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CAT
Critically Appraised Topic

Red blood cell algorithms in the diagnosis of red blood cell diseases and anaemia

Author: Lieselot Dedeene
Supervisor: Apr. Christine Van Laer
Search/methodology verified by: Apr. Christine Van Laer
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CLINICAL BOTTOM LINE

Anaemia is a major public health problem, which may, if not diagnosed or treated properly, cause irreversible organ dysfunction. Moreover, multiple pathologies may be at the root of anaemia, sometimes in a combined fashion, thereby complicating a prompt diagnosis and timely treatment. Screening algorithms based on red blood cell (RBC) parameters allow a quick differentiation between distinct types of anaemia/RBC diseases and may facilitate the differential diagnosis of anaemia. In order to improve the diagnostic work-up of anaemia in our laboratory (UZ Leuven), we (i) performed a literature search to document most relevant RBC algorithms supporting the differential diagnosis of anaemia and (ii) evaluated the diagnostic performance of a selection of relevant algorithms in our laboratory. To evaluate the diagnostic performance, a retrospective study was performed. All blood samples used in this study were measured on the Sysmex XN-9100 haematology analyzers. In this project, we focused on β-thalassaemia, iron deficiency anaemia, hereditary spherocytosis, pyruvate kinase deficiency, and microangiopathic haemolytic anaemia.

The algorithm of Urrechaga et al ([MicroRBC – Hypo-He – RDW-CV] > -5.1) turned out to be the best performing algorithm in our laboratory for the screening of β-thalassaemia and will soon be implemented. Algorithm-positive samples will be evaluated by the clinical biologist and a reflex test will be performed or a comment will be added if suspicious for β-thalassaemia. With respect to iron deficiency anaemia, none of the tested algorithms showed an adequate diagnostic performance in our laboratory. For hereditary spherocytosis, lab technicians will be trained to recognize the RET scattergram pattern indicative of spherocytes. For these samples, a smear review will be performed and if spherocytes are microscopically observed, a comment will be added to the lab report. For pyruvate kinase deficiency, the algorithm of Bobée et al (RET > 150*109/L, Ret/IRF > 9.5, MicroRBC < 5.5% and MicroRBC/Hypo-He < 6.0) will be implemented in our laboratory. To swiftly guide the clinician in the right direction, a comment will be added to algorithm-selected samples. Finally, with respect to microangiopathic haemolytic anaemia, the automated fragmented red cell count (FRC) shows a correlation with other RBC parameters (e.g., Hypo-He, RDW-CV and microRBC) resulting in a low specificity in the overall sample population. As such, FRC% should only be used supplementary to the microscopic assessment of schistocytosis, which is in line with the current ICSH guidelines on the assessment of schistocytosis.

Overall, this study showed the potential usefulness of the implementation of three screening tools in the context of anaemia and RBC pathologies in the clinical laboratory of UZ Leuven. Moreover, our results highlight the importance of evaluating the diagnostic performance of published screening algorithms using lab-specific sample populations and haematology analyzers.

CLINICAL/DIAGNOSTIC SCENARIO

Anaemia is a major public health problem, affecting approximately 25% of the entire world population [1]. A higher prevalence is observed in pregnant women and young children. According to the World Health Organization (WHO), anaemia is a condition in which the number of red blood cells or the haemoglobin (Hb) concentration within them is lower than normal. As such, the Hb concentration is a valuable parameter in the diagnosis of anaemia. The cut-off Hb values for anaemia are 13-14 g/dL for healthy men and 12 g/dL for healthy women. In people younger than 18 years, the cut-off value varies with age. Typical symptoms related to anaemia comprise fatigue, shortness of breath, tachycardia and headache [2]. If anaemia is not diagnosed or treated properly, a persistent decrease in oxygen supply may cause irreversible organ dysfunction. Therefore, detecting

anaemia and, importantly, the underlying cause in an early phase is highly demanded to ensure a timely and effective therapeutic intervention [2]. The latter is paramount to prevent long-term damage [2].

Identification of the underlying cause of anaemia should be guided at first instance by the patient history and clinical findings [3]. Nevertheless, multiple (sometimes mixed) pathologies may be the cause of anaemia, thereby complicating a prompt diagnosis and/or effective treatment. Iron deficiency is considered the most common cause of anaemia [1, 3]. Other common causes of anaemia include acute bleeding, vitamin B12/folate deficiency, chronic diseases, and hereditary red blood cell (RBC) diseases such as haemoglobinopathy, red cell membrane pathology and enzymatic deficiency [2–4]. Measurement of the mean corposcular volume (MCV) of the RBC allows to roughly distinguish between distinct types/causes of anaemia (**Table 1**). Additional RBC parameters, biochemical parameters and more specific (genetic) tests should be added to support a definite diagnosis (**Table 2**). Nevertheless, many of these parameters are somewhat ambiguous, are affected by acute phase reactions, or are only altered in a later stage, complicating matters further.

Table 1: Classification of anaemia based on the mean corpuscular volume (MCV) [2–4]**.**

Microcytic anaemia **MCV < 80 fL**	**Normocytic anaemia** **MCV: 80-100 fL**	**Macrocytic anaemia** **MCV > 100 fL**
Iron deficiency	Acute bleeding	VitB12/folate decifiency
Thalassaemia	Haemolytic anaemia:	Drug-induced
Chronic bleeding	• Enzyme defects (PKD, G6PD)	MDS
Haemolytic anaemia (HS)	• Hemoglobinopathies	Liver disease or alcohol use
	• Membrane defects (HS, PNH)	Hypothyroidism
Less common:	• Auto- and alloantibodies (AIHA)	
Chronic/infectious disease, MDS	• Microangiopathic (TTP, aHUS)	
	• Drug-induced	
	• Infections	
	• Toxic substances	
	• Metabolic disorders	
	Chronic/infectious disease	
	Hypersplenism	
	Less common:	
	Iron deficiency, VitB12/folate, MDS	

Abbreviations: HS, hereditary spherocytosis; MDS, myelodysplastic syndrome; PKD, pyruvate kinase deficiency; G6PD, glucose-6-phosphate dehydrogenase; PNH, paroxysmal nocturnal haemoglobinuria; AIHA, auto-immune haemolytic anaemia; TTP, thrombotic thrombocytopenic purpura; aHUS, atypical haemolytic uremic syndrome

Automated haematology analyzers offer a wealth of information. Next to the well-established RBC parameters (**Table 2**), which are commonly reported, advanced RBC parameters (referred to as RBC research parameters) usually do not leave the safe surroundings of the laboratory. Nevertheless, these parameters may offer additional information. Over the years, several types of screening algorithms using RBC research parameters were established allowing a quick differentiation between distinct types of anaemia/RBC diseases and, consequently, facilitating the differential diagnosis of anaemia. As such, samples are classified according to these algorithms and may be considered suspicious for a particular condition (eg., thalassaemia). Next, a reflex test should be performed to confirm the respective condition (eg., Hb electrophoresis). This way, the diagnostic work-up is sped up by avoiding waiting for the next doctor's consultation to obtain fresh blood samples. If reflex testing is not possible (e.g., not enough sample volume), the clinician can be swiftly guided into the right direction by adding a note to the lab report. Accordingly, unnecessary investigations and costs are avoided [5]. These algorithms might therefore favor a prompt diagnosis and efficient treatment strategy. Related to this, automated haematology analyzers (such as the Sysmex XN-9100 in UZ Leuven) provide several RBC suspect flags suggesting pathological samples (eg., "Fragments", "HGB defect", "Iron deficiency"). These flags, which are programmed by the company, are based on underlying algorithms using (research) RBC parameters. Of note, to profit from the aforementioned advantages, the sensitivity and specificity of adequate screening algorithms should be as high as possible to include all patients of interest and to avoid confirmatory tests in subjects suffering from other pathologies (false positives) [5]. Consequently, clinical laboratories should carefully assess the sensitivity and specificity of published algorithms using their own patient population and haematology analyzers.

Currently, RBC research parameters and related algorithms as well as the Sysmex RBC suspect flags are not considered in the clinical laboratory of UZ Leuven. Therefore, we now performed a literature search to document most relevant RBC algorithms which may support the differential diagnosis of several types of anaemia. Next, we aimed to evaluate the diagnostic performance of a selection of relevant algorithms and Sysmex RBC suspect flags in our laboratory. In this project, we focused on β-thalassaemia, iron deficiency anaemia, hereditary spherocytosis, pyruvate kinase deficiency, and microangiopathic haemolytic anaemia.

Table 2: **RBC parameters and lab tests supporting the differential diagnosis of several types of microcytic and normocytic anaemia** [2–4]**.**

	IDA - early phase	IDA – late phase	ACD	Thalas-saemia	HS	PKD	MAHA	Remarks
MCV	N	↓	N - ↓	↓	N - ↓	N	N	Morphological evidence is only detectable in a later phase
MCH	N	↓	N - ↓	↓	MCHC ↑	N	N	No early marker
RBC	↓	↓	↓	↑	↓	↓	↓	/
RDW	N	↑	N	N	N - ↑	N - ↑	N - ↑	Increased values are ambiguous
Ret-He	↓	↓↓	↓	↓↓	N	N	N	Not affected by acute phase reactions
Reticulocytes	N	N - ↓	↑	N - ↓	N - ↑	N - ↑	↑	Analytical interference might be present
BM hemosiderin	0	0	N - ↑	N - ↑	/	/	/	BM aspirate required
BM sideroblasts	N - ↓	↓	↓	N	/	/	/	BM aspirate required
Iron	N	↓	N - ↓	N	n.a.	n.a.	n.a.	Diurnal variations and day-to-day variations of iron concentration
Ferritin	↓	↓	N - ↑	N - ↑	n.a.	n.a.	n.a.	Positive acute phase reactant
Transferrin saturation	N - ↓	↓	↓	N	n.a.	n.a.	n.a.	Formula contains concentration of serum iron, which shows diurnal variation and day-to-day variation
Haptoglobin	N	N	N	N	N - ↓	N - ↓	↓	Positive acute phase reactant
Microscopy	/	Elliptocytes, pencil cells, target cells	/	Target cells, basophilic inclusions	Spherocytes	Potential morphological abnormalities	Schistocytes or sphero-cytes	Subjective report by visual reading, morphological evidence is later detectable than deviating biomarkers
Specific lab tests	/	/	/	Hemoglobin electrophoresis, genetic diagnostics	EMA, cryo-haemolysis, osmotic fragility, genetic diagnostics	PK activity, genetic diagnostics	Direct coombs test, ADAMTS13, …	Not part of routine blood count, on specific demand

N indicates normal levels; arrow indicates increased or decreased levels.
Abbreviations: IDA, iron deficiency anaemia; ACD, anaemia of chronic disease; HS, hereditary spherocytosis; PKD, pyruvate kinase deficiency; MAHA, microangiopathic haemolytic anaemia; BM, bone marrow

QUESTION(S)

1) *Which algorithms based on red blood cell (research) parameters are reported in literature to facilitate the diagnostic work-up of red blood cell diseases and anemia?*
2) *Which algorithms could be useful as a screening tool for distinct types of red blood cell diseases and anemia in our laboratory? What is the diagnostic performance of these algorithms in our laboratory?*

SEARCH TERMS

1) *MeSH Database (PubMed): MeSH terms*
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/), The National Institute for Clinical Excellence (http://www.nice.org.uk/), Cochrane (http://www.update-software.com/cochrane)*
4) *International organizations: e.g. 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)*
5) *UpToDate Online*

Question 1: Which algorithms based on red blood cell (research) parameters are reported in literature to facilitate the diagnostic work-up of red blood cell diseases and anemia?

1. Red blood cell indices and research parameters

In UZ Leuven, the automated haematology analyzer Sysmex XN-9100 is used to perform a complete blood count (CBC). The conventional RBC indices and available advanced RBC indices (i.e. research parameters) together with their proposed clinical use are listed in **Table 3**. Most of these parameters are derived from either the RBC histogram (RBC/PLT channel) or the reticulocyte scattergram (RET channel) (**Fig. 1**).

Table 3: Overview of reported RBC indices and RBC research parameters derived from the Sysmex XN-9100.

