New erythrocyte and reticulocyte parameters: Indicators for early diagnosis of iron deficiency anemia and anemia of chronic disease

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Abstract: *Introduction:* Iron deficiency anemia (IDA) is a global health burden with an increasing prevalence in children and adult females. Traditionally, absolute iron deficiency has been based on low serum iron and ferritin. In recent years, the development of new automated hematologyanalyzers has paved the way for analysis of new parameters for mature red blood cells and reticulocytes in diagnosis of anemia. *Materials and methods:* Blood samples of adult females of reproductive age group (15-45 years) were collected and processed within 6 hours of collection. Based on hematological and biochemical findings, patients were segregated into four subgroups: Iron deficiency anemia (IDA, n = 40), Anemia of chronic disease (ACD, n=40), Latent iron deficiency (n=40) and Controls (n=40). *Observations and results:* The novel hematological parameters-Reticulocyte hemoglobin content (Ret-He), percentage of microcytic red blood cells (MicroR), hemoglobin content of erythrocytes (RBC-He) and percentage of hypochromic erythrocytes (HypoHe) had a statistically significant difference between the four groups (p < 0.05). The correlation between reticulocyte indices and iron parameters depicted that levels of Ret-He among all study participants (160) increased as the serum iron and serum ferritin increased indicating a significant positive correlation. Ret-He and RBC-He demonstrated better predictive power as per area under curve of ROC as compared to MicroR and HypoHe indices for latent anemia identification. *Conclusion:* The present study emphasized upon the importance of novel and cost effective routine new parameters in early and accurate diagnosis of IDA and ACD. Moreover Ret-He emerged as a sensitive and specific marker for diagnosis of latent iron deficiency.

Keywords: Anemia of chronic disease, Iron deficiency anemia, Latent iron deficiency, Ret-He, RBC-He.

Introduction

Iron deficiency anemia (IDA) is a global health burden with an increasing prevalence in children and adult females. According to the World Health Organization (WHO), there are two billion people with anemia in the world and approximately half of the anemia is due to iron deficiency [1]. Iron balance is regulated by the rate of erythropoiesis and the size of iron stores [2]. The anemia that accompanies infection, inflammation and cancer and persists for >1 month duration is commonly termed as anemia of chronic disease (ACD), features apparently normal or increased iron stores. However, these patients have iron-restricted erythropoiesis (functional ID), an imbalance between the iron requirements of the

erythroid marrow and the actual iron supply. It leads to a reduction in red cell hemoglobinization, causing hypochromic microcytic anemia. The diagnosis is based on measuring the hemoglobin content of reticulocytes and percentage of hypochromic and microcytic cells (new hematological parameters) [3].

Anemia of chronic disease does not include anemia due to marrow replacement, blood loss, renal disease, hepatic dysfunction or endocrinopathy. IDA impairs substantial physical activity and cognitive performance in adults [4], thereby early diagnosis is essential. Iron-deficient erythropoiesis (latent iron deficiency), which is a transient stage of

cellular iron insufficiency occurs before the development of anemia. As reticulocytes have a shorter life span of a day or two, information related to its hemoglobincontent is a good indicator for iron availability and acts as an early marker of iron-deficient erythropoiesis [4]. Traditionally, absolute iron deficiency has been based on low serum iron and ferritin. In recent years, the development of new automated hematologyanalyzers has paved the way for analysis of new parameters for mature red blood cells and reticulocytes in diagnosis of anemia [5]. To the best of our knowledge, there is a paucity of literature (especially from India) on new parameters related to reticulocytes as well as red blood cells. The current study will focus on the role of these new hematological parameters in early diagnosis and differentiation of IDA and ACD as well as in early diagnosis of latent iron deficiency.

Material and Methods

The present case control study was conducted in the department of Pathology, ESIC Medical College and Hospital, Faridabad, Haryana over a period of 6 months (April to September 2018). It included adult females of reproductive age group (15-45 years). Patients on iron replacement therapy, patients with anemia due to marrow replacement, blood loss, renal disease, hepatic dysfunction or endocrinopathy and patients with any other type of anemias were excluded. The study was conducted after approval from Institutional Ethics Committee. Objective of the study was explained and confidentiality & anonymity was assured to the participants. Written informed consent was taken from the participants prior to sample collection.

