# Original Article

# Correlation of Microscopic and Automated Cell Counter Results of Newer Hematological Variables in Diagnosis and Recovery of Anemia

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## ABSTRACT

**Background:** The correct interpretation of new hematological parameters generated by Automated Cell Counters (ACCs) requires extensive knowledge for the clinical significance of these results. The present study will enlighten the vital role of the traditional & new parameters of the Complete Blood Count (CBC) in early diagnosis and follow up stage after treatment especially in the setting of anemia

**Aims:** To evaluate the vast & extended utility of newer hematological parameters generated by ACCs beyond their conventional role of CBC and Differential Leukocyte Count (DLC)

**Study Design:** This is a cross sectional observational study of results obtained from blood samples of 100 patients having various types of anemia diagnosed over a period of 18 months

**Subjects & Methods:** EDTA blood samples of the patient were collected under aseptic precautions and processed within 2 hours through new generation hematology analyzer i.e. ADVIAR 2120i. The peripheral blood film examination in each case was performed on Leishman-Geimsa stained smear. Second EDTA samples were taken on 2nd and 10th day of follow up of IDA and thalassemia cases respectively.

**Statistical Analysis Used:** The hematological, parameters obtained from ACCs/microscopic were assessed by Student's t-test, Fisher's exact test using the SPSS 21.0 software with considering p value < 0.05 as significant

**Results:** IRF and reticulocyte% can distinguish well between IDA and thalassemia major. CHr and MCVr are useful parameter for diagnosis of IDA/thalassemia and vitamin B12 deficiency anemia. CHr is the first parameter for monitoring response treatment of anemia.

*Keywords: Automated Cell Counters, Iron Deficiency Anemia, Thalassemia, Reticulocyte Count, Immature Reticulocyte Fraction, Mean Reticulocyte Volume, Mean Reticulocyte Hemoglobin Content, Fragmented RBC Count*

## Introduction

Automated Cell Counters (ACCs) are indispensible in view of accuracy and efficacy, however the manual age old Complete Blood Count (CBC) methodology is not yet obsolete due to economic considerations and non availability of ACCs, particularly in the smaller laboratory set ups in developing countries.

CBC primarily includes Red Blood Cells (RBCs), Hemoglobin (Hb) concentration, Mean Corpuscular Volume (MCV), White Blood Cells (WBCs), Platelet count etc which give rise to other parameters like Hematocrit (Hct), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), and Red cell Distribution Width (RDW). First blood counts and its aid to clinical diagnosis was established by Professor Karl Vierordt in 1852.[2] In 1896, George Oliver performed blood cells counting eye based measurement of light loss caused due to scattering and absorption in a test tube filled with diluted blood. This method could be considered the forerunner of automated blood count. In 1940s, Wallace Coulter evolved a simplified blood cell analysis tool for rapid screening of large number of blood samples.[7] Modern automated hematology technology works either on optical method (light scatter) or impedance Coulter method (changes in electrical current induced by blood cells flowing through an electrically charged opening) or a combination of both. Progressive improvement in these instruments led enumeration and evaluation of blood cells with great accuracy, precision, speed at low cost.

ACCs are available as three to seven-part differential systems which can count neutrophils, eosinophils, basophils, lymphocytes, monocytes large unstained cells (atypical lymphocytes) and large immature cells (blasts & immature granulocytes) based on its configuration. Inherent flagging systems, in built quality control programs and automated maintenance are additional qualities of ACCs.

The newer ACCs like Advia[R] 2120i and Sysmex XT-4000i provide parameters like Nucleated Red Blood Cells (NRBCs), Immature Reticulocyte Fraction (IRF), Mean Reticulocyte Volume (MCVr) and Mean Reticulocyte Hemoglobin Content (CHr), Fragmented RBCs count (FRBCs) and Immature Platelet Fraction (IPF). [Table 1] We will discuss the salient features of some of the most useful new parameters.