Parameter (unit)	What?	Channel	Proposed clinical use
MCV (fL)	Mean corpuscular volume = hematocrit/#RBC	RBC/PLT channel	Size of RBC is dependent on its hemoglobin content. If MCV decreased: failure to produce haemoglobin. If MCV increased: division of RBC precursor cells in the bone marrow is impaired.
MCH (pg)	Mean cell haemoglobin content = hemoglobin/#RBC	RBC/PLT channel	Decreased if hemoglobin synthesis is impaired.
MCHC (g/dL)	Mean cell haemoglobin concentration = haemoglobin/hematocrit	RBC/PLT channel	Increased in HS, spherocytes have a reduced surface/volume ratio.
RDW-CV (%) or RDW-SD	Red cell distribution width	RBC/PLT channel	Degree of anisocytosis. If increased: non-specific marker of RBC abnormality.
MicroRBC* (%)	Percentage of microcytic red cells (volume < 60 fL)	RBC/PLT channel	Useful in combination with other RBC parameters (mainly hypochromic RBC) to obtain discriminant indices for the differential diagnosis of microcytic anaemia.
MacroRBC* (%)	Percentage of macrocytic red cells (volume > 120 fL)	RBC/PLT channel	/
Reticulocytes (#/L or %)	Number of reticulocytes	RET channel	Reflects erythropoiesis in bone marrow (response to anaemia). Monitoring of anaemia therapy.
Ret-He (pg)	Reticulocyte hemoglobin equivalent	RET channel	Measure of direct iron availability. Decreased in (functional) iron deficiency. Independent of acute phase reaction. Monitoring of anaemia therapy.
Delta-He* (pg)	Difference in hemoglobin content between RBC and reticulocytes	RET channel	Most sensitive indicator of acute iron deficiency. Real-time marker for inflammation/acute phase reaction. Negative value indicates that hemoglobinisation of the newly formed reticulocytes is less than in mature erythrocytes.
Hypo-He* (%)	Percentage of hypochromic RBC (cellular haemoglobin content lower than 17 pg)	RET channel	Assessment of iron availability for erythropoiesis : reflects the iron status in the last 3 months.
Hyper-He* (%)	Percentage of hyperchromic red cells (cellular haemoglobin content higher than 49 pg)	RET channel	/
IRF (%)	Immature reticulocyte fraction = medium fluorescence reticulocytes (MFR) + high fluorescence reticulocytes (HFR)	RET channel	Sensitive marker for erythropoiesis.
FRC* (#/µL or %)	Percentage of fragmented RBC	RET channel	Reflects the presence of schistocytes.
RBC score*	1/(1 + exp (-(-7.6055 + 1.5873 * FRC(%) + 0.0402 * Reticulocytes 10^9 /L)))	RET channel	Part of the CBC-O concept, which tracks down the cause of elevated MCHC (eg., cold agglutination, lipaemia, haemolysis, RBC disease, …)

*Parameters indicated with an asterisk are non-reported RBC research parameters.

In short, the RBC/PLT channel uses impedance technology to measure the number and the size of RBC. The RBC go through the impedance channel, consisting of two electrodes of opposite charge, thereby generating an electrical pulse in correlation to their size. These data are gathered in a histogram representing the number of pulses/cells in function of the cell size **(Fig. 1)**. The haematocrit is calculated as the sum of all individual cell pulse heights. The haemoglobin concentration is measured photometrically following cell lysis with sodium lauryl sulphate. Next, MCV, MCH and MCHC are calculated based on the RBC count, Hb concentration and/or hematocrit. MicroRBC, macroRBC and RDW are directly derived from the RBC histogram **(Fig. 1)**.

Second, the RET scattergram is derived from the RET channel, which is based on fluorescence flow cytometry. Using a lysis reagent, the cell membrane of reticulocytes, platelets, RBC and white blood cells is perforated. Next, a fluorescent marker is able to enter the cells and subsequently labels the intracellular nucleic acids. The intensity of the fluorescence signal is directly proportional to the nucleic acid content. This allows the differentiation of cellular populations based on the fluorescence intensity (measurement of side fluorescence (SFL)) and size (measurement of the forward scatter (FSC)). The IRF reflects the relative number of immature reticulocytes and is calculated as the sum of medium fluorescence reticulocytes (MFR) and high fluorescence reticulocytes (HFR). Moreover, the haemoglobin content of RBC or reticulocytes is calculated based on the FSC using a proprietary algorithm. As such, the following parameters are derived from the RET scattergram: Ret-He, Hypo-He, Hyper-He and Delta-He **(Fig. 1)**. Finally, the FRC value, reflecting fragmented red cells and used in the RBC score, is derived from the RET scattergram as well **(Fig. 1)**.

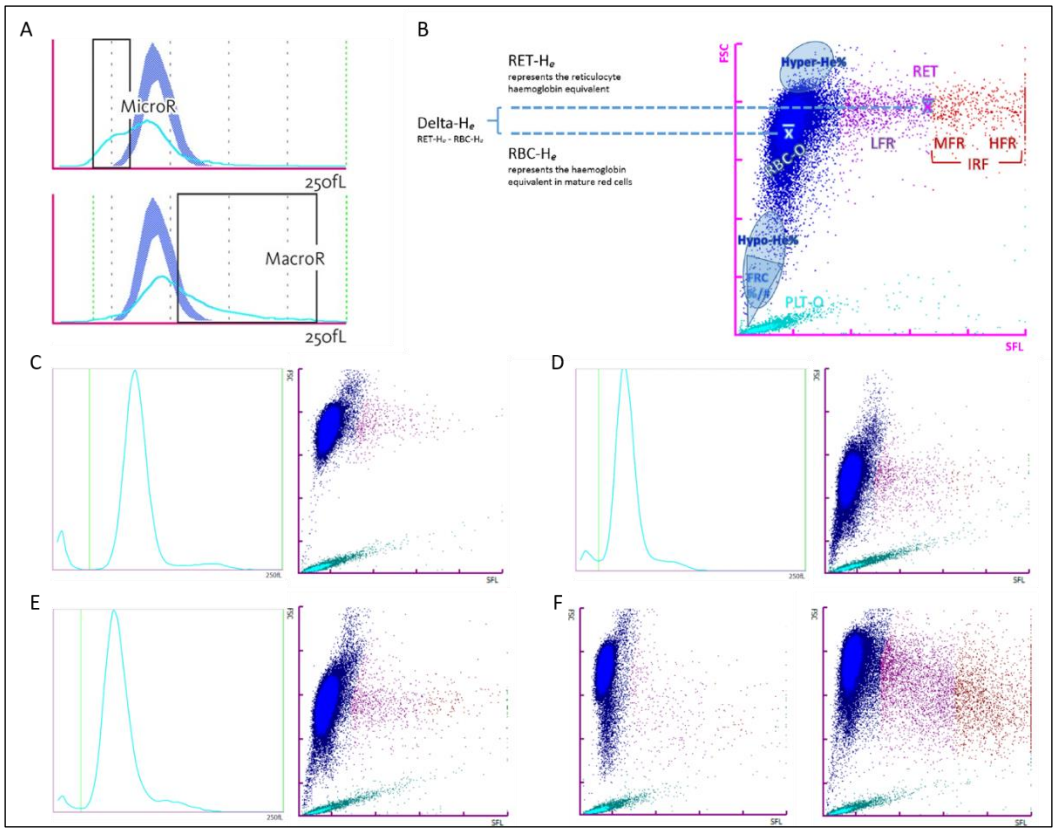

Figure 1: Advanced RBC indices (i.e. research parameters) derived from the Sysmex XN-9100. (A) MicroRBC and macroRBC are derived from the RBC histogram. **(B)** The RET scattergram provides the number of reticulocytes, Ret-He and IRF, and the following research parameters: hyper-He, hypo-He, delta-He (RBC-He) and FRC. (Figure: courtesy of Sysmex) **(C)** RBC histogram and RET scattergram of a healthy control: microRBC: 0.8%, Hypo-He: 0.1%, Delta-He: 2 pg, Hyper-He: 0.8%, MCV: 89.5 fL , Reticulocytes: 35*10^9/L. **(D)** RBC histogram and RET scattergram of a β-thalassaemia patient: microRBC: 74.2%, Hypo-He: 34.8%, Delta-He: -0.3 pg, Hyper-He: 0.1%, MCV: 56.3 fL , Reticulocytes: 61*10^9/L. **(E)** RBC histogram and RET scattergram of another β-thalassaemia patient: microRBC: 29.4%, Hypo-He: 8.8%, Delta-He: 0.5 pg, Hyper-He: 0.2%, MCV: 69.9 fL , Reticulocytes: 68*10^9/L. **(F)** Two RET scattergrams showing a decreased delta-He. Left: Hypo-He: 2.0%, Delta-He: -9.8 pg, Hyper-He: 0.8%, Reticulocytes: 31*10^9/L. Right: Hypo-He: 1.5%, Delta-He: -8.1 pg, Hyper-He: 4.3%, Reticulocytes: 408*10^9/L.

2. Screening algorithms

2.1 β-thalassaemia

Genetic thalassaemia mutations are present in approximately 1-5% of the global population and thalassaemia is known to be highly prevalent in the Mediterranean basin and countries of the Far East [6]. However, due to continued migration, thalassaemia and other inherited Hb disorders are currently considered as a global health concern [6, 7]. With respect to β-thalassaemia, patients are classified as minor, major or intermedia, based on the genotype (mild to severe mutations in the β-globin gene), the extent of anaemia, and the clinical symptoms [6]. In patients with β-thalassaemia, the production of β-globin chains is impaired and an excess of α-globin chains is present. Due to this imbalance in the globin chains, β-thalassaemia is typically characterized by the increase in HbA2 (α2γ2) levels (> 3.2%) (as measured by HPLC or electrophoresis methods). On the other hand, the formation and accumulation of unstable α-globin chain tetramers in erythroid cells results in ineffective erythropoiesis (cell death in the bone marrow) or peripheral haemolysis [6, 8]. The latter leads to acute complications (eg., cholelithiasis) and organ damage both on the short- and long-term [6]. The cornerstone of the treatment of thalassaemia patients is the management of anaemia (eg., transfusion and monitoring associated secondary complications such as alloimmunization and iron overload). The only disease-modifying treatment currently available is a haematopoietic stem-cell transplantation, which might be appropriate in thalassaemia patients with severe disease. Nevertheless, the associated risks should be kept in mind, such as the 5-10% mortality from transplant conditioning, graft-versus-host disease and graft failure [6]. Considering all this, adequate screening for β-thalassaemia is required to ensure an appropriate treatment (in order to prevent short- and long-term damage) and to ensure the timely genetic counseling of couples at risk.

β-thalassaemia and iron deficiency anaemia (IDA) are both characterized by microcytic, hypochromic anaemia (**Table 2**), complicating a prompt diagnosis (especially in case of ongoing acute phase reactions). Since 1973, several screening algorithms/formulas were developed to discriminate between (β-)thalassaemia (trait) and IDA using easy-accessible RBC parameters in order to optimize and facilitate the diagnostic workflow (for example: Sirdah et al [9], Ehsani et al [10], England et al [11] Green and King [12], Mentzer [13], Ricerca et al [14], Shine and Lal [15], and Srivastava and Bevington [16]) (**Table 4**). A recent study [17] evaluated all published discriminating formulas which are easy-to-use (*n*=25, the authors established their own, local cutoffs based on ROC analysis) and therefore have the potential to be used widespread as a universal screening tool. Moreover, they focused on the discriminating performance between IDA and thalassaemia in general as well as on α-thalassaemia and β-thalassaemia separately. In the latter study, the Jayabose (MCV × RDW/RBC) [18], Janel (combination of 11 existing formulas/indices into a single score: RBC, Mentzer, Shine and Lal, England and Frazer, Srivastava, Green and King, RDW, RDWI, Ricerca, Ehsani and Sirdah) [19] and Green and King (MCV^2 × RDW/100 * Hb) [12] formulas performed better than all other formulas. Furthermore, all investigated formulas performed better in distinguishing β- than α-thalassaemia from IDA [17].

Table 4: Published screening algorithms/formulas allowing the discrimination between (β-)thalassaemia and IDA.
Table adapted from Urrechaga et al [7].

Reference	Formula	Cut-off for thalassaemia
Ehsani et al [10]	MCV – (10*RBC)	<15
England et al [11]	MCV – RBC – 5*Hb – 3.4	<0
Green and King [12]	MCV^2 * RDW/100 * Hb	<65
Mentzer [13]	MCV/RBC	<13
Ricerca et al [14]	RDW/RBC	<4.4
Shine and Lal [15]	MCV^2 * MCH * 0.01	<1530
Sirdah et al [9]	MCV – RBC – 3 * Hb	<27
Srivastava and Bevington [16]	MCH/RBC	<3.8
Urrechaga et al [20, 21]	M/H (=microRBC/Hypo-He)	>3.7
Urrechaga et al [7]	MicroRBC – Hypo-He – RDW-CV	>-5.1 or >-7.6

As haematology analyzers evolved, additional information on individual RBCs and specific RBC subpopulations (eg., MicroRBC and Hypo-He (**Table 3**)) was provided. This enabled the detection of small changes in the amount of RBCs with poor hemoglobinization. Along the way, there was a renewed interest in screening formulas/algorithms for β-thalassaemia in an attempt to obtain a higher sensitivity and specificity. Interestingly, by means of a meta-analysis, Hoffman et al showed that the MicroRBC/Hypo-He (M/H) ratio was superior as

compared to other discriminating formulas using classical RBC parameters [21]. Furthermore, in 2011, Urrechaga et al [7] developed the following discriminating index using the Sysmex XE5000 analyzer: MicroRBC - Hypo-He – RDW-CV. They applied two cut-offs for β-thalassaemia (-5.1 and -7.6). In the latter study, this new formula reached a higher sensitivity and specificity in comparison to previously published algorithms using the original cut-offs (β-thalassaemia patients: *n*=270, IDA: *n*=250, apparently healthy subjects: *n*=90) (**Table 5**).

Table 5: Comparison of screening algorithms/formulas in terms of sensitivity and specificity. Table adapted from Urrechaga et al [7] and Schoorl et al [5].