Blood samples of adult females of reproductive age group (15-45 years) were collected and processed within 6 hours of collection. EDTA samples were analyzed using automated hematologicalanalyzer (Sysmex XN 1000) New erythrocyte and reticulocyte parameters like Reticulocyte hemoglobin content (Ret-He), percentage of microcytic red blood cells (MicroR), hemoglobin content of erythrocytes (RBC-He) and percentage of hypochromic erythrocytes (HypoHe) along with other basic parameters were obtained and analyzed. Biochemical parameters such as serum iron, serum ferritin and Total iron binding capacity

(TIBC) were processed on automated chemistry analyzer (RandoxDayTona).

Based on hematological and biochemical findings, patients were segregated into four subgroups: Iron deficiency anemia (IDA), Anemia of chronic disease (ACD), Latent iron deficiency and Controls.

- *Group I:* IDA- Serum Iron (SI) levels reduced (<30 µg/dl); Serum ferritin reduced (<13ng/ml); Hb<12g/dl.
- *Group II:* ACD- SI levels normal or reduced (Normal range in females- 30-145 µg/dl for women); SF- normal or high (Normal range in females 13-120 ng/ml); Hb<12g/dl.
- *Group III:* Latent iron deficiency: Ferritin <20 ng/ml, Hb>12g/dl.
- *Group IV:* Control group will comprise of healthy adult females with no clinical signs/symptoms of the disease and normal hematological and biochemical findings.

Statistical analysis: All data were compiled and analysis was done using Statistical Package for Social Sciences (SPSS) version 17 software; One-way Analysis of variance (ANOVA) were used for the analysis. A p value of <0.05 was considered significant at 95% confidence interval. The results are presented in mean $\pm$ SD and percentages. Receiver operator characteristic analysis (ROC) was used to evaluate the accuracy of the parameters to differentiate between different groups. Correlation among various parameters in different groups were estimated.

Results

The study population was segregated into 4 groups: Group I – Iron deficiency anemia (n=40), Group II – Anemia of Chronic Disease (n=40), Group III – Latent iron deficiency (n=40) and Group IV – Control (n=40). The mean age in group I (IDA) was 30.62$\pm$8.79 years. S. Iron was lowest (23.7$\pm$4.79) with highest TIBC levels (460.12$\pm$4.83) among the four groups.

The novel hematological parameters including hemoglobin content of reticulocytes (Ret-He) and RBC (RBC-He) were found to be very low and there was a marked increase in values

of MicroR and HypoHe with a mean of 25.3±20 and 34.35±6.79. In group II (ACD), mean age was 26.95+5.11. Iron profile was within normal limits however Ret-He and RBC-He were higher compared to IDA group. The group III (latent iron deficiency) had a mean age of 29.55+6.72. Hemoglobin and the red cell indices were within normal limits. S.ferritin was low however S.Iron was within the reference range. The values of Ret-He and RBC-He in latent group were intermediate between IDA and Control groups. Hematological parameters, Comparative analysis of Iron profile and Comparison of novel hematological parameters among all the four groups are depicted in Table I,II & III respectively. The novel hematological parameters- Ret-He, MicroR, RBC-He and HypoHe had a statistically significant difference between the four groups ($p < 0.05$) (Figure 1).

Fig-1: Comparison of novel hematological parameters among all the four groups
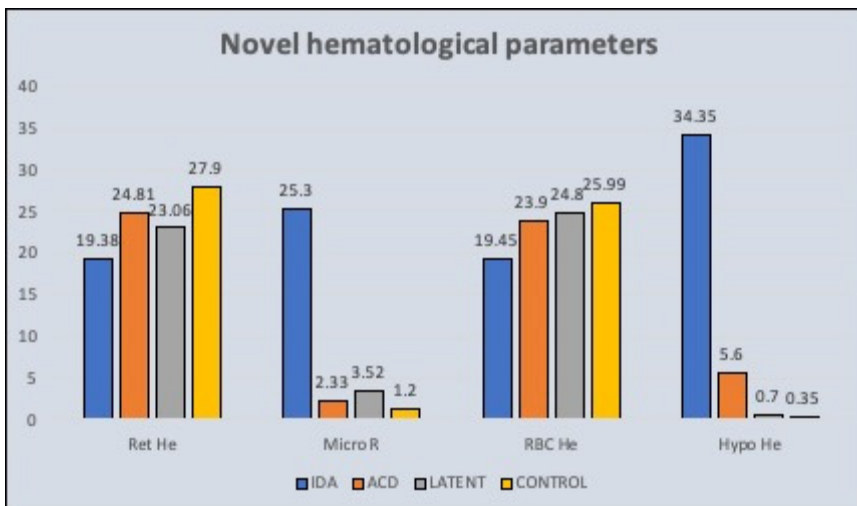

