8	-[N]	ND
4(M)[J]	3mon	8,7	75,2	35,4	199	8,8	22,6	16	3,8	4,2	ND	19,7	33,6	-	ND
5(M)	15y	16,1	84,9	38,2	426,6	2,8	152,4	4,1	0,3	13,7	10,22	19,8	27,6	ND	ND
6(M)	8y	11,4	74,9	36,8	303	4,2	72,1	16,1	1,3	12,4	1,3	48,9	43,1	-	ND
7(F)	2y 10mon	11,2	79,5	34,8	202	4,8	42,1	13,7	2	6,9	2,7	51	39,9	Spectrin deficiency	ND
8(M)	21y	13,6	95	33,9	514	10,7	48	5	1,2	4,2	8,12	56,3	34,3	Spectrin deficiency	ND
9(M)	27y	12	82,3	36,5	538	10,9	49,4	10	1,8	5,6	ND	ND	34	ND	ND
10 (F)	17y	12,5	87,6	37,7	125,1	0,4	312,8	1,6	0,2	8	4,94	ND	ND	Band 3 deficiency	ND
11 (M)	42y	17,2	82,7	35,5	81,9	1,7	48,2	3,3	0,2	16,5	0,48	ND	ND	ND	ND
12 (M)	57y	16,1	89,6	35,1	83,5	0,3	278,3	1,4	0,2	7	0,63	ND	ND	ND	ND
13 (M)	14y	12,5	87,4	37,7	288,8	4,4	65,6	3,2	0,3	10,7	3,81	ND	ND	Band 3 deficiency	ND
14 (M)	31y	13	93,2	33,8	178	4,7	37,9	1	0,3	3,3	8,4	ND	27	ND	ND
15 (M)	UA[O]	9,5	71,3	35,1	113,6	5,6	20,3	21,3	2,7	7,9	ND	13,1	34,9	ND	ND
16 (M)	34y	16,9	88,3	32,4	85,7	6,2	13,8	4,8	1,4	3,4	0,43	ND	32,8	ND	ND
17 (M)	52y	13,3	82,8	35,9	290	4,7	61,7	7,6	1,2	6,3	2,72	19,3	18,8	Band 3 deficiency	ND
18 (M)	8y 8mon	11,4	81,7	32,8	293,5	9,7	30,3	12,4	3,8	4,4	ND	ND	38	ND	ND
19 (M)	5y 10mon	12,5	84,7	38,2	84,9	4,1	20,7	1,8	0,1	18	0,6	ND	ND	ND	+[P]
20 (M)	2mon	7,6	79,2	35,7	181	11	16,5	10,2	2,6	3,9	4	ND	34	ND	ND
21 (M)	11y 5mon	9,2	75,3	35,1	378,6	13,8	27,4	20,8	2,9	7,2	4,3	ND	ND	ND	+
22 (F)	8y 11mon	11,8	81,2	36,5	345,1	5,5	62,7	11	1,7	6,5	4,9	ND	ND	ND	+
23 (F)	8y	11,4	86,8	32	584,3	8,4	69,6	10,6	4,1	2,6	ND	ND	ND	ND	+
24 (F)	1mon	6,6	81,7	36,9	158,1	15,4	10,3	6,1	1,4	4,4	3,1	ND	41,9	ND	ND
25 (M)	3y 10mon	5,1	65,8	32,3	209,5	21,7	9,7	40	15	2,7	2	ND	30,5	ND	ND
26 (M)	76y	10,1	96,3	35,7	285,2	15,8	18,1	50	25	2,0	2,6	ND	47,7	Band 3 and protein 4.2 deficiency	+
27 (M)	11mon	10,8	69,4	34,8	280,7	13,6	20,6	3,5	1,4	2,5	ND	ND	ND	ND	ND
28 (M)	2y 1mon	5,9	88,5	30,7	287,5	37,4	7,7	23,9	5,9	4,1	2	11,3	25,2	-	ND
29 (F)	37y	10,9	88,4	32,4	370,5	7,1	52,2	7,9	1,5	5,3	18,9	ND	33,9	ND	ND
30 (M)	17y	11,1	80,8	35,2	175,5	11,2	15,7	23,5	3,2	7,3	ND	ND	ND	Band 3 deficiency	ND
31 (M)	8d[Q]	10,8	112,3	32	363,6	1,5	242,4	ND	ND	ND	29,5	ND	ND	ND	+
32 (F)	2mon	6,6	77,9	34,7	279,4	33,4	8,4	ND	ND	ND	2,2	ND	ND	ND	+
33 (F)	1mon	5,7	81,9	37	211,9	24,2	8,8	ND	ND	ND	3,4	ND	ND	ND	+
34 (F)	2y 4mon	10,4	73,6	35,3	469,2	15	31,3	ND	ND	ND	3	ND	ND	ND	+
35 (F)	4y	10,6	78,8	37,6	222,3	14,8	15	ND	ND	ND	3	ND	ND	ND	+
36 (F)	7y 3mon	8,4	73,2	33,7	461,7	23,2	19,9	ND	ND	ND	4,8	ND	ND	ND	+
37 (F)	11mon	10,9	78,9	33,4	432,4	24,5	17,6	ND	ND	ND	ND	ND	ND	ND	+
38 (F)	6mon	9,3	73,6	33,8	471,1	23,5	20	ND	ND	ND	1,8	ND	ND	ND	+
39 (F)	5y 4mon	10,4	77,7	35	308,7	19,3	16	ND	ND	ND	0,7	ND	ND	ND	+
40 (F)	7mon	10,2	78,1	34,5	457,1	20,5	22,3	ND	ND	ND	ND	ND	ND	ND	+
41 (F)	3y 11mon	11,5	82	35	277,1	11,5	24,1	ND	ND	ND	2,7	ND	ND	ND	+
42 (F)	7mon	10,2	79,4	34	396,9	18,8	21,1	ND	ND	ND	ND	ND	ND	ND	+
43 (M)	1d	17,3	97,8	36,2	419,1	37	11,3	ND	ND	ND	21,8	ND	ND	ND	+
44 (F)	1y 10mon	9,8	78	32,6	584	28,5	20,5	ND	ND	ND	ND	ND	ND	ND	+
45 (F)	5mon	9,9	80,9	32,9	508,5	31	16,4	ND	ND	ND	ND	ND	ND	ND	+

