UZ LEUVEN

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

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What is anaemia?

- Major public health problem: **25%** of the entire world population
- WHO: “**decrease** in the number of red blood cells or Hb concentration”
- Persistent decrease in O2 supply → **irreversible** organ dysfunction

Detecting anaemia and the underlying cause
in an early phase is highly demanded!

Different types of anaemia

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

Screening algorithms

- **Facilitate** the differential diagnosis of **anaemia**
  Favor a **timely** diagnosis and treatment
- Based on **RBC parameters**
  - Conventional parameters (e.g., MCH, MCV, ...)
  - Research parameters (e.g., microRBC, Hypo-He,...)
- **Confirmatory tests** are required
- Adequate screening algorithm: **high sensitivity and specificity**
- **Sysmex RBC suspect flags:** “HGB defect?”, “Fragments?”, “Iron deficiency?”

Research questions

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 anaemia?

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?

With a focus on ...

1. β-thalassaemia
2. Iron deficiency anaemia
3. Hereditary spherocytosis
4. Pyruvate kinase deficiency
5. Microangiopathic haemolytic anaemia

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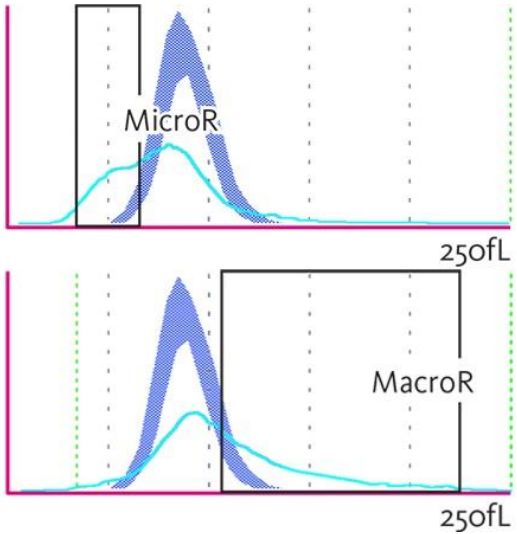
RBC research parameters

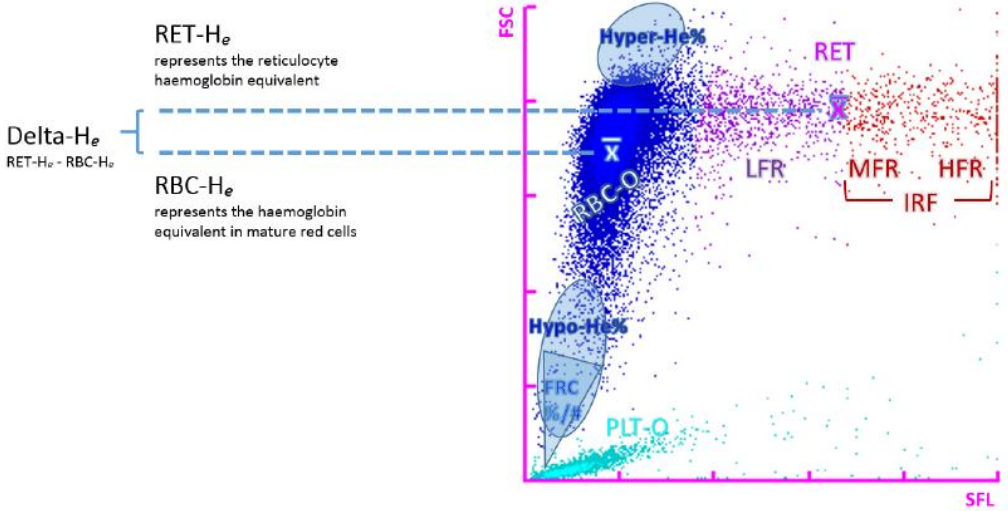
→ Sysmex XN-9100

RBC/PLT channel

MicroRBC (%)



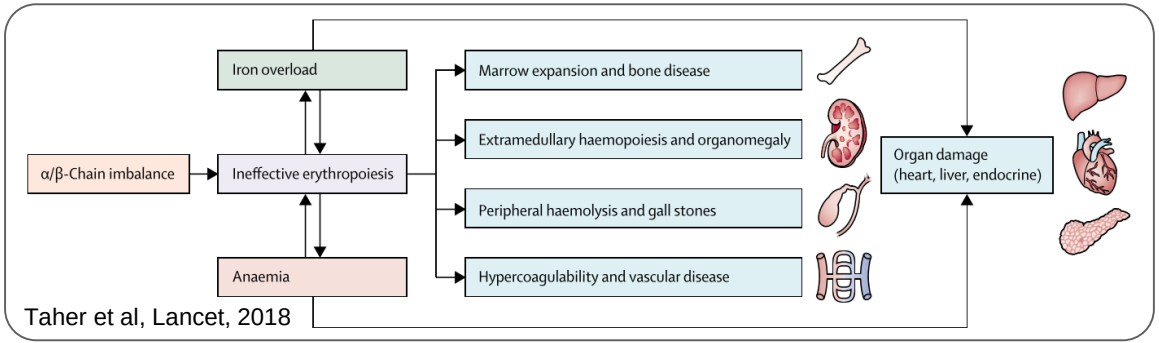
RET channel



β-thalassaemia

- **Minor, major or intermedia**
  - based on the genotype, the extent of anaemia, and the clinical symptoms

- Production of β-globin chains impaired + excess of α-globin chains:
  - **increase in HbA2** (α2δ2) levels (> 3.2%)
- Accumulation of **unstable α-globin chain tetramers** in erythroid cells
  - ineffective erythropoiesis and peripheral haemolysis



Taher et al, Lancet, 2018

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- Treatment:
  - **management of anaemia**
  - haematopoietic stem-cell transplantation (if severe disease)

β-thalassaemia: algorithms

- Microcytic, hypochromic anaemia (~Iron deficiency anaemia)
- Since 1973...