Nucleated Red Blood Cells are primarily produced and stored in bone marrow (BM) in response to erythropoietin as precursors to reticulocytes and mature erythrocytes. Usually NRBCs are found only in the circulation of fetuses and newborn infants upto 3rd or 4th day of life. Their presence in an adult's peripheral blood smear (PBS) is pathogenic and reflects very high demand for BM to produce RBCs leading to release of immature RBCs into circulation. NRBCs count may help in differential diagnosis of anemia and to determine transfusion needs. The reference value of mean NRBC by manual PBS is 0/100WBC and by Advia[R] 2120i range is 0-0.2x10$^3$ cells/cumm respectively. As NRBC has size similar to lymphocytes, many hematology analyzers misclassify them and may generate wrong total leukocyte and lymphocyte counts. Usually such samples are flagged by ACCs indicating the need to get corrected WBC count by microscopic analysis as; Corrected WBC = Total WBC x [100 ÷ (NRBC + 100)]

A schistocyte is an irregular, jagged, fragmented portion of RBC; formed due to mechanical destruction as in hemolytic anemia, mechanical artificial heart valves, hemolytic uremic syndrome, thrombotic thrombocytopenic purpura, Microangiopathic Hemolytic Anemia (MAHA), disseminated intravascular coagulation (DIC) and aortic stenosis. Owing to the abnormal shape, schistocyte easily undergoes hemolysis or get removed by macrophages in the spleen.[73] The reference value of schistocytes on microscopy is <2 schistocytes at 100x magnification and mean fragmented RBCs (FRC) by Advia[R] 2120i is 0.01-0.02 RBC fragmentsx10$^6$ cells/ul.[168] Overall the standard

method for a schistocyte count remains microscopic examination of PBS.[80]

The reticulocyte is immature, non-nucleated newly produced RBC that contains at least 2 granules of reticulum. Reticulocyte count in blood reflects the effective erythropoiesis.[87] In healthy individuals, reticulocytes circulate in the peripheral blood for 1-2 days after being released from BM. At times of increased erythropoietic demand, their life span in peripheral blood increases to >3 days due to premature release of immature/stress reticulocytes from the BM. This Immature Reticulocyte Fraction (IRF) is the quantitative proportion of all immature/younger reticulocytes and is calculated as a ratio of immature reticulocytes to the total number of reticulocytes. IRF is a very early and sensitive index of marrow erythropoietic activity and its fraction in excess of 5% is a reliable marker for hemopoietic recovery.[81] IRF value along with reticulocyte count can be utilized to differentiate various causes of anemia [Table 1].

Reticulocyte Production Index (RPI) give a snapshot of the functional iron available for incorporation into hemoglobin within RBCs over the previous 3-4 days. A decreased value generally reflects reduced cellular hemoglobin content and is the strongest predictor of IDA in children.[131]

Determination of mean reticulocyte hemoglobin content (CHr) provides an early measure of functional iron deficiency in adults and children because reticulocytes are the earliest erythrocytes released into blood and circulate for 1-2 days.[138] CHr value can be used as an early measure of the response to iron therapy, increasing within 2-4 days of the initiation of intravenous iron therapy. Various other biochemical parameters used to diagnose IDA include serum ferritin, transferrin saturation, total iron binding capacity and serum iron however are affected by many factors.

Mean Reticulocyte Volume (MCVr) is a specific new index that has potential to screen hereditary spherocytosis and to differentiate hemolytic anemia with megaloblastic anemia.

**Table1: Correlation of IRF with Absolute Reticulocyte Count & Clinical Conditions.**

| IRF | Absolute reticulocyte count | Clinical conditions |
|---|---|---|
| Low | Low | Aplastic anemia, chronic renal failure |
| High | Low | Repopulating bone marrow |
| Low to normal | Low | Early erythropoietic response after anemia or engraftment after BMT |
| High | High | Response to erythropoietin treatment or early acute hemorrhage or hemolytic anemia |
| High | Low to normal | Early response to iron therapy |