Table-1: Hematological parameters of all the groups					
Parameter	IDA	ACD	Latent	Control	p-Value
Hb	9.14±1.36	10.65±1.05	13.14±0.77	13.53±0.84	0.0016
MCV	73.95±9.38	91.14±11.24	91.97±5.55	94.57±6.72	0.0003
MCH	20.93±3.74	28.15±3.2	29.2±1.9	30.55±2.2	0.0010
MCHC	28.47±1.55	31.09±1.27	31.62±1.42	32.2±1.3	0.0001
RBC	4.62±0.83	3.86±0.63	4.46±0.33	4.44±0.43	0.0001

Table-2: Comparative analysis of Iron profile of all four groups					
Parameter	IDA	ACD	Latent	Control	p-Value
S. Iron	23.7+4.79	82.02+7.49	67.08+6.39	70.1+2.15	0.017
S.Ferritin	5.0+6.91	38.58+8.75	9.56+4.68	31.27+6.21	0.001
TIBC	460.12+4.83	377.8+6.6	401.2+8.98	362.4+8.38	0.017
Ret He	19.38+3.5	24.81+2.7	23.06+3.28	27.9+1.4	Ret He
Micro R	25.3+20	2.33+1.86	3.52+0.68	1.2+1.02	Micro R
RBC He	19.45+3.78	23.9+2.3	24.8+1.3	25.99+1.3	RBC He
Hypo He	34.35+6.79	5.6+1.5	0.7+0.62	0.35+0.33	Hypo He

Table-3: Cut off values for early detection of iron deficiency in the latent phase		
Parameter	Cut off	Sensitivity/ Specificity
Ret He	26.7	77.5/80
Hypo He	0.25	72.5/55
Micro Hr	0.75	77.5/57.5
RBC	25.8	90/52.5

The correlation between reticulocyte indices and iron parameters was also evaluated. Levels of Ret He among all study participants (160) increased as the serum iron and serum ferritin increased indicating a significant positive correlation (r: 0.44, p<0.0001& r: 0.52, p<0.0001 respectively) (Figure 1). There was a significant negative correlation between TIBC and Ret-He among all study participants (r:0.40, p<0.0001).

Fig-2: Correlation between Ret He and serum ferritin (r: 0.52, p<0.0001)
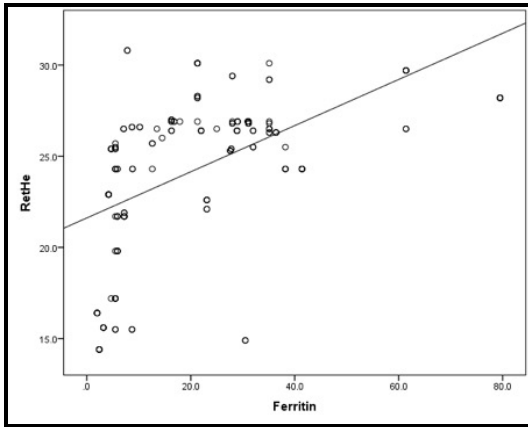
In both IDA and ACD, there was a positive correlation between Ret-He and RBC-He (r:0.94, p<0.0001 and r:0.93, p<0.0001 respectively) however there was negative correlation between Ret-He and MicroR among group I and II (r:0.46, p<0.002 and r:0.88, p<0.0001 respectively) (Figure 2).

The results of ROC analysis in latent group (Group III) were Ret-He (AUC: 0.837, P<0.001), RBC-He (AUC: 0.743, p<0.001), MicroR (AUC: 0.698, p=0.002). The sensitivity and specificity of novel hematological parameters to identify latent iron deficiency were Ret He (sensitivity 77.5% and specificity 80%), HypoHe (sensitivity 72.5% and specificity 55%), MicroR (sensitivity 77.5% and specificity 57.5%) and RBC-He (sensitivity 90% and specificity 52.5%) (Figure 3) (Table 3).