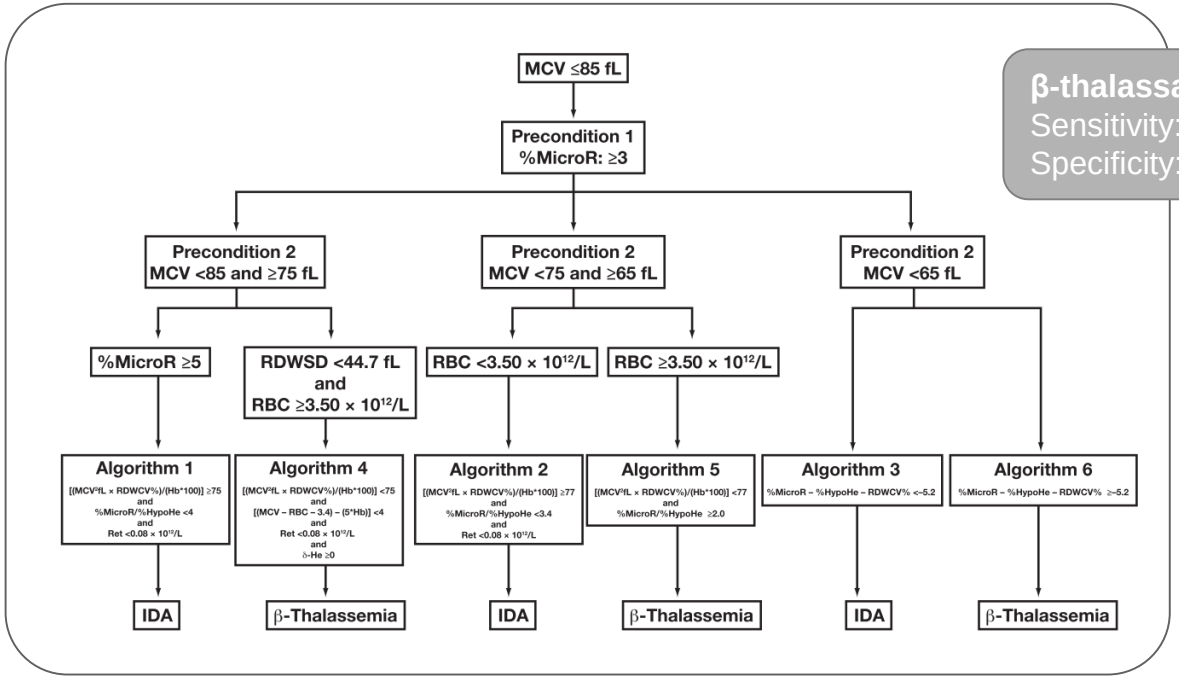
Reference	Formula	Cut-off for thalassaemia
Ehsani et al, 2009	MCV – (10*RBC)	<15
England et al,1973	MCV – RBC – 5*Hb – 3.4	<0
Green and King, 1989	MCV^2 * RDW/100 * Hb	<65
Mentzer, 1973	MCV/RBC	<13
Ricerca et al, 1987	RDW/RBC	<4.4
Shine and Lal, 1977	MCV^2 * MCH * 0.01	<1530
Sirdah et al, 2008	MCV – RBC – 3 * Hb	<27
Srivastava and Bevington, 1973	MCH/RBC	<3.8
Urrechaga et al, 2008	M/H (=microRBC/Hypo-He)	>3.7
Urrechaga et al, 2011*	MicroRBC – Hypo-He – RDW-CV	>-5.1 or >-7.6

*β-thalassaemia patients: n=270, IDA: n=250, apparently healthy subjects: n=90

β-thalassaemia: algorithms

Schoorl et al, 2012

β-thalassaemia patients: n=34, IDA: n=142, apparently healthy subjects: n=309

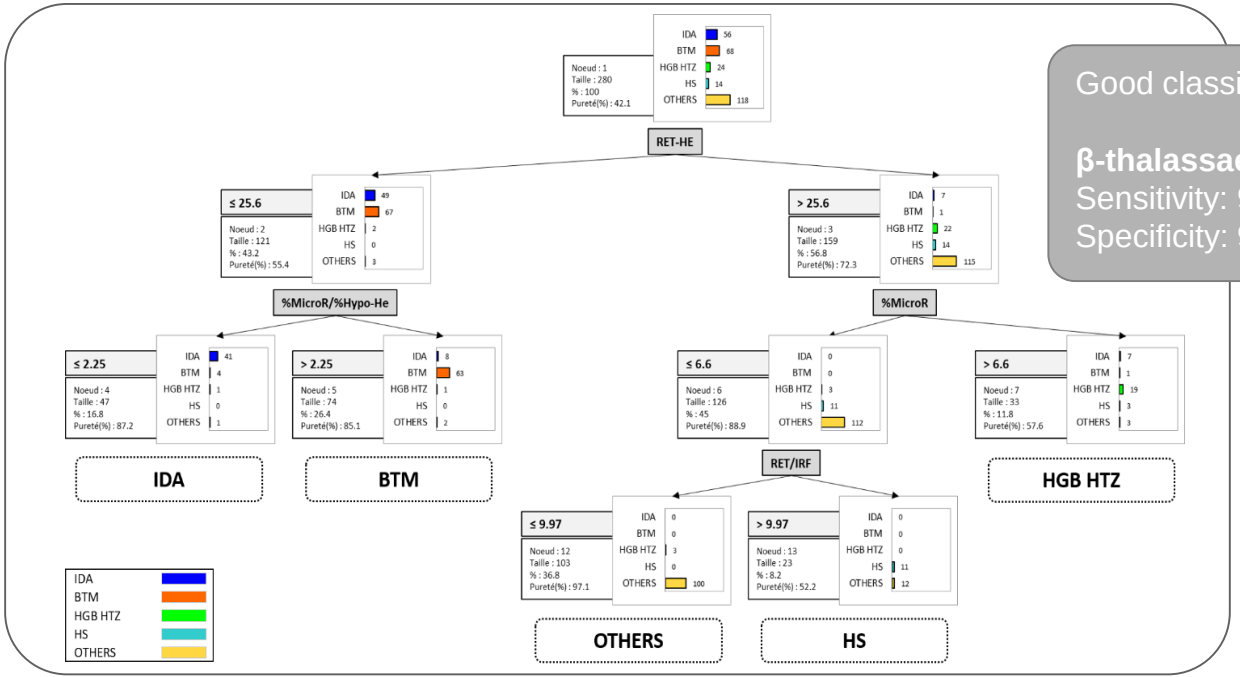


β-thalassaemia
Sensitivity: 74%
Specificity: 98%

β-thalassaemia: algorithms

Adam et al, LHUB-ULB (Belgium)

IDA (n=56), BTM (n=68), HS (n=14), heterozygote hemoglobinopathies (n=24) and others (n=118)



Good classification rate: 86.6%.

