# Application of Haemocytometry in Combination with Reticulocyte Count and Red Cell Zinc Protoporphyrin for Appropriate Classification of Microcytic Anaemia

Piet.C.M. BARTELS, Marianne SCHOORL, and Margreet SCHOORL

*Department for Clinical Chemistry, Haematology and Immunology, Medical Center Alkmaar, Alkmaar, The Netherlands*

*Haemocytometric parameters such as MCV, erythrocyte and reticulocyte counts, RDW-SD, and ZPP/Hb ratio are considered to be valuable for discriminating between iron deficiency anaemia and thalassaemia syndromes in subjects with microcytosis. ZPP/Hb ratio values are obviously increased in patients with iron deficiency and, to a lesser degree, in some subjects with α - or ß-thalassaemia trait. With regard to screening for detection of thalassaemia syndromes or iron deficiency MCV is recommended as an initial test. Subsequent application of the discriminant formula 2 × RDW (fL) - 5 × RBC(×10¹²/L) - 250 × reticulocytes (×10¹²/L) + 30 × ZPP/Hb ratio (µmol/mol Hb) yields appropriate classification in 90% of subjects with iron deficiency, α-thalassaemia or ß-thalassaemia.*

(Sysmex J Int 13 : 78-82, 2003)

**Key Words** Automted Hematology Analyzer, XE-2100, Iron Deficiency, Thalassemia, Erythrocytes, MCV, Reticulocytes, Red Cell Distribution Width (RDW), Zinc Protopohyrin/Hemoglobin (ZPP/Hb) Ratio

Received 1 September, 2003; Accepted 8 September, 2003

## INTRODUCTION

Particularly in children, young women and elderly people iron deficiency is a frequent cause of anaemia. Iron deficiency in children is mostly due to an unbalanced diet with respect to nutrients which contain insufficient amounts of iron. In adults, chronic loss of blood may be a causal factor for iron deficiency. Iron supplementation and insufficient availability of iron for heme synthesis in a hospital patients population may be complicated by pathophysiological abnormalities which are likely to be present simultaneously[1-4]. Evaluation of serum iron concentration in combination with transferrin concentration is indicated for clinical evaluation of the iron state. Clinical interpretation is rather complicated in case of anaemia of chronic disease or anaemia due to acute blood loss.

During erythropoiesis zinc protoporphyrin (ZPP) may accumulate as a result of reduced availability of iron for heme synthesis. A result of ZPP/Hb ratio within healthy reference limits is indicative of sufficient availability of iron in bone marrow for heme synthesis[5,6]. Haemocytometric parameters like erythrocyte count, haemoglobin concentration, MCV and reticulocyte count contribute to the differential diagnosis of microcytic anaemia.

In the case of microcytic anaemia additional evaluation of the red cell distribution width based on the standard deviation (RDW-SD), the reticulocyte count and the ZPP/Hb ratio is performed. Increased ZPP/Hb levels are indicative of iron deficiency but slightly elevated results are also demonstrated in some subjects with ß-thalassaemia trait and α-thalassaemia trait.

This study demonstrates the efficacy of haemocytometric parameters and the ZPP/Hb ratio to discriminate between patients with α-thalassaemia trait, ß-thalassaemia trait, iron deficiency and apparently healthy subjects.

## PATIENTS AND METHODS

Blood specimens were drawn into Vacutainer tubes, with $K_3$EDTA as anticoagulant (Becton Dickinson, Franklin Lakes, NJ, USA).

Haemocytometric investigations, including RDW-SD and reticulocyte counts, were determined by the Sysmex XE-2100 automated haematology analyzer (Sysmex Corporation, Kobe, Japan).

Measurement of ZPP/Hb ratio was performed on a dedicated haematofluorometer (AVIV Biomedical Inc., Lakewood NJ 08107, USA) by front surface illumination fluorometry[7]. The result of the ZPP/Hb ratio is expressed as µmol ZPP per mol Hb.

Haemoglobin electrophoresis was performed in order to diagnose subjects with ß-thalassaemia trait (Sanquin, Amsterdam, The Netherlands). Increased $HbA_2$ content (> 3.0 %) was considered to confirm ß-thalassaemia trait. If an abnormal haemoglobin variant was observed in the pattern of haemoglobin fractions and in case of an increased foetal haemoglobin fraction (HbF) in the electropherogram (> 1.5 %), the sample was excluded from the study.