Fig-3: Receiver operating characteristics (ROC) curves for Ret-He, MicroR, HypoHe and RBC-He in the diagnosis of latent iron deficiency
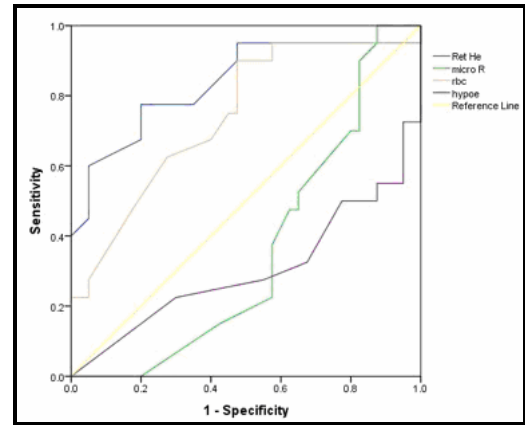
To correlate the findings, peripheral smears of all the four study groups were assessed as shown in Figure 4.

Fig-4: Peripheral smear a)- IDA, b)- ACD, c)-Latent Iron deficiency, d)-Control
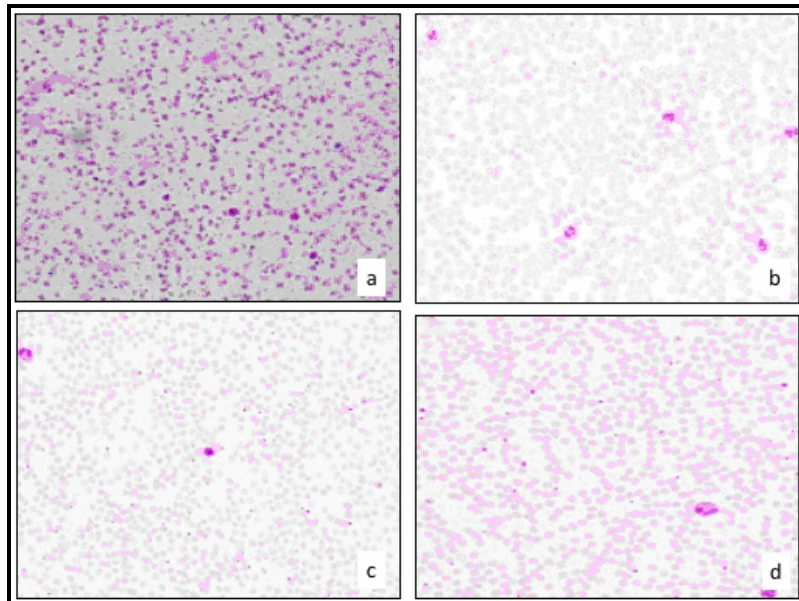
Discussion

Anemia is a global health burden involving 30% of world population with IDA being most common in both developed and underdeveloped countries [6]. Three important causes of anemia are blood loss, decreased production and increased destruction of RBCs. Although no population group is unaffected, the rates of iron deficiency are higher among young females and children in their first two years of life. It leads to cognition impairment thereby affecting memory, attention and concentration. The present study was planned among young reproductive age group females.

Hemoglobin estimation is an important parameter for the diagnosis of anemia with iron status assessment (serum iron, serum ferritin, TIBC, percentage transferrin saturation) playing a major role in diagnosing IDA and differentiating it from ACD, however they are time consuming. [7]Serum ferritin, being widely recognized as an acute phase reactant is affected in chronic infections/inflammation, liver disease, malignant neoplasm etc, thereby leading to wrong interpretation [7]. Latent iron deficiency, also known as iron deficient erythropoiesis, is a medical condition in which there is iron deficiency without anemia (normal hemoglobin level), characterized by Ferritin <20 ng/ml and Hb>12g/dl [8]. Studies have shown that iron deficiency without anemia can affect brain development and function [9].

Modern automated hematology analyzers enable determination of new hematological parameters during CBC evaluation which are not affected by acute or chronic inflammation unlike other biochemical parameters. Therefore, they may serve as an important tool in diagnosis of IDA and ACD [10]. Ret-He depicts the real-time information on the synthesis of hemoglobin by young erythrocytes. Reticulocytes are young RBC's, circulating in peripheral blood for one or two days, thereby information regarding hemoglobin content of young erythrocytes is a good indicator of iron availability and an early marker of iron-deficiency erythropoiesis [6].