Footnote:

A,Haemoglobin; B, Mean Corpuscular Volume; C, Mean Corpuscular Haemoglobin Concentration; D, Reticulocytes; E, Immature Reticulocytes Fraction; F, Microcytic erythrocytes (%); G, Hypochromic erythrocytes (%); H, Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis; I, Female; J, Male; K, Year ; L, Not Determined; M, Month; N, Negative; O, Unavailable; P, Positive; Q, Days.

Table 2. Hereditary spherocytosis diagnostic tool

Rule		Parameters		
Rule 1	Precondition	Ret ≥ 80000/µl and Ret/IRF > 7.7		
Rule 2	Severity	Trait or mild HS Hb > 12g/dl	Moderate HS 8 g/dl ≥ Hb ≤ 12g/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

Footnote:

Ret indicates Reticulocytes (/µl); IRF, Immature Reticulocytes Fraction (%); HS, Hereditary Spherocytosis; Hb, Haemoglobin; MicroR, Microcytic erythrocytes (%); Hypo-He, Hypochromic erythrocytes (%)

Table 3: 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 (10^3/µl)	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

Footnote:
AUC indicates Area Under the Curve; 95% CI, 95% Confidence Interval; PPV, Predictive Positive Value; NPV, Negative Predictive Value; MCHC, Mean Corpuscular Hemoglobin 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 (/µl*%); HS, Hereditary Spherocytosis

Figure 1. Reticulocytes channel on Sysmex XE-5000^{CM}

(a) Hereditary Spherocytosis: reticulocytosis with decreased Immature Reticulocytes Fraction (IRF)**.** Definition of Hypo-He: Hypochromic erythrocytes (%) and Hyper-He: Hyperchromic erythrocytes, is 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 17pg, corresponding to the low discriminator for RBC-H_e (RBC-He -LD), are classified as hypo-haemoglobinized cells, whereas the hyper-haemoglobinized red blood cell population contains cells with a haemoglobin content higher than to 49pg, the high discriminator for RBC-H_e (RBC-H_e -HD).