**Table 2: Clinical Correlation of Old & New Hematological Parameters in Anemia.**

| Parameter OLD RBC PARAMETER | Availability | Proposed clinical applications | Limitations |
|---|---|---|---|
| **Mean Corpuscular volume (MCV) (fL)**[92,93] | All analyzers | Anemia classification based on morphological approach | Affected by preanalytical variables (storage, temperature, time) |
| **Mean corpuscular Hemoglobin (MCH) (pg)**[94, 95] | All analyzers | Useful when hemoglobin synthesis is impaired as in iron deficiency anemia | Highly correlated with MCV |
| **Mean Corpuscular Hemoglobin Concentration (MCHC) (g/L)**[96, 97] | All analyzers | Increased in spherocytosis due to reduced surface/ volume ratio | With some impedance analyzers, the value is clamped around the mean |
| **Red cell  Distribution Width (RDW) (%)** [98–101] | All analyzers | Generic marker of abnormality when increased | Of little usefulness in the differential diagnosis of anemia. Reference intervals are method dependent |
| **NEW RBC  PARAMETERS** | | | |
| **Percentage of hypochromic red  cells** [102-105] | Hypo% (Siemens Advia 2120) | Assessment of iron availability (absolute or functional) for erythropoiesis based on iron status in last 3 months | Affected by preanalytical variables  (storage, temperature, time) |
| **Parameter** | **Availability** | Proposed clinical applications | Limitations |
| **Percentage of hyperchromic red  cells**[106, 107] | Hyper% (Siemens Advia 2120) | Diagnosis of  hereditary/ immune spherocytosis | Reference intervals and diagnostic thresholds are method dependent |
| **Percentage of  microcytic red cells**[108-111] | Micro% (Siemens Advia 2120); % micro-R (Sysmex XE/ XN) | Useful in Combination with other RBC Parameters (mainly hypochromic erythrocytes) to obtain discriminant indices for the  differential diagnosis of microcytic anemia | Reference intervals and diagnostic thresholds are method dependent |
| **RETICULOCYTE PARAMETERS** | | | |
| **Immature, reticulocyte fraction (IRF)** [112-122] | All analyzers | Classification of  anemias and monitoring of treatment. Verify aplastic anemia | Reference intervals and diagnostic cutoff  are method dependent |
| **Reticulocyte mean hemoglobin content (pg)** [122-125] | CHr (Siemens  Advia 2120); Ret- He (Sysmex  XE/XN) | Diagnosis of iron-deficient erythropoiesis. Early monitoring the response to iron therapies | Limited value in the presence of α or β thalassemia |
| **Mean reticulocyte volume (fL)**[121,124,126] | MCVr (Siemens Advia 2120) | Diagnosis of iron-deficient erythropoiesis. Early monitoring of  treatment with B12/folate/iron in nutritional anemia | Affected by preanalytical Variables (storage temperature, time) Reference intervals strictly method dependent |

**Table 3: Various Hematological Parameters in Anemia.**

| Parameters | Control (50) | IDA (45) | Megaloblastic Anemia (45) | CKD (40) | Thalassemia (10) |
|---|---|---|---|---|---|
| MCV (fl) | 85.2 ±4.6 | 71.3±48 | 110.4±8.2 | 82.9±6.1 | 67.8±4.1 |
| MCVr (fl) | 99.1±6.2 | 90.5±5.4 | 139.6±9.7 | 98.6±4.3 | 87.2±6 |
| MCVr/MCV ratio | 1.12 | 1.26 | 1.26 | 1.18 | 1.28 |
| CHCM (g/dl) | 40.2±1.9 | 29.4±2.1 | 34±2.1 | 29.9±2.4 | 31.6±1.1 |
| CHCMr (g/dl) | 31.7±1.4 | 23.4±3.2 | 26.1±2.9 | 23.7±2.2 | 24.4±1.6 |
| CHCMr/CHCM ratio | 0.79 | 0.79 | 0.77 | 0.79 | 0.77 |
| CH (pg) | 30.3±1.8 | 20.5±1.6 | 35.4±4.1 | 22.7±2.1 | 21.1±1.4 |
| CHr (pg) | 32.4±1.7 | 21.1±1.7[*] | 37.1±1.6[*] | 24.8±1.6 | 21.7±1.7[*] |
| CH /CHr ratio | 0.93 | 0.98 | 0.95 | 0.91 | 0.97 |
| Reticulocyte% | 1.19±0.15 | 0.34±0.06 | 0.34±0.07 | 0.66±0.08 | 1±0.15 |
| IRF | 5.2±0.6 | 0.62±0.18[**] | 0.66±0.19 | 0.74±0.20 | 2.9±0.5[**] |

