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# EVALUATION OF RETICULOCYTE SUBPOPULATIONS AND IMMATURE RETICULOCYTE FRACTIONS IN IRON DEFICIENCY ANEMIA

**Hina Tariq[1*], Ikramuddin Ujjan[2], Tahir Sultan Shamsi[3]**

[1*]Department of Clinical Research, National Institute of Blood Disease and Bone Marrow Transplantation, Street 2/A, Block 17, Gulshan-e-Iqbal, KDA Scheme 24, Karachi, Sindh, Pakistan. Email: htmamoon@gmail.com
[2]Department of Pathology, Liaquat University of Medical and Health Sciences, Deh Soun Valhar, Jamshoro, Sindh, Pakistan
[3]Department of Clinical Research, National Institute of Blood Disease and Bone Marrow Transplantation, Street 2/A, Block 17, Gulshan-e-Iqbal, KDA Scheme 24, Karachi, Sindh, Pakistan

***Corresponding author:** Hina Tariq
*Department of Clinical Research, National Institute of Blood Disease and Bone Marrow Transplantation, Street 2/A, Block 17, Gulshan-e-Iqbal, KDA Scheme 24, Karachi, Sindh, Pakistan. Email: htmamoon@gmail.com

**Abstract:**
**Rationale:** In addition to offering increased precision and accuracy, automated reticulocyte counts use flow cytometry to reliably assess mRNA content and cellular indices such as volume, Hb concentration, and content. Novel reticulocyte metrics have been employed in the identification and treatment of anemias. Studies on the clinical use of these unique metrics, the value of reporting such numbers, and their interpretation have been spurred by their discovery.
**Objective:** This research aims to analyze reticulocyte subpopulations and immature reticulocyte fractions in iron deficient patients.
**Methodology:** The present study included 288 subjects, divided into two groups: control subjects (n = 150), and subjects with iron deficiency anemia (n = 138). The results were analyzed by Student's t- test for comparison of means. The correlational analysis was performed to evaluate a relationship between various parameters and presence/absence of iron deficiency anemia Differences were considered significant when the two-tailed p-value was $< 0.05$.
**Results:** Patients with Iron deficiency anemia had significantly elevated levels of IRF, MFR, and HFR but decreased LFR in comparison to the controls. MCV had a high positive correlation (r=0.895) with the presence of iron deficiency anemia.
**Conclusion:** The reticulocyte immaturity indices are higher in the presence of iron deficit, indicating a lack of the essential components needed for the production of hemoglobin. As a result, these reticulocyte immaturity indices may be used as early indicators of anemia and iron deficit. These indices must be standardized in order to be used in laboratory tests and clinical practice.

**Keywords:** Iron Deficiency Anemia, Reticulocyte, Subpopulations, Immature Reticulocyte Fraction, Fluorescence Ratio

## INTRODUCTION

Anemia is defined by the World Health Organization (WHO) as an illness in which the hemoglobin (Hb) levels are lower than 12.0 g/dl and 13.0 g/dl in males and females respectively and irrespective of the underlying cause, red blood cell morphology or red blood cell function.[1] Anemia is one of the most frequent public health problems in the world with a total of 1.8 billion cases reported worldwide by WHO.[2] Anemia can target all stages of life but it is more prevalent in pregnant women and young children with major consequences for human health as well as socioeconomic development.[3] Iron deficiency anemia (IDA) is one of the major causes of anemia, as more than 50% of the cases (males: 66.1%, females: 56.8%) are due to insufficient iron intake which is an important micronutirent for various physiological functions which include oxygen transport, DNA synthesis, and cellular metabolism. It is considered a prevalent global health challenge that affects individuals across a diverse demographic domain including infants, preschool children, adolescents, and pregnant women, and also imposes a substantial burden on the the healthcare system.[4] The implications of untreated IDA are immense, ranging from fatigue, and diminished cognitive function to impaired immune response, particularly in more vulnerable populations such as children and pregnant women.

Keeping in mind the important role of iron in a myriad of physiological processes, the accurate estimation of iron status is very important in the timely intervention and thus the effective management of IDA. Conventional diagnostic methods such as hemoglobin levels, mean corpuscular volume (MCV), mean cell haemoglobin (MCH), corpuscular hemoglobin concentration (MCHC), and red cell distribution width (RDW), and serum ferritin. Although widely used, they exhibit certain limitations. For example, MCV and MCH help in the diagnosis and assessing trends over a period of weeks or months and cannot help in the assessment in the acute changes in the iron level.[5] Similarly, serum ferritin is a marker which helps in the identification of iron overload or to differentiate between iron-deficient or non-iron-deficient types of anemia. However, its levels are also grealty affected in the presence of inflammation which may then display a falsely high levels of ferritin and thus disguise the presence of iron deficiency anemia.[6] These limitations thus underscore the urgent need to discover alternative diagnostic methods that may play a complementary role to the existing mehtodologies and provide a more nuanced understanding of the iron status.

