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# Application of innovative hemocytometric parameters and algorithms for improvement of microcytic anemia discrimination

Margreet Schoorl, Marianne Schoorl, Johannes van Pelt, Piet C.M. Bartels

**Department of Clinical Chemistry, Hematology and Immunology, Medical Center Alkmaar, The Netherlands**

## Abstract

Hemocytometric parameters like red blood cell (RBC) count, mean red blood cell volume (MCV), reticulocyte count, red blood cell distribution width (RDW-SD) and zinc protoporphyrin (ZPP) are frequently established for discrimination between iron-deficiency anemia and thalassemia in subjects with microcytic erythropoiesis. However, no single marker or combination of tests is optimal for discrimination between iron-deficiency anemia and thalassemia. This is the reason why many algorithms have been introduced. However, application of conventional algorithms, only resulted in appropriate classification of 30-40% of subjects. In this mini-review the efficacy of innovative hematological parameters for detection of alterations in RBCs has been considered. It refers to parameters concerning hemoglobinization of RBCs and reticulocytes and the percentages microcytic and hypochromic RBCs, for discrimination between subjects with iron-deficiency anemia (IDA) or thalassemia as well as a combination of both. A new discriminating tool including the above mentioned parameters was developed, based on two precondition steps and discriminating algorithms. The percentage microcytic RBCs is considered in the first precondition step. MCV, RDW-SD and RBC count are applied in the second precondition step. Subsequently, new algorithms, including conventional as well as innovative hematological parameters, were assessed for subgroups with microcytic erythropoiesis. The new algorithms for IDA discrimination yielded results for sensitivity of 79%, specificity of 97%, positive and negative predictive values of 74% and 98% respectively. The algorithms for β-thalassemia discrimination revealed similar results (74%, 98%, 75% and 99% respectively). We advocate that innovative algorithms, including parameters reflecting hemoglobinization of RBCs and reticulocytes, are integrated in an easily accessible software program linked to the hematology equipment to improve the discrimination between IDA and thalassemia.

## Introduction

Anemia is a global public health problem affecting populations in both developing and developed countries. According to the World Health Organisation (WHO) anemia affects 1.62 billion people, which corresponds to approximately 25% of the world population. It is assumed that 50% of the cases of anemia are due to insufficient iron content in the diet, particularly in women in child-bearing age with increased menstrual blood loss or during pregnancy, young children and vegetarians.[1]

Iron-deficient erythropoiesis and thalassemia are both associated with mild to moderate microcytic anemia, which frequently results in an incorrect diagnosis.

It is important to discriminate between iron-deficiency anemia (IDA) and thalassemia, in order to avoid unnecessary iron therapy and to prevent the development of hemosiderosis, which may result in serious complications like cardiomyopathy, liver fibrosis or endocrine dysfunctions.[2,3]

Regarding discrimination between iron deficiency and thalassemia, this mini-review focuses on the expedience of innovative hematological parameters concerning red blood cell (RBC) hemoglobinization as well as RBC production.

## Iron deficiency

Nutritional deficiency as a result of inadequate iron, folic acid and vitamin B12 intake, will contribute to the development of anemia. Iron deficiency is considered to be the main cause for anemia: it is responsible for more than 50% of all cases. Iron deficiency results, amongst others, in impaired activity of several enzymes. As a consequence, iron depletion may result in serious health problems such as retarded growth, mental irritability, reduced resistance to infection and impaired intellectual development, particularly in case of infants in the growth phase. The causes for iron deficiency include decreased resorption of heme iron, increased consumption of phytate and phenolic compounds which inhibit iron absorption, increased requirement, and blood loss.[4-6]

According to WHO publications, the highest number of pre-school children, pregnant women and non-pregnant women suffering from IDA live in the Eastern Mediterranean countries, North-Africa and the Middle East.[7] Despite the start of iron fortification, the prevalence of iron deficiency is still rather high in the Middle East countries. The prevalence of IDA in the Middle Eastern countries is equal to the prevalence in developing countries (25-35%), which is much higher than in industrial countries (5-8%).[8,9]

## Disorders of hemoglobin synthesis

Hemoglobinopathy encompasses a group of genetic disorders which involve an abnormal structure of one of the globin chains of the hemoglobin molecule. In contrast, thalassemias are disorders of hemoglobin synthesis which usually results in decreased production of normal globin proteins, often due to mutations in regulatory genes.[2,3]

It is estimated that approximately 7% of the world's population carries mutations of globin genes. In several regions, the prevalence of thalassemia is even higher. With increasing migration of people, a genetic disease which was initially rare in northern Europe, Australia and North America is more common at the present time in these regions. The actual number of subjects with hemoglobinopathy and thalassemia worldwide is unknown and is probably underestimated.[3,7,10]

### Alpha-thalassemia

Alpha-thalassemias are the most prevalent disorders of hemoglobin synthesis.[3]

Subjects with α-thalassemia 2 heterozygos-

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ity (with three functional α-globin genes: αα/-α) are characterized by normal to moderately decreased mean cell volume (MCV) values with normal or slightly decreased hemoglobin concentrations.[11,12] Subjects with homozygous α-thalassemia 2 (-α/-α) and heterozygous α-thalassemia 1 (αα/--) are phenotypically very similar and cannot be distinguished by only blood count markers. Erythropoiesis with decreased MCV, frequently associated with mild anemia, is frequently observed.

