Research Article

Yeter Düzenli Kar* and Konca Altınkaynak

Reticulocyte hemoglobin equivalent in differential diagnosis of iron deficiency, iron deficiency anemia and β thalassemia trait in children.

[Retikülosit hemoglobin eşdeğerinin çocuklarda demir eksikliği, demir eksikliği anemisi ve β talasemi taşıyıcılığı ayırıcı tanısında yeri]

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Abstract

Objectives: This study investigated the diagnostic power of reticulocyte hemoglobin equivalent (Ret-He) in the differential diagnosis of hypochromic microcytic anemia to differentiate iron deficiency anemia (IDA) and thalassemia trait (TT) based on the traditionally used erythrocyte index and formulas.

Methods: Twenty-six children with iron deficiency (ID), 26 with IDA, 33 with β -TT, 41 healthy children were assessed. Complete blood count parameters, Ret-He, immature reticulocyte fraction (IRF), low-fluorescence ratio (LFR), Mentzer's indexes (MI) were evaluated. The diagnostic power of Ret-He in distinguishing between IDA and β -TT was investigated using ROC analysis.

Results: Ret-He levels were (median(Q1-Q3)) 20.6(19.7–21.5) pg in β -TT, 16.1(13.1–20) pg in IDA, 29.7(27.2–30.7) pg in ID, 30.5(29.8–31.7) pg in healthy controls. Based on ROC analysis, diagnostic power for distinguishing between IDA and β -TT was

determined as $RBC > MI > Ret-He > RDW > LFR > IRF$. The highest sensitivity and specificity for differential diagnosis was obtained when the Ret-He cut-off value was 18.2pg. The AUC (95%CI) value was calculated as 0.765(0.637–0.866), and a statistically significant difference was found between groups ($p < 0.0006$).

Conclusions: In patients with hypochromic microcytic anemia, $Ret-He \leq 18.2$ pg combined with $RBC \leq 5.3 \times 10^6/L$ and $MI > 10.42$ can be safely used to distinguish IDA from β -TT. In particular, patients with low Ret-He who don't respond to iron therapy should be examined for β -TT.

Keywords: iron deficiency; microcytic anemia; reticulocyte hemoglobin equivalent; thalassemia trait.

Öz

Amaç: Bu çalışmada, hipokrom mikrositer anemi ayırıcı tanısında, demir eksikliği anemisi (IDA) ve Talasemi taşıyıcılığı (TT) durumlarının ayırt edilmesinde retikülosit hemoglobin eşdeğerinin (RET-He), klasik olarak kullanılan eritrosit indeks ve formüllerine göre diagnostik gücü araştırılmıştır.

Gereç-Yöntem: Çalışmaya 26 demir eksikliği (ID), 26 IDA, 33 β -TT, 41 sağlıklı çocuk alındı. Hastaların tam kan sayımı parametreleri, Ret-He, immatür retikülosit fraksiyonu (IRF), low fluorescence ratio (LFR), Mentzer İndeksi (MI) değerlendirildi. Receiver operating characteristic (ROC) analizi ile Ret-He'nin IDA ve β -TT'ni ayırt etmede diagnostik gücü araştırıldı.

*Corresponding author: Yeter Düzenli Kar, MD, Department of Pediatrics, Division of Pediatric Hematology/Oncology, Erzurum Regional Training and Research Hospital, Erzurum, Turkey, E-mail: yeterduzenli@yahoo.com. <https://orcid.org/0000-0003-2917-7750>

Konca Altınkaynak, Department of Biochemistry, University of Health Sciences, Erzurum Regional Training and Research Hospital, Erzurum, Turkey

Bulgular: Çalışmamızda Ret-He düzeyi β -TT; median (Q1-Q3) 20.6 (19.7–21.5) pg, IDA; 16.1 (13.1–20) pg, ID; 29.7 (27.2–30.7) pg, sağlıklı kontroller; 30.5 (29.8–31.7) pg olarak tespit edildi. ROC analizine göre IDA ve β -TT'ni ayırt etmede diagnostik gücü en yüksekten en düşüğe doğru RBC>MI>Ret-He>RDW>LFR>IRF olarak tespit edildi. Ret-He cut-off değeri 18.2 pg alındığında IDA- β -TT ayırıcı tanısında AUC (95%CI) değeri 0.765(0.637–0.866) olarak en yüksek sensitive ve spesitiviteye sahipti ve her 2 grup arasında istatistiksel anlamlı fark olduğu bulundu ($p < 0.0006$).