Detection of α-thalassaemia trait was performed by application of DNA technology (CLB, Amsterdam, The Netherlands). Samples lacking the α-thalassaemia gene

at one or more α-chains of the haemoglobin molecule were classified as α-thalassaemia trait. Samples with the α,α/α,α-gene type were considered to be normal.
Determination of the serum iron concentration was performed by the Vitros 950 IRC Analyzer (Ortho-Clinical Diagnostics Raritan NJ, USA). Serum transferrin concentrations were measured with the BN-II nephelometer (Dade Behring, Dearfield, IL, USA).
The study design comprises 4 groups:

1. Apparently healthy subjects (Ref)
   The reference subjects group consists of 70 caucasian male and female blood donors.
2. Iron deficiency anaemia subjects (IDA)
   The iron deficient group consisted of 47 male and female patients, with MCV values = 80fL, iron concentrations < 8µmol/L combined with transferrin concentrations > 4mg/L.
3. Subjects with α-thalassaemia trait (α)
   The α-thalassaemia trait group consisted of 54 male and female patients. Abnormal DNA-analyses at the α-chain of haemoglobin were considered to be confirmatory for α-thalassaemia trait.
4. Subjects with ß-thalassaemia trait (ß)
   The ß-thalassaemia trait group consisted of 78 male and female patients. An increased $HbA_2$ content was considered to be confirmatory for ß-thalassaemia trait.

## Statistical analysis and algorithm for discrimination

For statistical evaluation the software package SPSS/PC, version 10.0 for Windows, was used.
In order to discriminate efficiently between subjects with microcytic erythrocytes, an algorithm is established from parameters which classify activity of erythropoiesis. An appropriate weight factor for each parameter is added by application of discriminant analysis.

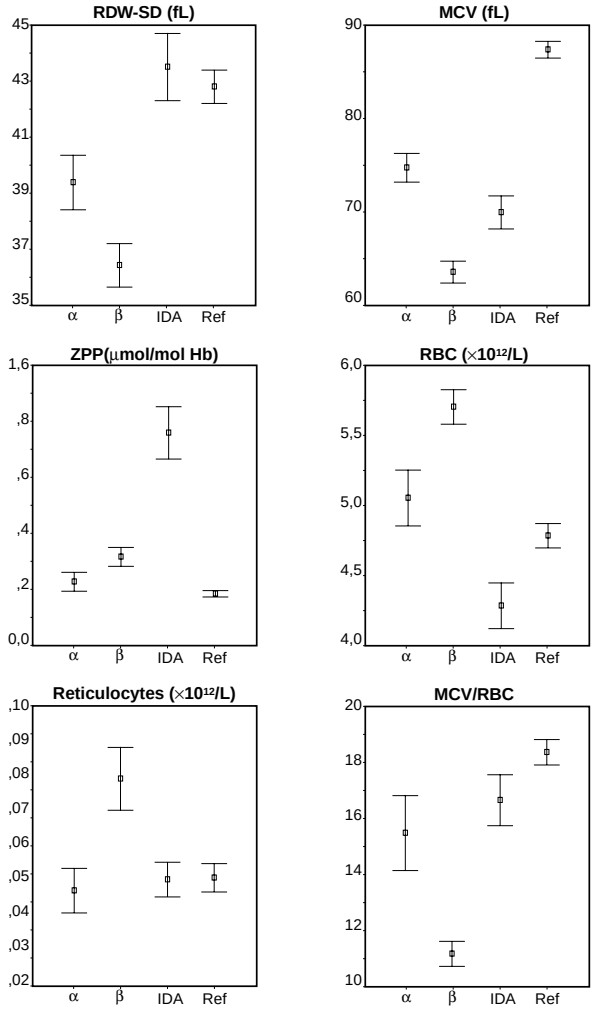


***Fig. 1*** *Error bar plot of results of single haemocytometric parameters MCV, erythrocytes, RDW-SD, reticulocytes and ZPP/Hb ratio. From the left to the right side bars represent 3 subject groups (α-thalassaemia trait, ß-thalassaemia trait, iron deficiency) and a reference group of apparently healthy blood donors. An error bar is centered on the mean of the distribution range and demonstrates the interval of standard errors of the mean.*

