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# Diagnostic performance of morphological analysis and red blood cell parameter-based algorithms in the routine laboratory screening of heterozygous haemoglobinopathies

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**Abstract**

**Objectives:** The aim of this study was to carry out a cross-analysis of the morphological abnormalities (MA) and the electrophoretic profile (EP) of blood samples suspect for heterozygous haemoglobinopathies (HTZ HGP). Screening for HTZ HGP was based on erythrocyte parameters provided by the Sysmex XN analysers.
**Methods:** A total of 596,000 blood samples was included in the study. According to the results of the mean corpuscular haemoglobin concentration (MCHC), the percentage of microcytes (Micro%) and the standard deviation of the red blood cell distribution width (RDW-SD), 842 different adults were screened as suspect for HTZ HGP and underwent simultaneous morphological analysis of red blood cells (RBCMA) and haemoglobin fraction analysis.
**Results:** The majority (72.8 %) of HTZ HGP suspects presented a pathological EP, mostly compatible with a confirmed β-thalassaemia trait (50.1 %) or a heterozygous β-haemoglobin variant (12.2 %). MA were identified in 360 (42.8 %) samples and 70 (8.3 %) of these had 3 or more MA. The most common MA was poikilocytosis (28.1 %). Patients with at least 1 MA detected were more likely to have a pathological EP (p=0.003). However, correlation between the number of MA detected and the type of EP was negligible.
**Conclusions:** Screening for HTZ HGP based on erythrocyte parameters measured on Sysmex XN analysers is a relevant tool with a positive predictive value of 72.8 % and definitely superior to microscopic RBCMA which now appears to be of low added value and obsolete in this indication.

**Keywords:** haemoglobin electrophoresis; haemoglobinopathy screening; red blood cell morphology; red blood cell parameter; Sysmex XN; thalassaemia

## Introduction

Haemoglobinopathies (HGP) refer to a group of inherited genetic disorders affecting one or more globin genes (*α1, α2, β, δ, γ1, γ2*). They are divided, on the one hand, into structural variants in which the genetic defect causes a structural change in haemoglobin (Hb) that can lead to a sickle cell syndrome, a haemolytic anaemia or an erythrocytosis and, on the other hand, into thalassaemias which are characterised by a quantitative reduction in the production of one of the globin chains and, consequently, of erythrocyte Hb [1]. To date, more than 500 thalassaemia variants and over 1,400 structural variants have been identified [2].

These disorders represent the leading cause of recessive monogenic disease worldwide, with more than 5.2 % of the world's population carrying a clinically significant Hb variant [3, 4]. Sickle cell syndromes are the most prevalent (42–97.8 cases per 100,000 inhabitants) and are increasing while thalassaemias follow a decreasing trend and are estimated at between 6 and 18.3 cases per 100,000 inhabitants [5–7]. Initially confined to the thalassaemic belt and certain regions of Africa, these pathologies, as a result of immigration from these endemic areas, have spread worldwide including in Europe where the frequency of HGP is now comparable across its northern, southern and western regions [8–11].

Individuals with heterozygous HGP (HTZ HGP) are generally asymptomatic, unlike homozygotes or compound

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heterozygotes whose clinical forms can vary greatly in severity and may be fatal without early management [12]. The search for these pathologies is particularly indicated when investigating chronic haemolytic anaemia, hypochromic microcytic anaemia that is not iron-deficient, polycythaemia or cyanosis [13]. However, in the absence of clinical signs, the identification of heterozygous carriers may easily be missed and is typically discovered following a preconceptional, prenatal, or neonatal screening programme, through the incidental finding of an Hb variant during glycated Hb measurement in a diabetic patient or an abnormal Kleihauer test result [1].

The biological diagnosis of HGP relies on various methods, including a morphological analysis of red blood cells (RBCMA) on a blood smear stained with May-Grünwald-Giemsa which can assist the laboratory to explore disturbances in erythrocyte parameters [14, 15]. This examination allows the identification of morphological abnormalities (MA) in size, colour, shape or the presence of inclusions which may be observed in HGP but are by no means specific [16].

An algorithm using erythrocyte parameters measured on the Sysmex XN-10 has been proposed and validated in adults aged 15 years or older by Nivaggioni et al. This red blood cell (RBC) tree allows, among other things, the detection of HTZ HGP based on the results of the mean corpuscular Hb concentration (MCHC), the percentage of microcytes (Micro%) and the standard deviation of the RBC distribution width (RDW-SD) [17].

The objectives of this study were to analyse the MA and the electrophoretic profile (EP) of blood samples suspect for HTZ HGP based on erythrocyte parameters provided by the Sysmex XN analysers, as well as to establish the added value of RBCMA compared to Hb separation techniques for the screening of HTZ HGP in daily practice.