Considering all these factors, there is a need to streamline the diagnostic approach for the prior detection and assessment of iron deficiency. An important clinical test for the evaluation of erythropoietic activity and diagnosis of bone marrow is the reticulocyte count. Reticulocytes are non-nucleated immature red blood cells in peripheral blood, containing ribosomal RNA residues which reaches maturity three days after being prodcued in the bone marrow and then released into the peripheral circulation one day after to become mature. The most common method used for the detection of reticulocytes is manual count which employs supravital staining for the observation of residual ribosomal RNA.[7] For the classification and monitoring of different types of anemia, reticulocyte count is considered one of the most frequeny hematological test as it determines the functionality of bone marrow.[8] In the past, when automated reticulocyte counters were not introduced, the RPI (Reticulocyte Production Index) was recognized and commonly accepted as an indicator of bone marrow response to anemia. These days, new parameters have been introduced in automated reticulocyte counts to evaluate marrow activity.[9]

Automated Flow cytometric analysis of reticulocytes has been incorporated into routine laboratory testing, in recent years, as a substitute for the manual approach. It is quick, more precise, and simple to use. It gives the quantity of reticulocytes as well as a number of indices that can be useful for evaluating bone marrow recovery and pathology diagnosis. These indices have not yet been utilized in clinical practice since they need to be standardized and reference values need to be defined.[10]

Low fluorescence ratio (LFR), medium fluorescence ratio (MFR), and high fluorescence ratio (HFR) are the three groups/subpopulations into which reticulocytes can be classified using the automated method based on their fluorescence intensity, which indicates maturity.[11] Immature reticulocyte fraction (IRF) evaluates reticulocyte maturation based on the intensity of the staining of reticulocytes, which reflects mRNA content and is the combination of MFR and HFR.[12] Hence, the

degree of reticulocyte maturation is closely correlated with intracellular RNA levels and fluorescence intensity.[11] By providing an additional dimension to the haematological assessments, these characteristics may be used as early indicators for the assessment of iron deficiency. This study aimed to analyze the reticulocyte maturity indices (LFR, MFR, HFR) and Immature Reticulocyte Fraction (IRF) in iron deficiency in all age groups and thus provide insights into their potential roles in the diagnosis and monitoring of IDA.

## MATERIALS AND METHODS

The study population consisted of individuals coming to the outpatient department of the National Institute of Blood disease and Bone Marrow Transplantation from August 2017 till August 2019. Inclusion criteria comprised individuals who had not received any iron or vitamin therapy. The study was approved by the internal review board of the National Institute of Blood disease and bone marrow Transplantation. Voluntary informed consent was then obtained from patients (<18 years), assent from patients (>10 years-<18 years, and parents or legal guardians for patients <10 years) after they had been explained the risks and benefits of the study. All the data was recorded in a pre-designed questionnaire.

Blood samples from individuals were obtained in ethylenediaminetetraacetic acid (EDTA) anticoagulant and gel tubes. Hematological analysis was performed on Sysmex XN-1000 while biochemistry investigation for the evaluation of the iron status was carried out using Vitros and Architect by Chemiluminescence technique. Specimens were analyzed within four hours of collection. The sample was divided in two groups i.e. control group (non-anemic - Hb> 11.0 g/dL) and the anemic (Hb< 11.0 g/dL). All control subjects had normal haemoglobin values (12-15 g/dl for women and 14-17 g/dl for men), mean corpuscular volume (76-96 fl), and mean corpuscular haemoglobin (28-32 pg). Patients were labeled as having iron deficiency anemia if they had Hb and ferritin below the normal i.e. < 11 g/dL and < 20 ng/ml respectively. Reticulocyte indices based on the fluorescence intensity were divided into three groups i.e. (LFR: 89.9%-98.4 %; MFR: 1.6%-9.5 %; HFR:0%-1.7% of the total reticulocytes).[13]

Statistical analysis of data was performed using the Statistical Package for the Social Sciences (SPSS) version 20. The normality of the data was determined using the Shapiro-Wilk test. As per the distribution of the data, the continuous variables were reported as mean (SD). Independent sample two test was used to compare the means between the control and iron-deficient groups. Spearman's correlational analysis was performed to evaluate the relationship between the various understudy parameters and the presence (patients) or absence (controls) of iron deficiency anemia. A p- value of <0.05 was considered as statistically significant.