Sonuçlar: Hipokrom mikrositer anemili hastalarda Ret-He $\leq$ 18.2 pg olması, beraberinde RBC $\leq$ 5.3 $\times 10^6$ /L olması ve MI >10.42 olması IDA'yı β -TT'nden ayırt etmede güvenle kullanılabilir. Özellikle Ret-He düşük saptanan ve demir tedavisine yanıt vermeyen hastalar β -TT açısından tetkik edilmelidir.

Anahtar Kelimeler: demir eksikliği; mikrositik anemi; retikülosit hemoglobin eşdeğeri; talasemi taşıyıcılığı.

Introduction

Anemia is a significant health problem that affects approximately a third of the world's population and has negative effects on maternal and child mortality rates, physical performance. Iron deficiency (ID) is responsible for anemia in approximately half of the world's population. The most common cause of iron deficiency anemia (IDA) is nutritional deficiency [1], and it is characterized by hypochromia and microcytosis [2]. ID and IDA are more common during child growth and menstruation in adolescent girls. Diet, poor socioeconomic status, and previous infections are factors contributing to IDA development [3]. The incidence of IDA in Turkey has been reported to be 15.2–62.5% in various studies [3–6]. Iron plays an important role in neural myelination, maintaining neurotransmitter functions, and development of the central nervous system. Especially in infancy, IDA is associated with poor neurodevelopment. Cognitive and behavioral performances may not be fully improved even with iron supplementation in severe cases of IDA [7].

β -thalassemia trait (β -TT) is the other most common cause of hypochromia and microcytosis, and is caused by a synthesis defect in the β chain of hemoglobin. After detecting hypochromia and microcytosis, the first step is to exclude ID by evaluating serum iron, total iron-binding capacity (TIBC), transferrin saturation, and ferritin levels. The next step is HbA₂ measurement for β -TT [8].

Differential diagnosis of hypochromia and microcytosis is clinically important. While ID can be easily

treated with iron supplementation, the failure to diagnose of thalassemia trait (TT) may lead to iron accumulation in patients. In regions with high TT incidence such as Turkey, the most important aspect in the correct diagnosis of TT is to determine whether mother and father candidates have TT. If both parents have TT, the risk of thalassemia major in the baby will be 25%; in such cases, genetic counseling and prenatal diagnosis can reduce the incidence of thalassemia major, and this disease can even be eradicated [9].

Reticulocyte hemoglobin equivalent (Ret-He) is a parameter that reflects the hemoglobin content of reticulocytes. Recent XN series hematology devices (Sysmex Corporation, Kobe, Japan) allow evaluation of new reticulocyte parameters including high fluorescence ratio (HFR), medium fluorescence ratio (MFR), and low fluorescence ratio (LFR) using flow cytometry based on the RNA content of Ret-He and other reticulocyte parameters in the reticulocyte channel [10–12]. Together, MFR and HFR are termed immature reticulocyte fractions (IRF) [11, 12]. Reticulocytes are immature non-nucleated erythrocytes containing ribosomal RNA residues that mature three days after being produced in the bone marrow and are released into the peripheral circulation to mature after one day in circulation [13]. In case of ID, hemoglobin synthesis decreases in reticulocytes first. Therefore, the Ret-He level is a reliable marker that can be used for evaluating both erythropoietic activity and bone marrow iron stores [1, 2, 10, 14].

The study aimed to calculate the diagnostic power of Ret-He in distinguishing between IDA and β -TT in children with hypochromic microcytic anemia and to calculate the cutoff values for the study population.

Materials and methods

Population selection

The study included children who were admitted to the Pediatric Hematology/Oncology Outpatient Clinic between December 2018 and July 2019 and were diagnosed with ID (n=26), IDA (n=26), and β -TT (n=33) alongside 41 healthy children. The patient files were analyzed retrospectively. Anemia was diagnosed as Hb $\leq$ 10.5 g/dL for children aged 0.5–2 years, Hb $\leq$ 11.5 g/dL for those aged 2–12 years, and Hb $\leq$ 12 g/dL for those aged 12–18 years [15]. Microcytosis was diagnosed with <mean corpuscular volume (MCV)+age in children aged <10 years and with 80 fL in children aged $\geq$ 10 years. Children with ferritin levels<12 μ g/L and transferrin saturation<16% were classified as having IDA [3]. Children with normal hemoglobin and MCV levels and serum ferritin levels<15 μ g/L were classified as having ID. Patients with hypochromic microcytic anemia for whom IDA was ruled out and in whom the HbA₂ value obtained through high-performance liquid chromatography (HPLC) was >3.5% were considered to have β -TT [8]. Alpha globulin gene analysis was performed for patients with

