

Evaluating RET-Hb vs Hb in Predicting Early Response in Oral Iron Therapy: A Prospective Interventional Study

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ABSTRACT

Introduction: Children are more vulnerable to anaemia due to their rapid growth, which requires an expanding erythroid mass and a high tissue iron demand. Anaemia can negatively impact psychomotor and cognitive development and can lead to a decline in immunity. Reticulocytes are precursors to erythrocytes and make up about 1% (ranging from 0.5% to 2.5%) of circulating erythrocytes. The reticulocyte content serves as a measurement of haemoglobin within the reticulocyte and correlates directly with the functional availability of iron in the bone marrow. Consequently, it is considered one of the strongest predictors of Iron Deficiency (ID). The Reticulocyte Haemoglobin Content (RET-Hb) level is useful for diagnosing anaemia before it clinically manifests in a patient.

Aim: To assess the effectiveness of RET-Hb content as a predictor for early response to oral iron therapy in comparison to haemoglobin levels.

Materials and Methods: This prospective interventional study was conducted in the Department of Paediatrics at a tertiary care Hospital in Thrissur, Central Kerala, India, over a period of 18 months, from December 2019 to June 2021. A total of 44 samples were included in the study, and socio-demographic details were collected using a semistructured proforma from patients with

haemoglobin levels below the mean reference value. Baseline haemogram parameters {Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC), Red Cell Distribution Width (RDW), Haemtocrit (HCT)}, reticulocyte indices, and ferritin levels were measured before administering oral iron therapy (T0). These parameters were reassessed after seven days (T1) and 30 days (T2) following the initiation of therapy. The Chi-square test was used to assess the association between various factors, and Pearson's correlation coefficient was utilised to find the correlation.

Results: The mean age of the participants was 3.46 ± 2.2 years, with 24 males and 20 females included in the study. The mean RET-Hb was 21.15 ± 1.71 pg before the initiation of treatment, which significantly increased to 23.37 ± 1.95 pg and 25.77 ± 1.56 pg at days 7 and 30, respectively. The haemoglobin level increased from 8.37 ± 1.25 g/L to 8.55 ± 1.19 g/L and 9.72 ± 0.98 g/L at T1 and T2, respectively, which was statistically significant. The Absolute Reticulocyte Count (RET#) also showed a significant early increase after initiating treatment.

Conclusion: These findings indicate that RET-Hb and RET# are reliable early predictors of response to iron therapy, alongside haemoglobin levels, and can be used as effective alternatives.

Keywords: Anaemia, Early marker, Haemoglobin, Haematology analysers, Iron deficiency, Reticulocyte haemoglobin content, Treatment response

INTRODUCTION

Anaemia is a public health problem. Infants, children, and pregnant women are particularly prone to anaemia due to their increased physiological requirements. Anaemia adversely affects psychomotor and cognitive development while also weakening immunity [1].

Iron Deficiency (ID) may manifest without anaemia, as haemoglobin levels can be maintained for a while after iron deposits are diminished [2]. The gold standard diagnostic test for ID is the staining of erythroid precursors and bone marrow macrophages using Prussian blue stain in a bone marrow aspiration. Nevertheless, this procedure is very costly and invasive [3]. Alternative biochemical tests to assess ID include serum ferritin concentration, serum iron level (Fe), Total Iron-binding Capacity (TIBC), and Transferrin Saturation (TSAT), but these tests may be influenced by certain conditions. Serum ferritin levels are proportional to iron stores, so a diminished value indicates ID. However, serum ferritin is also an acute-phase protein and may appear normal or elevated in cases of infections, inflammatory conditions, or malignancies [2,4].