### Beta-thalassemia

Mutations causing β-thalassemia result in a lack of β-globin production, which ranges from minimal (mild β+-thalassemia alleles) to a complete absence (β0-alleles).

Almost all heterozygous conditions (except the so-called normal HbA2-thalassaemias, *e.g.* δβ-thalassemia), are characterized by an increased HbA2 content (≥3.2%) and a marked degree of microcytosis, which is frequently associated with mild anemia. Subjects with homozygous β-thalassemia suffer from severe microcytic anemia.

### Hemoglobinopathies

Hemoglobinopathies encompass a heterogeneous group of inherited disorders which affect the structure of the hemoglobin molecule, resulting in microcytic erythropoiesis. Four abnormal hemoglobins (Hb), in particular HbS, HbC, HbE and HbD are rather common in various parts of the world, *e.g.* Africa, the Mediterranean area and Southeast Asia. Carriers of hemoglobinopathies, if not associated with α-thalassemia, usually do not suffer from anemia while results of MCV and mean red blood cell hemoglobin (MCH) are situated in the lower part of the reference range.

## Discrimination between iron deficiency and thalassemia syndromes

The discrimination between IDA and thalassemia has important clinical implications. A reliable diagnosis is important in order to reduce unnecessary laboratory testing and to avoid inappropriate treatment. A wide range of laboratory parameters is available to facilitate the discrimination between IDA and thalassemia. However, no single marker or combination of tests is optimal for discrimination between iron deficiency and thalassemia. In addition, iron deficiency often occurs in combination with other diseases which complicates the differential diagnosis. Diagnosing subjects with combined thalassemia minor and iron deficiency is even more challenging.

In order to facilitate the discrimination pro-

cedure a number of algorithms have been described, which combine routine RBC parameters and indices obtained from routine complete blood count. At least 40 of these algorithms are published. Well known conventional algorithms are those of Mentzer, England&Fraser, Green&King, Shine&Lal, Ricerca and Shrivastava (Table 1).[13-18] However, none of these algorithms is appropriate for a correct classification. For example, the algorithms of Mentzer, England&Fraser and Green&King only resulted in appropriate classification of 30-40% of subjects with β-thalassemia.

In the late 80s, the determination of ZPP in RBC was recommended to appropriately discriminate subjects with iron deficiency from subjects with thalassemia. ZPP is a biomarker for the detection of *long term* iron-deficient erythropoiesis.[19] ZPP results are obviously increased in patients with iron deficiency and, to a lesser degree, in subjects with α- or β-thalassemia trait.[20-22] In addition, introduction of discriminating algorithms including MCV, MCH, RBC and red blood cell distribution width (RDW) has advanced the differential diagnosis of iron deficiency and α- or β-thalassemia, but in several cases these algorithms still resulted in an inappropriate diagnosis.[23]

Over the past decade, discriminating procedures and algorithms have been improved *step-by-step*.

In 2003 the efficacy of a discriminating algorithm including the hematological parameters RBC, RDW-SD, ZPP and reticulocyte count has been established.[24] MCV is recommended as an initial test. Subsequently, appropriate classification was achieved in 90% of the subjects with iron deficiency, α-thalassemia or β-thalassemia by application of the algorithm 2×RDW (fL) - 5×RBC(×10$^{12}$/L) - 250×reticulocytes (×10$^{12}$/L) + 30×ZPP (μmol/mol Hb).[24]

The introduction of new generations of hematology equipment enabled the use of innovative hemocytometric parameters for hemoglobinization in reticulocytes and RBCs. The reticulocyte hemoglobin content (CHr) was initially determined on the Bayer H*3 hematology analyzer.[25] The CHr is determined

from measurements of light scatter at two different angles after isovolumetric sphering of oxazine-750 stained reticulocytes.[25,26] A few years later Sysmex Corporation (Tokyo, Japan) introduced parameters indicating the hemoglobin content of reticulocytes and RBCs, RET-He and RBC-He respectively. The hemoglobin content of RBCs and reticulocytes is established by means of the flow cytometric methodology. The mean forward light scattered intensity in the reticulocyte channel is measured as a parameter for estimation of the cell volume and to establish simultaneously hemoglobin content of RBCs and reticulocytes. Ret-He has demonstrated to be comparable with CHr.[27]