# Materials and methods

## Study design

All blood samples for which a complete blood count (CBC) was ordered were retrospectively analysed over a period of 29 consecutive months (from December 2021 to April 2024) after the implementation of the RBC tree algorithm in the Extended Information Processing Unit (E-IPU; Sysmex Corporation, Kobe, Japan) integrated validation software at the University Hospital Center of Liège (Belgium). This algorithm classifies samples as suspect for HTZ HGP when three criteria are simultaneously met, namely a MCHC of 365 g/L or less, a Micro% of 12.9 % or more and a RDW-SD of less than 38.5 fL, and if there is no recent patient history (i.e. a sample already suspect for HTZ HGP within the last 90 days). Samples classified as suspect for HTZ HGP then underwent simultaneous RBCMA and Hb fraction analysis, as recommended by Nivaggioni et al. [17]. To ensure data quality, duplicate samples (i.e. multiple samples from the same patient), samples from children aged under 15 years and incomplete samples (i.e. samples for which RBCMA and/or Hb fraction analysis could not be performed) were excluded (Figure 1). Alongside this study, children aged between 2 and 15 years who were classified as suspect for HTZ HGP using the same algorithm also underwent microscopic and electrophoretic evaluation, but were excluded from the analysis since the RBC tree algorithm was validated in adults aged 15 years or older. The local Institutional Review Board deemed the study exempt from review. Informed consent was therefore not applicable.

## Analytical methods

Both hospital and outpatient samples were collected in BD Vacutainer® $K_3$EDTA tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The CBC was performed within 8 h of sample collection using a SYSMEX 9100 line (Sysmex Corporation, Kobe, Japan). This system consists of XN-10 and XN-20 haematology analysers connected to two identical modules, each including a SP-50 for blood smear preparation, a CF-70 slide loader and a CellaVision® DI-60 digital light microscopy system (CellaVision AB, Lund, Sweden). Internal quality controls supplied by Sysmex were analysed daily as part of the laboratory's routine.

The RBCMA was performed using the CellaVision® RBC Advance software (CellaVision AB, Lund, Sweden). A total of 21 MA (i.e. abnormalities in size, colour, shape or the presence of inclusions) were scored semiquantitatively in accordance with the guidelines of the International Council for Standardisation in Haematology [16].

Hb fraction analysis was performed by capillary electrophoresis (CE) on a Capillarys-2 Flex Piercing analyser (Sebia, Lisses, France) using the Capillarys Hemoglobin kit (Sebia, Lisses, France), in accordance with the manufacturer's instructions. The confirmation of each presumed β-Hb variant, except for Hb Lepore, was carried out by tandem mass spectrometry as previously described [18, 19].

Patient diagnostic categorisation, based on mean corpuscular Hb (MCH) and CE results, was performed according to the relevant recommendations [12, 20–22]. The reference values used were adapted to those validated by our laboratory, namely a mean corpuscular volume (MCV) between 83.7 and 100.8 fL, a MCH greater than 27.9 pg, a $HbA_2$