Reticulocytes constitute about 1% (0.5-2.5%) of circulating erythrocytes. The haemoglobin content within reticulocytes reflects the functional availability of iron in the bone marrow, making it the most reliable predictor of ID [5-7]. Absolute Reticulocyte count

(RET#) and RET-Hb have been found to be useful early markers for the diagnosis of ID and IDA, both children and adults, according to studies by Brugnara C et al., Ullrich C et al., and Bakr AF and Sarette G., [8-10]. The analysis by Neef V et al., revealed that Ret-Hb significantly correlated with ferritin, TSAT, and soluble transferrin receptor (sTfR). It was also found that Ret-Hb can be used as a single screening parameter [11]. Parodi E et al., found that both absolute reticulocyte count and RET-Hb are good markers to predict the early haematological response to iron supplements after one week of therapy [12]. A similar prospective multicentric study by Russo G et al., also supported this finding [13]. Mittman N et al., and Brugnara C et al., observed an increase in RET-Hb within 48-72 hours of intravenous treatment [14,15]. Given the paucity of data comparing Hb with RET-Hb in Kerala, Authors believed the present study will offer a cost-effective approach to measure iron status directly from blood. This test can be integrated into routine Complete Blood Counts (CBC) without requiring additional blood draws or specialised procedures.

The RET-Hb is a parameter that can be identified well before an increase in haemoglobin occurs following pharmacotherapeutic interventions, making it useful for treatment follow-up. As there are not many similar studies, especially in India, the purpose of present study was to assess the effectiveness of RET-Hb content as a

predictor of early response to oral iron therapy in comparison to blood haemoglobin levels.

MATERIALS AND METHODS

The prospective interventional study was conducted over 18 months, from December 2019 to June 2021, in the Department of Paediatrics at a tertiary care hospital in Central Kerala, India. The study was approved by the Institutional Ethics Committee (64/19/IEC/JMMC&RI). Informed consent was obtained from the parents in a structured format and in their preferred language.

Inclusion criteria: All children between the ages of six months and 14 years who visited the hospital on an outpatient and inpatient basis with a haemoglobin level below the mean reference value for age and gender, as well as serum ferritin levels below the normal range, were included in the study through a consecutive sampling method [16].

Exclusion criteria: Patients already on iron supplementation, those with haemoglobinopathies, a history of blood transfusion within the past six months, or a history of chronic illness or carcinoma were excluded from the study.

Sample size calculation: Based on the proportion of complete early response observed in an earlier publication by Parodi E et al., with a 95% confidence level and a 20% relative allowable error, the minimum sample size was determined to be 50 [12]. The total number of initial samples collected for the study was 74. Total of 8 samples were, lost after the first follow-up, and 22 after the second follow-up, resulting in a total of 44 samples taken for further evaluation. Socio-demographic and dietary details were collected using a semistructured proforma. The socio-economic status of the children was assessed using the modified Kuppuswamy scale [17]. The weight of the children was recorded and classified according to the Indian Academy of Paediatrics (IAP) classification [18].

Study Procedure

A 3 mL blood sample {using Ethylene Diaminetetracetic Acid (EDTA) containers} was collected before administering oral iron therapy (T0) to evaluate the haemogram (MCV, MCH, MCHC, RDW, HCT), reticulocyte level-absolute Reticulocyte Count (RET#); RET-Hb; and ferritin. T0 served as a baseline parameter to assess the effectiveness of the iron therapy given to the patients and to monitor the changes in the parameters after the therapy. All parameters, except ferritin, were analysed within two hours of collection. Ferritin was analysed twice a week in the Institution. The reference values for haemoglobin were kept as shown in [Table/Fig-1] [16].

Age (in years)	Reference values
6 months-2 years	12.0
2-6 years	12.5
>6-12 years	13.5
>12-14 years (female)	14.0
12-14 years (male)	14.5

[Table/Fig-1]: Reference values for Haemoglobin (g/dL) [16].

