

## Discriminant value of %microcytic cells/%hypochromic cells ratio in the differential diagnosis of microcytic anemia

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### Abstract

The Mindray 6800 Plus analyzer reports red cells (RBC) extended parameters, which represent the subsets of erythrocytes. We aimed to evaluate the reliability of RBC extended parameters in the differential diagnosis of microcytic anemia. The learning set comprised samples from 250 patients with microcytic anemia mean cell volume <80 fL. MH ratio (%microcytic cells/%hypochromic cells) and other discriminant functions were calculated. Optimal cut offs were established using receiver operator curves. This value was used in the validation set of 135 patients 50 carriers and 85 with mild iron deficiency anemia (IDA). Area under the curve 0.945 (95% confidence interval 0.890 to 0.977), cut off >10 rendered the best Youden index (0.798), sensitivity 93.2%, specificity 86.2%. In the validation set using MH ratio >10, 45 in 50 patients were correctly classified as carriers. All of 40 beta carriers were correctly classified, while the 5 false negatives resulted to be alpha carriers. In the IDA group 5 patients had MH ratio >10 and thus considered carriers, but all of them had Hyper <3%. The combination of MH ratio >10 and %Hyper <3% correctly classified 100% of IDA patients. An algorithm derived from RBC extended parameters provided by the Mindray 6800 Plus analyzer could be a useful tool in the differential diagnosis of microcytic anemia.

### Introduction

The most commonly encountered disorders with mild microcytic anemia are iron deficiency anemia (IDA) and  $\beta$ -thalassemia trait (BTT).<sup>1</sup> The differentiation between IDA and microcytosis due to a genetic cause has important clinical implications, to improve the overall management of the disease, with an appropriate screening, detection of patients and counsel of couples at risk, and also to avoid unnecessary potentially harmful iron therapy in carriers.<sup>2</sup>

This differential diagnosis can be a diagnostic dilemma, initial evaluation

includes the complete blood count (CBC), since the advent of automation different discriminant formulas have been published derived from red cell parameters reported by counters.<sup>3-6</sup>

These indices, rapidly obtained and inexpensive, can be effective for use as a preliminary screening tool for selecting suspicious samples for further confirm the disease, using more expensive and sophisticated analysis. One of the simplest and yet most powerful discriminant functions is the ratio of microcytic to hypochromic red blood cells (RBC), the MH ratio.<sup>7</sup>

Measurement of microcytic and hypochromic RBC was first offered in Technicon hematology analyzers, now Advia (Siemens Medical).<sup>8</sup> The improvement of differentiation between BTT and IDA was soon recognized.<sup>9-11</sup>

A new software version was made available for the BC 6800 Plus analyzer (Mindray Diagnostics, Shenzhen, China) allowing the measurement of RBC subsets. Also a scatterplot (the so-called Mie map) is displayed, where each dot represents a single cell; in this graph cell volume values are plotted along the  $y$  axis and the cell Hb concentration along  $x$  axis.

Our aim was to prospectively evaluate the M/H ratio as measured on Mindray BC 6800 Plus as a discriminant index for the differential diagnosis of microcytic anemia. The MH ratio was compared with other formulas regarding their ability to screen those samples that would need additional tests to confirm the presumptive diagnosis of thalassemia.

### Patients and methods

#### Criteria for selecting the groups of patients

The criteria for inclusion was age older than 18 years, and no iron therapy nor transfusion in the previous month.

*Healthy subjects:* samples from apparently healthy subjects were obtained in the course of routine analysis. Blood cell counts and biochemical iron tests results were within the reference range.

*Subjects with mild IDA:* females Hb<120 g/L, males Hb<130 g/L, C reactive protein <5 mg/L, ferritin <15  $\mu$ g/L and/or transferrin saturation <20 %.

Patients with Hb<90 g/L were excluded, because these cases are not confused with BTT in our daily practice.

*$\beta$  thalassemia carriers:* samples from consecutive patients with a previous diagnosis of the disease.