	Urrechaga et al [7]		**Schoorl et al** [5]	
	Sensitivity (%)	**Specificity (%)**	**Sensitivity (%)**	**Specificity (%)**
Ehsani et al [10]	87.2	89.9	/	/
England et al [11]	78.6	98.4	51	96
Green and King [12]	91.0	99.1	64	97
Mentzer [13]	94.3	84.2	48	95
Ricerca et al [14]	100	13.7	/	/
Shine and Lal [15]	100	13.3	/	/
Sirdah et al [9]	81.3	97.9	/	/
Srivastava and Bevington [16]	70.8	91.3	/	/
Urrechaga et al [7] (cutoff >-5.1)	98.1	97.1	/	/
Urrechaga et al [7] (cutoff >-7.6)	100	92.6	69	89
Schoorl et al [5]	/	/	74	98

Schoorl et al [5] developed 6 different algorithms to discriminate β-thalassaemia from IDA using the Sysmex XE5000 analyzer (**Fig. 2**). In contrast to Urrechaga, preconditions were used including an MCV value ≤ 85 fL and a microRBC ≥ 3%. In this study, this new algorithm outperformed existing formulas in terms of specificity (β-thalassaemia patients: *n*=34, IDA: *n*=142, apparently healthy subjects: *n*=309). Therefore, these data show that the sensitivity and specificity of a particular formula/algorithm is strongly dependent on the study population (regional patient characteristics or inclusion and exclusion criteria e.g., mild/severe anaemia, with or without concomitant iron deficiency) and on analytical factors (e.g., type of haematology analyzers used). Indeed, several studies have shown that the sensitivity and specificity of discriminating formulas/algorithms is considerable lower if β-thalassaemia patients with concomitant IDA are used as a study group [17]. This highlights the importance of exploring and testing different algorithms in individual labs upon choosing the most suitable one.

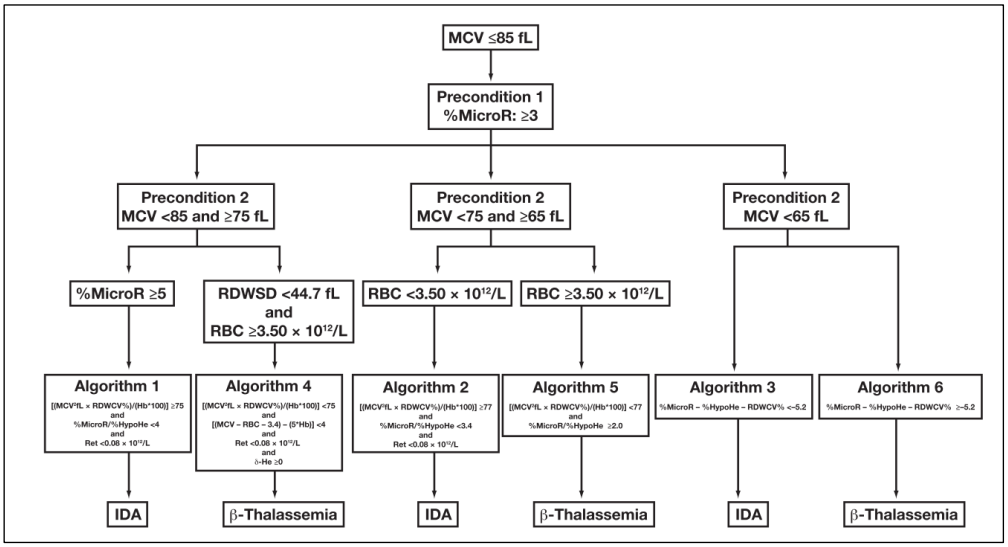

Figure 2: Screening algorithm allowing the differentiation between IDA and β-thalassaemia published by Schoorl et al [5]**.**

Interestingly, Adam et al recently presented a new algorithm on the Red blood cell disorders day on 25/02/2021 **(Fig. 3)** (Anne-Sophie Adam, LHUB-ULB (Belgium), contact information: AnneSophie.ADAM@lhub-ulb.be). The latter is applicable to Sysmex XN-9000 analyzers, but is still preliminary (amongst others not validated yet by an independent cohort and only applicable to adult patients). This algorithm enables the differentiation between IDA (*n*=56), BTM (*n*=68), HS (*n*=14), heterozygous hemoglobinopathy (*n*=24) and others (*n*=118), reaching a good classification rate of 86.6%. For the classification of BTM, a sensitivity of 92.6% and a specificity of 94.8% was reached.

Finally, Sysmex provides a RBC suspect flag "HGB defect?" to suggest hemoglobinopathies. This flag is based on an algorithm (RDW-CV < 15% and MCV < 75 fL), which cannot be changed by the user.

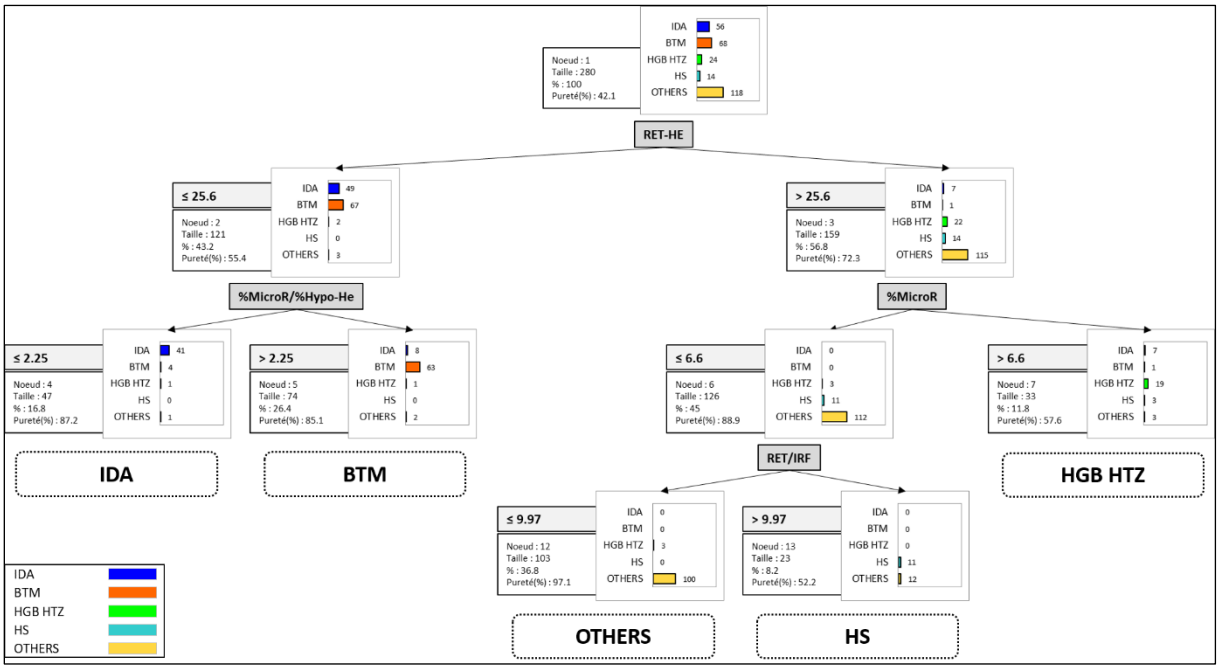

Figure 3: Screening algorithm established by Adam et al (Anne-Sophie Adam, LHUB-ULB (Belgium), contact information: AnneSophie.ADAM@lhub-ulb.be). Figure: courtesy of Anne-Sophie Adam.

2.2 Iron deficiency anaemia

Iron deficiency anaemia (IDA) is the most common cause of anaemia, counting for approximately 50% of all anaemia diagnoses [1]. IDA is usually driven by insufficient iron intake (eg., by malnutrition or during periods in life with higher demand i.e. pregnancy or growth) [1]. The gold standard to detect IDA is analyzing the bone marrow iron stores using Perls' Prussian blue staining [2]. However, this method requires an invasive procedure to obtain the bone marrow aspirate. Therefore, frequently used biochemical parameters include serum ferritin levels, transferrin levels/saturation (TSAT) and soluble transferrin receptors (**Table 2**) [2]. The main challenge in the diagnosis of IDA is the concomitant presence of inflammatory conditions, since ferritin and transferrin are acute phase reactants.

Recently, Ret-He, which is measured using the RET channel and represents the haemoglobin content of erytroid precursor cells, was proposed as a reliable and early parameter to detect iron deficiency. Indeed, Ret-He does not alter under inflammatory conditions and shows less biological variation as compared to TSAT and ferritin [2]. Moreover, reticulocytes survive only 1 to 3 days in the peripheral blood circulation and Ret-He therefore accurately reflects the actual iron availability in the bone marrow. The cut-off for Ret-He is approximately 28 pg, as suggested by several studies [22–24]. In a recent study using 12782 blood samples of Dutch healthy subjects, the reference value of Ret-He turned out to be 29.7 – 35.4 pg [2]. In UZ Leuven, the cut-off is set at 30.3 – 35.7 pg. Despite being a good indicator for IDA, Ret-He is also decreased in patients with β-thalassaemia. Therefore, discriminating formulas or algorithms including several RBC parameters can be used in the differential diagnosis of the microcytic anaemias IDA and β-thalassaemia, as mentioned above (**Table 4-5**).

Another challenge in the diagnosis of IDA is the differentiation between IDA and anaemia of chronic disease (ACD)/acute inflammation, which is the second most prevalent type of anaemia. Due to chronic (eg., renal insufficiency, malignancies) or acute (eg., sepsis) inflammatory conditions, complex biochemical processes are triggered leading amongst others to the production of hepcidin-25. Hepcidin-25 reduces the absorption of dietary iron, reduces the release of iron from the reticulo-endothelial system and increases the iron in macrophages [25]. As a consequence, hepcidin-25 causes a functional iron deficit despite normal or elevated ferritin levels (the latter in case of elevated CRP levels) [26]. Functional iron deficiency is, therefore, one component in the pathogenesis of ACD/inflammation-related anaemia. Other components include early erythroid precursor cell death, reduced erythropoietin (EPO) production and reduced sensitivity of the erytroid precursor cells to EPO, which is all caused by the actions of inflammatory cytokines [27, 28].

A first discriminating indicator between IDA and ACD is the MCV value, which may be unaltered in ACD (normocytic anaemia). Nevertheless, this parameter is useless in case of early IDA (in which MCV values are still normal) or in case of combined ACD/IDA. Therefore, multiple studies have sought to establish discriminating parameters/algorithms for IDA and ACD. Recently, Weimann and colleagues established a novel diagnostic plot, the so-called Haema-plot (**Fig. 4**), in order to discriminate between IDA, thalassaemia, ACD, and sepsis/acute inflammation and to monitor therapy [25]. They incorporated Ret-He in combination with Delta-He (=Ret-He – RBC-He) into the algorithm. Delta-He clearly reflects acute phase reactions in inflammation or ACD by a sudden decrease in Ret-He, whereas RBC-He remains normal (RBC survive approximately 120 days). Therefore, a decrease in Delta-He is indicative of ACD or sepsis/acute inflammation. Other authors suggested the Thomas plot, which is a four-bay diagnostic plot consisting of Ret-He and the ferritin index [soluble transferrin receptor/log(ferritin)] [26, 29] **(Fig. 4)**. This plot may assist the decision whether iron supplementation and/or erythropoietin application is the correct therapeutic strategy.

Recently, Nivaggioni et al established an umbrella algorithm allowing the differentiation between multiple types of anaemia (including IDA, heterozygous hemoglobinopathy, sickle cell disease, hereditary spherocytosis, and patients without RBC disease or IDA) using the Sysmex XN-10 analyzers (**Fig. 5**) [30]. Therefore, this algorithm might be more applicable to a routine setting, in which all types of patients are represented. The algorithm includes five RBC parameters (MCHC, RDW, microRBC, NRBC, IRF) and the RBC score (RBC score = 1/(1 + exp (-(-7.6055 + 1.5873 * FRC(%) + 0.0402 * RET 10^9 /L)))). 158/166 of the RBC hereditary disease patients and 114/120 IDA patients were classified correctly, thereby reaching a correct classification rate of 99.4%.

Furthermore, the aforementioned algorithm of Adam et al (cf. 2.1 β-thalassaemia and **Fig. 3**) enables the classification of IDA as well, thereby reaching a sensitivity of 73.2% and a specificity of 97.3% (Anne-Sophie Adam, LHUB-ULB (Belgium), contact information: AnneSophie.ADAM@lhub-ulb.be).