Ferrous sulphate preparations were administered at a dosage of 4 mg/kg [19]. Parents were advised to give the supplements on an empty stomach, avoiding milk or phytates immediately before or after administration. Adverse effects such as gastritis, black stools, and staining of teeth were explained to parents. The children were reviewed after seven days (T1) and 30 days (T2). During each visit, these parameters were reassessed, and compliance as well as any side effects were inquired about. Blood samples were analysed using an automated haematology analyser that employs the double hydrodynamic sequential system and the impedance method (Yumizen H2500 made by Horiba Medical Group from France). Ferritin was analysed using the Vitros 5600 OCD eCLIA two-step immunometric method. The cut-off values for ferritin, MCV, MCH,

MCHC, and RDW across different age groups and genders has been summarised in [Table/Fig-2] [16,20].

Parameters	Age group/gender	Cut-off value
Ferritin	Boys	15-300 ng/mL
	Girls	15-200 ng/mL
MCV	6 months-2 years	Mean: 78, Lower limit: 70
	>6-12 years	Mean: 86, Lower limit: 73
	>12-14 years girls	Mean: 90, Lower limit: 75
	>12-14 years boys	Mean: 88, Lower limit: 75
MCH	6 months-2 years	27 pg/cell
	>6-12 years	29 pg/cell
	>12-14 years girls	30 pg/cell
	>12-14 years boys	30 pg/cell
MCHC	6 months-2 years	33 pg/cell
	>6-12 years	34 pg/cell
	>12-14 years girls	34 pg/cell
	>12-14 years boys	34 pg/cell
RDW	Range	12.8±1.2%
RET-Hb	Range	25.1 to 33.3 pg
RET#	Range	0.039 to .093

[Table/Fig-2]: Cut-off range age and gender-wise distribution for biochemical parameters [16,20].

STATISTICAL ANALYSIS

Qualitative variables were expressed as frequencies and percentages, while quantitative variables were expressed as means and standard deviations. The Chi-square test was used to assess the association between various factors.

RESULTS

In the present study, 44 samples were included. Socio-demographic factors of children with ID anaemia are summarised in [Table/Fig-3]. The mean age of the participants was 3.46±2.2 years. The majority of the children, 33 (75%), were between the age group of 1 to 5 years. The study group had a slight male predominance,

Socio-demographic variables	n (%)	
Age (in years)	6 months-2 years	11 (25.0)
	2-6 years	26 (59.1)
	>6-12 years	7 (15.9)
	>12-14 years	0
Gender	Male	23 (52.3)
	Female	21 (47.7)
Religion	Hindu	21 (47.7)
	Christian	11 (25.0)
	Muslim	12 (27.3)
Socio-economic status	Upper	3 (6.8)
	Upper middle	17 (38.6)
	Lower middle	18 (40.9)
	Upper lower	6 (13.6)
Weight	Normal	19 (43.2)
	Grade-I	14 (31.8)
	Grade-II	8 (18.2)
	Grade-III	2 (4.5)
	Grade-IV	1 (2.3)
Diet distribution	Vegetarian	3 (6.8)
	Non vegetarian	41 (93.2)

[Table/Fig-3]: Socio-demographic variables in patients with Iron Deficiency (ID) anaemia.

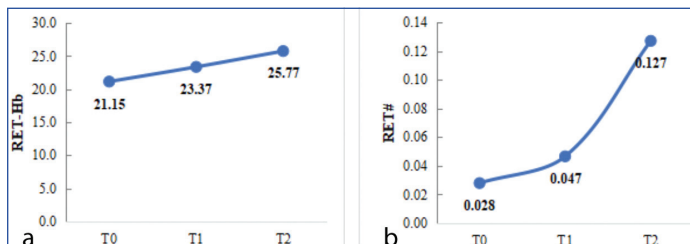
with 23 (52.3%) male patients. Socio-economic status was assessed using the modified Kuppuswamy scale, and the majority belonged to the lower middle class (Class III), comprising 18 patients (40.9%). When assessing weight according to the Indian Academy of Paediatrics (IAP) classification, it was found that the majority of the children, 19 patients (43.2%), had normal weight, while Grade 1 Protein Energy Malnutrition (PEM) was noted in 14 patients (31.8%). The average ferritin level was 9.49 ± 2.86 ng/mL. When dietary distribution was assessed, it was observed that among the 44 patients, 41 (93.6%) were non vegetarians.