## RESULTS

Results of single haemocytometric parameters are plotted in ***Fig. 1***. On the basis of MCV results patients with microcytic erythropoiesis are discriminated from the reference subjects group. Despite statistically significant deviations between subject groups with α-thalassaemia, ß-thalassaemia and iron deficiency ($p < 0.0001$), respectively, discrimination based on the separate results for MCV, RBC, RDW-SD, ZPP/Hb ratio and reticulocyte count cannot be unequivocally performed because of rather low sensitivity and specificity indices for separate single parameters (***Table 1***). With application of the ZPP/Hb ratio the iron deficiency group can be distinguished from the reference subjects group and the α- and ß-thalassaemia patients groups ($p < 0.001$). Subjects with ß-thalassaemia trait can be discriminated from iron deficiency and in most cases from α-thalassaemia by using the MCV/RBC ratio and RDW-SD results (***Fig. 1***). Sensitivity and specificity for these parameters cover a wide range (***Table 1***). In view of the low sensitivity single haemocytometric parameters are not thought to be satisfactory for discriminating between the several patient groups (***Table 1***).

In ***Fig. 2,*** the results are demonstrated after combination of several haemocytometric parameters in a discriminant function $2 \times$ RDW (fL) - $5 \times$ RBC ($\times 10^{12}$/L) - $250 \times$ reticulocytes ($\times 10^{12}$/L) + $30 \times$ ZPP/Hb ratio (µmol/mol Hb). By application of this algorithm patients with thalassaemia are successfully discriminated from patients with iron deficiency ($p < 0.001$). Subsequent discrimination

***Table 1*** *Sensitivity and specificity for haemocytometric parameters of 3 subject groups compared with the reference subjects group*

| | MCV (fL) | RBC ($\times 10^{12}$/L) | MCV/RBC | RDW-SD (fL) | ZPP/Hb ratio (µmol/mol Hb) | Reticulocytes ($\times 10^{12}$/L) |
|---|---|---|---|---|---|---|
| **α-thalassaemia trait** (n= 54) | | | | | | |
| 95% confidence interval | 59-88 | 3.09-6.36 | 9.0-31.8 | 33.5-53.5 | 0.07-0.61 | 0.011-0.135 |
| Sensitivity | 63 | 41 | 67 | 36 | 36 | 15 |
| Specificity | 81 | 86 | 92 | 85 | 85 | 78 |
| **β-thalassaemia trait** (n=78) | | | | | | |
| 95% confidence interval | 54-79 | 4.10-6.68 | 8.6-18.7 | 30.5-46.0 | 0.13-0.62 | 0.030-0.155 |
| Sensitivity | 90 | 60 | 96 | 83 | 49 | 34 |
| Specificity | 95 | 88 | 99 | 95 | 87 | 85 |
| **Iron deficiency** (n=47) | | | | | | |
| 95% confidence interval | 55-78 | 2.88-5.49 | 11.4-26.5 | 35.6-56.2 | 0.37-1.75 | 0.025-0.092 |
| Sensitivity | - | 37 | 27 | 10 | 100 | 3 |
| Specificity | - | 94 | 90 | 89 | 100 | 86 |
| **Reference group** (n=70) | | | | | | |
| 95% confidence interval | 79-95 | 4.09-5.62 | 14.5-21.8 | 38.5-49.0 | 0.10-0.29 | 0.026-0.091 |