After the requested tests had been completed, we used the residual samples for the

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Key words: Anemia; discriminant formulae; iron deficiency; thalassemia.

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present study. This practice is in accordance with the guidelines established by the Ethic Committee at Galdakao-Usansolo Hospital, so the study obtained the ethics approval from the Research Ethics Committee in Berruolde-Interior District IHO (Biscay, Spain).

### Laboratory methods

Venous blood samples were drawn into evacuated tubes containing K2-EDTA (Vacutainer™ Becton-Dickinson, Rutherford, NJ, USA), kept at ambient temperature and processed within 6 h from the time of blood collection. Extended RBC parameters were measured in the reticulocytes mode of a Mindray BC 6800 Plus. The analyzer was calibrated, controlled, and maintained according to the manufacturer's recommendations. Optical alignment was also performed and verified before the start of the study.

Biochemical tests of iron status (serum iron, transferrin, and ferritin) were measured using standard methods in a Cobas c711 analyzer (Roche Diagnostics, Mannheim, Germany). Hemoglobin-A2 was assayed using high pressure chromatography HPLC in an Arkray HA 8180 T analyzer (Menarini Diagnostics, Firenze, Italy).

### Statistical analysis

The Kolmogorov-Smirnov test was used for assessing normality of data distributions.

Differences among groups were examined using Kruskal-Wallis ANOVA; P values <0.05 were considered statistically significant. Bonferroni correction was applied for post-hoc comparisons of outcomes between each pair of groups.

Diagnostic performance of the indices in the learning set was determined using receiver operator characteristics (ROC) curves; ROC curves were compared using the DeLong method. In the validation set, we calculated the amount of correct classifications, using the results from the learning set and the final diagnosis.

All statistical procedures were performed using MedCalc Statistical Software, version 17.6 (MedCalc Software bvba, Ostend, Belgium).

## Results

During the learning phase of the study, 250 patients with microcytic anemia, 150 patients IDA 100 patients with BTT, and 100 healthy subjects were selected.

*Healthy subjects:* 58% female, 42% male; 22-62 years, mean 45 years.

*Subjects with mild IDA:* 28% male, 72% female; age 18-68 years, mean 38 years.

*β thalassemia carriers:* 45% male, 55% female; age 18-82 years, mean 53 years.

Differences of standard and extended RBC parameters among groups were found to be significant (P<0.0001).

Table 1 illustrates the Hematological and biochemical parameters in the study group. Table 2 presents the indices evaluated, including mathematical formulae and cut offs published by authors in their original papers. Table 3 summarizes the ROC curve analysis results, AUC comparison P<0.0001 (Figure 1).

MH ratio provided the best area under the ROC curve (AUC) of all indices 0.945 (95% confidence interval 0.890 to 0.977), cut off >10, sensitivity 93.2%, specificity 86.2% and the best Youden index (0.798) (Table 4). This cut off, MH ratio >10, was employed to classify the validation group, which included 135 patients with microcytic anemia, 50 carriers (40 β, 10 α) and 85 with IDA.

In the genetic anemia group, 45/50 patients were correctly classified as carriers; all of 40 β carriers were correctly classified, while 5 α carriers resulted false negative.

In the IDA group 5 patients had MH ratio >10 and thus considered carriers, but all of them had Hyper <3%. The combination of MH ratio >10 and %Hyper <3% correctly classified 100% of IDA patients.