hypochromic microcytic anemia for whom IDA or β -TT were ruled out. Patients with mutations detected by gene analysis were accepted as α -TT. Children excluded from the study included 10 patients treated with iron preparations, six patients who received blood transfusions in the last three months due to microcytic anemia, two patients who had IDA with TT, five patients with α -TT, and one patient who had HbS with α -TT.

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guidelines on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. The study was approved by the local ethics committee decision (no: 2020/04-52, dated:17.02.2020) of Erzurum Region Training and Research Hospital.

Blood sample collection and measurement of parameters

Blood samples were collected in ethylenediamine tetra acetic acid dipotassium salt (EDTA-2K) tubes and complete blood counts, Ret-He levels, IRF, MFR, HFR and LFR levels were determined using an automated hematology analyzer (XN-1500, Sysmex, Kobe, Japan). For complete blood count, hemoglobin (Hb), hematocrit (Hct), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), MCV, red blood cells (RBCs), and red blood cell distribution width (RDW) values were recorded.

Serum iron (sFe) and unsaturated iron-binding capacity (UIBC) were measured using an automated chemical analyzer, (Architect c16000, Abbott, USA). TIBC was automatically calculated using the same device by adding UIBC and sFe. Serum ferritin was measured using an automated chemical analyzer (Architect i12000, Abbott, USA). Transferrin saturation was calculated as serum iron $\times 100 / \text{TIBC}$. Mentzer's index (MI) was calculated as MCV / RBC [3, 8]. HbA2 was quantitatively measured using the HPLC system (HPLC, Variant II, Biorad). HPLC is used to separate hemoglobin variants such as HbA2, hemoglobin F, hemoglobin A0, hemoglobin S, hemoglobin C, and hemoglobin D, which consist of two phases (mobile and stationary), depending on the changes in ionic strength and/or buffer pH. Eluent buffers (buffer solutions) are used in the mobile phase, whereas a matrix column is used as the stationary phase. 5 μL of whole blood sample taken into the tube with EDTA is pipetted and this sample is automatically hemolyzed using the device and a hemolysate. The released hemoglobin molecules are gradually combined with eluent buffers. Each hemoglobin type has a different ionic charge. The prepared sample is injected into the stationary-phase column using pressure-controlled pump systems. While hemoglobin molecules in the stationary phase leave the column in parallel with their load amounts, the results are automatically printed spectrophotometrically [16].

Statistical analysis

The Shapiro–Wilk normality test results were used in the selection of hypothesis tests between diagnostic groups and quantitative medical parameters. Variance analysis (ANOVA) was used for quantitative parameters with normal distribution, and the Kruskal–Wallis test was used for parameters without normal distribution. In ANOVA application, Tukey's post hoc test was performed when variance homogeneity between groups was achieved, and the Tamhane post hoc test was

performed when variance homogeneity was not achieved. The Bonferroni corrected Dunn test was performed as a post hoc analysis following the Kruskal–Wallis test. To test the relationships between quantitative medical parameters, the Spearman correlation analysis was performed for data that was not normally distributed during the correlation analysis phase. During the receiver operating characteristic (ROC) analysis, statistical measurements and confidence intervals were calculated together. The confidence level of the study was 95%. All applications were performed using IBM SPSS (Statistical Package for the Social Sciences) 17 and MEDCALC 18; $p < 0.05$ was considered statistically significant.