β-thalassaemia
Sensitivity: 92.6%
Specificity: 94.8%

β-thalassaemia: retrospective study

Identification of β-thal patients

- **2000 - 2021**
- **β-thal.** : HbA2 levels (>3.2%, electrophoresis method) and a lab report indicating β-thalassaemia
- **No β-thal.:** HbA2 level < 3.2% and a lab report indicating “normal capillary zone electropherogram”, independent of the MCV, MCH and number of erythrocytes.

Samples with CBC derived from β-thal patients?

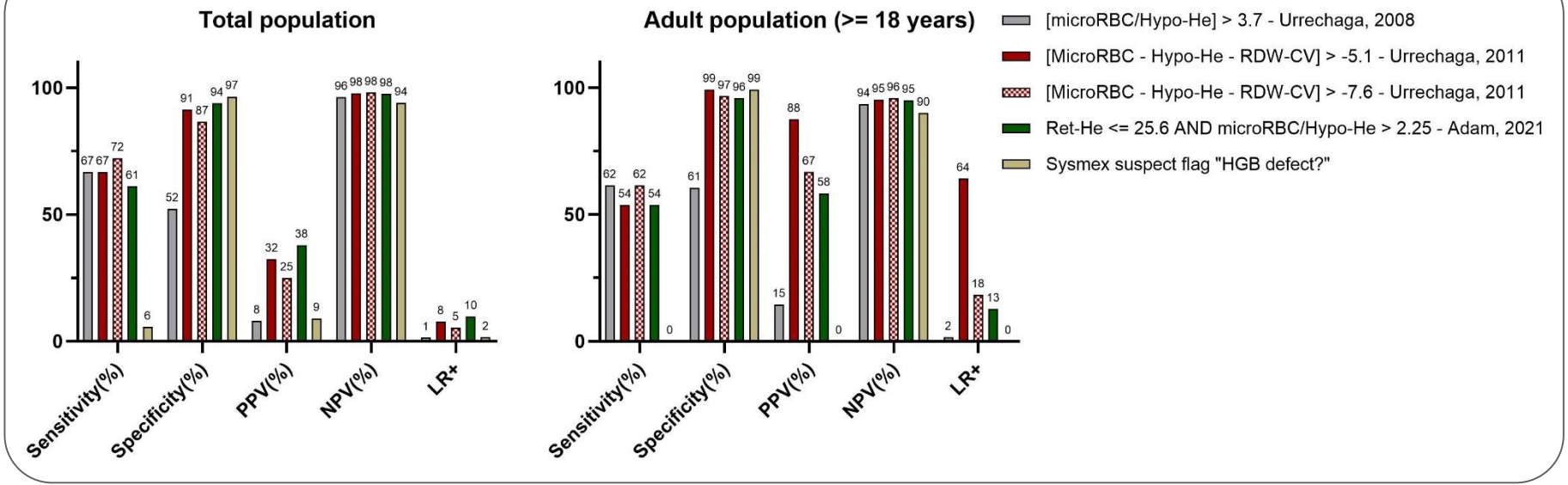
- **Three month period**
- **15376** blood samples were analyzed on the Sysmex XN-9100, including the RET channel
- **Total** population: **β-thal.: *n* = 18** samples; **No β-thal.: *n* = 289** samples
- **Adult** population: **β-thal.: *n* = 13** samples; **No β-thal.: *n* = 119** samples

Analysis

- M/H ratio: [MicroRBC/Hypo-He] > 3.7 - **Urrechaga et al, 2008** → Advia 2120
- [MicroRBC - Hypo-He - RDW-CV] > -5.1 of -7.6 - **Urrechaga et al, 2011** → Sysmex XE 5000 analyzer
- Sysmex **RBC suspect flag** “HGB defect?”: RDW-CV < 15% and MCV < 75 fL
- Ret-He ≤ 25.6 AND [MicroRBC/Hypo-He] > 2.25 - **Adam et al, 2021, LHUB-ULB** → Sysmex XN-9000

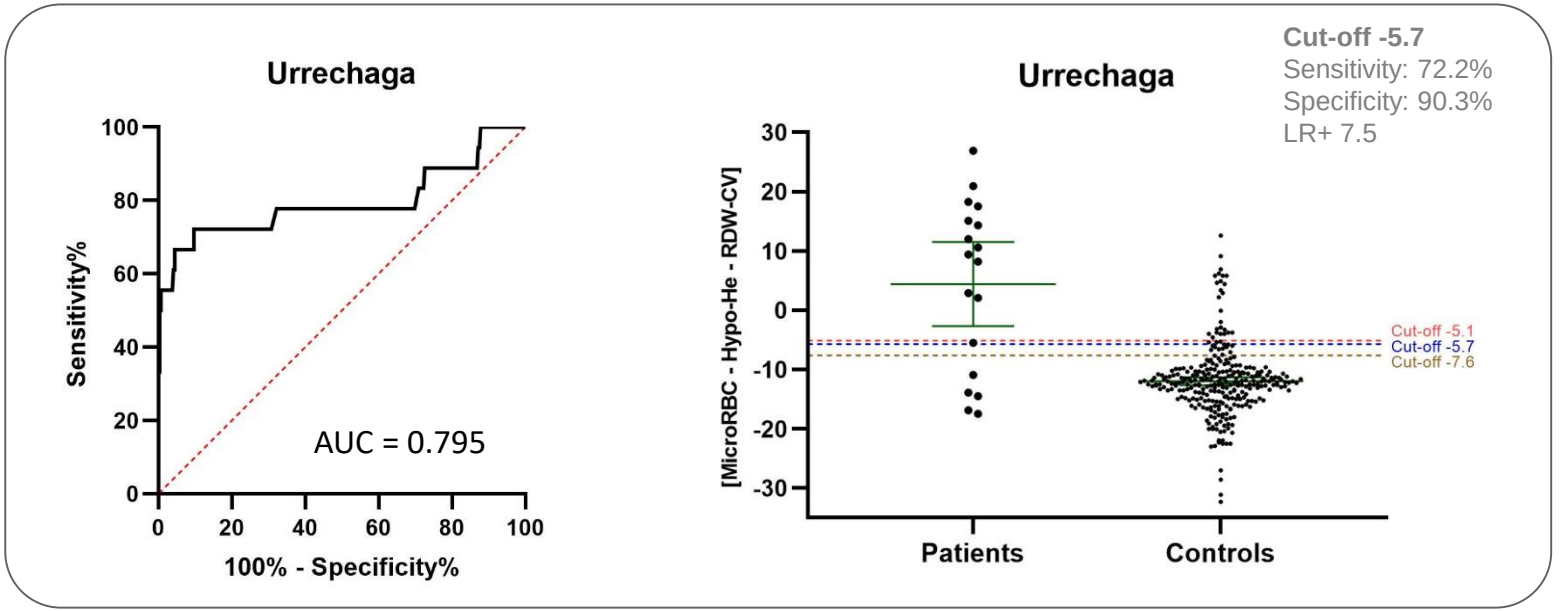
β-thalassaemia: retrospective study

Diagnostic performance



β-thalassaemia: retrospective study

- **ROC analysis** study population [MicroRBC - Hypo-He - RDW-CV]
  (β-thalassaemia *n*=18, controls *n*=289)