**Table 1. Hematological and biochemical parameters in the study group, healthy, β thalassemia carriers and iron deficiency (IDA) patients.**

	Healthy		IDA		Thalassemia	
	Mean	Range	Mean	Range	Mean	Range
RBC (10 <sup>12</sup> /L)	4.9	4.3-5.7	4.67	3.5-5.7	5.8	5.0-6.9
Hb (g/L)	154	141-169	105	98-118	126	100-140
MCV (fL)	91.1	81.6-95.3	70	64.3-79.1	66.1	62.2-74.5
MCH (pg)	31.3	27.4-35.4	21.8	14.6-27.5	21.5	19.3-24.5
MCHC (g/L)	343	331-35.4	315	285-344	337	323-363
RDW (%)	12.4	11.3-13.5	17.5	15.2-20.0	16.2	14.1-18.0
Microcytic (%)	0.41	0.2-2.7	10.8	1.0-10.9	26.4	19.6-39.1
Hypochromic (%)	0.15	0-0.4	3.2	1.2-18.8	1.1	0.6-2.5
Hyperchromic (%)	0.5	0-0.9	1.5	0.4-2.8	5.0	2.0-7.8
MH ratio			4.6	0.1-27.8	15.1	4.3-54

RBC, red blood cells; Hb, hemoglobin; MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; RDW, red cell distribution width; MH ratio, % microcytic/% hypochromic.

**Table 2. Indices evaluated, England and Fraser (E&F), Green and King (G&K), Mentzer (M), Ricerca (R), Mathematical formulae and cut offs as defined in the original published reports.**

INDICES	IDA	Thalassemia
E&F = MCV – RBC – 5xHb – 3.4	>0	< 0
G&K = MCV <sup>2</sup> x RDW / 100 x Hb	>65	<65
M = MCV / RBC	>13	<13
R = RDW / RBC	>4.4	<4.4

RBC, red blood cells, Hb, hemoglobin; MCV, mean cell volume; MCH, mean cell hemoglobin; RDW, red cell distribution width.

**Table 3. Comparison of discriminant functions for identifying thalassemia carriers in a set of patients with microcytic anemia in the learning group: 150 patients with mild iron deficiency anemia and 100 β thalassemia carriers.**

INDEX	AUC	95 %CI	Cutoff	Sensitivity	Specificity
Englang Frazer	0.899	0.882-0.930	0	80.0	87.5
Green King	0.909	0.891-0.936	65	90.0	83.7
Mentzer	0.905	0.869-0.922	13	86.7	84.2
Ricerca	0.838	0.799-0.83	4.4	83.3	78.8
MH ratio	0.945	0.890-0.977	10	93.2	86.2

AUC, area under curve; CI, confidence interval.

**Table 4. Sensitivity and specificity MH ratio at various cut off values**

Cut off	Sensitivity %	95% CI	Specificity %	95% CI
>6.0	100.0	91.8 – 100.0	65.1	54.1 – 75.9
>6.5	95.3	84.2 – 99.4	78.8	66.4 – 88.0
>8.5	95.3	84.2 – 99.4	82.7	73.2 – 90.0
>9.0	93.0	80.9 - 98,5	82.7	73.2 – 90.0
>10	93.0	80.9 – 98.5	86.8	77.1 – 92.7
>10.5	90.7	77.9 – 97.4	86.8	77.1 – 92.7
>11,0	90.7	77.9 – 97.4	87.3	78.5 – 93.5
>11.5	83.7	69.3 – 93.2	87.3	78.5 – 93.5
>12.0	83.7	69.3 – 93.2	94.2	87.1 – 98.1

CI, confidence interval.

## Discussion and Conclusions

The advantages of technology in characterizing red cells allowed the introduction of additional parameters. The application of these parameters and their impact on diagnosis and patient management has been established in certain clinical conditions such as microcytic anemia.<sup>12</sup>

Flow cytometry provides information about individual cell characteristics, in addition to calculated average values of the total RBC: cell by cell individual optical signals are measured.<sup>13</sup> The forward-scattered (or low angle) light is directly proportional to the cell size. The side-scattered (or high angle) light indicates the internal cell structure and complexity of the cells.<sup>14</sup> Good separation of the cell subpopulations allows an accurate quantitation of RBC subsets leading to RBC extended parameters, along with standard CBC, based in impedance.