Results

A total of 126 participants were enrolled in the study, including 26 children with ID, 26 with IDA, and 33 with β -TT and 41 healthy children. The median (Q1–Q3) age of patients was 6 (3.2–15) years in the β -TT group, 2 (1–15) years in the IDA group, 4.7 (2.5–15) years in the ID group, and 6.5 (3–10) years in the healthy control group. There were no differences among the four groups in terms of age, sex, and white blood cell and reticulocyte counts (%) ($p > 0.05$ for all). In patients with hypochromic anemia, Hb, RBC, serum iron, transferrin saturation, and ferritin were significantly lower; RDW and TIBC were significantly higher; and MI was significantly lower in the IDA group than in the β -TT group ($p < 0.05$). There were no significant differences between the β -TT and IDA groups in terms of MCV, MCH, MCHC, IRF, MFR, HFR, and Ret-He values. Hb, MCV, MCH, MCHC, and RET-He values in both these groups were significantly lower, whereas IRF, MFR, and HFR values were significantly higher than those in the ID group and healthy controls ($p < 0.05$) (Table 1). Table 1 shows the clinical and laboratory findings of the β -TT, IDA, and ID groups and healthy controls.

Figure 1 shows the distribution of Ret-He levels in the β -TT, ID, and IDA groups and the healthy controls.

In the IDA group, a strong positive correlation of Ret-He level was observed with Hb concentration ($r = 0.810$, $p < 0.001$) and MCHC ($r = 0.719$, $p < 0.001$) (Table 2). Table 2 shows findings from the correlation analysis between Ret-He and other parameters according to diagnostic groups.

The discriminant efficiency of RBC measurements provided by Sysmex XN-1500 was low with the exception of the RBC count; ROC analysis showed an area under curve (AUC) of 0.994 and sensitivity and specificity of 100 and 96.97%, respectively, for differentiating β -TT and IDA. Table 3 shows the cutoff values, sensitivity, specificity, LR + and LR–, and Youden's Index results, which have been determined at 95% confidence interval of the

Table 1: Characteristics and laboratory parameters of iron deficiency (ID), iron deficiency anaemia (IDA), beta thalassemia traits (β -TT), and normal controls.

Characteristics	β -TT (n=33)	IDA (n=26)	ID (n=26)	Normal controls (n=41)
Age (year) median (Q1–Q3)	6 (3.2–15)	2 (1–15)	4.7 (2.5–15)	6.5 (3–10)
Sex (female)	16(48.5)	13(50)	16(61.5)	18(43.9)
White blood cell, /mm ³ Mean $\pm$ SD	8035 $\pm$ 1867	7863 $\pm$ 2813	7912 $\pm$ 1872	7685 $\pm$ 2123
Hb, g/dL median (Q1–Q3)	11.3 (10.9–11.9) ^{a,b,c}	7.2 (5.2–10.2) ^{d,e}	13 (12–13)	13.2 (12.9–14.2)
RBC, 10 ⁶ /L median (Q1–Q3)	6.2 (5.8–6.3) ^{a,b,c}	4.6 (3.6–4.9) ^e	4.9 (4.7–5.1)	5 (4.8–5.2)
MCV, fL median (Q1–Q3)	58.6 (56.3–61) ^{b,c}	58.5 (52.8–64.8) ^{d,e}	77.4 (73.9–79.2)	78 (75.6–79.2)
MCH, pg median (Q1–Q3)	18.5 (17.9–19.3) ^{b,c}	15.9 (14–20) ^{d,e}	25.9 (24.5–26.7)	26.6 (25.9–27.3)
MCHC, % median (Q1–Q3)	31.8 (31.4–32.1) ^{b,c}	27.1 (25.9–31.1) ^{d,e}	33.5 (33–34.4)	34.4 (33.9–35)
RDW, % Mean $\pm$ SD	18.09 $\pm$ 1.6 ^{a,b,c}	20.4 $\pm$ 3.24 ^{d,e}	14.3 $\pm$ 1.54 ^f	12.83 $\pm$ 0.7
Serum iron, mg/dL Mean $\pm$ SD	75.2 $\pm$ 27.5 ^a	21.4 $\pm$ 12.7 ^{d,e}	57.7 $\pm$ 28.4 ^f	83 $\pm$ 34
Transferrin saturation(%) median (Q1–Q3)	27.1 (18.8–52.4) ^{a,b}	3.6 (2.8–7.4) ^{d,e}	16.3 (12.2–28.8) ^f	28.8 (20.9–46.7)
TIBC, μ g/dL Mean $\pm$ SD	235 $\pm$ 63.4 ^{a,b}	427.8 $\pm$ 28.2 ^{d,e}	314.1 $\pm$ 52.8 ^f	248 $\pm$ 38.3
MI median (Q1–Q3)	9.6 $\pm$ 0.9 ^{a,b,c}	14.4 $\pm$ 4.3	15.5 $\pm$ 0.96	15.5 $\pm$ 1.2
Ferritin, ng/mL median (Q1–Q3)	39 (24–54) ^{a,b}	3.3 (1.2–6) ^e	7.9 (6–10) ^f	31 (23–37)
IRF, % median (Q1–Q3)	11.9 (8.5–14.2) ^{b,c}	13.8 (10–19.1) ^{d,e}	7.2 (5.4–10.5) ^f	4.2 (3.3–5.8)
MFR, % Mean $\pm$ SD	10.3 $\pm$ 3.4 ^{b,c}	12.6 $\pm$ 4.9 ^{d,e}	7.2 $\pm$ 3.6 ^f	4.3 $\pm$ 1.9
HFR, % median (Q1–Q3)	1.6 (0.9–2) ^{b,c}	2.6 (1.3–3.7) ^{d,e}	0.6 (0.2–1.07)	0.3 (0.1–0.5)
Reticulocyte count, % median (Q1–Q3)	1.14 (0.8–1.6)	1.08 (0.8–1.3)	1.03 (1.03–1.3)	1.06 (1.06–1.27)
Ret-He, pg median (Q1–Q3)	20.6 (19.7–21.5) ^{b,c}	16.1 (13.1–20) ^{d,e}	29.7 (27.2–30.7)	30.5 (29.8–31.7)