(b) Other Haemolytical Anaemias: Reticulocytosis with normal IRF

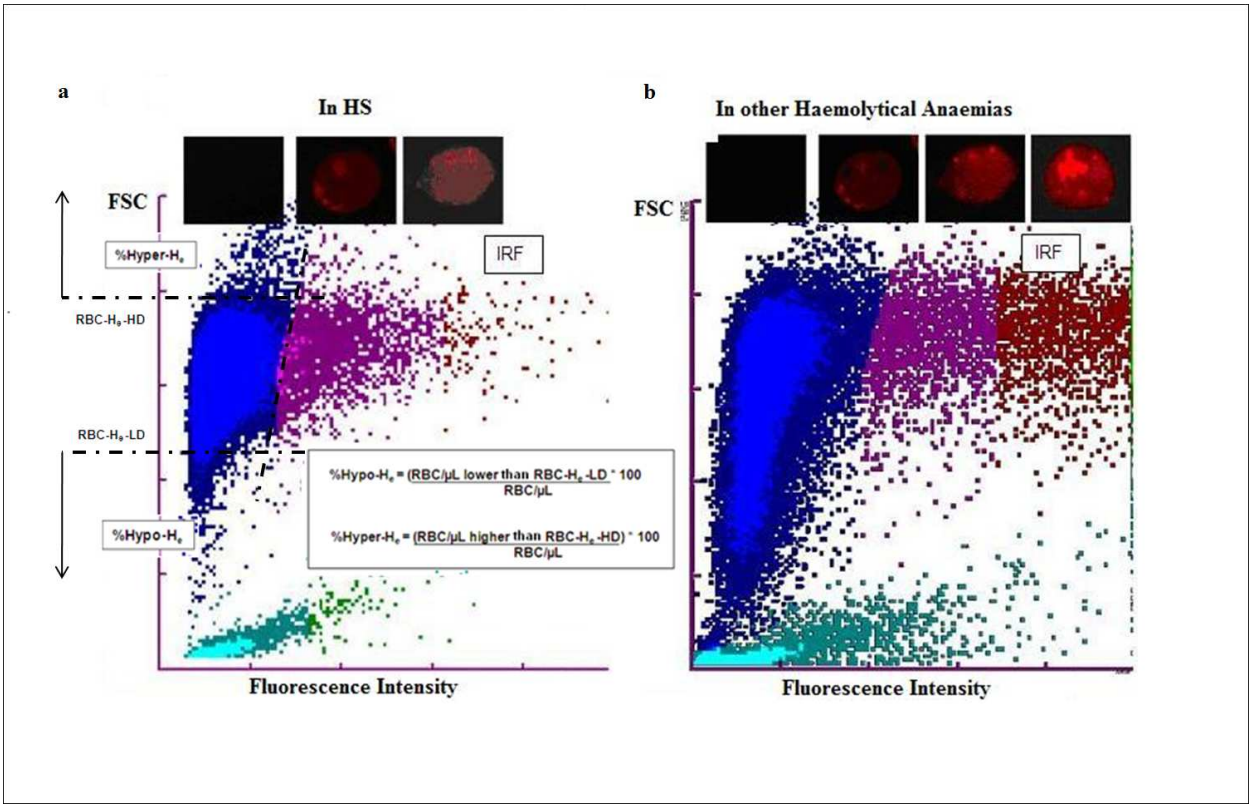

Figure 2: Distribution of Ret-IRF ratios (mean +/- SEM) among trait or mild HS (n=12), moderate HS (n=27), severe HS (n=6), haemolytic disorders (n=108), iron deficiency (n=93), healthy subjects (n=61) and routine haematological database (n=1230). The cut-offs defined in the diagnostic tool are also shown: 7,7 for rule 1 (continuous line) and 19,9 for rule 2 (dotted line).