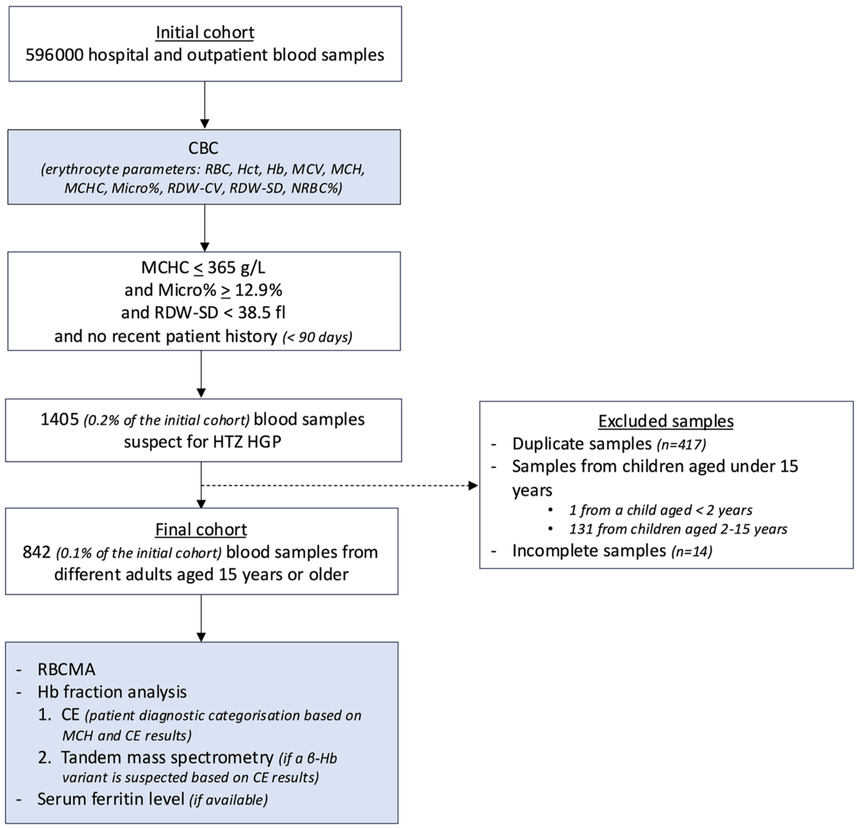


**Figure 1:** Sample selection flow chart.

level between 1.8 and 3.0 % and a fetal Hb (HbF) level lower than 1.2 % [3, 12, 21]. The description of the categorisation method used is available in Supplementary Table S1.

Serum ferritin was measured by a chemiluminescent microparticle immunoassay using an Alinity® I analyser (Abbott Laboratories, Abbott Park, IL, USA). When this data was available, iron deficiency (ID) was defined as a serum ferritin level of less than 15 µg/L [23].

## Statistical analysis

Data expressed as percentages refer to the total cohort, unless otherwise stated. For the purposes of this study, adults are defined as patients aged 15 years or older while children are defined as those under 15 years of age.

Regarding data on diagnostic categories and MA, 2 × 2 contingency tables were created and the chi-square test (or Fisher's exact test, depending on expected frequencies) was used to assess differences in their distribution between groups or categories. When relevant, diagnostic performance indicators (i.e. sensitivity, specificity, positive predictive value [PPV] and negative predictive value [NPV]) were determined to assess the ability of the RBCMA to predict diagnostic categories. In addition, Cohen's kappa coefficient was calculated to assess the degree of diagnostic agreement between EP and RBCMA and was interpreted according to the guidelines of Watson et al. [24].

Data related to MCV, MCH and serum ferritin levels were presented according to their median, range and interquartile range. The Mann-Whitney test was used to assess differences in their distribution between diagnostic categories.

Statistical analyses were performed using the R statistical software version 4.0.3 (R Foundation for Statistical Computing, Vienna, Austria) and the Kappa Statistic Calculator from GraphPad (GraphPad Software, San Diego, CA, USA) [25]. A p-value less than 0.05, or adjusted using the Bonferroni method when multiple comparisons were made, was considered statistically significant. The figure was created using Microsoft® PowerPoint version 16.92 (Microsoft Corporation, Redmond, USA).

# Results

## Patients

Of the 596,000 blood samples analysed, 1,405 (0.2 % of the initial cohort) were classified as suspect for HTZ HGP. After

**Table 1:** Demographic data of patients suspected of heterozygous haemoglobinopathy.

| Demographic data of the total cohort | |
|---|---|
| n | 842 |
| Mean age, years (±sd) | 46.0 (±18.5) |
| Sex | |
| Woman, n (%) | 455 (54.0) |
| Men, n (%) | 387 (46.0) |

sd, standard deviation.

excluding 563 samples (i.e. 417 duplicates, 1 from a child aged under 2 years, 131 from children aged between 2 and 15 years and 14 incomplete samples), the final cohort of this study included 842 (0.1 % of the initial cohort) blood samples from different adults aged 15 years or older and suspected of HTZ HGP. The mean age of these patients was 46.0 (±18.5) years, with a slight female predominance (54.0 %) (Figure 1 and Table 1).

## Diagnostic categories

The majority (72.8 %) of patients suspected of HTZ HGP presented a pathological EP. The most frequently detected abnormal profiles were confirmed (50.1 %) or possible (8.1 %) β-thalassaemia traits (BTT), followed by heterozygous β-Hb variants (12.2 %) which were mainly represented by HbS traits (8.6 %). Other identified pathological EP accounted for only 2.4 % of patients. In contrast, 27.2 % of patients displayed a normal EP (Table 2).

## RBCMA

MA were identified in 360 (42.8 %) samples suspect of HTZ HGP and 70 (8.3 %) of these had 3 or more MA. Patients with a positive RBCMA (i.e. at least 1 MA detected) were more likely to have a pathological EP than those without MA (78.1 vs. 68.9 %, respectively for each morphological group; p=0.003). This distinction was not specifically due to any particular subcategory of pathological EP (p=0.093). Similarly, patients with a strongly positive RBCMA (i.e. at least 3 MA detected) showed a higher frequency of pathological EP compared to those with less than 3 MA (84.3 vs. 71.8 %, respectively for each morphological group; p=0.024). This distinction was also not specifically due to any particular subcategory of pathological EP (p=0.178) (Table 3).