β-thalassaemia: retrospective study

- Total number of samples **indicated as positive** using the distinct algorithms

02/02/2021 – 02/05/2021	Total number of samples	M/H [MicroRBC/Hypo-He] > 3.7	Urrechaga [MicroRBC-Hypo-He-RDW-CV] > -5.1	Urrechaga [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%)

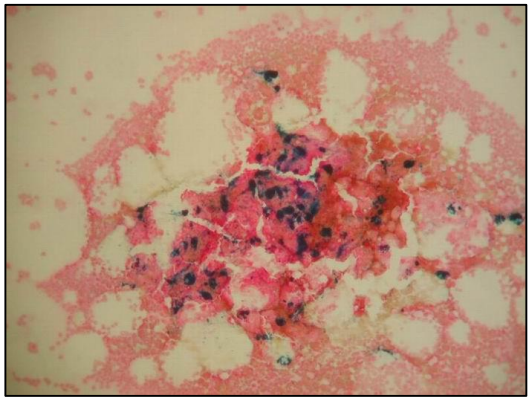
→ ± 2 samples/day

β-thalassaemia

Implementation – to do:

- [MicroRBC - Hypo-He - RDW-CV] > -5.1 (Urrechaga et al, 2011)
- Samples will be forwarded to a **separate worklist** within the LIS.
- The clinical biologist checks the list **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.

Iron deficiency anaemia



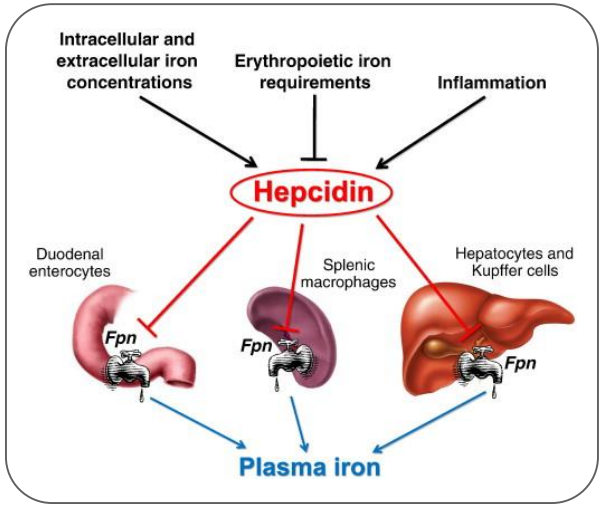
- **Most common** cause of anaemia
- Driven by **insufficient iron intake**
  - by malnutrition
  - during periods in life with higher demand i.e. pregnancy or growth
- **Bone marrow iron stores** using Perls' Prussian blue staining = **gold standard**, but requires invasive procedure
- **Biochemical parameters**:
  serum **ferritin** levels, **transferrin** levels/saturation (TSAT) and soluble transferrin receptors
  → **acute phase reactants!**

Iron deficiency anaemia

- Reticulocytes survive 1 to 3 days in the peripheral blood circulation
  → **Ret-He accurately reflects** the actual iron availability in the bone marrow

- Ret-He is also decreased in patients
  - with **β-thalassaemia**
  - with **ACD/acute inflammation**
    (= functional iron deficit mainly due to hepcidin-25)

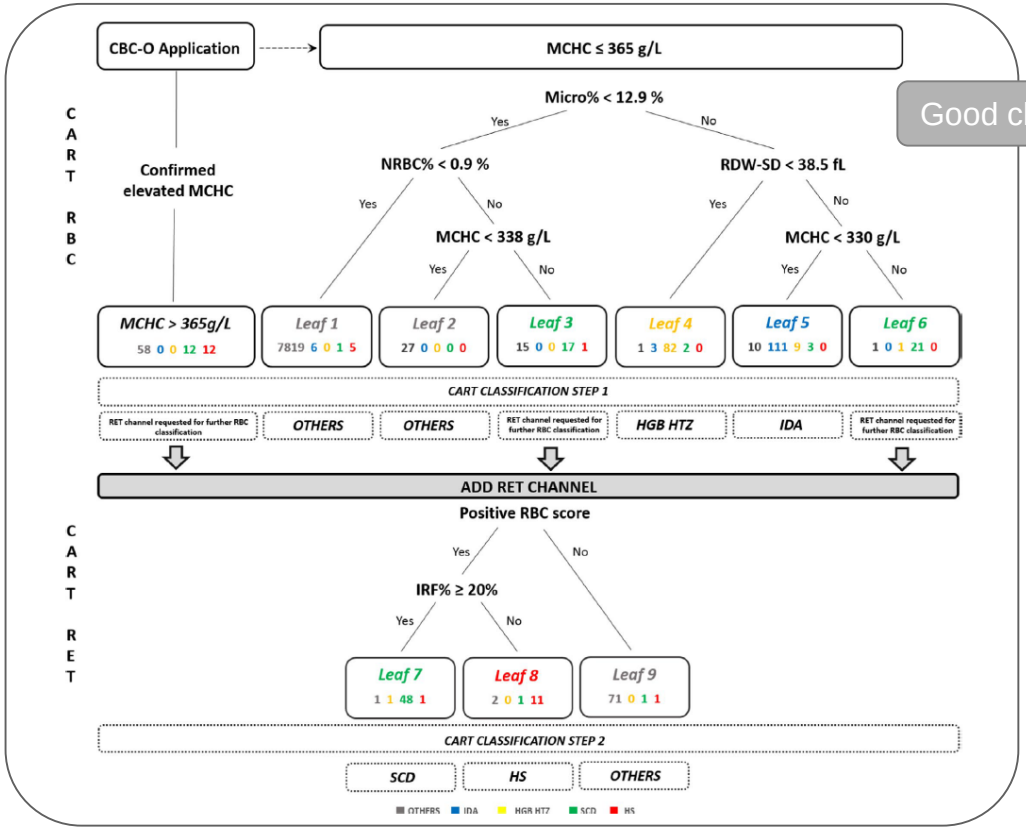
- **Discriminating formulas or algorithms!**