## Materials & Methods

This cross sectional observational prospective study was conducted in the Department of Laboratory Sciences & and Molecular Medicine at a "Tertiary Care Super-Speciality Hospital" from Oct 2015 to Mar 2017 and included 100 and 10 cases of anemia and thalassemia respectively; to analyze different conventional and newer hematological parameters. The relevant clinical data was accrued from the data register in the blood collection centre/wards/concerned departments or by direct patient interaction. Pregnant women were not included in the study as the results might interfere due to initiation of hematinics in them during early pregnancy. Blood samples of selected cases were collected under aseptic precautions in EDTA vacutainer and processed within 2 hours of collection through new generation hematology analyzer i.e. ADVIA$^R$ 2120i. Leishman-Geimsa stained peripheral blood film examination was performed by two experienced technologists followed by confirmation by a hematopathologist.

Additional tests like reticulocyte count, cytochemical evaluation and Hb electrophoresis were resorted wherever needed. Second EDTA samples were taken on 2nd and 10th day of follow up in cases of IDA and thalassemia respectively. The association between hematological, morphological and clinical features were tested using Student's t-test for continuous variables and the chi-square test (or Fisher's exact test) for qualitative variables. All statistical analyses will performed using the SPSS 21.0 software considering p value <0.05 as significant.

## Results

Total 110 patients comprising of 100 cases of anemia (IDA: 45, Megaloblastic: 45 MAHA: 10) and 10 cases

of thalassemia were included in the index study. Table 2 summarizes hematologic findings and reticulocyte parameters in the normal controls, IDA, megaloblastic anemia group, chronic kidney disease and thalassemia major group.

All ten patients of thalassemia major had increased mean NRBC by manual peripheral blood smear ranged from 25 NRBC/100WBC to 106 NRBC/100WBC and by Advia$^R$ 2120i value ranged from $1.21 \times 10^3$/cumm to $6.1 \times 10^3$/cumm. Statistically significant correlation for calculating NRBCs was observed between both the methods (p value <0.05).

All ten 10 patients of MAHA found to have increased schistocytes by microscopy as >2 per 100x magnification. Mean value of fragmented RBCs (FRC) provided by Advia$^R$ 2120i was $1.33 \times 10^6$ cells/uL with control value of $0.045 \times 10^6$ cells/uL and cut off value of $>0.02 \times 10^6$ cells/uL. Statistically significant correlation for measuring schistocytes was observed between both methods (p value <.0.05).

Reticulocyte% was significantly decreased in IDA (0.34±0.06) followed by megaloblastic anemia ( 0.34±0.07) whereas was near normal only in thalassemia (1±0.15) as compared to control group value of 1.19±0.15.

IRF was done in cases of nutritional anemia, follow up cases of nutritional anemia, anemia of chronic disease and thalassemia. In IDA and megaloblastic anemia follow up was done on 2nd and 10th day and in case of thalassemia IRF was done at the time of diagnosis. Cases with IDA, ACD, megaloblastic anemia, with their respective post-treatment iron therapy, erythropoietin and vitamin $B_{12}$ therapy showed increased IRF. Cases of thalassemia had

decreased IRF value of 2.9±0.5 as compared to control group value of 5.2±0.6.

CHr value was lowest in IDA (21.1±1.7 pg) as compared to thalassemia (21.7±1.7 pg), megaloblastic anemia (37.1±1.6 pg) and control group (32.4±1.7 pg). CHr value of < 26.2pg favours IDA. CHr from the baseline value of 21pg rose upto normal value of 31pg within 2 days to 32pg on 28th day post treatment in IDA, while IRF value returned in 10-14 days. CHr was the first parameter that responded to iron therapy even before MCV and IRF.

IDA & thalassemia patients had lower MCV, CHCM, CHCMr, CH, CHr values as compared to healthy and vitamin B12 deficiency group. CH/CHr ratio was maximally increased in IDA (0.98) followed by thalassemia (0.97), megaloblastic anemia (0.91) with control group value of 0.93.