Hemoglobin content in reticulocytes reflects a *short term* indication of the functional availability of iron for erythropoiesis and the response to iron supplementation. Reticulocyte maturation occurs with progressive decrease in RBC volume and hemoglobin content. If compared with interpretation of Hb content of mature RBCs, interpretation of Hb content of reticulocytes yields additional information concerning decreased functional availability of iron for hemoglobinization of erythroid precursors.[28,29]

Thomas *et al*. introduced a diagnostic model including the hemoglobin content of reticulocytes in combination with the soluble transferrin receptor/log ferritin ratio (sTfR-F index) for the purpose of monitoring the progression of iron deficiency, regardless of acute phase response.[28]

In 2006 the additional value of the innovative hematological parameters in case of subjects with IDA and thalassemia has been explored. Ret-He and RBC-He results were decreased in the subgroups with IDA and α- or β-thalassemia, if compared with the group of apparently healthy subjects. For the Ret-He/RBC-He ratio obviously decreased results were demonstrated in subjects with IDA (1.02±0.08, mean±SD) and to a lesser extent in subjects with β-thalassemia (1.06±0.04) if compared with the group of healthy reference subjects (1.11±0.02) and subjects with α-thalassemia (1.11±0.07). A combination of results

**Table 1. Overview of discrimination formulas including cut-off levels for iron deficiency anemia and for β-thalassemia.**

| Authors | Formulas | Iron deficiency anemia | Thalassemia |
|---|---|---|---|
| England & Fraser[14] | MCV - RBC - 5×Hb - 3.4) | >0 | <0 |
| Green & King[15] | (MCV$^2$×RDW-CV)/(Hb×100) | >65 | <65 |
| Mentzer[13] | MCV/RBC | >13 | <13 |
| Shine & Lal[16] | MCV$^2$×MCH×0.01 | >1530 | <1530 |
| Ricerca[17] | RDW/RBC | >4.4 | <4.4 |
| Shrivastava[18] | MCH/RBC | >3.8 | <3.8 |

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

for Ret-He, Ret-He/RBC-He ratio in relation to MCV results yields an appropriate method for obtaining insight into the degree of hemoglobinization and RBCs characteristics for proper clinical interpretation in the case of subjects with IDA or thalassemia. Cut-off levels for Ret-He of <29.5 pg and for Ret-He/RBC-He ratio of <1.02 are advocated as indicators of iron-deficient erythropoesis.[30]

More recently, innovative methods for establishment of microcytic and hypochromic RBCs have been introduced. Measurement of microcytic and hypochromic RBCs was first available on Technicon hematology analyzers (currently Advia®, Siemens Medical Solutions, Malvern, USA), applicating multidimensional light scatter by sphered RBCs, following the Mie theory principles.[31-33] Already in 1992 d'Onofrio et al. reported on the ratio between the percentage of microcytes and the percentage of hypochromic cells, the so-called M/H-ratio.[34] Iron-deficient erythropoiesis is characterized by the production of RBCs with a severely decreased Hb concentration, while microcytic RBCs in subjects with β-thalassemia are generally smaller, with a more preserved Hb concentration. In subjects with β-thalassemia, the percentage of microcytic RBCs is higher than the percentage of hypochromic RBCs. On the contrary, in subjects with IDA, the percentage of hypochromic RBCs is higher than the percentage of microcytic RBCs.[34] Measurement of the percentage hypochromic and microcytic RBCs, has demonstrated to be useful for detection of rather small changes in the amount of RBCs with inadequate haemoglobinisation.[28,35,36]

In 2008 Urrechaga et al. reported on improved discriminating formulas including the percentage hypochromic and microcytic RBCs (indicated as %Hypo-He and %MicroR respectively) combined with RDW-CV.[37]

The parameter %HypoHe reflects the percentage of hypochromic RBCs with a Hb content of less than 17 pg. %MicroR indicates the percentage of microcytic RBCs with a volume of less than 60 fL.[37,38]

In cases of β-thalassemia, obviously increased results for %MicroR are observed (mean±SD, 37.8±11.4) if compared with the groups of healthy subjects (1.1±0.44) and subjects with IDA (19.1±9.7). In cases of IDA, results of %Hypo-He are increased (mean±SD, 15.7±11.5) compared with the groups of healthy subjects (0.3±0.16) and subjects with β-thalassemia (11.9±7.2). In the same study, the efficacy of application of algorithms is explored such as %MicroR minus %Hypo-He (M-H). The cut off value which provides the best results for application of Youden index for M-H was 11.5 (β-thalassemia >11.5, IDA<11.5).[37,38]