Poikilocytosis was observed in 28.1 % of samples suspect for HTZ HGP and was the most frequent MA within each diagnostic category. Other commonly encountered morphological atypia included hypochromia (13.1 %), ovalocytes (12.2 %), microcytes (7.7 %) and target cells (5.6 %). Of the 21 abnormalities searched for, 7 were not observed in any of the samples, namely acanthocytes, helmet cells, sickle cells, Howell-Jolly bodies, Pappenheimer bodies, basophilic stippling and parasites.

The frequency of poikilocytosis (p<0.001), hypochromia (p=0.001), schistocytes (p=0.025) and dacrocytes (p=0.042) was higher in patients with a pathological EP compared to normal, while that of macrocytes (p=0.038) was lower. Confirmed BTT showed a higher proportion of poikilocytosis

**Table 2:** Prevalence of diagnostic categories in patients suspected of heterozygous haemoglobinopathy.

| Diagnostic category | Diagnostic subcategory | Diagnostic subclass | n (%) | | | |
|---|---|---|---|---|---|---|
| **Normal EP** | | | **229 (27.2)** | | | |
| **Pathological EP** | BTT | Confirmed BTT | **613 (72.8)** | 490 (58.2) | 422 (50.1) | |
| | | Possible BTT | | | 68 (8.1) | |
| | Heterozygous β-Hb variants | HbS | | 103 (12.2) | 72 (8.6) | |
| | | Isolated HbS | | | | 31 (3.7) |
| | | HbS possibly associated with iron deficiency or α-thalassemia minor | | | | 36 (4.3) |
| | | HbS with an indeterminate association with iron deficiency or α-thalassemia | | | | 5 (0.6) |
| | | Other variants | | | 31 (3.7)[a] | |
| | Other pathological EP | Thalassemic or sickle cell syndrome related to a β-Hb variant | | 20 (2.4) | 6 (0.7)[b] | |
| | | Acquired/genetic causes of increased HbF | | | 7 (0.8) | |
| | | Common causes of decreased $HbA_2$ | | | 7 (0.8) | |
| | | Heterozygous δβ-thalassemia | | | 0 | |
| | | HbH disease | | | 0 | |

The proportions refer to the number of patients in the total cohort (n=842). [a]Includes 15 (1.8 %) HbC, 7 (0.8 %) HbE, 1 (0.1 %) HbO, 4 (0.5 %) Hb Lepore and 4 (0.5 %) heterozygous variants with uncertain identification. [b]Of which 3 (0.4 %) are related to HbE, 2 (0.2 %) are related to HbC and 1 (0.1 %) is a compound heterozygous for HbS and HbC. EP, electrophoretic profile; BTT, β-thalassemia trait. The names and results of the “diagnostic category” were set in bold.

**Table 3:** Distribution of diagnostic categories according to the number of morphological abnormalities detected in patients suspected of heterozygous haemoglobinopathy.

| Diagnostic category or subcategory | Number of MA, n (%) | | | | | |
|---|---|---|---|---|---|---|
| | 0 (negative RBCMA) n=482 (57.2 %) | ≥1 (positive RBCMA) n=360 (42.8 %) | p-Value | <3 (non strongly positive RBCMA) n=772 (91.7 %) | ≥3 (strongly positive RBCMA) n=70 (8.3 %) | p-Value |
| Normal EP | 150 (31.1) | 79 (21.9) | **0.003**[a] | 218 (28.2) | 11 (15.7) | **0.024**[b] |
| Pathological EP | 332 (68.9) | 281 (78.1) | | 554 (71.8) | 59 (84.3) | |
| Confirmed BTT | 217 (45.0) | 205 (56.9) | 0.093[c] | 382 (49.5) | 40 (57.1) | 0.178[d] |
| Possible BTT | 38 (7.9) | 30 (8.3) | | 58 (7.5) | 10 (14.3) | |
| Heterozygous β-Hb variants | 67 (13.9) | 36 (10.0) | | 97 (12.6) | 6 (8.6) | |
| Other pathological EP | 10 (2.1) | 10 (2.8) | | 17 (2.2) | 3 (4.3) | |

The p-value corresponds to the statistical differences (i.e. chi-square or Fisher's exact test result) in the distribution of [a, b]the overall result of the EP (normal vs. pathological) or [c, d]the diagnostic subcategories of pathological EP according to morphological groups (negative vs. positive RBCMA or strongly positive vs. non strongly positive RBCMA). A p-value less than 0.05, or adjusted using the Bonferroni method when multiple comparisons were made, was considered statistically significant and highlighted in bold. MA, morphological abnormality; RBCMA, morphological analysis of red blood cells; EP, electrophoretic profile; BTT, β-thalassemia trait.