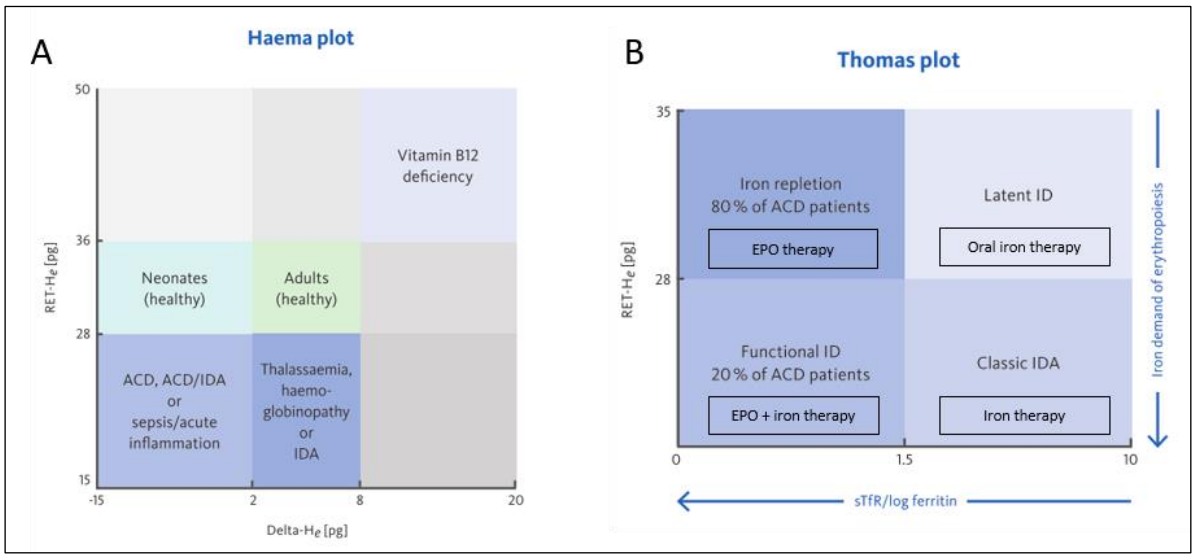

Figure 4: The Haema-plot and Thomas-plot. (A) The haema-plot aids in the differentiation between IDA, thalassaemia, ACD, and sepsis/acute inflammation and in monitoring therapy. **(B)** The Thomas plot is developed as a model for differentiating iron-deficient states and predicting the response to EPO therapy. Figures adapted from [2, 25]**.**

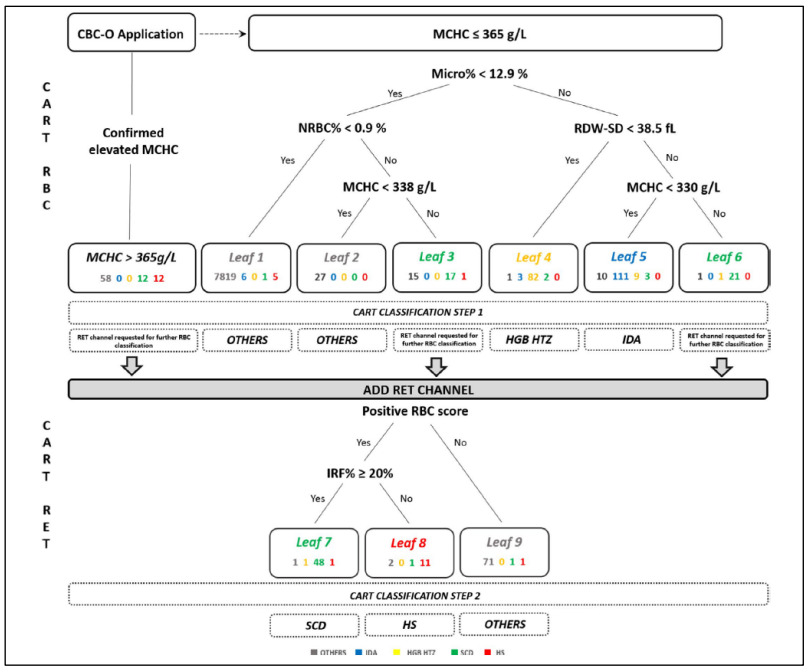

Figure 5: Algorithm established by Nivaggioni et al [30]**.** This algorithm allows the differentiation between IDA, heterozygous hemoglobinopathy, sickle cell disease, hereditary spherocytosis, and patients without RBC disease or IDA.

2.3 Hereditary spherocytosis

Hereditary spherocytosis (HS) is a type of haemolytic anaemia with a normocytic appearance. The highest prevalence of HS is in Northern Europe (prevalence 1 in 2000) [31]. Mutations in genes encoding distinct proteins of the RBC membrane or cytoskeleton may be the cause of HS. The most common affected proteins include spectrin (α/β), ankyrin, anion channel protein (Band-3 protein), protein 4.1 and protein 4.2 [31, 32]. In particular, these proteins play a role in the linkage of the RBC inner membrane skeleton and the outer lipid bilayer. Alterations in these linkage associations affect the elastic deformability and contribute to the spherocytic shape of HS RBC. The shortened lifespan of spherocytic RBC is a consequence of the impaired passage and consequent sequestration of the spherocytic RBC within the splenic cord, which promotes successive phagocytosis (resulting in further membrane loss) and intrasplenic destruction [32, 33]. HS can be classified as trait, mild, moderate and severe HS, dependent on severity of anaemia and markers of haemolysis (i.e. reticulocytes and bilirubin).

Several laboratory tests support the diagnosis of HS. The osmotic fragility test is the most traditional method, however, suffering from a low sensitivity and specificity [31]. Moreover, no clear difference is observed between spherocytosis resulting from HS or from other (acquired) conditions (e.g., auto/allo-immune haemolytic anaemia). Second, the observation of spherocytes on a blood smear together with a strong family history, may strongly suggest hereditary spherocytosis. However, the absence of spherocytes on the blood smear does not exclude HS. In addition, neonates often present with spherocytic cells independent of HS, complicating its diagnostic value further. The flow cytometry-based eosin-5'-maleimide (EMA)-binding test or the cryohaemolysis test are worthwhile alternatives with higher sensitivity and specificity scores and should definitely be considered in case of unclear results based on the osmotic fragility test and blood smear review. Other options include: sodium dodecyl sulfate–poly acrylamide gel electrophoresis (SDS-PAGE), ektacytometry and molecular testing [34]. Of note, auto-immune haemolysis should always be excluded using the direct antiglobulin test. Despite the diversity of available lab tests supporting the diagnosis of HS, all tests suffer from specific limitations including low sensitivity and specificity, time-consuming procedures, or scarce availability (eg., ektacytometry is only performed in specialized labs).

Several authors have tried to establish easy-accessible screening algorithms/diagnostic tools to identify HS. MCHC is well-known to be increased in HS patients, but not always reproducible as an accurate parameter (probably due to differences in analyzers) [35]. Interestingly, a reduced membrane surface area is already observed in circulating reticulocytes in HS patients due to defects in membrane-modelling, whereas acquired spherocytosis is not characterized by affected reticulocytes [33]. Reticulocyte parameters might therefore reflect the presence of affected reticulocytes (e.g., by insufficient entry of the fluorescence dye into the defective

reticulocytes), enabling the differentiation between hereditary and acquired spherocytosis. Mullier et al, for example, established a two-step algorithm using a precondition (Ret ≥80*10^9/L and Ret/IRF >7.7) and an increased Ret/IRF ratio (>19) to discriminate trait and mild HS from patients with other haemolytic disorders, IDA, and healthy individuals (Sysmex XE-2100 and XE-5000 analyzers). Furthermore, an increased MicroRBC (≥3.5%) and MicroRBC/Hypo-He ratio was used to discriminate moderate (MicroRBC/Hypo-He ≥ 2.5) and severe HS patients (MicroRBC/Hypo-He ≥ 2) [34]. This algorithm showed a sensitivity of 100%, specificity of 99.3%, PPV of 75% and NPV of 100%. Later on, the increase in the Ret/IRF ratio was implemented in the International Council for Standardization in Haematology (ICSH) guidelines for the lab diagnosis of HS [33]. The screening tool of Mullier et al was recently validated using the Sysmex XN-9000 analyzer and the cut-off of the Ret/IRF ratio in the mild HS population was adjusted [36] **(Table 6)**. As such, they confirmed the applicability of this screening tool for HS, especially in nonsplenectomized patients. Persijn et al [37] reported a lower diagnostic performance of this screening tool in their laboratory (UZ Gent) (sensitivity of 76%, specificity of 98%, PPV of 26.8% and NPV of 99.8%), which they assign to the presence of more severe pathologies in a university hospital population. In order to increase the diagnostic performance in their laboratory, they adapted the cut-off for reticulocytes (≥ 100*10^9/L) and microRBC (≥ 2.6%) [37].

Furthermore, Bobée and colleagues recently established an easy-to-use screening tool for HS using the Sysmex XE-5000 [35]. They included 47 HS patients, 23 G6PD and 17 PK deficiencies, 30 β-thalassaemia minor patients, 28 sickle cell trait, 30 sickle cell disease and 489 routine samples. The algorithm is based on the number of reticulocytes, Ret/IRF ratio, microRBC, microRBC/Hypo-He ratio, and hemoglobin concentration (**Table 7**). Finally, Adam et al recently published an update on the latter algorithm using the Sysmex XN-9000 analyzer (**Table 7**) [38].

In conclusion, screening tools based on RBC parameters seem to be useful in the diagnostic work-up of HS. However, these algorithms should be combined with a confirmation lab test (eg., EMA, osmotic fragility, …) for patients without a family history of HS, as recommended in the guidelines for the laboratory diagnosis of hereditary red cell membrane disorders [33, 36].

Table 6: Screening tool for HS proposed by Mullier et al [34] **and adapted by Sottiaux et al** [36] **using the Sysmex XN-9000 analyzers.**

Rule 1: precondition	(*n*=20 HS, 15 neonates, 18 adults (e.g., thalassaemia, G6PD, chronic bleeding, sickle cell disease,…))		
Reticulocytes	≥ 80*10^9/L		
Ret/IRF	> 7.7		
Rule 2: severity	**Hb > 12 g/dL** (*n*=14)	**8 g/dL ≤ Hb ≤ 12 g/dL** (*n*=5)	**Hb < 8 g/dL** (*n*=1)
Ret/IRF	≥ 14	/	/
MicroR	/	≥ 3.5%	≥ 3.5%
MicroRBC/Hypo-He	/	≥ 2.5	≥ 2
Sensitivity		94.1%	
Specificity		96.7%	
PPV		94.1%	
NPV		96.7%	

Table 7: Screening tools for HS proposed by Bobée et al [35] **and Adam et al** [38]**.**

Parameters		**Bobée et al** [35] (*n*= 47 HS, 17 PKD, 118 other (e.g. sickle cell, thalassaemia) and 489 routine samples)	**Adam et al** [38] (*n*=29 HS, 58 non-HS)
Reticulocytes		> 80*10^9/L	> 101*10^9/L
Ret/IRF		> 9.1	> 8.35
Hb < 12 g/dL:	MicroRBC	> 2.2%	/
Hb < 12 g/dL:	MicroRBC/Hypo-He	/	≥ 1.6
Hb > 12 g/dL:	MicroRBC/Hypo-He	≥ 3.5	≥ 3.8
Sensitivity		100%	100%
Specificity		92.1%	97.3%

2.4 Pyruvate kinase deficiency

Pyruvate kinase deficiency (PKD) is, in similarity to HS, another type of haemolytic anaemia with a normocytic appearance. Despite being the most common type of nonspherocytic haemolytic anaemia, PKD is rather rare with an estimated prevalence of 1/20 000 in the general Caucasian population [39, 40]. PK catalyzes the conversion of phosphoenol pyruvate to pyruvate, thereby generating 50% of the RBC total ATP. This is the only pathway for the production of ATP in RBC. Decreased ATP levels reduce the RBC lifespan by removal of the RBCs from the circulation [39]. Interestingly, the consequent accumulation of an upstream byproduct (2,3-diphosphoglycerate) reduces the affinity of hemoglobin to oxygen, and consequently increases oxygen delivery to tissues [41]. This phenomenon contributes in some patients to a well-tolerated anaemia and may therefore hamper the suspicion of PKD in those patients [41]. In addition, since PKD is an autosomal recessive disorder lacking a prominent parental history, the diagnosis of PKD is often overlooked. Approximately 260 mutations in the *PK-LR* (PK liver and RBC) gene have been reported causing PKD [42]. In PKD patients with mild anaemia, no treatment is required, whereas for patients with severe aneamia, supportive therapy (e.g., blood transfusions) and/or splenectomy (to reduce the need for transfusions) is the only option [39]. Currently, no disease-modifying treatment is available.

The diagnosis of PKD relies on the measurement of PK enzyme activity using spectrophotometric analysis [41]. Of note, normal levels should be interpret with caution, since several factors may influence PK levels (e.g., transfusion, increased reticulocytosis with higher enzyme levels in immature RBCs, …) [41]. Alternatively, *PK-LR* molecular testing can be performed. Nowadays, combining both tests is recommended for a definite diagnosis of PKD and adequate patient management [41]. Recently, Bobée et al proposed the first screening algorithm based on RBC parameters to facilitate the diagnosis of PKD (study population: 47 HS patients, 23 G6PD and 17 PK deficiencies, 30 β-thalassaemia minor patients, 28 sickle cell trait, 30 sickle cell disease and 489 routine samples) [35]. The algorithm is based on the same RBC indices as for the HS screening tool (number of reticulocytes, the Ret/IRF ratio, the microRBC, and the microRBC/Hypo-He ratio) (**Table 8**). Moreover, the Ret/IRF ratio reaches similar levels as in HS patients. To explain the high Ret/IRF ratio in PKD, the authors hypothesized that the lower levels of ATP affect the RBC membrane at the Na+/K+ ATP-dependent pump, thereby modifying the entry of the fluorescence dye.

Table 8: Screening tool for PKD proposed by Bobée et al.

Parameters	**Bobée et al** [35] (*n*= 47 HS, 17 PKD, 118 other (e.g. sickle cell, thalassaemia) and 489 routine samples)
Reticulocytes	$> 150*10^9$/L
Ret/IRF	> 9.5
MicroR	< 5.5%
MicroRBC/Hypo-He	< 6.0
Sensitivity	100%
Specificity	96.5%

In conclusion, a screening algorithm based on RBC parameters could facilitate the diagnosis of PKD, which is a rather underestimated disorder due to several factors (cf. supra). Based on the algorithm and clinical history, suspected samples should be tested for PK enzyme activity (and subsequent molecular testing). This multi-faceted approach might increase the number of diagnosed PKD patients and thereby improve patient management (including genetic counselling) and prevent complications.