β -TT, beta thalassemia trait; IDA, iron deficiency anemia; ID, iron deficiency; SD, standart derivation; Hb, hemoglobin; RBC, red blood cells; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red blood cell distribution; TIBC, total iron binding capacity; MI, Mentzer Index; IRF, immature reticulocyte fraction; MFR, medium fluorescence ratio; HFR, high fluorescence ratio; Ret-He, reticulocyte hemoglobin equivalent. P Statistical significance between groups: ^a Statistical significance between β -TT and IDA, $p < 0.05$, ^b Statistical significance between β -TT and ID, $p < 0.05$, ^c Statistical significance between β -TT and normal controls, $p < 0.05$, ^d Statistical significance between IDA and ID, $p < 0.05$, ^e Statistical significance between IDA and normal controls, $p < 0.05$, ^f Statistical significance between ID and normal controls, $p < 0.05$.

Figure 1: Reticulocyte hemoglobin equivalent (Ret-He) in the β -TT, iron deficiency (ID), iron deficiency anemia (IDA), and normal control (NC) groups.**Table 2:** Correlations between reticulocyte hemoglobin equivalent and routine blood indexes.

	Ret-He		
r value	β -TT	IDA	ID
Hb, g/dL	0.519	0.810 ^{***}	0.492 ^{**}
RBC, 10 ⁶ /L	-0.139	0.508 ^{**}	0.098 [*]
RDW, %	-0.255 ^{***}	-0.496 ^{***}	-0.018 ^{**}
MCV, fL	0.619 ^{***}	0.530 ^{**}	0.643 ^{***}
MCH, pg	0.665 ^{***}	0.619 ^{***}	0.652 ^{***}
MCHC, %	0.278	0.719 ^{***}	0.246 [*]
Ferritin, ng/mL	0.012	0.484 [*]	-0.151
Transferrin saturation (%)	-0.100	0.462 ^{***}	0.140 [*]
TIBC, μ g/dL	0.196	-0.129 ^{**}	-0.380 ^{**}
IRF, %	-0.212 ^{***}	-0.293 ^{**}	-0.294
LFR, %	0.214 ^{***}	0.293 ^{**}	0.294
Reticulocyte count, %	0.067	-0.136	-0.017

Ret-He, reticulocyte hemoglobin equivalent; β -TT, beta thalassemia trait; IDA, iron deficiency anemia; ID, iron deficiency; Hb, hemoglobin; RBC, red blood cells; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red blood cell distribution; TIBC, total iron binding capacity; IRF, immature reticulocyte fraction; LFR, low fluorescence ratio; Reticulocyte count (%). ^{*} $p < 0.05$, ^{**} $p < 0.01$, ^{***} $p < 0.001$. Bold values: a strong positive correlation implies.

Table 3: Receiver operating characteristic curve analysis results and sensitivity, specificity, predictive value of each erythrocyte index and formula according to revised cut-off values in the IDA, β -TT group.