(p<0.001), hypochromia (p=0.015), ovalocytes (p=0.005) and elliptocytes (p=0.019) than heterozygous β-Hb variants, whereas microcytes (p=0.019) and macrocytes (p=0.007) were less common. It should be noted that no patient with a normal EP showed schistocytes or dacrocytes, no confirmed BTT exhibited macrocytes and no heterozygous β-Hb variant had elliptocytes (Table 4).

## MCV, MCH and serum ferritin levels

All samples suspected of HTZ HGP were microcytic and had reduced Hb content, as evidenced by the maximum values of MCV and MCH found (75.8 fL and 27.1 pg, respectively). Patients with a pathological EP showed lower MCV (p<0.001) and MCH (p<0.001) than those with a normal EP,

**Table 4:** Distribution of the different types of morphological abnormalities by diagnostic categories in patients suspected of heterozygous haemoglobinopathy.

| Type of MA | Diagnostic category or subcategory | | | | | | |
|---|---|---|---|---|---|---|---|
| | Normal EP, n (%) n=229 | Pathological EP, n (%) n=613 | p-Value[a] | Confirmed BTT, n (%) n=422 | Heterozygous β-Hb variants, n (%) n=103 | p-Value[b] | Total cohort, n (%) n=842 |
| Poikilocytosis | 42 (18.3) | 195 (31.8) | **<0.001** | 149 (35.3) | 18 (17.5) | **<0.001** | 237 (28.1) |
| Hypochromia | 15 (6.6) | 95 (15.5) | **0.001** | 68 (16.1) | 7 (6.8) | **0.015** | 110 (13.1) |
| Ovalocytes | 35 (15.3) | 68 (11.1) | 0.099 | 52 (12.3) | 3 (2.9) | **0.005** | 103 (12.2) |
| Microcytes | 16 (7.0) | 49 (8.0) | 0.626 | 25 (5.9) | 13 (12.6) | **0.019** | 65 (7.7) |
| Target cells | 10 (4.4) | 37 (6.0) | 0.348 | 24 (5.7) | 9 (8.7) | 0.253 | 47 (5.6) |
| Elliptocytes | 6 (2.6) | 26 (4.2) | 0.274 | 20 (4.7) | 0 | **0.019** | 32 (3.8) |
| Stomatocytes | 5 (2.2) | 20 (3.3) | 0.412 | 13 (3.1) | 5 (4.9) | 0.369 | 25 (3.0) |
| Schistocytes | 0 | 13 (2.1) | **0.025** | 8 (1.9) | 0 | 0.365 | 13 (1.5) |
| Dacrocytes | 0 | 11 (1.8) | **0.042** | 8 (1.9) | 1 (1.0) | 1.000 | 11 (1.3) |
| Anisocytosis | 2 (0.9) | 6 (1.0) | 1.000 | 6 (1.4) | 0 | 0.603 | 8 (1.0) |
| Macrocytes | 5 (2.2) | 3 (0.5) | **0.038** | 0 | 3 (2.9) | **0.007** | 8 (1.0) |
| Spherocytes | 0 | 5 (0.8) | 0.331 | 5 (1.2) | 0 | 0.589 | 5 (0.6) |
| Echinocytes | 2 (0.9) | 2 (0.3) | 0.299 | 2 (0.5) | 0 | 1.000 | 4 (0.5) |
| Polychromatophilia | 1 (0.4) | 0 | 0.272 | 0 | 0 | 1.000 | 1 (0.1) |

The p-value corresponds to the statistical differences (i.e. chi-square or Fisher's exact test result) in the distribution of each type of MA according to (a) the overall result of the EP (normal vs. pathological) or (b) confirmed BTT vs. heterozygous β-Hb variants subcategories. A p-value less than 0.05 was considered statistically significant and highlighted in bold. As MA are not mutually exclusive, multiple MA can occur simultaneously within the same sample. Indeed, the sum of the different types of MA does not correspond to the total number of patients belonging to each category/subcategory. MA, morphological abnormality; EP, electrophoretic profile; BTT, β-thalassemia trait.