2.5 Microangiopathic haemolytic anaemia

Microangiopathic haemolytic anaemia (MAHA) represents a type of non-immune intravascular haemolysis charachterized by structural damage to the RBC membrane (e.g., by mechanical shearing) and the formation of red cell fragments or schistocytes. Additionally, under severe conditions, serum might color brown (due to the presence of methemoglobin) or pink (due to the presence of oxyhemoglobin) and free hemoglobin might be excreted in the urine, showing a darkish color. Moreover, several lab parameters are indicative (but not specific) to MAHA, such as decreased haptoglobin levels, increased LDH levels, increased indirect bilirubin levels and a negative Coombs test. Several conditions may cause MAHA, including shear stress (eg., defective mechanical

hearth valves, abnormalities in the microvasculature), thrombotic microangiopathies (TMA – hereditary or acquired) (eg., TTP, HUS, drug-induced TMA, and complement-mediated TMA), and other conditions (eg., pregnancy-associated syndromes, severe hypertension, systemic infections and malignancies, …). TMAs are life-threatening medical urgencies. TTP, for example, has a mortality rate of 90% if not treated promptly [43]. Therefore, plasma exchange is crucial in the treatment of TTP (which is a type of TMA due to ADAMTS13 deficiency) and is often initiated before the results of ADAMTS13 levels are available in case TTP is strongly suspected. This all highlights the importance of a fast diagnosis.

The observation of schistocytes on a peripheral blood smear is a decisive marker in the diagnostic-workup of MAHA or TTP, and is, currently, the golden standard. According to the ICSH recommendations, a schistocyte count > 1% on peripheral blood smear has a definite clinical value for the diagnosis of TMA in the absence of additional severe red cell shape abnormalities [44]. Despite many efforts improving the accuracy and reproducibility of schistocyte counting, this method is highly prone to inter-observer variations and is rather time-consuming (blood smear need to be prepared and stained) [44]. Haematology analyzers provide an automated count of fragmented red cells (FRC - absolute and relative count). FRC is deduced from the reticulocyte scattergram and used as a research parameter (**Fig. 1**). FRC events are present in the gate with a volume smaller than RBC (forward scatter) and with an RNA content lower than platelets (fluorescence intensity) [45]. However, the sensitivity and specificity of this parameter, designed to reflect the number of schistocytes, remains ambiguous [46–50]. Several authors established FRC references ranges in a population of healthy subjects and/or cut-offs for schistocytosis in pathological populations (**Table 9**) [46, 49, 51]. A notable disadvantage is the correlation of FRC values with Hypo-He values, resulting in falsely elevated FRC levels in hypochromic samples [46, 52]. Remarkably, some studies show a high correlation between microscopically counted schistocytes and FRC (although a slight under- or overestimation of the automated FRC count was marked), whereas other studies show rather discordant results between microscopy and FRC [44, 49]. This might, at least in part, be due to differences in the Sysmex analyzers used. Nevertheless, despite these controversial results, Sysmex implemented a trigger based on the FRC value and other parameters (RDW-SD, PLT upper discriminator, MCV, RBC lower discriminator, MCHC, MP) to suggest samples suspicious for the presence of fragmented RBC (Sysmex RBC suspect flag “Fragments”).

In conclusion, automated schistocyte counts based on the FRC parameter may serve as a qualitative indicator. Further optimization is required in order to use FRC as an accurate quantitative measure of schistocytosis. Therefore, current guidelines recommend the use of automated FRC counting as a useful complement to the microscopic evaluation (eg., as a screening tool) [44].

Table 9: FRC% values in normal and pathological study populations on Sysmex haematology analyzers.

Publication	Sysmex counter	Study population*	FRC(%)	
			Normal population	Cut-off for schistocytosis
Lesesve et al, 2012 [53]	XE-2100	1405 normal samples	< 0.5%	n.a.
Lesesve et al, 2015 [46]	XN-9000	1366 normal samples	0.00 – 4.01%**	n.a.
Abe et al, 2009 [51]	XE-2100	230 patients (of which 20 TMA or TTP) and 120 healthy subjects	Median (95% CI): 0.04% (0–0.205%)	> 1.2% (sensitivity: 90%, specificity: 96%, PPV: 90%, NPV: 90%)
Hantaweepant et al, 2020 [49]	XN-3000	62 suspected TMA and 35 thalassaemia	n.a.	> 0.6% (sensitivity: 86.1%, specificity: 77.8%, PPV: 94.4%, and NPV: 56%)
Hantaweepant et al, 2020 [49]	XN-3000	62 suspected TMA and 35 thalassaemia	n.a.	> 0.8% (sensitivity: 84.8%, specificity: 77.8%, PPV: 94.4%, and NPV: 53.8%)
Hantaweepant et al, 2020 [49]	XN-3000	62 suspected TMA and 35 thalassaemia	n.a.	> 1.0% (sensitivity: 78.5%, specificity: 83.3%, PPV: 95.4%, and NPV: 46.9%)
Hantaweepant et al, 2020 [49]	XN-3000	62 suspected TMA and 35 thalassaemia	n.a.	> 2.0% (sensitivity: 55.7%, specificity: 83.3%, PPV: 93.6%, and NPV: 30%)
Govindarjan et al, 2021 [50]	XN-1000	28 children (range: 2-140 months) with HUS	n.a.	> 1.49% (sensitivity: 95.4%, specificity: 50%)

*normal samples have a CBC count within reference ranges; **97% of the samples showed FRC < 1%

Question 2: Which algorithms could be useful as a screening tool for distinct types of red blood cell diseases and anemia in our laboratory? What is the diagnostic performance of these algorithms in our laboratory?

1. β-thalassaemia

An adequate screening algorithm shows a high sensitivity and specificity, is easy to use and well-established within the field. Moreover, the use of RBC research parameters reflecting small changes in RBC subpopulations rather than general RBC characteristics will, in our opinion, enhance the specificity and sensitivity. As described above, many discriminating formulas are easy-to-use and several are based on RBC research parameters. In this study, we compared the MicroRBC/Hypo-He (M/H) ratio [20, 21], the formula from Urrechaga et al (MicroRBC - Hypo-He - RDW-CV) [7] and the Sysmex RBC suspect flag "HGB defect?". Moreover, since geographical regional patient characteristics are also shown to play a role in the efficiency of a screening algorithm [21], we included a fourth algorithm established by Adam et al (LHUB-ULB, Belgium) using the Sysmex XN-9000 haematology analyzers **(Fig. 3)**. It has been shown that the diagnostic utility of most algorithms is dependent on the patient's age and most algorithms are validated in the original study using an adult population. However, since β-thalassaemia already occurs in childhood, we evaluated the performance of the selected algorithms in an adult population (≥18 years) as well as in the total sample population (< 18 years and ≥18 years).

To identify β-thalassaemia patients, all hemoglobin electrophoresis tests performed between 01/01/2000 and 02/05/2021 in UZ Leuven were extracted from the laboratory information system (LIS). Next, β-thalassaemia patients were selected based on increased HbA2 levels (>3.2%, electrophoresis method) and a lab report indicating β-thalassaemia (excluding HbS carriers, α-thalassaemia, carriers of other hemoglobin variants, combined HbS/β-thalassaemia, questionable β-thalassaemia (eg., normal blood count),…). Furthermore, patients without β-thalassaemia were selected based on a HbA2 level < 3.2% and a lab report indicating "normal capillary zone electropherogram", independent of the MCV, MCH and number of erythrocytes.

Over a three-month period (02/02/2021 – 02/05/2021), 15376 blood samples were analyzed on the Sysmex XN-9100, including the RET channel. Of these 15376 samples, 678 samples were derived from patients for whom a Hb electrophoresis was performed in our laboratory somewhere during the past 20 years. Of these 678 samples, 18 samples were derived from 13 different patients with β-thalassaemia (defined as described above) and 289 samples were derived from patients with a normal electropherogram (defined as described above). These samples were used to estimate the sensitivity, specificity, PPV, NPV and positive likelihood ratio (LR+) of the distinct screening algorithms in the total and adult sample population **(Table 10)**. As a result, all algorithms show a similar sensitivity, except for the Sysmex RBC suspect flag. A high specificity was observed in all algorithms, except for the M/H ratio. The LR+ was the highest for the algorithm of Urrechaga et al (cut-off -5.1) and Adam et al. The Sysmex RBC suspect flag "HGB Defect?" and the M/H ratio have a very low LR+ and PPV, and are therefore considered inadequate for detecting β-thalassaemia in our laboratory. With respect to the algorithms of Urrechaga et al and Adam et al, a lower sensitivity, but higher specificity and LR+ was observed in the adult sample population as compared to the total sample population. The sensitivity is considerably lower as compared to the original study (cut-off -5.1: 98.1%; cut-off -7.6: 100%) [7], but comparable to the performance of the Urrechaga algorithm in the study of Schoorl et al (cut-off -7.6: 69%) [5]. Altogether, the algorithm of Urrechaga et al (cut-off -5.1) and Adam et al outperformed the other algorithms and Sysmex flag in our laboratory. Upon comparing these two best performing algorithms, nearly all samples derived from known β-thalassaemia patients that are classified as positive by the algorithm of Urrechaga et al (cut-off > -5.1), are also classified as positive using the algorithm of Adam et al **(Fig. 6)**.

Since the discriminating algorithm of Urrechaga et al is a one-step formula, we performed a ROC analysis (AUC: 0.795) using our retrospective data-set **(Fig. 7)**. The cut-off value was chosen as the value for which the sum of sensitivity and specificity was the highest and turned out to be -5.7. For this cut-off, the sensitivity was 72.2%, the specificity 90.3% and the LR+ 7.5. As such, the LR+, sensitivity and specificity is quite comparable to the published cut-off of > -5.1. In addition, since we used a lower number of β-thalassaemia patients as compared to the original study of Urrechaga et al, we did not adjust the cut-off of this algorithm.

Fig. 6: **Venn diagram showing the overlap of β-thalassaemia samples classified as positive (= true positives) by the two best performing algorithms.** The algorithm of Urrechaga et al with a cut-off of -5.1 was compared to the algorithm of Adam et al in the total and adult sample population.

Table 10: Diagnostic performance of the distinct algorithms to screen for β-thalassaemia (retrospective study).

Total population (≥ 18 years AND < 18 years)											
02/02/2021 – 02/05/2021		**M/H** [20] [MicroRBC/Hypo-He] > 3.7		**Urrechaga** [7] [MicroRBC - Hypo-He - RDW-CV] > -5.1		**Urrechaga** [7] [MicroRBC-Hypo-He-RDW-CV] > -7.6		**Adam** Ret-He ≤ 25.6 AND [MicroRBC/Hypo-He] > 2.25		**Sysmex flag "HGB defect?"**	
	Total number	**Pos.**	**Neg.**	**Pos.**	**Neg.**	**Pos.**	**Neg.**	**Pos.**	**Neg.**	**Pos.**	**Neg.**
B-thal.	18	12	6	12	6	13	5	11	7	1	17
No B-thal.	289	138	151	25	264	39	250	18	271	10	279
Sens. (%)		66.7		66.7		72.2		61.1		5.6	
Spec. (%)		52.2		91.3		86.5		93.8		96.5	
PPV (%)		8.0		32.4		25.0		37.9		9	
NPV (%)		96.2		97.8		98.0		97.5		94	
LR+		1.4		7.7		5.4		9.8		1.6	
Adult population (≥ 18 years)											
02/02/2021 – 02/05/2021		**M/H** [20] [MicroRBC/Hypo-He] > 3.7		**Urrechaga** [7] [MicroRBC - Hypo-He - RDW-CV] > -5.1		**Urrechaga** [7] [MicroRBC-Hypo-He-RDW-CV] > -7.6		**Adam** Ret-He ≤ 25.6 AND [MicroRBC/Hypo-He] > 2.25		**Sysmex flag "HGB defect?"**	
	Total number	**Pos.**	**Neg.**	**Pos.**	**Neg.**	**Pos.**	**Neg.**	**Pos.**	**Neg.**	**Pos.**	**Neg.**
β-thal.	13	8	5	7	6	8	5	7	6	0	13
No β-thal.	119	47	72	1	118	4	115	5	114	1	118
Sens. (%)		61.5		53.8		61.5		53.8		0.0	
Spec. (%)		60.5		99.2		96.6		95.8		99.2	
PPV (%)		14.5		87.5		66.7		58.3		0	
NPV (%)		93.5		95.2		95.8		95.0		90	
LR+		1.6		64.1		18.3		12.8		0.0	

In an attempt to increase the number of β-thalassaemia patients for whom reticulocyte parameters are available, we checked whether β-thalassaemia patients (identified as described above) received a CBC (including the RET channel) in UZ Leuven between 01/10/2020 and 01/02/2021. We did not include RBC parameters of samples analyzed before October 2020, since the Sysmex XN-9100 analyzers are in routine use in our laboratory since the end of September 2020. During this period, we received 12 blood samples derived from 12 different patients with β-thalassaemia (5 patients < 18 years old). Seven out of these 12 samples (58.3%) met the criteria of the algorithm of Urrechaga et al (cut-off -5.1) and 5 out of 12 (41.7%) were positive according to the algorithm of Adam et al (these 5 samples were also indicated by Urrechaga et al). As a result, the algorithm of Urrechaga et al detected 2 additional samples as compared to the algorithm of Adam et al. Moreover, the sensitivity (58.3%) is similar as in the retrospective analysis during the three-month period **(Table 10)**.