Statistically significant changes were noted in Haemoglobin (Hb), Haematocrit (HCT), and Red Cell Distribution Width (RDW) at both seven and 30 days, while Mean Corpuscular Volume (MCV) and Mean Corpuscular Haemoglobin (MCH) changes became evident only after 30 days. No significant change in Mean Corpuscular Haemoglobin Concentration (MCHC) was observed. It was noted that both Reticulocyte Haemoglobin (RET-Hb) and RET# significantly increased after seven days and after 30 days [Table/Fig-4]. Changes in reticulocyte indices are also denoted in [Table/Fig-5].

Among the 44 samples, the majority of the children had normalised their parameters (Hb, RET-Hb, and RET#) after one

Haematological indices		Mean	p-value
Haemoglobin	T0	8.368±1.251	
	T1	8.550±1.195	<0.001
	T2	9.723±0.976	<0.001
Haematocrit	T0	25.49±3.710	
	T1	26.14±3.498	0.002
	T2	29.66±2.872	<0.001
Mean Corpuscular Volume (MCV)	T0	59.21±5.802	
	T1	59.10±6.047	0.788
	T2	62.45±5.047	<0.001
Mean Corpuscular Haemoglobin (MCH)	T0	19.47±2.056	
	T1	19.26±2.067	0.059
	T2	20.43±1.607	<0.001
Mean Corpuscular Haemoglobin Concentration (MCHC)	T0	32.66±0.882	
	T1	32.65±1.159	0.965
	T2	32.74±0.665	0.549
Red cell Distribution Width (RDW)	T0	19.00±3.021	
	T1	19.43±2.913	<0.001
	T2	25.67±2.924	<0.001
Reticulocyte Haemoglobin content (RET-Hb)	T0	21.15±1.712	
	T1	23.37±1.952	<0.001
	T2	25.77±1.584	<0.001
Absolute Reticulocyte Count (RET#)	T0	0.028±0.011	
	T1	0.047±0.014	<0.001
	T2	0.127±0.206	<0.001

[Table/Fig-4]: Haematological Indices at baseline (T0), 7 days (T1) and 30 days (T2). *Chi-square test



[Table/Fig-5]: a) RET-Hb has significantly increased after 7 days (23.37 ± 1.952) and further increased after 30 days (25.77 ± 1.584) compared to RET-Hb at T0 (21.15 ± 1.712). b) RET# has also significantly increased after 7 days (0.047 ± 0.014) and further increased after 30 days (0.127 ± 0.206) compared to RET# at T0 (0.028 ± 0.011).

month of iron therapy [Table/Fig-6]. A strong correlation was observed between Hb and RET-Hb, with a Pearson's correlation coefficient of $r=0.668$ and a p-value of <0.001 . However, a weak correlation was noted between haemoglobin and RET# (Pearson's correlation coefficient $r=0.183$ and a p-value of 0.234) [Table/Fig-7,8]. A strong and positive correlation indicates that RET-Hb increases with an increase in Hb and decreases with a decrease in Hb.

Age (in years)		6 months-2 years	>2-6 years	>6-12 years	>12-14 years
Hb (Normal)	T0	0	0	0	NA
	T1	0	0	0	NA
	T2	0	2	0	NA
RET-Hb (Normal)	T0	0	0	0	NA
	T1	1	2	0	NA
	T2	5	13	3	NA
RET# (Normal)	T0	0	0	0	NA
	T1	0	0	0	NA
	T2	0	3	0	NA

[Table/Fig-6]: Distribution of patients with normalised laboratory parameters with treatment to iron therapy.