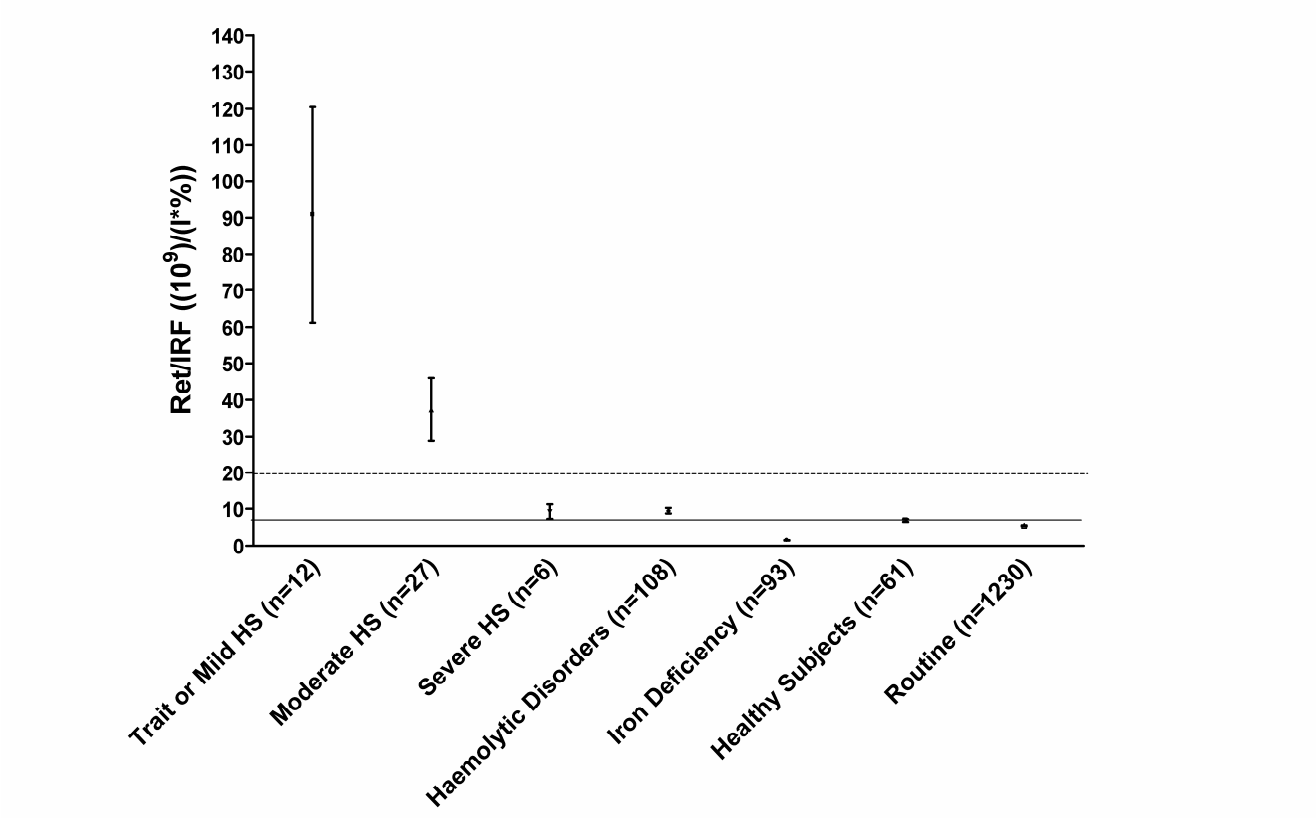

Figure 3: Distribution of microR/hypoHe ratios (mean+/-SEM) among trait or mild HS (n=11), moderate HS (n=15), severe HS (n=4), haemolytic disorders (n=108), iron deficiency (n=93), healthy subjects (n=61) and routine haematological database (n=1230). The cut-offs defined in the diagnostic tool are also shown: 2,5 for moderate HS (continuous line) and 2,0 for severe HS (dotted line).