**Table 5:** Distribution of MCV, MCH and serum ferritin levels by diagnostic categories in patients suspected of heterozygous haemoglobinopathy.

| | | Normal EP n=229 | Pathological EP n=613 | p-Value[a] | Confirmed BTT n=422 | Heterozygous β-Hb variants n=103 | p-Value[b] | Total cohort n=842 |
|---|---|---|---|---|---|---|---|---|
| MCV | Available data, n (%) | 208 (90.8) | 554 (90.4) | | 422 (100.0) | 91 (88.3) | | 762 (90.5) |
| | Median, fL (IQR) | 71.8 (4.1) | 65.3 (6.1) | **<0.001** | 64.5 (4.5) | 70.0 (5.6) | **<0.001** | 67.0 (7.9) |
| | Range, fL | [57.7–75.8] | [54.4–75.4] | | [54.4–74.2] | [54.4–75.4] | | [54.4–75.8] |
| MCH | Available data, n (%) | 208 (90.8) | 555 (90.5) | | 422 (100.0) | 91 (88.3) | | 763 (90.6) |
| | Median, pg (IQR) | 22.6 (2.2) | 20.5 (2.4) | **<0.001** | 20.2 (1.6) | 22.8 (2.6) | **<0.001** | 21.1 (2.8) |
| | Range, pg | [16.4–26.8] | [16.2–27.1] | | [16.2–25.5] | [16.9–27.1] | | [16.2–27.1] |
| Serum ferritin | Available data, n (%) | 97 (42.4) | 237 (38.7) | | 178 (42.2) | 37 (35.9) | | 334 (39.7) |
| | Median, µg/L (IQR) | 42.6 (71.1) | 107.2 (174.4) | **<0.001** | 113.2 (176.8) | 66.4 (142.8) | **0.016** | 76.9 (141.0) |
| | Range, µg/L | [2.0–683.3] | [2.0–1897.5] | | [4.5–1897.5] | [4.3–456.1] | | [2.0–1897.5] |
| | ID, n (%) | 35 (36.1) | 20 (8.4) | **<0.001** | 9 (5.1) | 6 (16.2) | **0.027** | 55 (16.5) |

The proportions of available data refer to the number of patients belonging to each diagnostic category/subcategory, which is indicated in the title row of each column, while those concerning ID refer to the number of patients for whom serum ferritin data were available within that category/subcategory. The p-value corresponds to the statistical differences of the median levels of MCV, MCH, serum ferritin (i.e. Mann-Whitney test result) or in the proportion of ID (i.e. chi-square or Fisher's exact test result) according to [a]the overall result of the EP (normal vs. pathological) or [b]confirmed BTT vs. heterozygous β-Hb variants subcategories. A p-value less than 0.05 was considered statistically significant, with significant differences highlighted in bold. EP, electrophoretic profile; BTT, β-thalassemia trait; ID, iron deficiency.

while their serum ferritin levels were higher ($p<0.001$). The proportion of patients with ID was higher when the EP was normal than when it was pathological (36.1 vs. 8.4 %, respectively for each category; $p<0.001$). Similarly, in the case of a pathological EP, patients with a confirmed BTT had lower MCV ($p<0.001$) and MCH ($p<0.001$) than those with a heterozygous β-Hb variant, whereas their serum ferritin levels were higher ($p=0.016$). ID was more common in patients with a heterozygous β-Hb variant than in those with a confirmed BTT (16.2 vs. 5.1 %, respectively for each category; $p=0.027$) (Table 5).

## Diagnostic performance and agreement between RBCMA and diagnostic categories

When a patient was suspected of HTZ HGP, the presence of a positive RBCMA had a sensitivity, specificity and PPV of 45.8, 65.5 and 78.1 %, respectively, for predicting a pathological EP. Notably, the presence of a strongly positive RBCMA showed a 95.2 % specificity for predicting a pathological EP, although the corresponding PPV was 84.3 %. The maximum Cohen's kappa coefficient obtained from the correlation studies between a positive or strongly positive RBCMA and the type of EP finally identified was 0.084 (95 % CI: 0.029–0.139), which, at best, corresponded to a slight agreement between the two methods (Table 6).

# Discussion

The objectives of this study were to evaluate the MA and the EP found in samples suspect for HTZ HGP, as well as to establish the added value of the RBCMA when a patient is suspected of carrying this pathology.

The implementation of this algorithm allowed for the identification of 613 patients with a pathological EP, representing 72.8 % of those initially suspected of HTZ HGP, and enabled a definitive diagnosis to be made in 63.0 % of the cases. Most of these confirmed cases were indeed diagnosed with HTZ HGP, namely a confirmed BTT (50.1 %) or a heterozygous β-Hb variant (12.2 %). In contrast, a small proportion of these patients were diagnosed with a thalassaemic or sickle cell syndrome (0.7 %) which are, by definition, homozygous or compound heterozygous, and were therefore misclassified as suspected of HTZ HGP (see Table 2 and Supplementary Table S1).