Hypo-He defines those red cells having MCH<17 pg. It is an important variable for assessment of hypochromia [11]. A value of > 6% is found to be better compared to soluble transferrin receptor (sTfR), ferritin and TIBC in differentiating between iron sufficient and iron deficient patients.

Urrechaga et al have stated that increased percentage of hypochromic erythrocytes and a reduced hemoglobin content of reticulocytes are the best combination of hematological indices for iron deficiency, akin to our study however it was not statistically significant [12]. Torino et al performed a study on 117 adult patients with anemia and classified them into four groups: IDA, ACD, ACD with IDA and heterozygous beta thalassemia and found erythrocyte and reticulocyte indices as moderately good parameters. Among these parameters, percentage of hypochromic erythrocytes was the best with sensitivity and specificity of 72.7% and 70.4% respectively [5]. Bovy et al [13] and Scherer et al [14] found a significant increase in HypoHe in iron deficient patients and considered it a good marker of iron deficiency. However, in the current study, the increase was not significant.

It's a well-known fact that hypochromia typically follows microcytosis in IDA however hypochromia precedes microcytosis in ACD [15]. In the present study, these findings are very well reflected in the novel hematological parameters, MicroR and HypoHe. A positive correlation between reticulocyte indices and iron deficiency anemia was also observed similar to other authors [10, 16-18].

Malczewska-Lenczowska et al [2] performed a study on 219 female athletes and observed significant changes in Ret-He &MicroR in patients with IDA while in latent iron deficiency, significant changes (p<0.001) were observed in red cell indices, thereby concluding that reticulocyte markers were useful for early diagnosis of latent iron deficiency. Thomas et al[3](2002) studied 442 patients with various disease-specific anemias and 154 nonanemic patients and have concluded that the combination of the hematologic indices (reticulocyte hemoglobin content and proportion of hypochromic red cells) provides an important tool for diagnosis and monitoring of functional ID.

Brugnara C et al [16] analyzed the importance of measurement of reticulocyte hemoglobin in iron deficiency states and found area under curve for Ret-He to be 0.913 (p<0.0001) using ROC analysis. They concluded that Ret He is a reliable marker of cellular hemoglobin content which can aid in diagnosis of iron deficiency with a sensitivity of 93.3%, and a specificity of 83.2%.

Toki Y et al [6] found significantly (p<0.001) lower Ret-He levels in IDA patients with AUC as 0.902 and a specificity of more than 90%. In the current study Ret-He is found to be lowest in IDA group and a significant parameter in differentiating IDA with ACD and latent iron deficiency similar to the findings of Toki et al. [6] In contrast, Torino et al[5] found that Ret-He was not better than classic indices (MCV and MCHC) in differentiating these groups.

Jarc et al [10] found significantly (p<0.0001) decreased Ret-He and significantly (p<0.0001) increased HypoHe levels in patients with IDA. Ret-He (AUC =0.893) and HypoHe (AUC-0.938) were found to be better predictors of iron deficiency identification using ROC analysis. In the index study, Ret-He (AUC: 0.837, p<0.001) and RBC-He (AUC: 0.743, p<0.001) demonstrated better predictive power as per area

under curve of ROC as compared to MicroR and HypoHe indices for latent anemia identification.

The limitations of the present study are the small sample size and single institution based study. Moreover transferrin and soluble transferrin receptor/log ferritin ratio were not calculated.

Conclusion

Evaluation of new hematological parameters [Reticulocyte hemoglobin content (Ret-He), percentage of microcytic red blood cells (MicroR), hemoglobin content of erythrocytes (RBC-He) and percentage of hypochromic erythrocytes (HypoHe)] is rapid, cost-effective and does not require additional sampling other than complete blood count.

The present study emphasized upon the importance of these novel markers in early and accurate diagnosis of IDA and ACD. Moreover Ret-He emerged as a sensitive and specific marker for diagnosis of latent iron deficiency. Multi-institutional studies on a larger sample size are required to establish the role of these novel parameters and determine the cut-off values for diagnosis.

Conflicts of interest: There are no conflicts of interest.

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