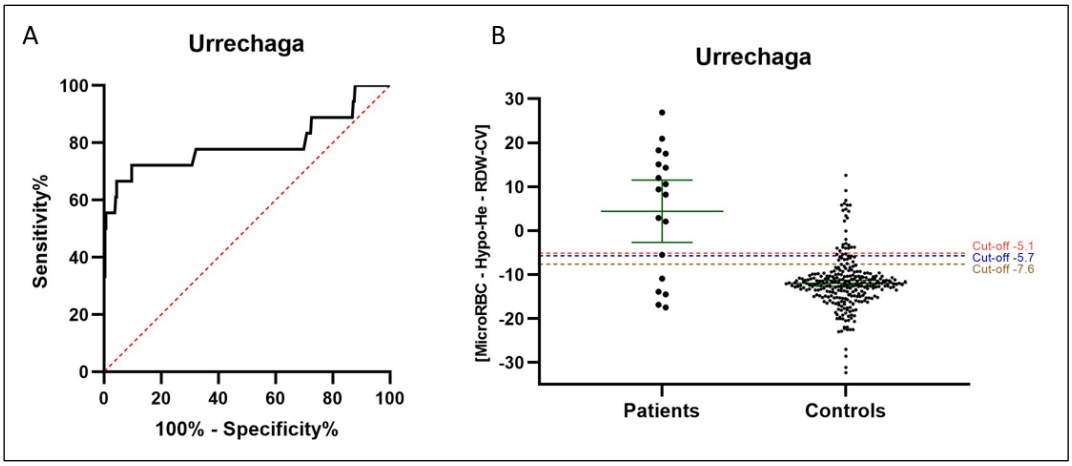

Fig. 7: ROC analysis of the algorithm of Urrechaga et al [MicroRBC-Hypo-He-RDW-CV]. (A) ROC curve of the algorithm of Urrechaga et al based on our retrospective data-set. The AUC is 0.795. **(B)** Graph showing the Urrechaga score of 18 β-thalassaemia patients and 289 controls. Error bars indicate mean ± 95% confidence interval. Dashed lines represent the two published cut-offs (-5.1 and .7-6) and the in-house cut-off based on the ROC analysis (-5.7).

Next, we estimated the number of samples that would be suspicious for β-thalassaemia per day according to the distinct algorithms in our laboratory. Over a three-month period (02/02/2021 to 02/05/2021), 15376 patient samples (< 18 years and ≥ 18 years) were analyzed on our haematology analyzers (including the RET channel). An overview of the number of samples indicated as positive by the distinct algorithms during this time period is represented in **Table 11**. Regarding the algorithm of Urrechaga et al and Adam et al, the average number of samples that would have to be checked for β-thalassaemia per day (2-5 samples/day) is acceptable.

Table 11: Retrospective analysis: total number of samples indicated as positive using the algorithm in the total and adult sample population over a three-month period.

02/02/2021 – 02/05/2021	Total number of samples	M/H [20] [MicroRBC/Hypo-He] > 3.7	Urrechaga [7] [MicroRBC-Hypo-He-RDW-CV] > -5.1	Urrechaga [7] [MicroRBC-Hypo-He-RDW-CV] > -7.6	Adam Ret-He ≤ 25.6 AND [MicroRBC/Hypo-He] > 2.25	Sysmex flag "Hgb defect?"
Total population (< 18 and ≥ 18 years)	15376	7153 (47%)	195 (1%)	409 (3%)	181 (1%)	39 (0.3%)
Adult population (≥ 18 years)	11567	5050 (44%)	34 (0.3%)	71 (0.6%)	89 (0.8%)	4 (0.03%)

In conclusion, the discriminating formula of Urrechaga et al (cut-off -5.1) is the best performing screening algorithm for β-thalassaemia in our laboratory (despite a rather low sensitivity). Although the screening algorithm of Adam et al provided similar results, the latter requires a two-step calculation and is less established within the field.

To do:
- Samples that are positive for the formula of Urrechaga et al (cut-off -5.1) should be forwarded to a separate worklist within the LIS.
- The clinical biologist should check the list with suspected β-thalassaemia patients on a daily basis. If suspicious for β-thalassaemia (based on the patient's history, blood parameters and clinical file), a reflex test for hemoglobin electrophoresis should be performed or, if sample volume is too low, a comment should be added to the lab report.
- The diagnostic value of this measure should be evaluated after a few months and after one year.

2. Iron deficiency anaemia

With respect to IDA, we preferred the recent algorithm of **Nivaggioni et al** [30] since they included a more representative population covering several RBC diseases and since the reticulocyte channel is not required for this algorithm. As an alternative, the **algorithm of Adam et al (Fig. 3)** was explored (representing a Belgian patient population, Sysmex XN-9000). Since both algorithms are validated using an adult population, we excluded patients < 18 years for this retrospective analysis.

From 02/02/2021 to 02/05/2021, 12627 patient samples (≥ 18 years) are available which were analyzed on the haematology analyzers and analyzed for ferritin on the same prescription. For 2479 out of these 12627 samples, parameters derived from the RET channel were available. In accordance with the study of Nivaggioni et al and Adam et al, iron deficiency was defined as serum ferritin levels < 15 µg/L and MCV < 80 fL. The retrospective analysis is summarized in **Table 12.** The sensitivity and specificity in this study is comparable to the study of Adam et al, but somewhat lower as compared to the study of Nivaggioni et al. In our study, the sensitivity and specificity is similar for both algorithms. The LR+ is clearly higher for the algorithm of Nivaggioni et al. Moreover, since the algorithm of Nivaggioni et al does not require reticulocyte parameters, this algorithm detects a higher number of samples with IDA **(Fig. 8)**.

Next, we estimated the number of samples that would be suspicious for IDA per day according to the algorithm of Nivaggioni et al in our laboratory. Over a three-month period (02/02/2021 to 02/05/2021), 84362 patient samples (≥ 18 years) were analyzed using the haematology analyzers (with and without RET channel). Of these 84730 samples, 1226 samples (1.5%) are suspicious for IDA according to the algorithm of Nivaggioni et al. This implies an average of 14 samples per day in our routine sample population.

In summary, the sensitivity and specificity of both algorithms (Nivaggioni et al and Adam et al) seem to be adequate. The LR+ is higher for the algorithm of Nivaggioni et al. However, the PPV is rather low (29.4%). Moreover, the sensitivity, specificity, PPV and NPV were calculated based on a sample population that was probably already suspicious for IDA (since iron parameters were requested by the clinician), wherefore the real values might be even lower in the total sample population. Therefore, we decided to not implement this algorithm in our laboratory.

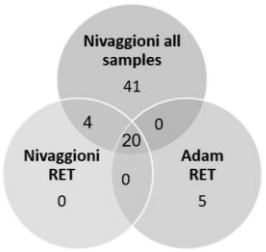

Figure 8: Venndiagram showing the overlap of IDA samples classified as positive by the different algorithms. Nivaggioni et al with and without reticulocyte parameters ("Nivaggioni all samples"): *n*=65, Nivaggioni et al with reticulocyte parameters ("Nivaggioni RET"): *n*=24, Adam et al with reticulocyte parameters (Adam RET"): *n*=25.

Table 12: Diagnostic performance of the algorithms of Nivaggioni et al and Adam et al in the screening of IDA.

02/02/2021 – 02/05/2021			Nivaggioni et al [MicroRBC ≥ 12.9% AND RDW-SD ≥ 38.5 fL AND MCHC < 33 g/dL]		Adam et al [Ret-He ≤ 25.6 AND MicroRBC/Hypo-He ≤ 2.25]	
		Total number	**Positive**	**Negative**	**Positive**	**Negative**
Samples **with and without** reticulocyte parameters	**Number of samples with IDA** ("ferritin < 15 µg/L and MCV < 80 fL")	91	65	26	n.a.	n.a.
	Number of samples without IDA ("ferritin > 15 µg/L and MCV > 80 fL" or "ferritin < 15 µg/L and MCV > 80 fL" or "ferritin > 15 µg/L and MCV < 80 fL")	12536	156	12380	n.a.	n.a.
	Sensitivity (%)		71.4		n.a.	
	Specificity (%)		98.8		n.a.	
	PPV (%)		29.4		n.a.	
	NPV (%)		99.8		n.a.	
	LR+		57.4			
Samples **with** reticulocyte parameters	**Number of samples with IDA** ("ferritin < 15 µg/L and MCV < 80 fL")	32	24	8	25	7
	Number of samples without IDA ("ferritin > 15 µg/L and MCV > 80 fL" or "ferritin < 15 µg/L and MCV > 80 fL" or "ferritin > 15 µg/L and MCV < 80 fL")	2447	35	2412	96	2351
	Sensitivity (%)		75.0		78.1	
	Specificity (%)		98.6		96.1	
	PPV (%)		40.7		20.7	
	NPV (%)		99.7		99.7	
	LR+		52.4		19.9	

3. Hereditary spherocytosis

In UZ Leuven, the osmotic fragility test and morphological assessment of spherocytes are the only two implemented lab tests in order to diagnose spherocytosis. Samples with high suspicion for HS and inconclusive results are forwarded to an external lab for the EMA-binding test, cryohaemolysis, ektacytometry and SDS-PAGE. Therefore, the use of an algorithm as a screening tool or as an additional test complementary to the aforementioned lab tests could be an asset to the diagnostic work-up of HS in our laboratory. We included the following algorithms in this study: the adapted algorithm of Mullier et al (Sysmex 9000 analyzers) [34, 36], the algorithm of Bobée [35] and the algorithm of Adam et al [38]. Unfortunately, an appropriate group of HS patients is not available in our laboratory to evaluate the diagnostic performance of these algorithms.

First, over a three-month period (02/02/2021 until 02/05/2021), we retrospectively estimated the number of samples that would have been indicated as positive using the distinct algorithms (total number of samples = 16550), which is summarized in **Table 13**. Only a minority of the samples are derived from patients who received an osmotic fragility test in our hospital somewhere between 01/01/2009 and 05/05/2021 **(Table 13)**. Moreover, there is a rather low overlap between the distinct algorithm-selected samples **(Fig. 9)**. Based on this retrospective analysis, we expect that these algorithms will yield a high number of false positive (non-HS) patients in our laboratory. In the absence of an appropriate HS patient group, we were not able to adapt cut-off values.

Table 13: Retrospective analysis: total number of samples indicated as positive according to the algorithm over a three-month period.

02/02/2021 - 02/05/2021	Mullier et al adapted			Bobée et al		Adam et al	
	Hb > 12	**8 > Hb > 12**	**Hb < 8**	**Hb > 12**	**Hb < 12**	**Hb > 12**	**Hb < 12**
Number of samples (total *n*=15325)	107	45	2	318	77	169	126
Samples tested for Osmotic Fragility	0	1	0	1	3	0	6

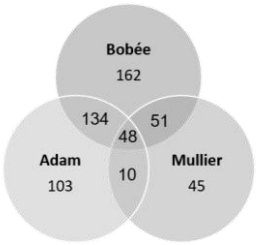

Figure 9: Venndiagram showing the overlap of all samples indicated as positive by the distinct algorithms.

To further explore the different algorithms, the best option would comprise a smear review of samples that are selected by the algorithm (prospective study). This requires a daily retrieval of the RBC parameters from the lab information system and making a blood smear of the selected samples within 24 hours from reception time (to avoid morphological artefacts). However, in our laboratory, lab technicians are trained to interpret the reticulocyte scattergram in the presence of the 'abnormal RET scattergram' analyzer flag with rejection of the reticulocyte parameters in case the reticulocytes are not properly gated and/or separated from the RBC gate. As a consequence, the reticulocyte parameters are not exported to the LIS and not reported to the clinicians. Instead, the following comment is reported: "unmeasurable due to analytical interference". With respect to this, it is known that spherocytes contribute to an abnormal RET scattergram (**Fig. 10**), resulting in analytical interference. Therefore, we performed a smear review of 28 samples (derived from 28 different patients) that appeared as "analytical interference" on the report (independent of the RET scattergram pattern). During this experiment, three patients with known auto-immune hemolytic anaemia and three patients with known HS were identified. Smear review of these samples was positive for spherocytes. Moreover, these samples all showed the typical spherical pattern in the RET scattergram indicative of the presence of spherocytosis (**Fig. 10**). In case of analytical interference, reticulocyte parameters are neither reported nor forwarded to the LIS, but still available in the database (extended IPU) of the analyzers. Using these non-reported parameters, the three aforementioned samples derived from patients with HS were indicated as positive using the different algorithms.

In conclusion, since inaccurate RET scattergrams are rejected in our laboratory, algorithms using parameters extracted from the LIS will only select samples without the morphological presence of spherocytes. However, a smear review of samples with a RET scattergram showing the typical spherocyte pattern, may identify samples with the clear morphological presence of spherocytes. As such, the diagnosis of HS or auto-immune haemolytic anaemia (it is not possible to differentiate based on the RET scattergram pattern) might be facilitated in a small group of patients. Especially patients lacking a strong family history of HS might benefit from this pro-active measure. Alternatively, RET parameters could be reported with reservations (by adding a comment to the lab report) in case of an inaccurate RET scattergram. This way, the screening algorithm could be implemented in the LIS. Since, in our opinion, reporting erroneous results should be avoided, we did not choose this option.

<u>To do:</u>
- Lab technicians should be trained to recognize RET scattergram patterns characteristic for the presence of spherocytes.
- A smear review should be performed on suspicious samples and in case spherocytes are present, a comment should be added to the lab report.
- Since HS/AIHA is not that prevalent, the added value of this measure should be evaluated over a one-year period.