Iron deficiency anaemia

Nivaggioni et al, 2020

IDA (n = 120),
heterozygous haemoglobinopathy (n = 92),
SCD (n = 56),
HS (n = 18),
other patients (n = 7931)



Good classification rate: 99.4%

Iron deficiency anaemia

Adam et al, LHUB-ULB (Belgium)

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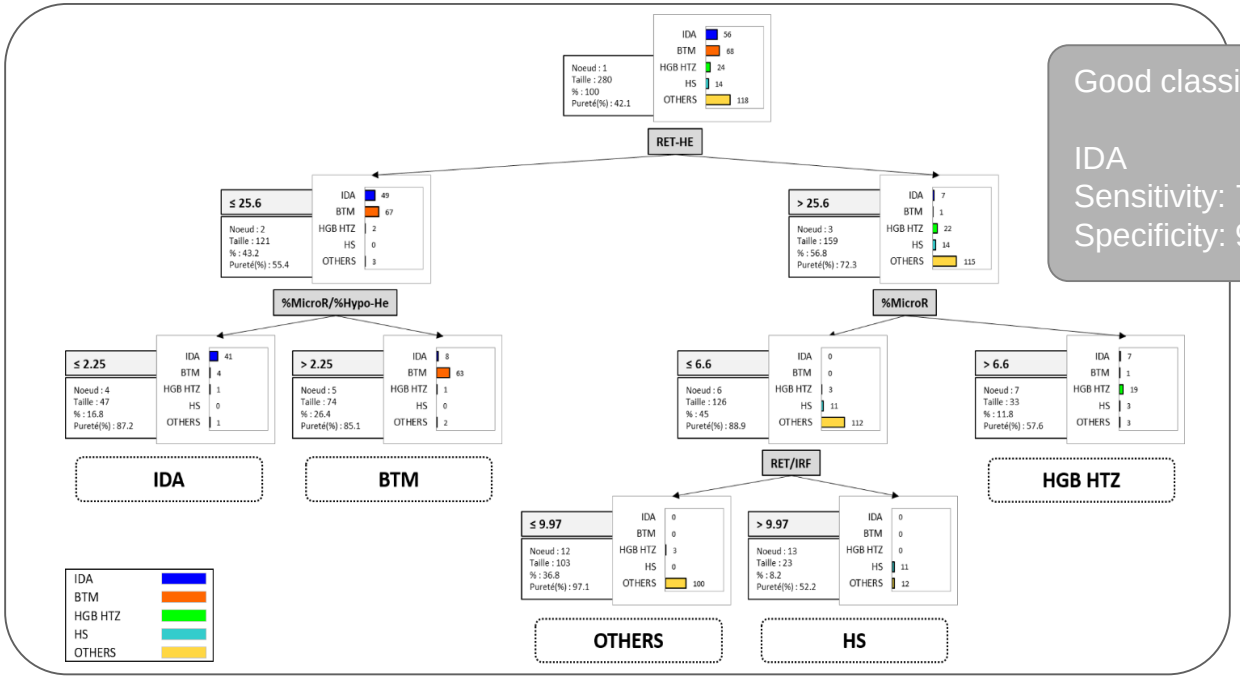


Good classification rate: 86.6%.

IDA
Sensitivity: 73.2%
Specificity: 97.3%

IDA: retrospective study

Definition iron deficiency

- **IDA:** serum ferritin levels < 15 µg/L and MCV < 80 fL
- **No 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”

Request for iron parameters and CBC on same prescription

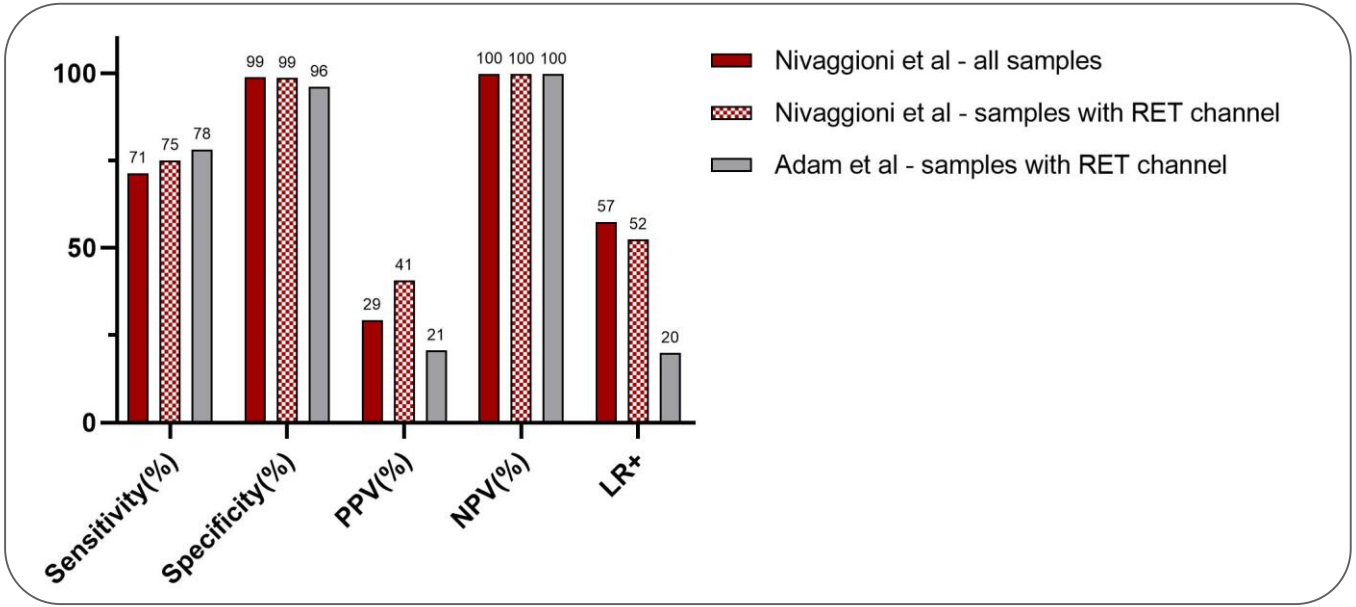
- Three month period - only **≥ 18 years**
- **12627** blood samples were analyzed on the Sysmex XN-9100 and ferritin of which 2479 with RET channel
- All samples: **IDA *n* = 91, no IDA *n* = 12536**
- Samples with **RET channel: IDA *n* = 32, no IDA *n* = 2447**