## RESULTS

As per the inclusion criteria, a total of 288 individuals were enrolled in the study which displayed a male predominance (54.5%; n=157). The study cohort was divided into two groups i.e. the control group (n =150) while the remaining 138 had the diagnosis of iron deficiency anemia.

The comparison of the demographic and clinical characteristics of the two groups is shown in Table 1. The group of patients with iron deficiency anemia had a statistically significant decrease in the values of Hemoglobin (Hb), Mean Corpuscular volume (MCV), Mean Corpuscular Hemoglobin (MCH), and Mean Corpuscular Hemoglobin Concentration. Regarding the reticulocyte indices, LFR was significantly lower while the rest of the parameters i.e. IRF, MFR, and HFR were observed to be higher as compared to their control counterparts **(Table 1)**. The correlational analysis of all the parameters between the controls and the iron-deficient patients revealed a strong positive correlation for mean corpuscular Hemoglobin (r=0.895; p-value: 0.000) **(Table 2)**.

**Table 1: Demographic characteristics of the control group and iron deficiency anemia group**

| Parameter | | Frequency | Mean (±SD) | p-value |
|---|---|---|---|---|
| **Age** | Controls | 150 | 34.2 ± 15.1 | 0.073 |
| | IDA | 138 | 30.8 ± 201. | |
| **RBC (10^12/L)** | Controls | 150 | 4.63 ± 0.39 | **0.000*** |
| | IDA | 138 | 3.47 ± 0.77 | |
| **Hb (g/dL)** | Controls | 150 | 13.6 ± 1.12 | **0.000*** |
| | IDA | 138 | 6.46 ± 1.65 | |
| **HCT (%)** | Controls | 150 | 40.25 ± 3.38 | **0.000*** |
| | IDA | 138 | 22.47 ± 4.6 | |
| **MCV (fL)** | Controls | 150 | 86.93 ± 3.34 | **0.000*** |
| | IDA | 138 | 65.49 ± 9.0 | |
| **MCH (pg)** | Controls | 150 | 29.37 ± 1.18 | **0.000*** |
| | IDA | 138 | 18.84 ± 4.16 | |
| **MCHC (g/dL)** | Controls | 150 | 33.8 ± 0.94 | **0.000*** |
| | IDA | 138 | 28.51 ± 3.31 | |
| **RDW-CV (%)** | Controls | 150 | 13.63 ± 1.02 | **0.000*** |
| | IDA | 138 | 22.49 ± 4.71 | |
| **RET-He (pg)** | Controls | 150 | 30.71 ± 2.32 | **0.000*** |
| | IDA | 138 | 16.52 ± 4.07 | |
| **MicroR (%)** | Controls | 150 | 2.66 ± 1.72 | **0.000*** |
| | IDA | 138 | 45.56 ± 23.44 | |
| **MacroR (%)** | Controls | 150 | 3.9 ± 0.4 | **0.000*** |
| | IDA | 138 | 1.66 ± 1.1 | |
| **RBC-He (pg)** | Controls | 150 | 26.48 ± 2.31 | **0.000*** |
| | IDA | 138 | 17.0 ± 4.78 | |
| **Delta-He (pg)** | Controls | 150 | 4.21 ± 2.14 | **0.000*** |
| | IDA | 138 | -0.33 ± 2.75 | |
| **IRF (%)** | Controls | 150 | 7.3 ± 4.38 | **0.000*** |
| | IDA | 138 | 22.06 ± 14.52 | |
| **LFR (%)** | Controls | 150 | 92.48 ± 4.58 | **0.000*** |
| | IDA | 138 | 78.03 ± 14.31 | |
| **MFR (%)** | Controls | 150 | 6.59 ± 3.5 | **0.000*** |
| | IDA | 138 | 13.79 ± 6.03 | |
| **HFR (%)** | Controls | 150 | 0.93 ± 1.42 | **0.000*** |
| | IDA | 138 | 8.05 ± 8.97 | |

*MCH: mean corpuscular hemoglobin; MCV: mean corpuscularvolume; MCHC: mean corpuscular hemoglobin concentration; RDW: red cell distribution width; IRF: Immature Reticulocyte fraction LFR: low fluorescence ratio; MFR:medium fluorescence ratio; HFR: high fluorescence ratio; *statistically significant*