Indices	Cut off value	AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	LR+(95% CI)	LR-(95% CI)	Youden indeksi	p-Value
RBC, $10^6/L$	IDA/ β -TT (≤ 5.3)	0.994 (0.928–1.000)	100.0 (86.8–100.0)	96.97 (84.2–99.9)	33.0 (4.8–227.4)	∞ –	0.9697	<0.0001
MI	IDA/ β -TT (>10.42)	0.953 (0.864–0.991)	92.31 (74.9–99.1)	87.88 (71.8–96.6)	7.62 (3.0–19.2)	0.088 (0.02–0.3)	0.8019	<0.0001
Ret-He, pg	IDA/ β -TT (≤ 18.2)	0.765 (0.637–0.866)	65.38 (44.3–82.8)	96.97 (84.2–99.9)	21.58 (3.1–151.7)	0.36 (0.2–0.6)	0.6235	0.0006
RDW, %	IDA/ β -TT (>20.4)	0.755 (0.626–0.858)	65.38 (44.3–82.8)	93.94 (79.8–99.3)	10.79 (2.7–42.5)	0.37 (0.2–0.6)	0.5932	0.0008
LFR, %	IDA/ β -TT (≤ 879)	0.672 (0.537–0.789)	65.38 (44.3–82.8)	66.67 (48.2–82.0)	1.96 (1.1–3.4)	0.52 (0.3–0.9)	0.3205	0.0164
IRF, %	IDA/ β -TT (>12.8)	0.671 (0.537–0.788)	65.38 (44.3–82.8)	66.67 (48.2–82.0)	1.96 (1.1–3.4)	0.52 (0.3–0.9)	0.3205	0.0168

AUC, area under curve; 95% CI, 95% confidence interval; Sensitivity, true positive/(true positive+false negative); Specificity, true negative/(true negative+false positive); LR–, likelihood ratio negative (1– sensitivity)/(specificity); LR +, likelihood ratio positive (sensitivity)/(1– specificity); β -TT, beta thalasemia trait; IDA, iron deficiency anemia; RBC, red blood cells; Ret-He, reticulocyte hemoglobin equivalent; RDW, red blood cell distribution; MI, Mentzer Index; IRF, immature reticulocyte fraction; LFR, low fluorescence ratio.

parameters used in the differential diagnosis of IDA and β -TT and the ROC analysis. From highest AUC value to the lowest, the parameters used to distinguish IDA and β -TT were ranked as RBC>MI>Ret-He>RDW>LFR>IRF. When the cutoff value of Ret-He was 18.2 pg, the diagnostic power for distinguishing IDA and β -TT was lower than that for RBC and MI and higher than that for RDW, LFR, and IRF.

Discussion

The gold standard in the diagnosis of IDA is the evaluation of bone marrow iron deposition or the increase in Hb and MCV following iron treatment [3, 17]. Serum iron decreases first in the bone marrow, but bone marrow aspiration is not routinely used as it can be invasive [3, 17, 18]. In regions such as Turkey, where hypochromic microcytic anemia is common, parameters used in daily practice for IDA diagnosis include complete blood count parameters (Hb, RBC, MCH, MCHC, MCV, and RDW), MI, and serum iron parameters (serum iron level, TIBC, transferrin saturation and serum ferritin) [19]. However, recent studies have reported that Ret-He has a high diagnostic power for both ID and IDA diagnoses [1, 2, 10, 13, 14, 20–24]. Reportedly, Ret-He can be used as a potential marker to detect ID, which can be evaluated using the same EDTA tube for complete blood count. This method provides results in less than 2 min with full blood count parameters [25].

Studies have previously investigated the cutoff value and reliability of Ret-He in assessing iron levels in children [10, 22] and adults [1, 2, 13, 14, 24] with IDA and ID.