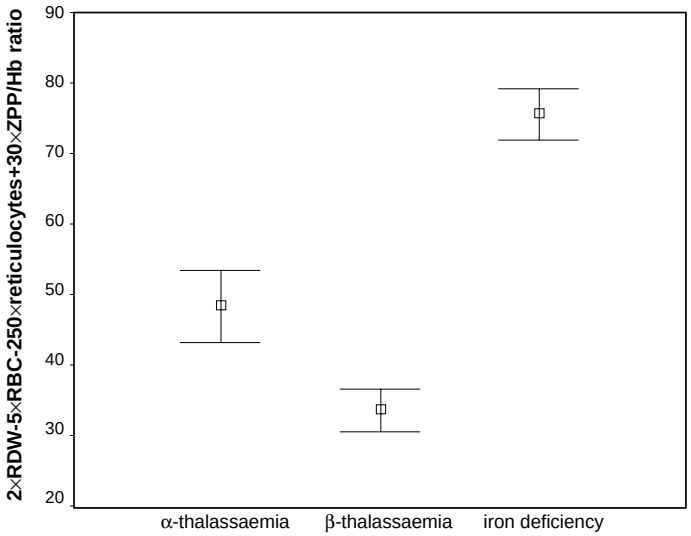


***Fig. 2*** *Error bar plot representing results of the discriminant algorithm function combining haemocytometric parameters erythrocytes ($\times 10^{12}$/L), RDW-SD (fL), reticulocytes ($\times 10^{12}$/L) and ZPP/Hb ratio (µmol/mol Hb) for the 3 respective patient groups.*
*An error bar is centered on the mean of the distribution range and demonstrates the interval of standard errors of the mean.*

***Table 2*** *Use of the discriminant function (2 × RDW-5 × RBC-250 × reticulocytes+30 × ZPP/Hb ratio) for 3 subject groups and a reference subjects group.*

| | 95% confidence interval | Sensitivity | Specificity |
|---|---|---|---|
| α-thalassaemia trait | 32 - 66 | 86% | 94% |
| β-thalassaemia trait | 20 - 50 | 97% | 99% |
| Iron deficiency | 54 - 94 | 43% | 88% |
| Normal subjects | 38 - 76 | - | - |

between α-thalassaemia trait and ß-thalassaemia trait is performed appropriately ($p < 0.001$). If compared with the reference subjects group, sensitivity and specificity indices were calculated as 86% and 94%, respectively, for subjects with α-thalassaemia trait, and 97% and 99% for ß-thalassaemia trait (***Table 2***). In ***Table 2*** the ranges (95% confidence interval) for results of the discriminant function are shown for each patient group and the reference subjects group.

## DISCUSSION

Discrimination between an iron deficient state and haemoglobin disorders is often requested for subjects with microcytic anaemia, particularly for people originating from the Mediterranean area or South East Asia. The diagnosis of microcytic anaemia is initially based on a decreased MCV value. Mild erythrocytosis and a marked degree of microcytosis are characteristic features for heterozygous ß-thalassaemia and α-thalassaemia with genotype -,-/αα or -α/-α.

Results within the reference interval for apparently healthy subjects or only slightly decreased MCV values are characteristic of α-thalassaemia with genotype -α/αα. In cases of iron deficiency RBC count and MCV value are more or less decreased, depending on whether the anaemia is of acute or chronic origin[8].

Already for many years, the use of red cell indices has been recommended to discriminate subjects with iron deficiency from subjects with a thalassaemia syndrome [9-11]. However, application of the Mentzer formula (MCV/RBC), the Shine & Lal formula ($MCV^2 \times MCH/1000$) and the England & Fraser formula (MCV-5 × Hb-RBC) resulted in only about 30-40 % of subjects being appropriately classified.

Application of the erythrocyte protoporphyrin content was published already many years ago in order to discriminate appropriately subjects with iron deficiency from subjects with thalassaemia in case of microcytic anaemia[12,13]. Later, ZPP/Hb ratio was recommended for the classification of microcytic erythrocyte disorders [2,5,6,8,14,15].

Results of investigations concerning screening procedures for ß-thalassaemia based on RBC indices originating from a mixed population of subjects with ß-thalassaemia, subjects with iron deficiency and apparently healthy subjects are described by Akai[16]. When the combined criteria of MCV = 80fL and RDW-SD = 32fL were evaluated in order to detect ß-thalassaemia, sensitivity and specificity amounted to 88% and 93%, respectively. However, in a population which also concerned patients with α-thalassaemia sensitivity of 64% and specificity of 84% were achieved with identical cut off values for MCV and RDW-SD[17].

The reticulocyte count is used as a parameter to follow therapy in patients with iron deficiency and with the anaemia of chronic disease. Increase of reticulocyte counts is frequently observed after effective oral iron therapy. Use of the reticulocyte count for screening for thalassaemia is not yet common.