Correlation between Hb and Reticulocyte Haemoglobin Content (RET-Hb)			
Variables	Mean	r	p-value
Hb	8.368±1.251	0.668	<0.001
RET-Hb	21.15±1.712		

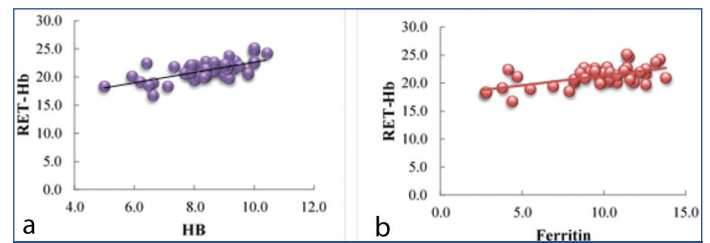
Correlation between Hb and Absolute Reticulocyte Count (RET#)			
Variables	Mean	r	p-value
Hb	8.368±1.251	0.183	0.234
RET#	0.028±0.011		

Correlation between Ferritin and Reticulocyte Haemoglobin Content (RET-Hb)			
Variables	Mean	r	p-value
Ferritin	9.491±2.858	0.586	<0.001
RET-Hb	21.15±1.712		

Correlation between Ferritin and Absolute Reticulocyte Count (RET#)			
Variables	Mean	r	p-value
Ferritin	9.491±2.858	-0.081	0.600
RET#	0.028±0.011		

[Table/Fig-7]: Correlation of Haemoglobin (Hb) and Ferritin with Reticulocyte Indices (RET-Hb, RET#).

*r: Pearson's correlation coefficient



[Table/Fig-8]: Correlation of haemoglobin and Ferritin with Reticulocyte Haemoglobin Content (RET-Hb). a) RET-Hb has a positive correlation with Hb and increases with increase in Hb and decreases with decrease in Hb. b) RET-Hb has a positive correlation with Ferritin and increases with increase in ferritin and decreases with decrease in ferritin.

Regarding the correlation between ferritin and reticulocyte indices, a strong correlation was observed between ferritin and RET-Hb (Pearson's correlation coefficient $r=0.586$ and a p-value of <0.001), while a weak correlation was noted between ferritin and RET# (Pearson's correlation coefficient $r=-0.081$ and a p-value of 0.600) [Table/Fig-7,8]. A strong and positive correlation indicates that RET-Hb increases with an increase in ferritin and decreases with a decrease in ferritin.

DISCUSSION

Early detection of anaemia and timely treatment will accelerate a child's path to recovery. The focus of the study was to determine the role of RET-Hb in predicting the effectiveness of oral iron therapy in comparison to haemoglobin.

In present study, the majority of children who presented with ID Anaemia (IDA) were in the age group of 1-5 years (75%), with a slight male preponderance (52.3%). This finding is consistent with studies conducted by John JJ et al., Gompakis N et al., and a National Nutrition Survey conducted between 2016 and 2018 by Kulkarni B et al., which indicated that younger children are more prone to developing IDA [21-23]. The current study showed that male and female children have approximately equal susceptibility to developing IDA, which aligns with the findings of Rothman JA [19]. This may be due to the majority of the children in present study being in the younger age group, as adolescent females are generally more prone to developing IDA. The study by George KA et al., reports that IDA is more common in females, while Manoj S and Meppadath IM report that males are more susceptible [24,25].

The findings of present study highlight that the majority of children (40.9%) diagnosed with IDA belong to the middle-class socio-economic status. The fact that this demographic more frequently approaches present hospital could reflect this finding. Although the lower strata of society are generally found to be at a higher risk of developing IDA, it is also observed among affluent groups, as supported by the studies of Rothman JA et al., and Plessow R et al., [19,26].