However, a limited number of studies have investigated the diagnostic power of Ret-He in the differential diagnosis of hypochromic microcytic anemia in children [10] and adults [2, 14, 24]. In our study, the Ret-He level was found to be slightly lower for IDA [1, 2, 10, 13, 22–24] and TT [10, 24] than the levels reported in other similar studies. Kadegasem et al. [10] investigated Ret-He levels in children and reported that the level of Ret-He was 25.4 ± 2.7 pg for those with IDA, 26.7 ± 2.4 pg for those with TT, 29.0 ± 2.9 pg for those with ID, and 30.8 ± 1.7 pg for healthy controls. In the present study, while Ret-He levels were similar to those in the study by Kadegasem et al. [10] in the ID and healthy control groups, Ret-He levels in the TT and IDA groups were lower. This may be explained by the Hb value in both the IDA and TT groups as it was lower than that in their study population (the present study: TT group Hb: 11.4 ± 0.8 g/dL, IDA group Hb: 7.6 ± 2.6 g/dL; Kadegasem et al. [10]: TT group Hb: 12.8 ± 1.0 g/dL, IDA group Hb: 11.7 ± 0.6 g/dL). In the study by Lian et al. [14] it was reported that the Ret-He value varied by the severity of anemia in both TT and IDA groups. They reported Ret-He levels of 20.83 ± 1.88 pg for TT and 22.45 ± 3.23 pg for IDA in mild anemia group (Hb 9–11 g/dL in women, 9–12 g/dL in men), and 19.26 ± 2.83 pg for TT and 17.38 ± 2.44 pg for IDA in moderate anemia (Hb 6.0–9.0 g/dL). These results signify that Ret-He values change in proportion to the Hb value of the study population. Although anemia is mild in children with TT, Ret-He level is lower than that in healthy controls due to reduced synthesis [14].

In the present study, a strong positive correlation of Ret-He level was observed with Hb and MCHC in the IDA

group [26]. Lian et al. [14] reported that there were strong correlations of Ret-He with Hb, MCV, MCH, and MCHC in the IDA group. These findings can be explained by the decrease in Hb concentration and MCHC due to decreased synthesis of Ret-He and an indirect decrease in Ret-He levels caused by the absence of iron in IDA states. In our study, there was a strong correlation between Ret-He and Hb in IDA group and a moderate correlation between Ret-He and Hb in TT group. This can be explained by the fact that anemia was more moderate in the TT group in this study population.

In the present study, the parameters with the highest sensitivity and specificity according to the ROC curve analysis in distinguishing IDA and β -TT were $RBC > MI > Ret-He > RDW > LFR > IRF$. The highest sensitivity and specificity for differential diagnosis was obtained when the Ret-He cutoff value was 18.2 pg, the AUC (95% CI) value was calculated as 0.765 (0.637–0.866), and a statistically significant difference was found between the groups ($p < 0.0006$) (Table 3). Buttarello et al. [2] reported Ret-He levels of 24.4 ± 4.8 pg in the IDA group and 21 ± 1.9 pg in the β -TT group, with a significant difference between the groups ($p < 0.0006$). Kadegasem et al. [10] reported that when they used a RET-He cutoff value of ≤ 27 pg, AUC value was 0.904 with a sensitivity of 91.7% and a specificity of 81%, and the diagnostic power for IDA was high. They reported that the diagnostic power of Ret-He for IDA was higher than MCH, MCHC, RDW, transferrin saturation, RDW, and ferritin, but they did not evaluate the diagnostic power of Ret-He in distinguishing between IDA and β -TT. Similar to the results of the present study, Lian et al. [14] performed an ROC analysis for the differential diagnosis of IDA and β -TT and concluded that when 20.9 pg was considered as the Ret-He cutoff value, the AUC value was 0.726 with a specificity of 67.06% and a sensitivity of 76.92% in the mild anemia group, and when 19.1 pg was considered as the Ret-He cutoff value, the AUC value was 0.714 with a specificity of 84.09% and a sensitivity of 68.42% in the moderate anemia group. Similar to the present study, studies investigating the erythrocyte index and formulas used to differentiate IDA and β -TT have also reported that the sensitivity, specificity, and discriminative power of RBC and Mentzer's index were higher than those of RDW [26–28].

Study limitations included a small sample size and the use of a single medical center. Furthermore, Ret-He could not be studied in all complete blood count devices.

In conclusion, Ret-He is an important parameter in terms of demonstrating ID and can be studied from the same EDTA tube for complete blood count in order to provide results in a short period of time. Ret-He levels were

low in both IDA and TT as a result of decreased synthesis. The diagnostic power of Ret-He in differentiating IDA and β -TT is increased when used together with RBC and MI. In patients with hypochromic microcytic anemia, Ret-He ≤ 18.2 pg with $RBC \leq 5.3 \times 10^6/L$ and $MI > 10.42$ can be safely used to distinguish IDA from β -TT. In particular, patients with low Ret-He who do not respond to iron therapy and have RBC levels $> 5.3 \times 10^6/L$ should be further examined for β -TT.

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