Apart from these three categories, the diagnosis of the other patients (37.0 %) remains only presumptive since the EP found is not fully specific to a particular type of HGP. This group of patients with an uncertain diagnosis consists mainly of individuals with a normal EP, but also includes rare patients affected by either a pathology that causes a decrease in $HbA_2$ level or an increase in HbF level, or a possible BTT. If the $HbA_2$ level is 'borderline-raised' (i.e. between 3.0 and 4.0 %), these possible BTT require

**Table 6:** Diagnostic performance and agreement between red blood cell morphological analysis and diagnostic categories in patients suspected of heterozygous haemoglobinopathy.

| Number of MA | Diagnostic category | Sensitivity, % | Specificity, % | PPV, % | NPV, % | Cohen's kappa (95 % CI) |
|---|---|---|---|---|---|---|
| ≥1 (positive RBCMA) | Pathological EP | 45.8 | 65.5 | 78.1 | 31.1 | 0.084 (0.029–0.139) |
| ≥3 (strongly positive RBCMA) | Pathological EP | 9.6 | 95.2 | 84.3 | 28.2 | 0.028 (0.007–0.049) |

Diagnostic performance indicators and Cohen's kappa coefficient were calculated if a diagnostic category was significantly differently distributed according to the number of MA (see Table 3). MA, morphological abnormality; RBCMA, morphological analysis of red blood cells; EP, electrophoretic profile.

genetic analysis to search for a silent BTT once other causes that could raise the $HbA_2$ level have been excluded [12].

Since all samples suspect for HTZ HGP are microcytic and had decreased MCH, the pathologies that can manifest simultaneously with a normal EP include ID, α-thalassaemias, « false negatives » of BTT (i.e. BTT associated with δ-thalassaemia, severe iron deficiency anaemia (IDA) or certain variants of the α or δ globin chain) and rare cases of heterozygous $(\varepsilon\gamma\delta\beta)^0$-thalassaemias [12]. The iron metabolism workup shows that these electrophoretically normal patients have lower serum ferritin levels and a higher proportion of ID than those with a pathological EP. Therefore, we confirm that there is a risk of misclassifying certain ID among samples suspect for HTZ HGP which, in the presence of a normal EP, justifies testing of iron and inflammatory panels in order to exclude it. Additionally, genetic analysis of the globin genes could also prove useful for these samples as ID may coexist with thalassaemia, leading to misdiagnosis by underestimating the $HbA_2$ level. Furthermore, the definitive confirmation of microcytic hypochromic anaemia, once ID has been ruled out, can only be achieved through molecular analysis [12, 22].

It should also be emphasised that the categorisation of patients as suspected of HTZ HGP partly relies on the presence of a low RDW-SD. This particularity explains the low frequency of anisocytosis detected by microscopy (1.0 %) as well as the absence of an EP compatible with a heterozygous δβ-thalassaemia. Indeed, this disorder presents a higher degree of anisocytosis than BTT, and this type of thalassaemia is especially rare in Central Europe [22, 26] (see Tables 2 and 4).

However, screening for HTZ HGP using an algorithm based on the erythrocyte parameters MCHC, Micro% and RDW-SD remains relevant. In fact, it shows a PPV of 72.8 % for identifying a pathological EP and provides the possibility of making a definitive diagnosis of HTZ HGP in more than half of the initially suspected cases. Furthermore, it may provide a complementary tool for the traditionally HGP pre-screening using MCV and MCH, which may sometimes be considered too restrictive in today's multiethnic societies [27].

In the literature, certain RBC features are associated with HTZ HGP, such as poikilocytosis, hypochromia, microcytosis, target cells, basophilic stippling, schistocytes, dacrocytes, anisocytosis, polychromasia, irregularly contracted RBC and various specific inclusions depending on the underlying HGP [12, 28, 29]. Most of these microscopic abnormalities are found in patients with a pathological EP, although a notable prevalence of ovalocytes (11.1 % of pathological EP) and elliptocytes (4.2 % of pathological EP) is observed. The increased frequency of ovalocytes and elliptocytes, typically seen in cases of ID, is likely explained by the fact that 8.4 % of patients with a pathological EP have concomitant ID (see Tables 4 and 5).

The explanation for the higher prevalence of poikilocytosis and strongly positive RBCMA in patients with a pathological EP is probably due to the fact that patients with BTT typically exhibit more pronounced poikilocytosis than those with IDA [29]. Indeed, confirmed BTT accounts for more than two-thirds of pathological EPs in our cohort and, as previously mentioned, ID is more common among individuals with a normal EP.