Analysis

- [MicroRBC ≥ 12.9% AND RDW-SD ≥ 38.5 fL AND MCHC < 33 g/dL] - **Nivaggioni et al, 2020**
- [Ret-He ≤ 25.6 AND MicroRBC/Hypo-He ≤ 2.25] - **Adam et al, LHUB-ULB, 2021**

IDA: retrospective study

- **Diagnostic performance**



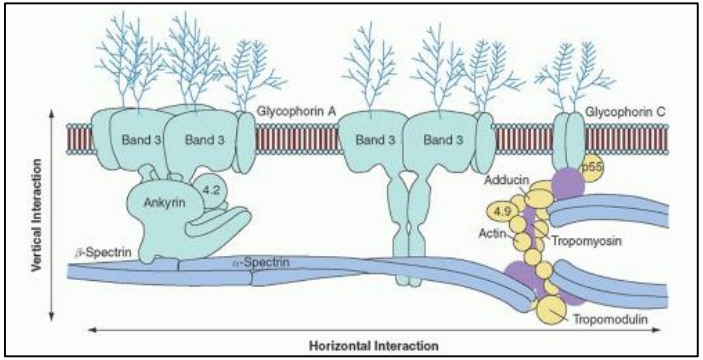
IDA: retrospective study

No implementation:

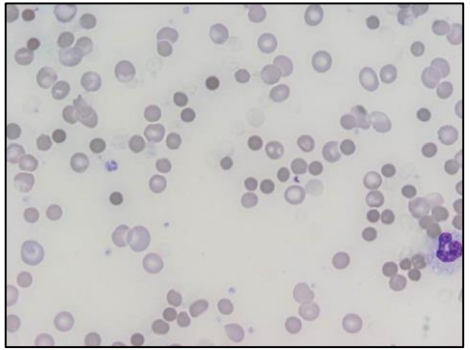
- sensitivity and specificity adequate
- PPV is rather low (29.4%)
- ± 14 positive samples per day → only 4 with IDA

Hereditary spherocytosis

- **Haemolytic** anaemia with a **normocytic** appearance
- Highest prevalence of HS is in **Northern Europe** (1 in 2000)
- Mutations in genes encoding distinct **proteins of the RBC membrane or cytoskeleton**
  - **elastic deformability** is affected
  - **shortened lifespan** of spherocytic RBC



Hereditary spherocytosis



- **Laboratory tests:**
  - the **osmotic fragility** test: low sensitivity and specificity
  - the observation of spherocytes on a **blood smear**.
  - The flow cytometry-based eosin-5′-maleimide (**EMA**)-binding test
  - the **cryohaemolysis** test
  - **SDS-PAGE**
  - **ektacytometry**
  - **molecular testing**

- **Specific limitations:**
  - low sensitivity and specificity, time-consuming procedures, or scarce availability
  - screening tools could have an added value

Hereditary spherocytosis

➢ Screening algorithms

Parameters	Bobée et al, 2018 (n= 47 HS, 17 PKD, 118 other (e.g. sickle cell, thalassaemia) and 489 routine samples)	Adam et al, 2020 (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%

HS: retrospective study

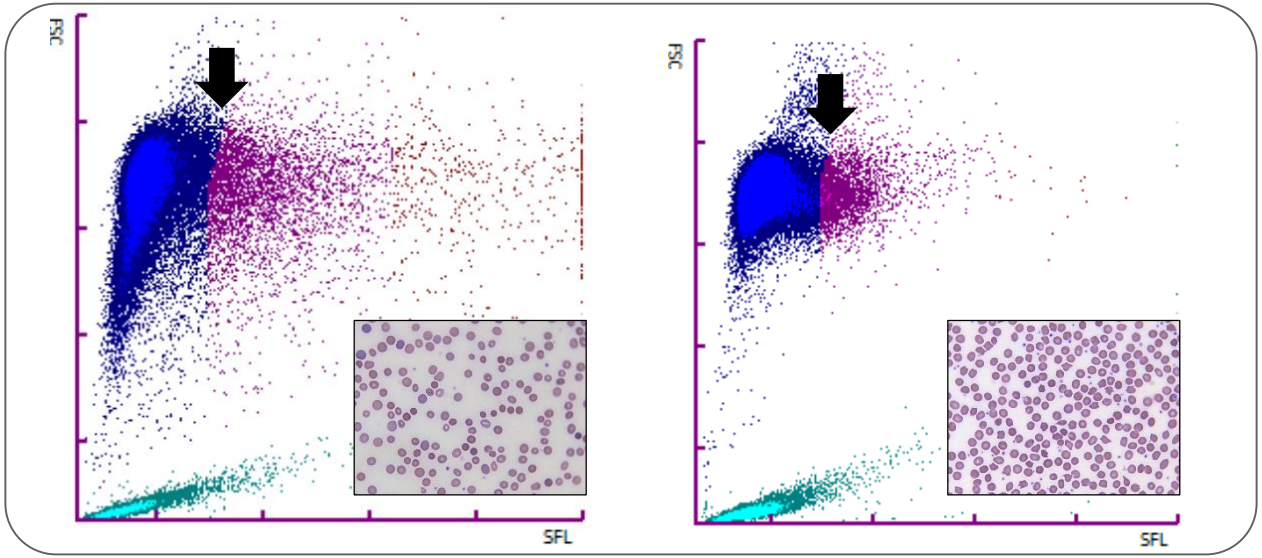
- Interpretation of the reticulocyte scattergram in the presence of the '**abnormal RET scattergram**' analyser flag
  - **rejection** if the reticulocytes are not properly gated and/or separated from the RBC gate
  - "**unmeasurable due to analytical interference**"

- **Spherocytes contribute to an abnormal RET scattergram**
  - Algorithms using parameters extracted from the LIS will only select samples without the morphological presence of spherocytes

HS: retrospective study

Smear review of 28 samples with “analytical interference”

- three patients with known auto-immune hemolytic anaemia
- three patients with known HS