**Table 2: Correlation of various blood parameters between the controls and patients with iron deficiency anemia (IDA)**

| | **Correlation coefficient** | **p-value** |
|---|---|---|
| **Age (years)** | 0.024 | 0.781 |
| **RBC (10^12/L)** | -0.044 | 0.612 |
| **Hb (g/dL)** | -0.115 | 0.180 |
| **HCT (%)** | -0.108 | 0.207 |
| **MCV (fL)** | 0.038 | 0.659 |
| **MCH (pg)** | 0.895 | **0.000*** |
| **MCHC (g/dL)** | 0.117 | 0.171 |
| **RDW-CV (%)** | -0.113 | 0.191 |
| **RET-He (pg)** | -0.011 | 0.898 |
| **MicroR (%)** | -0.029 | 0.740 |
| **MacroR (%)** | -0.106 | 0.216 |
| **RBC-He (pg)** | 0.048 | 0.575 |
| **Delta-He (pg)** | 0.105 | 0.223 |
| **IRF (%)** | 0.065 | 0.447 |
| **LFR (%)** | 0.117 | 0.170 |
| **MFR (%)** | 0.090 | 0.291 |
| **HFR (%)** | 0.103 | 0.230 |

## DISCUSSION

Iron deficiency anemia, considered as a one of the most prevalent health challenge on the global level, necessitates precise diagnostic markers which would thus help in its timely intervention. Investigations into the numerous reticulocyte markers i.e. LFR, MFR, HFR, and IRF have produced significant insights into their prospective utilization in the identification of IDA. The present study analyzed the reticulocyte maturity indices (LFR, MFR, and HFR) in individuals with iron deficiency where the fluorescence intensity demonstrated the immaturity of reticulocytes and the bone marrow activity.[8] In our study, the individuals with Iron Deficiency Anemia showed a statistically significant increase in the proportion of MFR, HFR, and IRF and a decrease in the proportion of reticulocytes with LFR **(Table 3)**. These findings were in concordance to the findings reported by various other studies conducted on the estimation of the reticlulocyte subpopulations in the iron deficient patients.[11, 14, 15] However, the value of LFR in the current study which was observed to be low contradicted the findings of Choi et al.[9] who reported an increase in LFR. A study conducted in India showed an increase in Immature reticulocyte fraction (IRF) in individuals with Iron Deficiency as compared to controls which are in accordance with our results.[15] One percent of circulating red blood cells corresponds to the daily renewal of erythrocyte mass and the average life span of red blood cells is about four months. Thus the abnormalities in anemic individuals may take weeks to be revealed.[11] IRF is an early and sensitive marker of erythropoiesis and has great clinical efficacy in the classification of anemias. Its utility has been reported in monitoring the therapy in nutritional anemias where IRF precedes the increase in total reticulocyte count by several days.[16] Thus the reticulocyte indices may serve as early markers of Iron Deficiency and anemia.

**Table 3: Reticulocyte parameters in comparison to other studies in individuals with Iron Deficiency Anemia**

| **Reticulocyte Parameters** | **Current study (n= 101)** | **Sunkara et al.[15] (2016) (n=58)** | **Wollmann et al.[11] (2014) (n= 39)** | **Joao et al.[14] (2008) (n=96)** | **Choi et al.[9] (2001) (n=374)** |
|---|---|---|---|---|---|
| | **Pakistan** | **India** | **Brazil** | **Brazil** | **Korea** |
| IRF | 22.06 ± 14.52 | 17.89±9.00 | - | 15.02+9.70 | - |
| LFR | 78.03 ± 14.31 | - | 87.4 ± 5.0 | 84.83+9.65 | 97.42 ± 1.26 |
| MFR | 13.79 ± 6.03 | - | 10.3 ± 4.7 | 12.69+6.69 | 2.45 ± 0.41 |
| HFR | 8.05 ± 8.97 | - | 2.3 ± 0.9 | 1.45 | 0.12 ± 0.05 |

***IRF: Immature Reticulocyte fraction LFR: low fluorescence ratio; MFR: medium fluorescence ratio; HFR: high fluorescence ratio***

The body's reaction to iron deficiency anemia is markedly highlighted by an increase in immature reticulocytes in the blood of an IDA patient, if the medullary tissues and cortical elements required for the erythropoiesis cycle are preserved. This could be one explanation for the results of our study. It is also believed that anemic hypoxia causes the kidneys to release more erythropoietin, which thus increases cell differentiation and proliferation.[17] It also underscores the importance of the assessment of the reticulocyte maturity indices and IRF during initial evaluation and subsequent monitoring of the response to therapy which can guarantee the presence of a functioning erythropoiesis cycle as well as erythropoietin's reaction to anaemic hypoxia.