Surprisingly, confirmed BTT show morphologically more hypochromia (9.3 % more; p=0.015) but fewer microcytes (6.7 % fewer; p=0.019) compared to heterozygous β-Hb variants, although they have quantitatively lower MCH and MCV values. This divergence may be due to two factors. On the one hand, patients with BTT have uniformly small erythrocytes, which explains why they typically have a low degree of anisocytosis and makes it difficult to detect microcytosis in the absence of normal-sized RBC for comparison [17, 26]. On the other hand, with regard to HbS traits which are the most common variant in our cohort, half are potentially associated with ID or minor α-thalassaemia according to their EP (see Tables 2, 4 and 5).

Other MA are rarely observed, with the exception of target cells (5.6 %) which, moreover, show a similar distribution across the different diagnostic categories. This is not surprising since this cellular atypia can be found in thalassaemias and IDA, both of which we have previously mentioned as being present among patients suspected of HTZ HGP, but also in HbC traits which account for 14.6 % of heterozygous β-Hb variants in our cohort [28, 29]. It is also worth noting that the presence of schistocytes ($p=0.025$) or dacrocytes ($p=0.042$) does not generally support an underlying normal EP (see Table 4).

Alongside these findings, it is important to emphasise that the various MA identified in this study are by no means specific to HTZ HGP and can therefore be observed in many other clinical situations. Consequently, morphology alone may raise suspicion of thalassaemia but is almost never diagnostic [29]. Furthermore, despite the standardisation of blood smear preparation thanks to modern haematology analysers, RBCMA remains time-consuming, subjective and requires thorough training although the CellaVision® RBC Advance software provides valuable assistance to technical staff. Finally, the cross-analysis of microscopic and electrophoretic results demonstrates that the number of MA detected by RBCMA presents, at best, moderate PPV but is neither sensitive nor correlated with the type of EP finally identified due to its high rate of false positive and false negative results. For these reasons, RBCMA should no longer be used to screen for HTZ HGP. Therefore, only Hb electrophoresis should be systematically performed as the first-line test, in accordance with the recommendations of Nivaggioni et al. [17].

The RBC tree algorithm was validated in adults aged 15 years or older, which explains why children have been excluded from the present study [17]. However, it is worth mentioning that, of the 596,000 blood samples analysed, 131 children aged between 2 and 15 years were also classified as suspect for HTZ HGP. The majority of these children presented a normal EP (67.2 % of children), which corresponds to a PPV of 32.8 % for identifying a pathological EP in this age group, and 77.9 % had a negative RCMA. These results show that the XN algorithm is of little added value for children and should be adapted with specific pediatric thresholds (Supplementary Table S2).

This study has several limitations. First, the categorisation of patients was based on erythrocyte parameters and electrophoretic data without further genetic confirmation. Nevertheless, our cohort is predominantly composed of patients with EP compatible with a confirmed BTT or carriers of a heterozygous β-Hb variant confirmed by mass spectrometry, neither of which requires secondary genetic confirmation [12, 30]. Second, samples that were not initially classified as suspect for HTZ HGP were not further evaluated. As a result, the diagnostic accuracy (i.e. sensibility, specificity) of HTZ HGP categorisation and its prevalence within the overall population could not be determined. Furthermore, since no comparison of diagnostic efficacy with the recommendations regarding the screening for HTZ HGP in low-prevalence areas has been conducted, the superiority of the algorithm tested in this study over these recommendations has not been evaluated. Therefore, it represents a complementary tool to the currently recommended screening which remains to identify women at risk based on erythrocyte parameters (i.e. MCV and MCH), ethnic origin and family history during the preconceptional or early prenatal period [12, 30]. Third, the values of $HbA_2$ and HbF used for the diagnostic categorisation may be subject to debate. In order to control this potential selection bias as much as possible, the recommendations based on the current literature, together with an explanation of the potential false positives and false negatives for each of these diagnostic categories, have been made available in Supplementary Table S1. In addition, the reference values used were adapted to those validated by our laboratory, as recommended [3, 12, 21]. Finally, we took into account the fact that the definition of a "borderline-raised" $HbA_2$ level, which suggest a possible BTT, varies widely in the literature ranging from 3.0 to 4.0 %. This is one of the reasons why samples with this $HbA_2$ level were classified as possible BTT [3, 31].

## Conclusions

An algorithm based on the erythrocyte parameters MCHC, Micro% and RDW-SD measured on Sysmex XN analysers represents a relevant tool with a PPV of 72.8 % for identifying a pathological EP in adults, thereby enabling an efficient screening of HTZ HGP. In contrast, microscopic RBCMA now appears to be of low added value and obsolete in this indication, given its poor sensitivity and the lack of reliable correlation between the number of MA detected and the type of EP finally identified. Consequently, only Hb electrophoresis should be systematically performed as the first-line test.

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