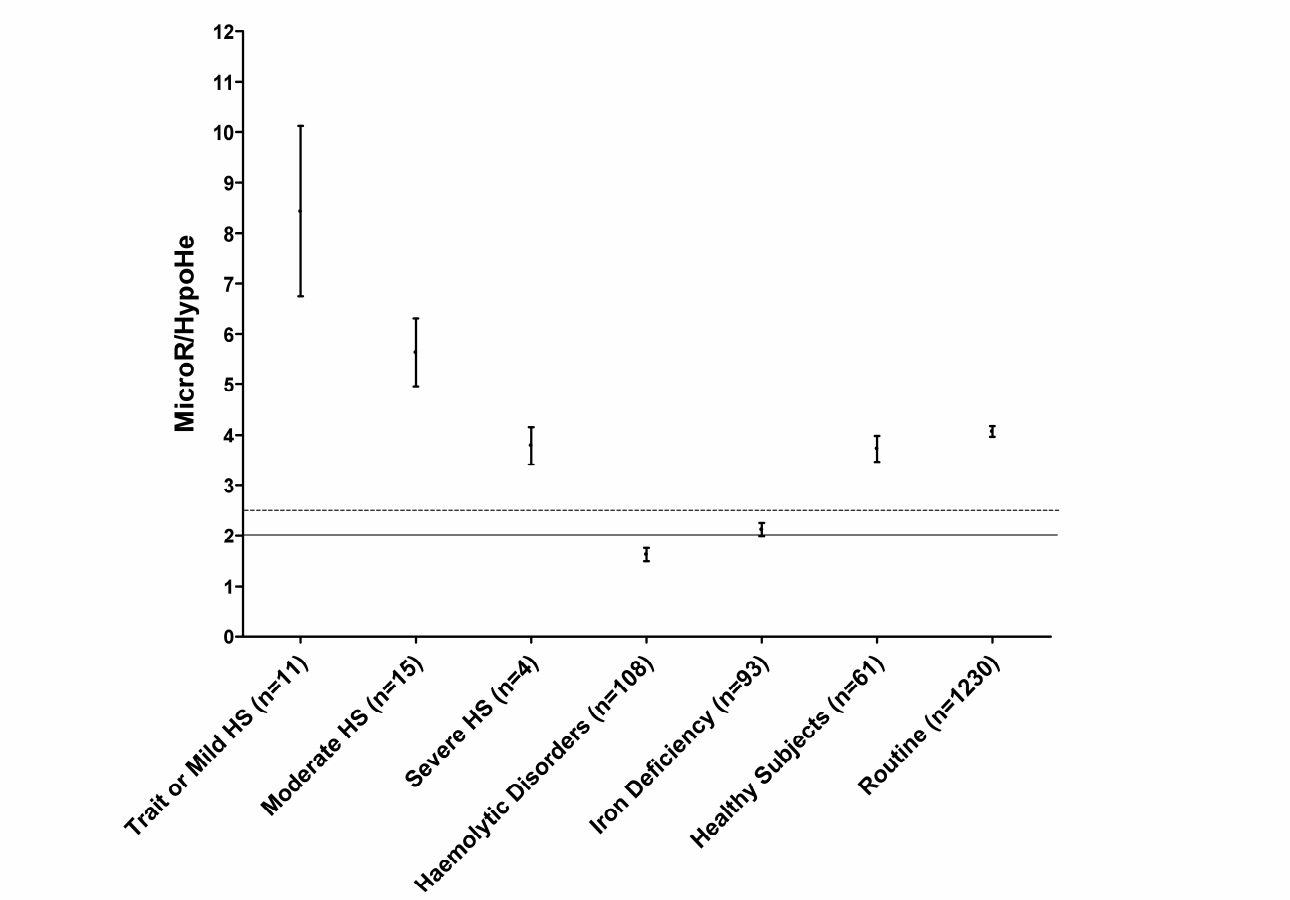

Figure 4: Distribution of MicroR (mean+/-SEM) among trait or mild HS (n=11), moderate HS (n=15), severe HS (n=4), haemolytic disorders (n=108), iron deficiency (n=93), healthy subjects (n=61) and routine haematological database (n=1230). The cut-off defined in the diagnostic tool is also shown: 3,5 for moderate and severe HS (dotted line).