As previously exposed, the first hematology analyzer to introduce the optical measurements using multidimensional light scatter by sphered RBC, following Mie theory principles, was the Technicon H\*1, nowadays the Advia series (Siemens Healthcare GmbH, Erlangen, Germany) (Siemens).<sup>8</sup>

Later, other manufacturers started to

offer similar parameters in their analyzers. One of these is the CELL-DYN Sapphire analyzer (Abbott Diagnostics, Santa Clara, CA, USA), which reports RBC and reticulocyte parameters that show a high degree of correlation with those of the Advia, although the absolute values differ because of differences in technology,<sup>15</sup> which include the laser wavelength, the number and degrees of the angles of diffracted light, the algorithms applied to translate the optical signals.

Due to the mentioned differences and the lack of international standardization, instrument-specific reference ranges and clinical decision values for each analyzer brands are mandatory; this implies that laboratories using other equipment than Advia need to establish the performance of their own analyzer's extended parameters.<sup>15</sup>

This need is evident when analyzing the values of MH ratio published as the best cut offs over decades and employing different instruments.

The first attempt to use the RBC extended parameters rendered a cut off  $>1$ , which means a higher amount of microcytes than hypochromic cells.<sup>10</sup> The best cut off for Advia counter was found to be MH ratio  $>3.7$ , while for CELL DYN Sapphire MH ratio  $>6.4$  performed best.<sup>16-18</sup> The results in the present study show better correlation

with the MH ratio values reported in the latter, and the percentages of microcytic and hypochromic cells in both groups resulted to be similar.

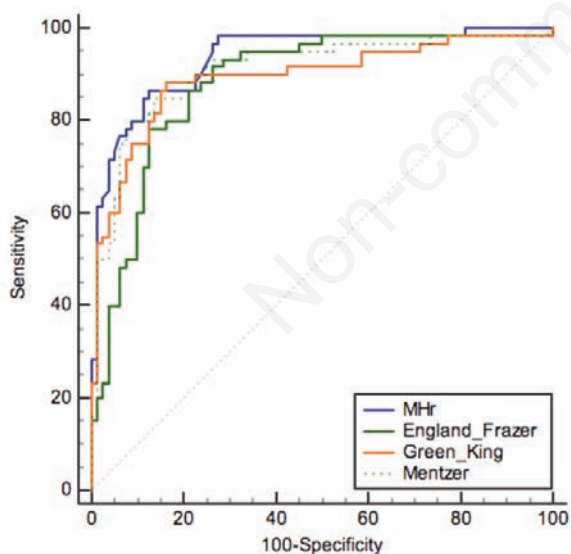
Cut offs by Technicon and Siemens analyzers are rather different to the value found in the present study: MH ratio  $>10$ , ten folds the first report, the main discrepancy is the hypochromic cells count.

The fact that the previous reports included patients with severe and mild anemia can explain the different hypochromic cells count and thus MH ratio.<sup>10</sup>

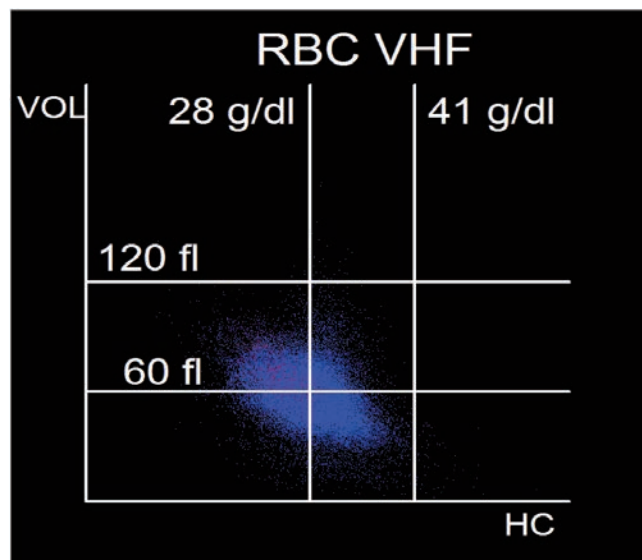
Also the technical differences and calculation algorithms used in the diverse counters can play a part in these different cut off values reported.