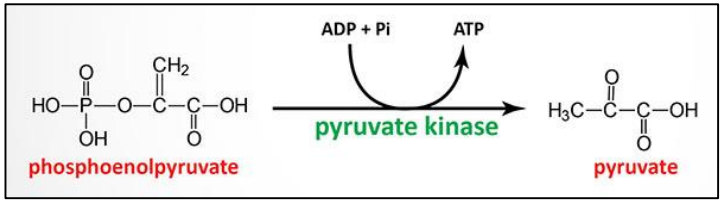
HS: retrospective study

Implementation – to do:

- lab technicians should be trained to recognize **RET scattergram patterns indicative 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**

Pyruvate kinase deficiency

- **Haemolytic** anaemia with a **normocytic** appearance
- 1/20 000 in the general Caucasian population
- PK generates 50% of the RBC total ATP
  → **decreased ATP levels reduce the RBC lifespan**



- **autosomal recessive disorder** often lacking a prominent parental history

Pyruvate kinase deficiency

- **Laboratory tests:**
  - measurement of **PK enzyme activity** using spectrophotometric analysis
  - *PK-LR* **molecular** testing
  - combining both tests is recommended for a definite diagnosis of PKD and adequate patient management

→ a **screening algorithm** based on RBC parameters could facilitate the diagnosis of PKD and increase awareness to the clinicians

Pyruvate kinase deficiency

- **Screening algorithm** for PKD

Parameters	Bobée et al, 2018 (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%

PKD: prospective study

- **Prospective study:**
  10 samples indicated as positive by the algorithm, with anaemia and without known haematological disorders (eg., acute leukemia)

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**

PKD: retrospective study

- **Retrospective study:**

01/10/2021 – 02/05/2021

- **36** routine samples were analyzed for both PK activity and reticulocyte parameters (same prescription): **normal PK activity** (> 3.8 U/g Hb)
- **non-suspicious for PKD** based on the algorithm

02/02/2021 – 02/05/2021

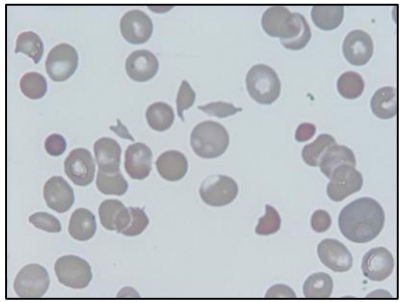
- 92 out of the 16550 (0.6%) samples are suspicious for PKD according to the algorithm **(± 1 sample/day)**

PKD: retrospective study

Implementation – to do:

- Positive samples will be forwarded to a separate worklist in the LIS
- The clinical biologist will check the list on a daily basis
- If appropriate, a **comment** will be added to the lab report
  - 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

Microangiopathic haemolytic anaemia



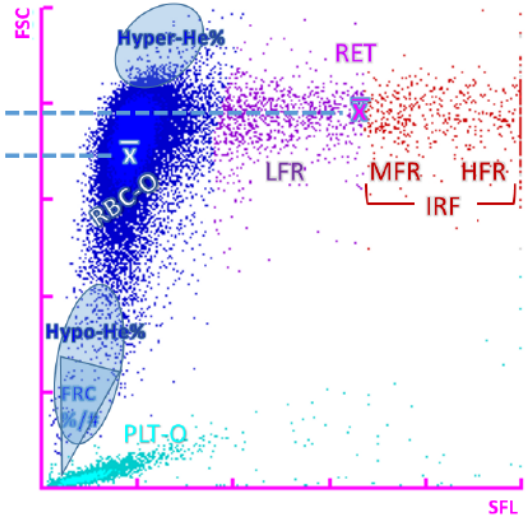
- Non-immune **intravascular haemolysis**
  - structural damage to the RBC membrane
  - formation of **red cell fragments or schistocytes**

- Shear stress
  - defective mechanical hearth valves, abnormalities in the microvasculature
- Thrombotic microangiopathies (TMA)
  - TTP, HUS, drug-induced TMA, and complement-mediated TMA
- Other conditions
  - pregnancy-associated syndromes, severe hypertension, systemic infections and malignancies, …

- TMAs are **life-threatening** medical urgencies: fast diagnosis is important!