In megaloblastic anemia, vitamin B12 therapy results in increased reticulocyte% count and IRF and decreased MCVr, CHCMr and CHr in 21 to 28 days. [Table 3]

## Discussion

Greek word 'Anemia' means an=not, naime=blood i.e. not having blood and functionally defined as an insufficient RBCs mass to adequately deliver oxygen to peripheral tissues. World Health Organization defines the lower limit of normal Hb concentration at sea level to be 14.0 gm/dl in adult men and 12.0 gm/dl in adult women whereas in individuals of >65 years) to be 8.5 gm/dl.

This study was performed to evaluate, analyze the clinical applications of traditional, microscopic results with newer hematological parameters generated by ACC and to explore their role in the course of diagnosis, hematological recoveries of IDA, thalassemia, ACD and megaloblastic anemia.

All ten patients of thalassemia major were analyzed for increased mean NRBC by microscopy and Advia[R] 2120i. The correlation of our results between both methods was statistically significant (p value <0.05) and corroborating well with other studies.[166,167] As manual counting of NRBC is a time consuming process, so use of Advia[R] 2120i is recommended.

For fragmented RBCs, we included ten patients of MAHA. Value of FRCs by Advia[R] 2120i was significantly high in patients with raised schistocytes on microscopy (p value <0.05). Our result was in concordance with J-F Lesesve et al.[169,170] So though both methods have depicted the schistocytes, however manual counting of schistocytes is a time consuming process, so Advia[R] 2120i can be used.

In anemia cases, the reticulocyte count is an indicator of effective erythropoiesis. The recent development of automated reticulocyte counts with the help of Advia[R] 2120i made it easy to calculate the proportion of IRF and permitted more precise counting of reticulocytes. Our results of IRF as sensitive index of marrow erythropoietic activity are similar to Łuczyński W et al.[171] Other reticulocytes parameters generated by Advia[R] 2120i guided us in differential diagnosis of IDA, thalassemia, vitamin B12 deficiency and CKD. Our results of IRF were statistically significant to distinguish IDA and thalassemia (p value <0.05).

CHr found to be more significant parameter than MCV, reticulocyte% and IRF in the diagnosis of IDA. Our results CHr as first parameter for monitoring response to intravenous iron therapy in IDA were in concordance with Buttarello M et al[178] CHr was decreased in IDA, CKD and thalassemia major group as compared to control and increased in vitamin B12 deficiency group and these results were similar to C. Ceylan.[181] The vitamin B12 deficiency group had significantly increased MCVr value as compared to control and other diseased group (p value <0.05) indicating its utility to diagnose megaloblastic anemia more precisely. Among microcytic anemias, only IRF and reticulocyte% are significant parameters between IDA and thalassemia major (p value <0.05) so can be utilized to distinguish them. CH/CHr ratio was maximally increased in IDA, but not as significant with thalassemia (p value >.05). Other ratios like MCVr/MCV and CHCMr/CHCM were also not as significant to compare IDA and thalassemia (p value >0.05).

Manual differential counting is considered as the gold standard for the accurate identification of cells in the peripheral blood,[184] however it is labor and time-intensive.[181,182] Therefore, CBCs and WBC differentials conducted using ACCs has replaced the traditional manual differential count method for the initial screening and detection of hematologic abnormalities in most laboratories.

## Conclusion

Results of NRBC, FRCs by automated Advia[R] 2120i were in excellent correlation with microscopic NRBC and schistocytes counts respectively. As our study has few such relevant cases, therefore additional studies with larger patients group are required for validation of clinical applications. IRF and reticulocyte% showed significant value to distinguish IDA and thalassemia major. CHr and MCVr are useful parameter for diagnosis of IDA/ thalassemia and vitamin B12 deficiency respectively with control groups. CHr was the first parameter that was responded to iron therapy even before MCV and IRF thus

representing their role as non-invasive, inexpensive and objective indicator of a patient's bone marrow response. Moreover, it should be remembered that despite the essential role of automation, microscopy of pathologic samples remains essential.

**Informed Consent:** A written consent in the language the patients understands was taken from all the subjects being enrolled after explaining the objectives and benefits of the study to them.

**Ethical Clearance:** The study was then undertaken after due approval of the hospital ethics committee.

**Funding Resources:** No funding was obtained from any external source.

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