An European multicenter study established the additional value of new discriminating algorithms which include the parameters for hemoglobinization of reticulocytes (Ret-He), Delta-He (RET-He minus RBC-He) and the percentages of microcytic RBCs (%MicroR) and hypochromic RBCs (%HypoHe) for discrimination between IDA and β-thalassemia.[39] The study established the sensitivity and specificity of the novel discriminating algorithms between these groups and a control group of healthy subjects and compared the algorithms with currently available algorithms for discrimination. To illustrate the decision making process for discriminating between IDA and β-thalassemia, pre-selection criteria and algorithms are depicted in Table 2. Application of the new algorithms for discriminating IDA yielded in an area under the curve 0.88, a sensitivity of 79%, a specificity of 97% and positive and negative predictive values of 74% and 98%, respectively. For the detection of β-thalassemia similar results were achieved (0.86, 74%, 98%, 75%, and 99%, respectively).[39] Evaluation of the diagnostic efficacy of the algorithms demonstrated excellent performance, if compared with conventional discriminating algorithms described by England&Fraser, Green&King and Mentzer.[13-15] Nevertheless, none of the algorithms provided 100% sensitivity and 100% specificity in discrimination between subjects with iron deficiency and subjects with β-thalassemia. Therefore, after screening with the six algorithms, confirmatory testing should be performed for proper diagnosis.[39]

## Anemia discrimination in subjects with combinations of iron-deficiency anemia and thalassemia

When facing the challenge of a discriminating algorithm for subjects with combinations of β-thalassemia and IDA, application of Ret-He and Ret-He/RBC-He ratio as additional parameters is recommended. In this particular group of subjects, Ret-He and Ret-He/RBC-He ratio are obviously decreased (respectively 29.5 pg and <1.02).[30]

In subjects with combinations of β-thalassemia and IDA, also a combination of %Hypo-He and M-H index seems to be promising.[40] In this group of subjects markedly increased %Hypo-He (>20) occur in combination with a decreased M-H index (<11.5) (personal communication). Further investigation is required.

## Critical remark

Several manufacturers of automated hematology equipment facilitate establishment of innovative parameters like hemoglobin content of RBCs and reticulocytes. However, the comparability of these parameters is a matter of serious concern. Particularly in multicenter-settings, calibration and adjustment of the measuring channels is an important issue. In case of multicenter evaluation studies involving hematology equipment from different suppliers, validation and mutual alignment is required.

## Conclusions

It is strongly advised to use discriminating algorithms as a tool for anemia discrimination in order to reduce diagnostic testing for confir-

Table 2. Preconditions and algorithms for discrimination microcytic anemia on iron deficiency anemia (IDA) and beta-thalassemia (THAL).

| Precondition 1 | Precondition 2 | Algorithm | |
|---|---|---|---|
| %MicroR≥3 | MCV<85 and 275 AND %MicroR≥5 | %MicroR / %HypoHe <4 AND [ (MCV$^2$ × RDW-CV) / (Hb*100) ] ≥75 AND Reti#<0.08 | IDA |
| %MicroR≥3 | MCV<75 and ≥65 | %MicroR / %HypoHe <3.4 AND [ (MCV$^2$ × RDW-CV) / (Hb*100) ] ≥77 AND Reti#<0.08 | IDA |
| %MicroR≥3 | MCV<65 | %MicroR − %HypoHe − RDW-CV < -5.2 | IDA |
| %MicroR≥3 | MCV<85 and ≥75 AND RDW-SD<44.7 AND RBC≥3.50 | [ (MCV$^2$ × RDW-CV) / (Hb*100) ] <75 AND [ (MCV-RBC -3.4 - (5*Hb) ] <4 AND Reti#<0.08 AND Delta-He≥0 | THAL |
| %MicroR≥3 | MCV<75 and≥65 AND RBC≥3.50 | [ (MCV$^2$ × RDW-CV) / (Hb*100) ] <77 AND %MicroR / %HypoHe ≥2.0 | THAL |
| %MicroR≥3 | MCV<65 | (%MicroR - %HypoHe − RDW-CV) ≥ -5.2 | THAL |

%MicroR, percentage microcytic red blood cells; MCV, mean red blood cell volume; %HypoHe, percentage hypochromic red blood cells; RDW, red blood cell distribution width. Parameters used in the algorithms are expressed in the following units: red blod cells (10$^{12}$/L), hemoglobin (g/dL), MCV (fL), RDW-SD (fL), RDW-CV (%), Reti# (10$^{12}$/L).

mation and to properly diagnose the underlying cause(s) in the patients.

It is advocated that innovative algorithms, including parameters reflecting hemoglobinization of RBCs and reticulocytes, are integrated in an easily accessible software program linked to the hematology equipment, preferably provided by the manufacturer of the equipment to facilitate this interaction, to improve the discrimination between IDA and thalassemia.

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