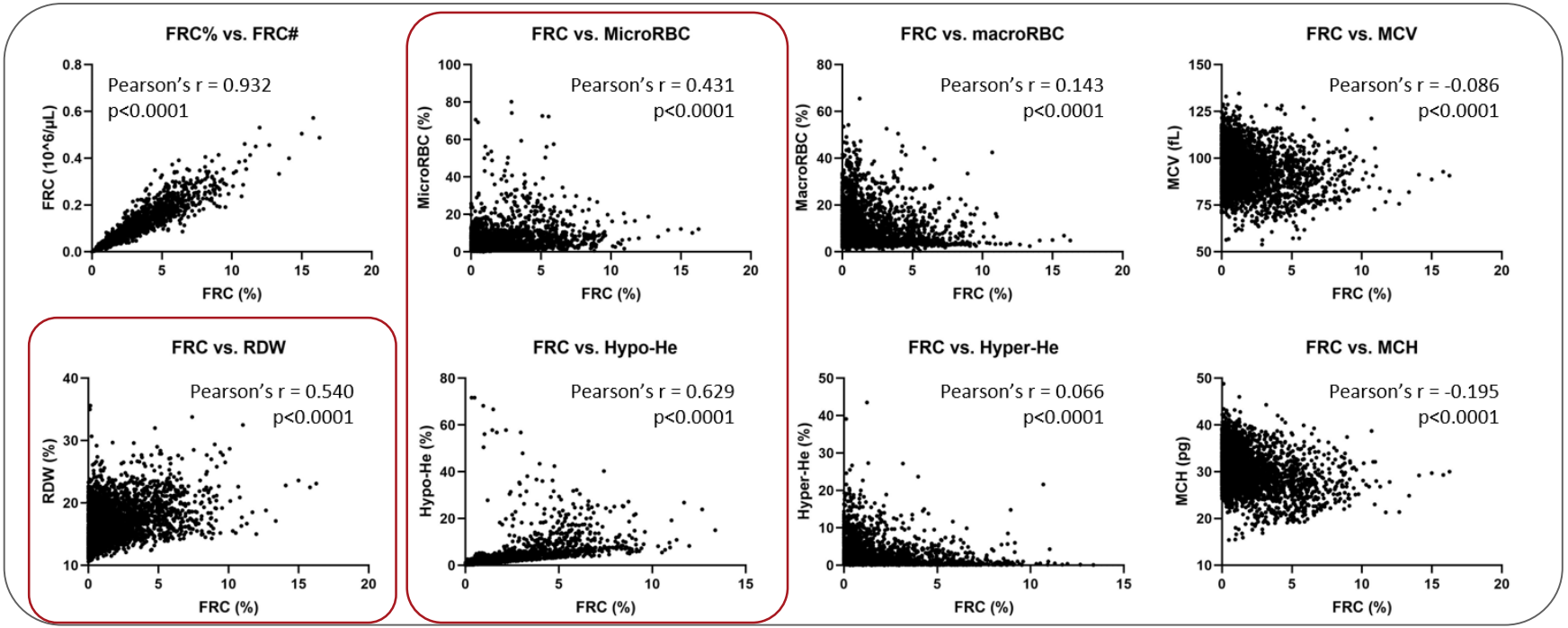
Microangiopathic haemolytic anaemia

- Golden standard: **smear review**
  - ICSH: 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
  - inter-observer variations and time-consuming
- Haematology analyzers: automated count **FRC**
  - sensitivity and specificity remains ambiguous
- **Sysmex suspect RBC flag: “Fragments?”**
  - combination of FRC, RDW-SD, PLT upper discriminator, MCV, RBC lower discriminator, MCHC, MP



MAHA: retrospective study

- 11/03/2021 and 10/05/2021: *n*=11180 samples

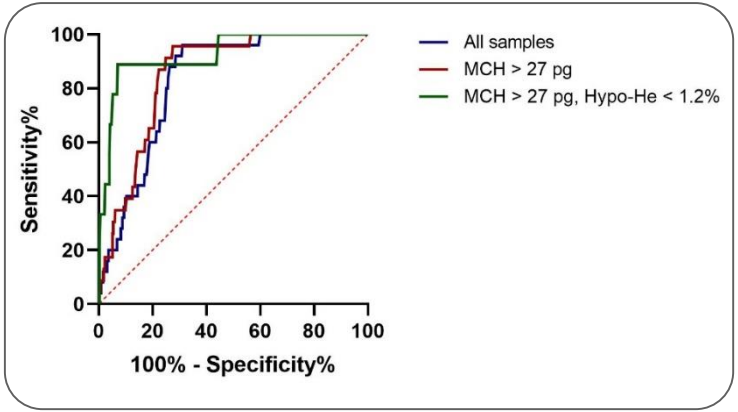


MAHA: retrospective study

- 11/03/2021 and 10/05/2021: *n*=571 samples

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

- **ROC analysis**

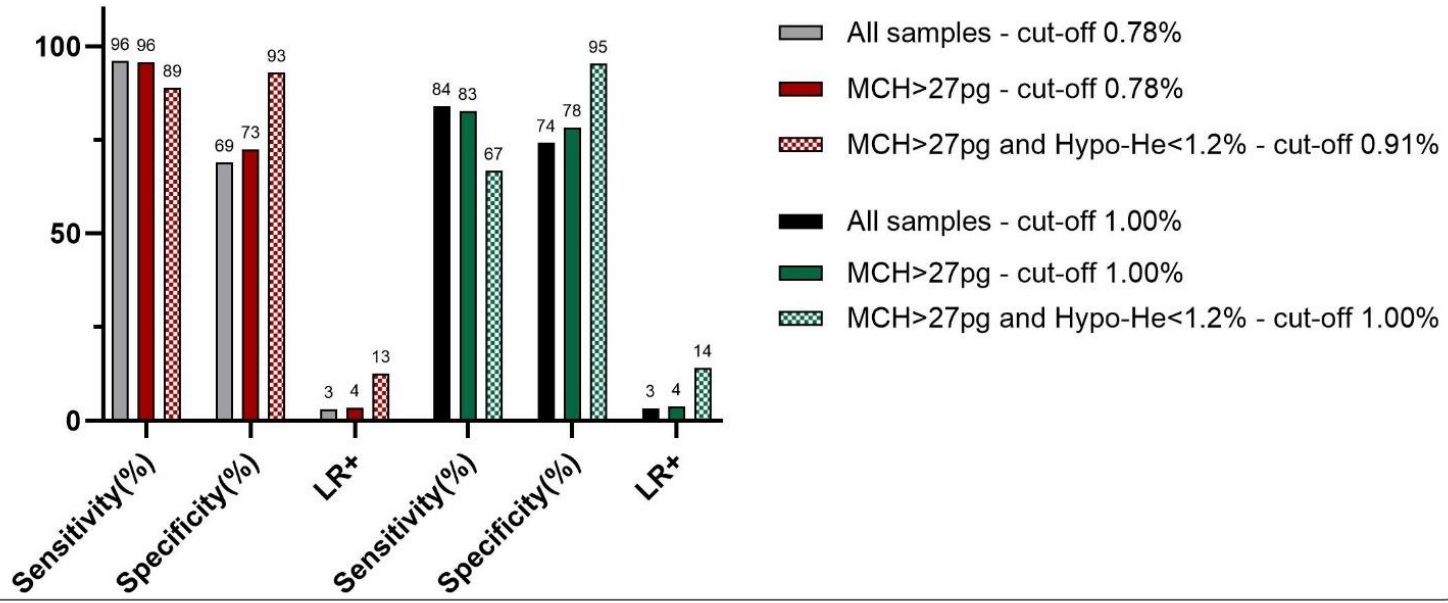


Schistocytosis: schistocyte score ≥ 2

Pre-condition	Number of samples with score ≥ 2	Number of samples with score < 2	AUC
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

MAHA: retrospective study

➢ **Diagnostic performance** (Schistocytosis: schistocyte score ≥ 2)



MAHA: retrospective study

- **RBC suspect flag “Fragments?”:**
  Only 10 out of 25 (**40%**) with score ≥ 2
  → insufficient

- Number of **selected samples** using the distinct FRC% cut-offs

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)

MAHA: retrospective study

No implementation as a screening tool:

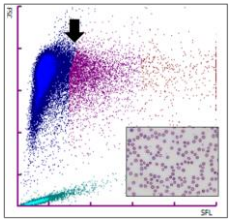
- Specificity too low
- Too much samples that would have to be screened (35 samples/day)
- FRC% may be used **supplementary** to the microscopic assessment of schistocytosis. This is in line with the current **ICSH guidelines** on the assessment of schistocytosis.

To do/actions

- **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



- Lab technicians should be trained to recognize the RET scattergram pattern indicative of **HS**.

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

General conclusion

- ✓ 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**

- ✓ Our results highlight the importance of **evaluating the diagnostic performance** of published screening algorithms using **lab-specific sample populations and haematology analyzers.**

Thank you!

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