In the present study, the majority of children were found to have a normal weight (43.2%) when classified according to IAP guidelines, while Grade I Protein-Energy Malnutrition (PEM) was observed in 31.8% of the children. Overweight and obese individuals are also seen to have a higher risk of ID than those with normal body weight. This may be partially explained by the fact that obesity is considered a low-grade chronic inflammatory state, which can alter iron parameters [27,28].

Regarding the parameters used to evaluate IDA and the response to treatment, present study noted that MCH and MCV were nearly the same after seven days of treatment, while they showed a statistically significant increase after 30 days of treatment. However, no significant increase was noted in MCHC values. Early changes in these parameters were also not observed in the studies conducted by Suega K and Bakta IM, and Uçar MA et al., [29,30].

One of the initial parameters of anaemia is an increase in RDW, which is accompanied by a fall in MCV. These changes are due to a higher proportion of microcytic red blood cells in the bloodstream. With iron treatment, the marked reticulocytosis that occurs in the first four weeks following therapy is manifested as a sudden increase in RDW, sometimes exceeding 30% [31]. This marked increase in RDW after the initiation of therapy helps confirm the diagnosis. In our study, RDW showed a statistically significant rise at both seven and 30 days of treatment. However, Brugnara C report a slower rise in these values [32].

Present study showed a mean haemoglobin value of 8.37 ± 1.25 g/dL before the initiation of treatment. It increased to 8.55 ± 1.19 g/dL and 9.72 ± 0.98 g/dL at T1 and T2, respectively, which was found to be statistically significant. The RET-Hb also significantly increased from 21.15 ± 1.71 pg to 23.37 ± 1.95 pg and 25.77 ± 1.58 pg at days 7 and 30, respectively. studies by Parodi E et al., Suega K and Bakta IM and Uçar MA et al., found a mild rise in haemoglobin in children after seven days of treatment, but it was not statistically significant [12,29,30]. Another study by Parodi E et al., found that there was no response at three days, but a response in haemoglobin values was noted at 14 days [33]. All these studies, however, observed that RET-Hb was the first to increase after iron therapy, as early as 48 hours, making it a better predictor of response. Studies by

Mittman N et al., Brugnara C et al., Auerbach M et al., and Mehta S et al., also support this finding. RET# was significantly increased in present study after seven days of treatment, which also marks it as a good predictor of response, similar to the findings of Parodi E et al., [14,15,33-35].

A strong positive correlation was noted between RET-Hb and haemoglobin, thus proving that it is a reliable marker for diagnosing ID anaemia and evaluating its response. A similar response was also noted in the study by Auerbach M et al., [34]. Early identification of response to treatment will help differentiate patients with no improvement, allowing for additional diagnostic tests and management strategies to be offered.

Limitation(s)

The small sample size and large number of attritions make it difficult to generalise the results. The number of samples and attrition were significantly affected by the Coronavirus Disease (COVID)-19 pandemic. The evaluation of the first response was conducted after seven days, resulting in a significant increase in both haemoglobin and RET-Hb levels. Evaluating the early response over a shorter duration might show that RET-Hb is a better predictor than haemoglobin. This emphasises the need for future studies in this field.

CONCLUSION(S)

The RET-Hb is a good early predictor of response to iron therapy, along with haemoglobin, and can be used as an alternative. The absolute reticulocyte count also shows a significant early increase after the initiation of treatment. This early identification helps in recognising patients who do not improve, allowing for timely additional diagnostic tests and management strategies. Consequently, using RET-Hb and absolute reticulocyte count can enhance patient outcomes by facilitating early intervention and personalised treatment plans. Future studies should validate the predictive value of RET-Hb and absolute reticulocyte count across diverse populations and explore their cost-effectiveness and long-term outcomes. However, an earlier evaluation of reticulocyte parameters would be a better predictor than haemoglobin levels. This represents an area for future study and ultimately benefits patients by enabling alternative diagnosis for appropriate management.

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