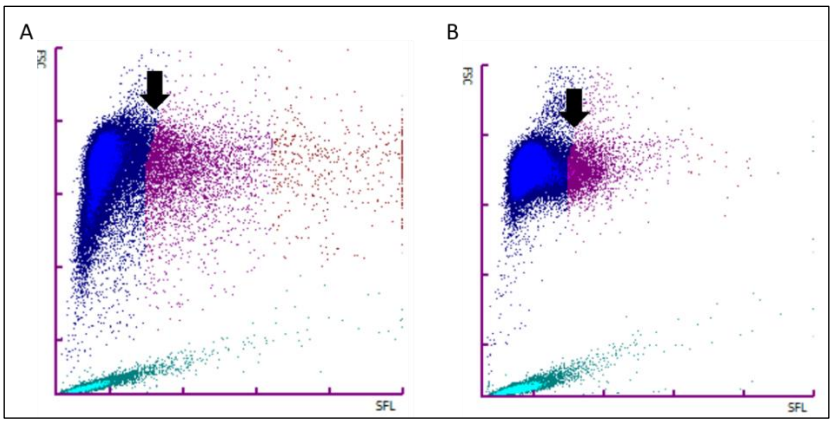

Figure 10: The reticulocyte scattergram of two samples with the morphological presence of spherocytes. (A) RET scattergram of a sample derived from a recently diagnosed HS patient (without splenectomy). Spherocytes were microscopically observed. **(B)** RET scattergram of a sample derived from a HS patient with splenectomy. Spherocytes were microscopically observed. **(A-B)** The scattergrams show an inaccurate separation between the red blood cells and the reticulocytes. A spherical pattern is typically observed (arrow).

4. Pyruvate kinase deficiency

Since PKD is underestimated in the general population, the implementation of a screening algorithm for PKD seems an attractive attempt to increase awareness to the clinicians. Hitherto, one single algorithm for PKD is available (Bobée et al) [35]. First, over a three-month period (02/02/2021 until 02/05/2021), we retrospectively estimated the number of samples that would have been indicated as positive using this algorithm (total number of samples = 16550). In total, 92 out of the 16550 (0.6%) samples are suspicious for PKD according to the algorithm (± 1 sample/day).

As a second step, we analyzed PK activity in 10 samples indicated as positive by the algorithm, showing anaemia and without known haematological disorders (eg., acute leukemia). In a previous study at our laboratory, PK activity was shown to be stable for at least 21 days at 4-8°C [54]. All samples in our study were measured within 20 days from reception date and were stored at 4-8°C until analysis. Nine patients showed normal PK activity levels (reference values: 3.8 U/g Hb – 12 U/g Hb), whereas one patient showed a decrease in PK activity **(Table 14)**. This patient did not show other signs of haemolysis (normal LDH and bilirubin, haptoglobin not measured).

Furthermore, since the implementation of our new haematology analyzers (1/10/2021), 36 routine samples were analyzed for both PK activity and reticulocyte parameters (same prescription). All samples showed a normal PK activity (> 3.8 U/g Hb). Interestingly, all these samples were considered as non-suspicious for PKD based on the algorithm. Of note, only three samples out of 242 samples analyzed for PK activity between 1/01/2020 and 10/5/2021 showed a low PK activity (< 3.8 U/g Hb), illustrating the low prevalence of PKD in our laboratory.

In conclusion, PKD is a rather rare disorder and is estimated to be frequently overlooked. To increase awareness to the clinicians, we decided to implement the algorithm in our LIS. Since only one out of the ten selected samples showed a low PK activity in this study, we prefer to add a comment to algorithm-selected samples rather than performing a reflex test for PK activity. This way, the clinician may be guided swiftly in the right direction and should assess whether the measurement of PK activity is appropriate or not in a particular patient. Another argument in favor of a comment instead of a reflex test, is the rather high volume of blood that is required to perform the PK activity analysis (approximately 1 mL). Since PKD is a rather rare disorder, the efficacy of this newly implemented algorithm will be evaluated over a one-year-period.

Table 14: Measurement of PK activity in ten algorithm-selected samples.

Patient n°	Age (y)	Gender (m/f)	Hb (g/dL)	PK activity (U/g Hb)
1	33	m	8,7	4,8
2	64	f	13,0	6,6
3	90	m	11,0	10,9
4	68	m	7,3	10,0
5	65	f	9,2	7,2
6	38	f	8,9	8,3
7	29	f	10,9	7,5
8	76	m	11,8	6,7
9	91	f	11,5	6,1
10	41	f	10,9	0,2

5. Microangiopathic haemolytic anaemia

The diagnostic performance of the Sysmex FRC parameter is highly debated. In this study, we evaluated the diagnostic usefulness of FRC% in our laboratory.

First, all samples analyzed between 11/03/2021 and 10/05/2021 for which the FRC (relative and absolute) value is available, were extracted from the LIS (*n*=11180 samples). In this population, the relative FRC value ranged from 0.00% to 16.26%. A good correlation between the relative FRC and absolute FRC count was observed (Pearson's r = 0.932; p<0.0001) **(Fig. 11)**. Therefore, we exclusively use the relative FRC value (%) in the next part of this study. Next, since several studies show a correlation between FRC and Hypo-He, we investigated the correlation of FRC with Hypo-He and other RBC parameters (including microRBC, macroRBC, Hyper-He, MCV, MCH, RDW) in our cohort **(Fig. 11)**. Interestingly, all parameters significantly correlated with the relative FRC value (Pearson's test). The highest pearson correlation coefficient was obtained for Hypo-He and RDW, closely followed by microRBC, confirming previously published studies [52]. In support of this, Hypo-He and FRC are situated in the same region of the RET scattergram **(Fig. 1, Fig. 12)**.

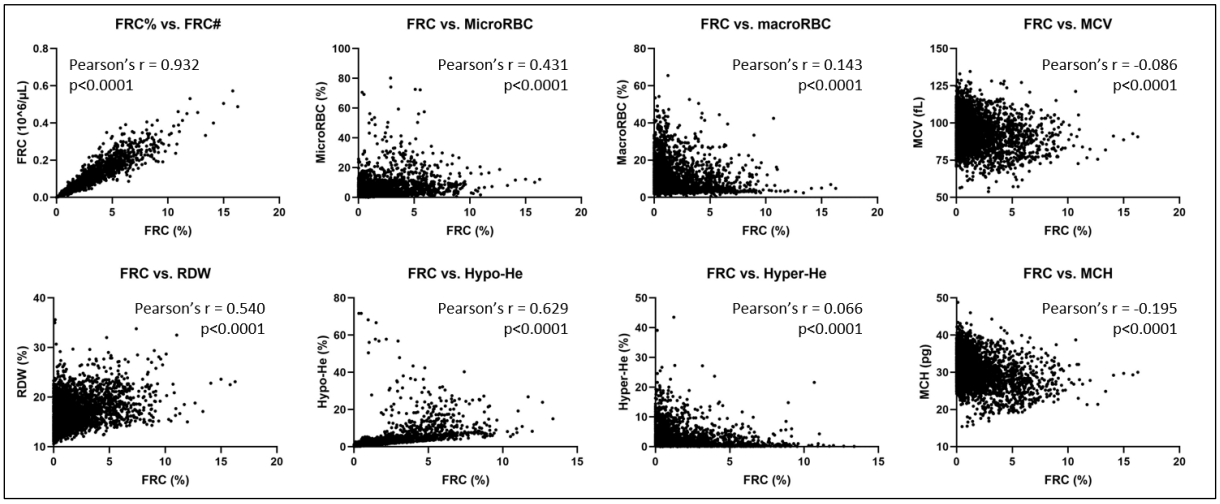

Figure 11: Correlation of FRC% with several RBC parameters. The FRC% value shows a significant correlation with all RBC parameters analyzed. The highest Pearson correlation coefficient was observed for Hypo-He and RDW, closely followed by MicroRBC.

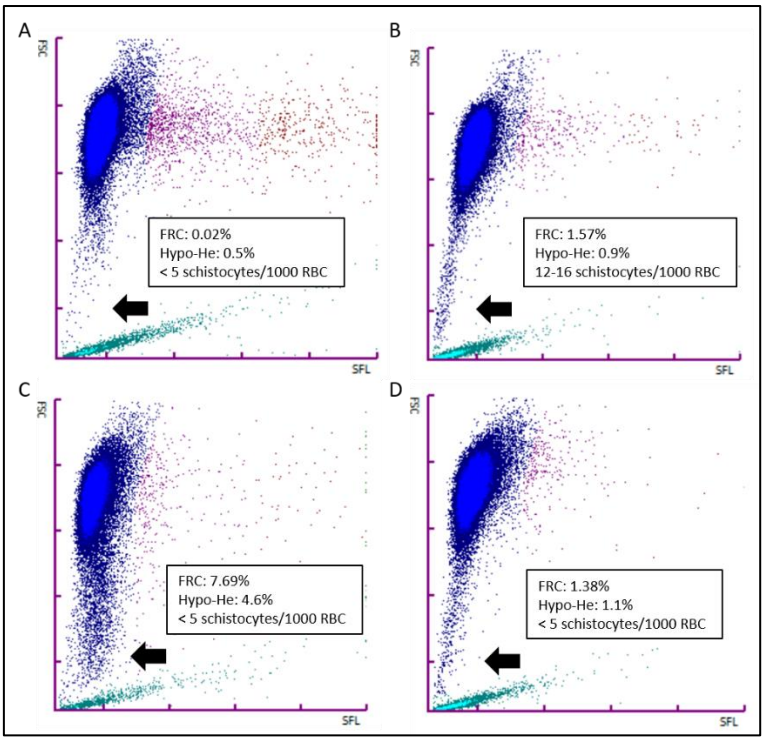

Figure 12: FRC value is derived from the RET scattergram and correlates with Hypo-He. (A) RET scattergram of a normochromic blood sample (MCH: 30.4 pg, Hypo-He: 0.5%) showing a low FRC value (0.02%), in accordance with the absence of microscopically observed schistocytes (true negative). **(B)** RET scattergram of a normochromic blood sample (MCH: 29.2 pg, Hypo-He: 0.9%) showing an increase in FRC (1.57%), reflecting the increase in microscopically assessed schistocytes (12-16 schistocytes/1000 RBC) (true positive). **(C)** RET scattergram of a normochromic blood sample with elevated Hypo-He (MCH 29.7 pg, Hypo-He 4.6%) showing an increase in FRC (7.7%) (false positive). No excess of schistocytes was microscopically observed. **(D)** RET scattergram of a normochromic blood sample (MCH 31, Hypo-He 1.1%) showing an increase in FRC (1.38%). However, no excess of schistocytes was microscopically observed (false positive).

Next, we aimed to explore the diagnostic performance of FRC%. For this, we used all samples analyzed between 11/03/2021 and 10/05/2021 wherefore FRC% and a microscopically schistocyte count was available. To enlarge our sample population, we included some additional samples (*n*=45) which were not analyzed for schistocytes in routine analysis (25 samples with a high FRC% value (> 4%) and 20 samples with Hypo-He < 1.5%, MCH > 27 pg and FRC > 1%) and performed a smear review of these samples within 24 hours from reception time. For the microscopic assessment of schistocytes, the number of schistocytes in five to ten microscopic fields (50x magnification, one field represents approximately 1000 RBC) was counted and the minimum and maximum number of schistocytes observed (range) was reported. For statistical analysis, this schistocyte range was converted to a semi-quantitative score as indicated in **Table 15**. Considering the positive correlation between FRC% and Hypo-He, we performed ROC analysis in three distinct sample populations (using pre-conditions):

1. All samples
2. MCH > 27 pg
3. MCH > 27 pg and Hypo-He < 1.2%

With respect to Hypo-He, several reference ranges have been previously reported: 0.0-0.2% (male) and 0.0-0.4% (female) [2], 0.0 -0.6% [55], and 0.1-0.5% (male) and 0.1-1.1% (female) [46, 56]. Mild hypochromia is considered as from Hypo-He 1.2–5.2%, and severe hypochromia as from Hypo-He 5.3–35.4% [52]. Therefore, we considered all samples with Hypo-He < 1.2% as normochromic samples in the third condition.

Table 15: Semiquantitative schistocyte score. Schistocytes are reported in our lab as a range of schistocytes counted in 5-10 microscopic fields (1 field contains approximately 1000 RBC). The scoring system is based on the maximum number of schistocytes within that range.

Microscopic assessment: the maximum number of schistocytes counted	Score
≤ 6 schistocytes/ 1000 RBC	0
7-10 schistocytes/ 1000 RBC	1
11-20 schistocytes/ 1000 RBC	2
> 20 schistocytes/ 1000 RBC	3

The ROC analysis was performed twice using two different definitions of schistocytosis:

1) A schistocyte score ≥ 1 (microscopically observed) was considered as schistocytosis (since we consider this score as mild schistocytosis in our laboratory).
2) A schistocyte score ≥ 2 (microscopically observed) was considered as schistocytosis (since the ICSH guidelines consider a schistocyte count > 1% on peripheral blood smear as a definite clinical value for the diagnosis of TMA in the absence of additional severe red cell shape abnormalities).