Analytical interferences can result in a falsely high count of hypochromic cells; the higher count in the Technicon analyzer could tribute to the measurement of cells or particles along with hypochromic cells: RBC fragments, cell debris, platelets, ghost cells.

The superiority of the MH ratio over other discriminant indices has been objectively proven.<sup>7</sup> This discriminant power can be explained because percentages of hypochromic erythrocytes and microcytes expand information at a cellular level: RBC subsets give insights of physiology underlying microcytic anemia, and a significant



**Figure 1.** Comparison of discriminant functions for identifying thalassemia carriers in a set of patients with microcytic anemia: 150 patients with mild iron deficiency anemia and 100  $\beta$ -thalassemia carriers.



**Figure 2.** On the V/HC cytogram, Mie map, Hb concentration is plotted along the x axis and cell volume is plotted along the y axis. Only red blood cells appear on this cytogram. Markers organize the cytogram into 9 distinct areas of red blood cell morphology. On the x axis, Hb concentration markers are set at 28.0 g/dL and 41.0 g/dL. RBC with a Hb concentration  $< 28.0$  g/dL are hypochromic, while cells with a Hb concentration  $> 41.0$  g/dL are hyperchromic. On the y axis, RBC volume markers are set at 60 fL and 120 fL. RBC with a volume  $< 60.0$  fL are microcytic, while cells with a volume  $> 120.0$  fL are macrocytic.



alteration in these parameters reflects erythropoiesis status.

Although IDA and thalassemia are microcytic anemias, hypochromia and microcytosis exhibit opposite trends in both diseases, then the ratio enhances the difference improving the performance in differential diagnosis.

Thalassemia is characterized by erythrocytosis, as a result of the chronic increase in erythropoiesis. The underlying pathology in thalassemia has no fluctuations and the bone marrow produces a constantly uniform microcytic erythrocytes.<sup>19</sup>

In contrast, Iron-deficient erythropoiesis is characterized by the production of RBC with a decrease in Hb content, so a high percentage of hypochromic cells is present, and they tend to be more microcytic along the process of depletion. Because of their long life span, several cohorts of normocytic and microcytic RBCs coexist in the peripheral blood leading to anisocytosis.<sup>20</sup>

Microcytes in case of  $\beta$  thalassemia are generally smaller, with more preserved Hb concentration, and a percentage of apparent hyperchromia can be detected: due to the disbalance in Hb content and very low cell volume a percentage of hyperchromic red cells can be measured in thalassemia carriers, in contrast to patients suffering microcytic hypochromic anemia due to lack of iron.<sup>21</sup>

Despite the different optimal cut offs reported, the differential characteristics of erythropoiesis are highlighted in the same way by all the counters evaluated over the decades. Also the characteristic pattern typical of carriers on Mie map cytogram remains constant: cells clustering in the lower microcytic area and slight shift towards the hyperchromic area; visual inspection can help in the recognition of carriers, as previously highlighted using different brand analyzers<sup>22,23</sup> (Figure 2).

Mie map and MH ratio can be effective for use as a preliminary screening tool in the investigation of microcytic anemias, rendering typical patterns, so can be used as laboratory based criteria for the selection of samples for accurate diagnosis of IDA and BBT.

Findings from further studies should confirm our results on prospectively collected population of patients with microcytic anemia and assess the pattern of micro-

cytic and hypochromic red cells in to other types of anemia.