In cases of severe anemia, the maturation time of reticulocytes may accelerate in the bone marrow.[18] This is due to a release of higher number of immature reticulocytes released into the peripheral blood, where they remain for an estimated 48 hours or longer until they mature into mature red blood cells. As a result, there are more immature reticulocytes in the peripheral blood.[19] Reticulocyte immaturity-related indices are elevated in IDA, indicating a decrease in the precursors needed to produce hemoglobin and convert reticulocytes into mature red blood cells. Our study's results are consistent with those of previous worldwide research that found that individuals with anemias, including IDA, have higher MFR and HFR.

Without requiring additional biochemical studies, Sunkara et al.[15] indicated that if different reticulocyte parameters are used, the diagnosis of IDA can be made useful. The study reported that immature reticulocyte fraction and immature reticulocyte sub-populations were considerably greater in IDA due to inadequate erythropoiesis.[15]According to Wollmann et al., reticulocyte indices associated with maturity may be used as early indicators of anemia and IDA. They found that IDA patients had statistically significant higher MFR and HFR than the control group.[11] Reticulocyte maturity indices and iron-related characteristics were compared in females who were iron-deficient and healthy, according to Choi et al.[9] The results of the study showed that when serum ferritin and iron levels were low, moderate and high fluorescence ratios began to rise. When iron deficit was evident, reticulocyte maturity indices increased the most.[9] Zhao et al. evaluated anemias by comparing different red blood cell and reticulocyte parameters.[20] They discovered that in all cases of anemia, the medium and high fluorescent reticulocyte subpopulations increased, while the low fluorescent reticulocyte subpopulation significantly decreased when compared to the control population. The study proposed the use of characteristics related to reticulocyte maturity, red cell distribution width, and mean corpuscular volume for the assessment of different anemias.[20] Velasco-Rodríguez et al.[21] evaluated and associated different reticulocyte parameters with the appropriate pathophysiologic aspects in delta-beta thalassemia trait, beta thalassemia trait, and IDA. The results indicated that compared to beta thalassemia trait, patients with IDA had significantly more immature reticulocytes and a reduced absolute reticulocyte count. The study proposed that reticulocyte maturity indices and red cell indices might be used in conjunction to distinguish between the three clinical entities.[21]

The standard renewal of RBC mass corresponds to 1% of RBCs in circulation since the average lifecycle of an RBC in peripheral circulation is approximately 120 days. Consequently, it may take weeks to months to identify abnormalities in the routine hemoglobin indices of patients with IDA.[14, 15] Conversely, reticulocyte maturity indicators benefit from automation and greater precision, allowing for the early detection of alterations. These factors make reticulocyte maturity indices useful as early and significant indicators of anemias, such as IDA.

The present study had certain limitations. One of them was the small sample size. Secondly, there was no follow-up of patients with Iron Deficiency Anemia after receiving of the iron therapy to observe the betterment in the indices. The results of this study call for more research in various age groups and larger sample sizes of both healthy people and those at high risk of developing IDA at various centers in Pakistan to benefit from the automation and newer reticulocyte parameters by introducing these tests into routine haematology laboratory investigations.

## CONCLUSION

Measurement of LFR, MFR, HFR, and IRF in patients with iron deficiency anemia has provided new insights into diagnosis by highlighting the dynamic changes in reticulocyte populations during iron deficit. Low LFR is a sign of impaired erythropoiesis, whereas high MFR, HFR, and IRF indicate bone marrow compensatory mechanisms. These reticulocyte fractions have the potential to improve treatment approaches and refine IDA diagnosis when incorporated into clinical evaluations. A better knowledge of reticulocyte dynamics in IDA would surely aid in the development of hematological diagnostic paradigms as this field of study advances, improving patient outcomes.

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Not applicable

## CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**AUTHOR CONTRIBUTIONS:** HT collected and analyzed samples, and wrote the initial draft; IU and TSS critically reviewed the manuscript.

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**ETHICS STATEMENT:** The study was carried out with the approval of the Institutional Review Board of the National Institute of Blood Disease & Bone Marrow Transplantation.

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