Multivariant discriminant analysis concerning MCH, RBC, MCV and RDW, is useful for the diagnosis of α- or ß-thalassaemia and iron deficiency[18]. Sensitivity for screening subjects for α-thalassaemia was 70%, for ß-thalassaemia 72% and IDA 92% in patients with microcytic anaemia.

As discrimination of thalassaemia trait from IDA has important clinical implications a reliable diagnosis is important to reduce unnecessary testing and inappropriate treatment.

In case of microcytic erythrocytes (MCV = 80fL) iron deficiency can be differentiated from apparently healthy subjects and subjects with thalassaemia with application of the ZPP/Hb ratio. As a result of our study a discriminant function is recommended using the haematological parameters RDW-SD, RBC, ZPP/Hb ratio and reticulocyte count. Using the discriminant function 2 × RDW - 5 × RBC - 250 × reticulocytes + 30 × ZPP/Hb ratio it is possible to discriminate between α-thalassaemia and ß-thalassaemia trait and to separate thalassaemia from iron deficiency with a rather high level of accuracy.

***References***


1) *Vercelotti GM: A balanced budget - Evaluating the iron economy. Clin Chem, 42: 657, 1996.*
2) *Hastka J, et al.: Laboratory tests or iron status: correlation or common sense? Clin Chem, 42: 718-724, 1996.*
3) *Sherwood RA, Pippard MJ, Peters TJ: Iron homeostasis and the assessment of iron status, Ann Clin Biochem, 35: 693-708, 1998.*
4) *Baynes RD: Assessment of iron status. Clin Biochem, 29: 209-215, 1996.*
5) *Labbé RF, Vreman HJ, Stevenson DK: Zinc protoporphyrin: a*

*metabolite with a mission. Clin Chem, 45:2060-2072, 1999.*

6) *Labbé RF, Dewanji A, McLaughlin K: Observations on the zinc protoporphyrin/haeme ratio in whole blood. Clin Chem, 45: 146-148, 1999.*
7) *Blumberg WE, et al.: Principles and applications of haematofluorometry. J Clin Lab Autom, 4:29-42, 1984.*
8) *Harthoorn-Lasthuizen EJ, Lindemans J, Langenhuijsen MMAC: Combined use of erythrocyte zinc protoporphyrin and mean corpuscular volume in differentiation of thalassemia from iron deficiency anemia. Eur J Hematol, 60: 245-251, 1998.*
9) *Mentzer WC: Differentiation of iron deficiency from thalassaemia trait. Lancet, i:882, 1973.*
10) *England JM, Fraser PM: Differentiation of iron deficiency from thalassaemia trait by routine blood count. Lancet, i: 449-452, 1973.*
11) *Shine I, Lal S: A strategy to detect beta thalassaemia minor. Lancet, ii: 692-694, 1977.*
12) *Stockman JA, et al.: The measurement of free erythrocyte porphyrin (FEP) as a simple means of distinguishing iron deficiency from beta thalassemia trait in subjects with microcytosis. J Lab Clin Med, 85: 113-119, 1975.*
13) *Koenig HM, Lightsey AL, Schanberger JE: The micro-measurement of free erythrocyte protoporphyrin as a means of differentiation alpha thalassemia trait from iron deficiency anemia. J Pediatr, 86: 539-541, 1975.*
14) *Hershko C, et al.: Combined use of zinc protoporphyrin (ZPP), mean corpuscular volume and haemoglobin measurements for classifying microcytic RBC disorders in children and young adults. Clin Lab Haematol, 7: 259-269, 1985.*
15) *Han P, Fung KP, Teo CP, Tam LP: Alpha-thalassaemia and beta-thalassaemia traits: biological difference based on red cell indices and zinc protoporphyrin. Clin Lab Haematol, 12: 169-176, 1990.*
16) *Akai Y, et al.: A study of ß-thalassemia screening using an automated hematology analyzer. Sysmex J Int. 8: 110-114, 1998.*
17) *Akai Y, et al.: A study of ß-thalassemia screening using an automated hematology analyzer (2nd report). Sysmex J Int, 9: 160-163, 1999.*
18) *Eldibany MM, Totonchi KF, Joseph NJ, Rhone D: Usefulness of certain red blood cell indices in diagnosing and differentiating thalassemia trait from iron deficiency anaemia. Am J Clin Pathol, 111: 676-682, 1999.*