## References

- Hallberg L. Iron requirements comments on methods and some crucial concepts in iron nutrition. *Bio Trace Elements Res* 1992;35:25-45.
- Weatherall DJ, Clegg JB. Inherited haemoglobin disorders: an increasing global health problem. *Bull World Health Organ* 2001;79:704-12.
- England JM, Bain BJ, Fraser PM. Differentiation of iron deficiency from thalassaemia trait. *Lancet* 1973;1:1514.
- Mentzer WC Jr. Differentiation of iron deficiency from thalassaemia trait. *Lancet* 1973;1:882.
- Ricerca BM, Storti S, d'Onofrio G, et al. Differentiation of iron deficiency from thalassaemia trait: a new approach. *Haematologica* 1987;72:409-13.
- Green R, King R. A new red cell discriminant incorporating volume dispersion for differentiating iron deficiency anemia from thalassemia minor. *Blood Cells* 1989;5:481-95.
- Hoffmann JJML, Urrechaga E, Aguirre U. Discriminant indices for distinguishing thalassemia and iron deficiency in patients with microcytic anemia: a meta-analysis. *Clin Chem Lab Med* 2015;53:1883-94.
- Mohandas N, Kim YR, Tycko DH, et al. Accurate and independent measurement of volume and hemoglobin concentration of individual red cells by laser light scattering. *Blood* 1986;68:506-13.
- Robertson EP, Pollock A, Yau KS, et al. Use of Technicon H\*1 technology in routine thalassaemia screening. *Med Lab Sci* 1992;49:259-64.
- d'Onofrio G, Zini G, Ricerca BM, et al. Automated measurement of red blood cell microcytosis and hypochromia in iron deficiency and beta-thalassemia trait. *Arch Pathol Lab Med* 1992; 116:84-9.
- Jimenez CV, Minchinela J, Ros J. New indices from the H\*2 analyser improve differentiation between heterozygous beta or delta beta thalassaemia and iron-deficiency anaemia. *Clin Lab Haematol* 1995;17:151-5.
- Archer NM, Brugnara C. Diagnosis of iron-deficient states. *Crit Rev Clin Lab Sci* 2015;52:256-72.
- Urrechaga E, Borque L, Escanero JF. Biomarkers of hypochromia: the contemporary assessment of iron status and erythropoiesis. *Biomed Res Int* 2013: 603786.
- Kim YR, Ornstein L. Isovolumetric spherizing of erythrocytes for more accurate and precise cell volume measurement by flow cytometry. *Cytometry* 1983;3:419-27.
- Hoffmann J, van den Broek N, Curvers J. Reference values of CELL-DYN Sapphire extended red blood cell parameters. *Clin Chem Lab Med* 2012; 50:941-8.
- Urrechaga E. Discriminant value of % Microcytic / % Hypochromic ratio in the differential diagnosis of microcytic anemia. *Clin Chem Lab Med* 2008;46: 1752-8.
- Urrechaga E. Red blood cell microcytosis and hypochromia in the differential diagnosis of iron deficiency and  $\beta$ -thalassaemia trait. *Int Jnl Lab Hematol* 2009;31:528-34.
- Urrechaga E, Hoffmann JJML, Izquierdo S, et al. Differential Diagnosis of Microcytic Anemia: the Role of Microcytic and Hypochromic Erythrocytes. *Int Jnl Lab Hematol* 2015;37:334-40.
- Rund D, Rachmilewitz E. Beta-thalassemia. *N Eng J Med* 2005;353:1135-46.
- Juncà J, Flores A, Roy C, et al. Red cell distribution width, free erythrocyte protoporphyrin, and England-Fraser index in the differential diagnosis of microcytosis due to iron deficiency or beta-thalassemia trait. A study of 200 cases of microcytic anemia. *Hematol Pathol* 1991;5:33-6.
- Urrechaga E, Borque L, Escanero JF. Erythrocyte and reticulocyte parameters in iron deficiency and thalassemia. *J Clin Lab Analysis* 2011;25:223-8.
- Gibbs G. Separating thalassemia trait and iron deficiency by even simpler inspection. *Am J Clin Pathol* 2009;132: 643-4.
- Urrechaga E, Boveda O, Hoffmann JJML. Red cell cytogram in CELL-DYN® Sapphire: a ready-to-use function for recognizing thalassemia trait. *Thal Rep* 2016;6:5260.