The number of samples included per group is shown in **Table 16**. The cut-off value was, on the one hand, chosen as the value for which the sum of sensitivity and specificity was the highest **(Table 16, Fig. 13-14)**. On the other hand, since other authors have shown that 97% of the samples (total *n*=1366) displayed a FRC < 1% [46], we compared the sensitivity and specificity at a FRC cut-off of 1% in every condition **(Table 16, Fig. 13-14)**. The third sample population (MCH > 27 pg and Hypo-He < 1.2%) consistently showed the highest AUC, specificity and LR+, which is plausible since samples with a high value for Hypo-He (which have possibly falsely elevated FRC values) are excluded in this population. Interestingly, the sensitivity and specificity for a cut-off > 1% (if schistocytosis = score ≥ 1) is similar as previously published [49]. As expected, when schistocytosis was defined as a schistocyte score ≥ 2, the sensitivity was a bit higher, but the specificity somewhat lower. Furthermore, we checked the number of samples with microscopically assessed schistocytosis that were indicated by the Sysmex RBC suspect flag "Fragments". As a result, only 20 out of 56 (36%) samples with score ≥ 1 and 10 out of 25 (40%) with score ≥ 2 were flagged by the haematology analyzers. Therefore, we consider the Sysmex flag as insufficient for the screening of schistocytosis.

Table 16: Diagnostic performance of FRC% using three different pre-conditions and two definitions of schistocytosis.

Schistocytosis: score ≥ 1				
Pre-condition	**Number of samples with score ≥ 1**	**Number of samples with score < 1**		**AUC ROC curve**
1. All samples	56	515		0.8223
2. MCH > 27 pg	49	461		0.8496
3. MCH > 27 pg and Hypo-He < 1.2%	23	368		0.8956
Pre-condition	**Cut-off value FRC%**	**Sensitivity**	**Specificity**	**LR+**
1. All samples	> 0.91	85.7	74.6	3.4
2. MCH > 27 pg	> 0.91	85.7	78.3	3.9
3. MCH > 27 pg and Hypo-He < 1.2%	> 0.91	73.9	95.1	15.1
1. All samples	> 1.00	78.6	77.1	3.4
2. MCH > 27 pg	> 1.00	77.6	81.1	4.1
3. MCH > 27 pg and Hypo-He < 1.2%	> 1.00	56.5	97.0	18.8
Schistocytosis: score ≥ 2				
Pre-condition	**Number of samples with score ≥ 2**	**Number of samples with score < 2**		**AUC ROC curve**
1. All samples	25	546		0.8286
2. MCH > 27 pg	23	487		0.8519
3. MCH > 27 pg and Hypo-He < 1.2%	9	382		0.9254
Pre-condition	**Cut-off value FRC%**	**Sensitivity**	**Specificity**	**LR+**
1. All samples	> 0.78	96.0	68.9	3.1
2. MCH > 27 pg	> 0.78	95.7	72.5	3.5
3. MCH > 27 pg and Hypo-He < 1.2%	> 0.91	88.9	92.9	12.6
1. All samples	> 1.00	84.0	74.2	3.3
2. MCH > 27 pg	> 1.00	82.6	78.2	3.8
3. MCH > 27 pg and Hypo-He < 1.2%	> 1.00	66.7	95.3	14.2

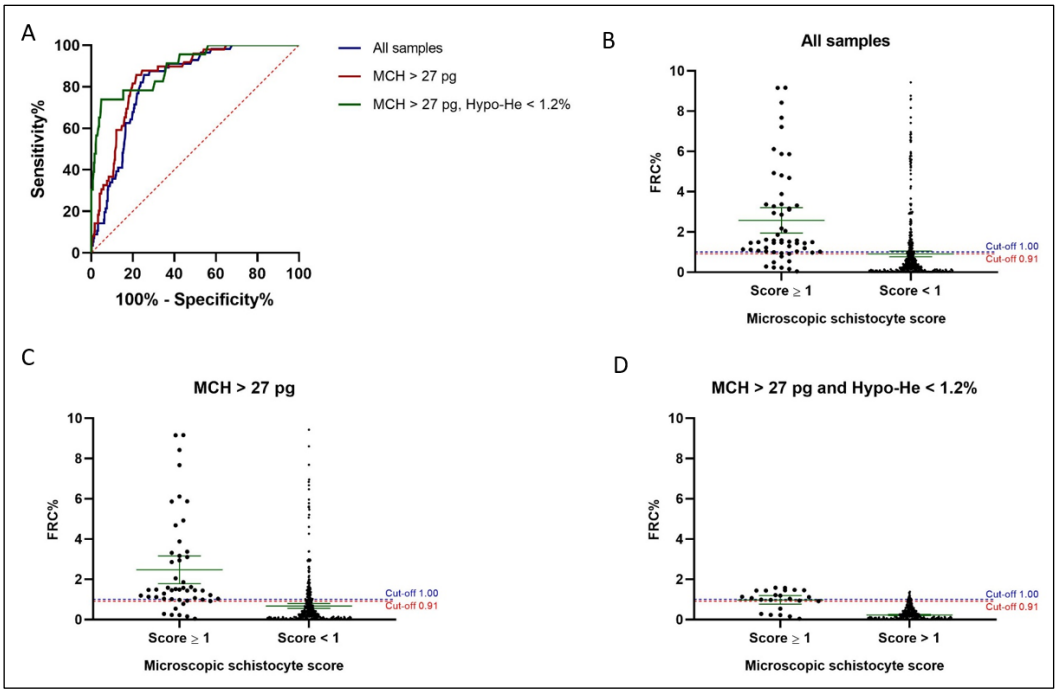

Figure 13: ROC analysis of FRC% when schistocytosis is defined as a schistocyte score ≥ 1. (A) ROC analysis of FRC% using three different sample populations (pre-conditions). **(B)** All samples: graph showing the FRC% of 56 samples with a microscopic schistocyte score ≥ 1 and 515 samples with a microscopic scistocyte score <1. **(C)** MCH > 27 pg: graph showing the FRC% of 49 samples with a microscopic schistocyte score ≥ 1 and 461 samples with a microscopic scistocyte score <1. **(D)** MCH > 27 pg and Hypo-He < 1.2%: graph showing the FRC% of 23 samples with a microscopic schistocyte score ≥ 1 and 368 samples with a microscopic scistocyte score <1. **(B-D)** Error bars indicate the mean ± 95% confidence interval. Dashed lines represent the in-house established cut-off based on the ROC analysis (0.91) and the cut-off based on literature (1.00).

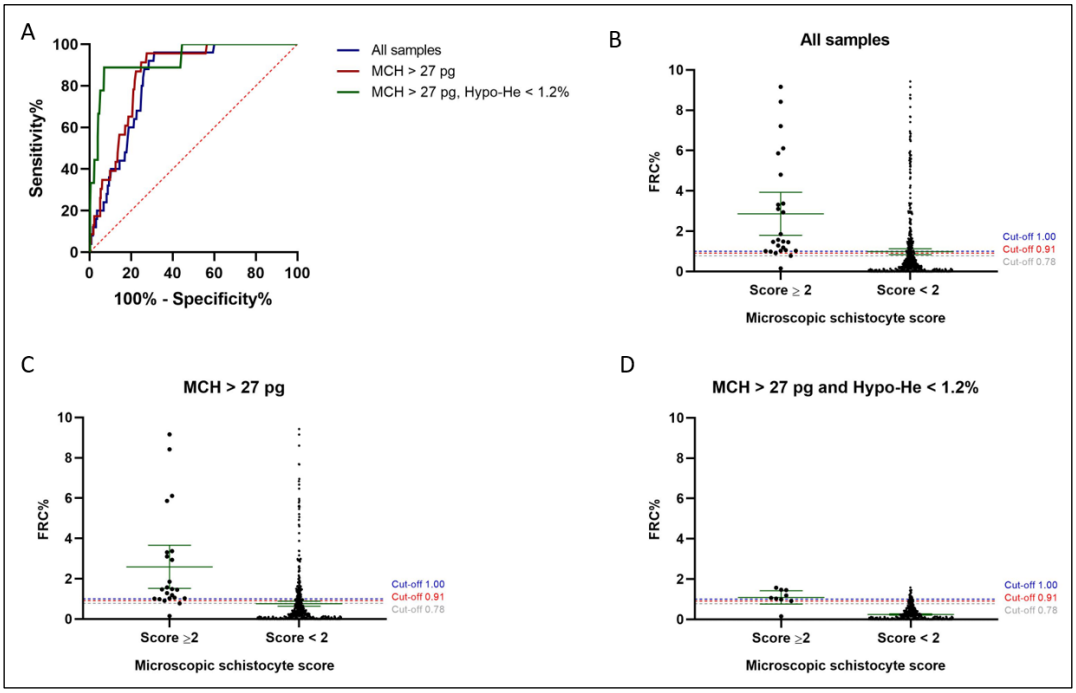

Figure 14: ROC analysis of FRC% when schistocytosis is defined as a schistocyte score ≥ 2. (A) ROC analysis of FRC% using three different sample populations (pre-conditions). **(B)** All samples: graph showing the FRC% of 25 samples with a microscopic schistocyte score ≥ 2 and 546 samples with a microscopic scistocyte score < 2. **(C)** MCH > 27 pg: graph showing the FRC% of 23 samples with a microscopic schistocyte score ≥ 2 and 487 samples with a microscopic scistocyte score < 2. **(D)** MCH > 27 pg and Hypo-He < 1.2%: graph showing the FRC% of 9 samples with a microscopic schistocyte score ≥ 2 and 382 samples with a microscopic scistocyte score < 2. **(B-D)** Error bars indicate the mean ± 95% confidence interval. Dashed lines represent the in-house established cut-off based on the ROC analysis (0.91 and 0.78) and the cut-off based on literature (1.00).

Next, we retrospectively assessed the number of samples that would be indicated as schistocytosis using the aforementioned FRC cut-off values in the entire sample population (including samples with unknown microscopic schistocyte count), using the aforementioned pre-conditions (**Table 17**).

Table 17: Retrospective evaluation of the number of selected samples using the distinct FRC% cut-offs (11/03/2021-10/05/2021).

Pre-condition	Cut-off	Number of samples
1. All samples (*n*=11180)	> 0.78	2328 (21%, ± 39 samples/day)
2. MCH > 27 pg (*n*=10002)	> 0.78	1731 (17%, ± 29 samples/day)
3. MCH > 27 pg and Hypo-He < 1.2% (*n*=8410)	> 0.78	352 (4%, ± 6 samples/day)
1. All samples (*n*=11180)	> 0.91	2100 (19%, ± 35 samples/day)
2. MCH > 27 pg (*n*=10002)	> 0.91	1534 (15%, ± 26 samples/day)
3. MCH > 27 pg and Hypo-He < 1.2% (*n*=8410)	> 0.91	220 (3%, ± 4 samples/day)
1. All samples (*n*=11180)	> 1.00	1949 (17%, ± 33 samples/day)
2. MCH > 27 pg (*n*=10002)	> 1.00	1400 (14%, ± 23 samples/day)
3. MCH > 27 pg and Hypo-He < 1.2% (*n*=8410)	> 1.00	154 (2%, ± 3 samples/day)

Overall, there are several ways to cope with these data:

(1) FRC% could be used as a screening tool. All samples with an FRC value higher than the established cut-off should be microscopically screened for schistocytosis. If applying this rule to all samples, this would imply the microscopic assessment of approximately 35 samples per day. We consider this as too much for our laboratory. If applying this rule to all normochromic samples with Hypo-He < 1.2%, this would imply the screening of approximately 4 samples per day. Despite a very good specificity in this sample population, the sensitivity is rather low (56.5%). Moreover, a considerable number of samples with schistocytosis is excluded in the latter population. Considering all this and considering that MAHA is marked by strong clinical and biochemical signs, we do not contemplate using FRC as a screening tool for schistocytosis in our laboratory.

(2) FRC% could be used to report probable schistocytosis upon demand. This could reduce the turn-around-time for schistocytosis in specific patients highly suspicious for MAHA based on clinical signs. When a schistocyte score ≥ 2 (microscopically observed) was considered as schistocytosis, a sensitivity of 96% could be reached. As such, in patients with a clinical suspicion of MAHA, this parameter could be reported quickly awaiting the microscopic assessment of schistocytosis. Nevertheless, the specificity (69%) is, in our opinion, not yet high enough to enable the confident reporting of schistocytosis based on the FRC value. Nevertheless, this measure could definitely be an option if the specificity of the FRC parameter is further optimized on next generation haematology analyzers.

(3) FRC% may be used supplementary to the microscopic assessment of schistocytosis. Upon the microscopic assessment of schistocytosis, the FRC value can be checked by the observer (eg., in difficult samples with poikilocytosis) with keeping all above-mentioned issues in mind. This is in line with the current ICSH guidelines on the assessment of schistocytosis [44].

TO DO/ACTIONS

1) Implementation of the following screening algorithms:

   - Formula of Urrechaga et al to screen for **β-thalassaemia**:
     [MicroRBC – Hypo-He – RDW-CV] > -5.1
   - Formula of Bobée et al to screen for **PKD**:
     [RET > 150*109/L, Ret/IRF > 9.5, MicroRBC < 5.5% and MicroRBC/Hypo-He < 6.0]

   Positive samples will be forwarded to a separate worklist in the LIS for evaluation by the clinical biologist. If appropriate, a reflex test will be performed or a comment will be added to the lab report.

2) Lab technicians should be trained to recognize the RET scattergram pattern indicative of **HS**.
   If a RET scattergram pattern indicative of spherocytes is observed, a smear review will be performed.
   If spherocytes are microscopically observed, a comment will be added to the lab report.

3) Evaluation of the usefulness of these screening tools a few months and/or one year following the implementation.

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